STUDIES ON ALLERGY AND INFLAMMATION

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SUMMARY

The enclosed papers have been classified into five major sections whose content is as follows:

Section A - The eosinophil leucocyte

The first works are studies on the mechanisms of eosinophil accumulation following antigen-antibody reactions in guinea pig skin.[1,2] These were extended to observations on eosinophil chemotaxis *in vitro* in relation to the complement system.[3,4]

The eosinophil chemotactic factor of anaphylaxis (ECF-A) was first described in 1971.[5,6] Interactions between ECF-A and complement-derived eosinophilotactic factors were reported later[7] as were investigations on the chemical characterisation of ECF-A.[8] Inhibition of eosinophil chemotaxis by an agent related to disodium cromoglycate is also described.[9] Other eosinophil chemotactic agents thought to participate in eosinophil accumulation *in vivo* include material derived from Hodgkin's lymph node cells,[10] histamine and one of its major catabolites, imidazole acetic acid.[11] The interactions of these agents both *in vitro* and *in vivo* were extensively studied[12,13] and extended to investigations on the response of eosinophils to an ECF-A tetrapeptide and histamine in various disease states[14] as well as the capacity of these agents to mobilise eosinophils in the skin of atopic and non-atopic human volunteers.[15]

Evidence was provided that one of the functions of

the eosinophil in allergic tissue reactions may be its capacity to inhibit mast cell 'regranulation'.[16]

Membrane receptors for IgG and complement (C4, C3b and C3d) on human eosinophils and neutrophils and their relation to eosinophilia were described[17] and this work led to the observation that the ECF-A tetrapeptides and histamine both selectively enhance human eosinophil complement receptors.[18]

This work, and those of others, was reviewed in several articles.[19-26]

Section B - Mediators of hypersensitivity

In 1974 it was shown that both slow reacting substance of anaphylaxis (SRS-A) and ECF-A were released from passively sensitized skin following interaction with specific antigen.[27] The inactivation of SRS-A by the arylsulphatase contained in various tissues was described[28] and later it was shown that appreciable amounts of human SRS-A were present in lung as a pre-formed mediator.[29]

A number of miscellaneous papers relating to mediators are contained in this section: these include an observation on complement activation by Corynebacterium parvum;[30] some chemical and physical properties of synthetic human fibrinopeptides[31] and the description of a primate macrophage-cytophilic antibody.[32]

A review of the various biological pathways associated with the inflammatory response as they relate to complications of blood transfusion were described in a review article.[33]

Section C - Studies on chemotaxis

This section contains papers on chemotaxis of neutrophils, monocytes and basophils.[34] Particular attention was given to the identification of chemotactic agents associated with Hageman factor-dependent pathways,[35] fibrin formation[36,37] and fibrinolysis.[38,39]

There is a study on the relation between neutrophil accumulation *in vivo* and agents that are chemotactic *in vitro*.[40]

Alterations in monocyte chemotaxis in bronchial carcinoma were described.[41]

Much of this work, especially the relation between chemotaxis and haemostasis, was reviewed in 1975.[42]

Section D - Clinical studies

This section contains reports on alterations in the complement systems in bronchial asthma[43-45] and the significance of immunoglobulins and complement in pleural effusions associated with bronchial carcinoma.[46] Studies on mediators of hypersensitivity in chronic bronchitis and asthma and their modulation by pharmacological agents are also described.[47,48]

Detailed immunological investigations of two clinical cases, one of chronic benign neutropenia[49] and the other on the effect of transfer factor in chronic mucocutaneous candidiasis,[50] are described.

A consideration of how coagulation and other biological systems may interrelate in the context of 'Stroke' was discussed in a review article.[51]

Section E - Methodology

This section contains two articles on methodology, one on the preparation of transfer factor[52] and the other on tests of immune function.[53]

SECTION A - THE EOSINOPHIL LEUCOCYTE

Studies on eosinophil leucocyte migration

I. EOSINOPHIL AND NEUTROPHIL ACCUMULATION FOLLOWING ANTIGEN–ANTIBODY REACTIONS IN GUINEA-PIG SKIN

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STUDIES ON EOSINOPHIL LEUCOCYTE MIGRATION

I. EOSINOPHIL AND NEUTROPHIL ACCUMULATION FOLLOWING ANTIGEN–ANTIBODY REACTIONS IN GUINEA-PIG SKIN

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(*Received* 7 *July* 1969)

SUMMARY

Eosinophil leucocytes migrated into the site of PCA reactions mediated by those fractions of 7S guinea-pig IgG containing IgG_1; neutrophils were associated with IgG_1 and IgG_2. Maximal eosinophil infiltration is seen at 12 hr and was associated with degranulation.

Intradermal histamine was not eosinophilotactic in guinea-pigs.

Preformed antigen–antibody complexes of IgG_1 and IgG_2 both promoted eosinophil and neutrophil migration in guinea-pig skin but slightly more eosinophils were seen following injections of complexes containing IgG_1. Local eosinophilia and PCA activity were mediated by a relatively heat-stable element since these effects were demonstrable even after prolonged heating of fractions containing IgG_1. Eosinophils were seen following injections of Compound 48/80 and this was accompanied by low mast cell counts; however, there was also some associated tissue destruction.

INTRODUCTION

Eosinophils are associated with certain 'allergic' tissue reactions particularly immediate-type (Type 1) hypersensitivity. Various mediators of an eosinophilia have been proposed including histamine (Kline, Cohen & Rudolph, 1932; Archer, 1963), antigen–antibody complexes (Litt, 1964) and sensitized tissue treated with antigen (Samter, Kofoed & Piper, 1953; Parish & Coombs, 1968).

Experiments *in vitro* using Boyden's millipore technique indicate that the eosinophil and neutrophil chemotaxis generated by antigen–antibody complexes require the presence of fresh serum and that the chemotactic activity is probably mediated by intermediate complement components or their fragments (Keller & Sorkin, 1969; Ward, 1969).

In the present study an initial experiment showed that following passive cutaneous anaphylaxis (PCA) reactions in the guinea-pig eosinophils and neutrophils accumulated

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in large numbers around the reaction site 24 hr following challenge with antigen. Investigations were, therefore, carried out to determine the relationship of eosinophil to neutrophil migration following PCA reactions using fractions of 7S guinea-pig immunoglobulins. In further experiments the eosinophilia was specifically studied in order to determine more precisely the mechanism of eosinophilotaxis.

MATERIALS AND METHODS

Animals

Male albino guinea-pigs weighing between 200 and 400 g were used throughout this study.

Guinea-pig antisera

Antisera to ferritin (Koch-Light) and bovine serum albumin (BSA, Armour Laboratories) were raised separately in two sets of eight guinea-pigs according to the following schedule:

Day 1. One hundred micrograms of the antigen was given in Freund's complete adjuvant to each hind footpad.

Day 14. One hundred micrograms of antigen in Freund's incomplete adjuvant was given in two subcutaneous areas in the neck.

Day 28. One hundred micrograms of antigen was introduced intradermally into two areas of the back. This process was repeated at weekly intervals until the development of a strong

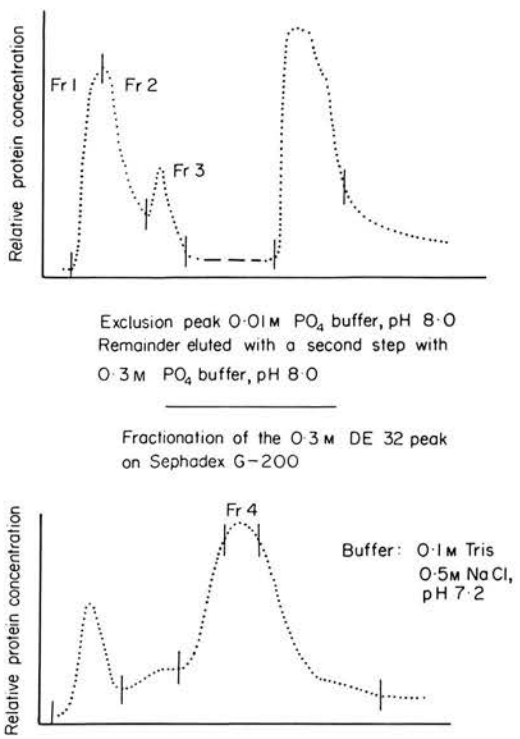

FIG. 1. Chromatographic fractionation of guinea-pig anti-ferritin. Virtually identical patterns were obtained with antiserum to BSA.

Arthus reaction. The course was usually complete within 6–8 weeks. The animals were bled 1 week after the last injection and the anti-ferritin and anti-BSA antisera were pooled separately.

Preparation of guinea-pig IgG_1 and IgG_2 immunoglobulins

The procedures followed were essentially those of Forsgren (1968). Sixty millilitres of guinea-pig anti-ferritin was precipitated with 33% saturated ammonium sulphate and the precipitate was redissolved in distilled water. The process was repeated four times and the final precipitate was redissolved in 10 ml of water and dialysed against 0·01 M phosphate buffer at pH 8·0. The sample was applied to a column of DEAE-cellulose (DE 32—Whatman) 3·5 × 26·5 cm, which had been previously equilibrated with 0·01 M phosphate buffer, pH 8. A second small peak followed the main exclusion peak (Fig. 1). The rest of the sample was eluted with a 0·3 M phosphate buffer, pH 8. The fractions were pooled as indicated in Fig. 1 and concentrated using a UM 1 Amicon-Diaflow membrane. The 0·3 M peak was then applied to a column of Sephadex G-200 (3·5 × 95 cm) in order to eliminate IgM and to further purify IgG_1. The purity of the fractions was tested using gel-diffusion and immunoelectrophoresis as described below. The guinea-pig anti-BSA sera gave virtually similar chromatographic patterns as the anti-ferritin sera (Fig. 1).

Preparation of anti-sera to guinea-pig immunoglobulins

(1) *Rabbit anti-guinea-pig 7S IgG.* The second Sephadex G-200 peak [Fraction (Fr) 4] of the guinea-pig anti-BSA was precipitated at equivalence with the antigen and the precipitate was washed eight times in 0·01 M EDTA–saline at +4°C. Half a milligram of the washed

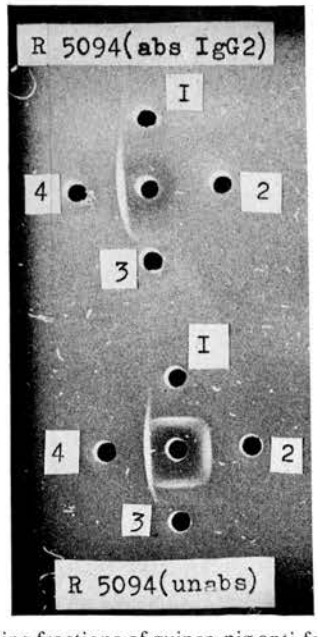

FIG. 2. Gel-diffusion studies using fractions of guinea-pig anti-ferritin. 1, 2, 3 and 4 correspond to Fr 1, 2, 3 and 4, respectively. The R5094 (abs IgG_2) gives an antisera specific for IgG_1. Similar results were obtained with R5095.

precipitate was suspended in 1 ml of saline and emulsified with 1 ml of FCA and injected intramuscularly into rabbits (R5048 and R5049). The injections were repeated 14 days later and the animals were subsequently given 0·25 mg of the precipitate intravenously in saline at weekly intervals for 2 weeks following the second injection. The animals were bled 1 week following the last injection.

(2) *Specific anti-guinea-pig IgG$_1$*. The ascending part of the DEAE exclusion peak of the guinea-pig anti-BSA (Fr 1) which gave a single line on gel diffusion and a single γ_2 line on immunoelectrophoresis was polymerized with ethyl chloroformate for use as an immunoabsorbent as described by Avrameas & Ternynck (1967). The rabbit anti-IgG$_1$, also containing IgG$_2$ (R5048 and R5049), was absorbed with the polymerized IgG$_2$ (Fr 1) to give an antisera specific for IgG$_1$. The results of the gel-diffusion studies are shown in Fig. 2. Fr 1 contains only IgG$_2$ and gives a line of identity with the other fractions. Fr 4, therefore, contains some IgG$_2$ in addition to the spuring IgG$_1$. The late peak of IgG$_2$ (Fr 3) shows bending of the IgG$_1$ line and therefore contains a little IgG$_1$.

Preparation of antigen–antibody complexes

Samples of anti-ferritin Fr 1 and Fr 4 (IgG$_2$ and IgG$_1$) were precipitated at equivalence with ferritin following a standard optimal proportions titration. The complexes were washed four times in 0·01 M EDTA–saline and resuspended in saline. The protein content of the final suspension was measured by the Folin method.

Passive cutaneous anaphylaxis

The recommendations of Brocklehurst (1967) were observed in all PCA reactions with the exception that 0·1-ml volumes of fractions were injected intradermally under sterile conditions. Following sensitization 1 ml of 0·25% Evans Blue containing 1 mg of ferritin was injected intravenously.

Quantitation of eosinophil and neutrophil infiltration following PCA reactions

At intervals following challenge with antigen and dye the animals were killed and the injection sites, previously marked, were immediately biopsied using a circular punch of 1 cm diameter. The biopsy included all layers of the skin including the panniculus carnosus muscle. Following fixation in formal saline, or Susa's medium in the case of mast cell experiments, sections were taken from the site and approximately 2 mm either side of the intradermal injection. The sections were wax embedded, cut and stained in the usual way using haematoxylin and chromotrope 2R. Chromotrope 2R was found to be a satisfactory stain for eosinophils as background staining was minimized. For mast cell experiments aqueous toluidine blue was used. From each section a total of fifteen random high power fields were counted in 0·3-mm strips between the junction of the epidermis and dermis and the upper limit of the panniculus carnosus using a previously calibrated graticule and the high power ($\times 40$) objective (Fig. 5). The cell count is expressed as a total of fifteen strips which represents the mean of the three sections counted, i.e. forty-five strips. Preliminary experiments showed that sections from the anterior abdominal wall were unsatisfactory due to difficulties in obtaining complete sections of the entire skin thickness. The biopsy studies reported below were prepared from multiple, well spaced sites on the back which reached laterally to the mid axillary line. Biopsies from these areas gave the most satisfactory results.

RESULTS

The association of eosinophil and neutrophil infiltration with passive cutaneous anaphylaxis

An initial experiment showed that large numbers of eosinophils and neutrophils infiltrated into the site of PCA reactions when the skin was passively sensitized with a dilution of 1:50 of the unfractionated guinea-pig antiserum to ferritin. Skin injected with saline or dye alone and the uninjected sites all showed minimal cellular infiltration.

Isolated fractions of 7S IgG showed that PCA reactions were only associated with those fractions containing IgG_1 (Fr 3 and 4), but not with Fr 1 and 2 which by gel diffusion and immunoelectrophoresis had been shown to contain IgG_2 only. Biopsies of PCA reactions using isolated fractions of IgG (Fr 1, 3 and 4) taken 24 hr after antigen challenge showed that eosinophil infiltration is only associated with those fractions containing IgG_1 and giving positive blueing reactions (Fig. 3). A substantial skin eosinophilia was noted with a

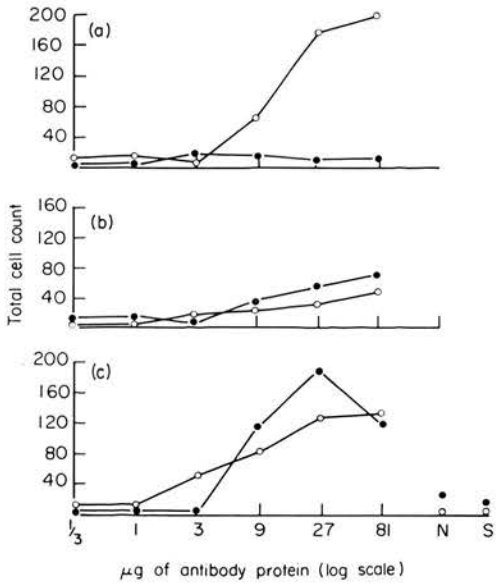

FIG. 3. Eosinophil (●) and neutrophil (○) accumulation into guinea-pig skin following passive cutaneous anaphylaxis with isolated fractions of guinea-pig IgG anti-ferritin. IgG_2 (a), IgG_2 (late peak) (b) and IgG_1 (c) are fractions 1, 3 and 4, respectively. Four hours sensitization period. Biopsies taken 24 hr following antigen challenge. N, Normal skin; S, saline.

dose as low as 9 μg of antibody protein. A few eosinophils were seen in normal skin but neutrophils were almost always entirely absent. Neutrophils appear in large numbers following treatment with IgG_1 or IgG_2 in doses as low as 9 μg of antibody protein. The results in Fig. 3 represent the counts from one animal given randomly spaced injections of the various dilutions of antibody protein. Normal skin and saline injections represent controls in the same animal implying that the migration of eosinophils and neutrophils is directional to the reactive sites. The experiment in this form was repeated once and gave essentially similar results. In further investigations a dose of 27 μg of antibody protein was chosen for fractions 1 and 4, respectively, and as seen from Figs. 4 and 7 and Table 1, the

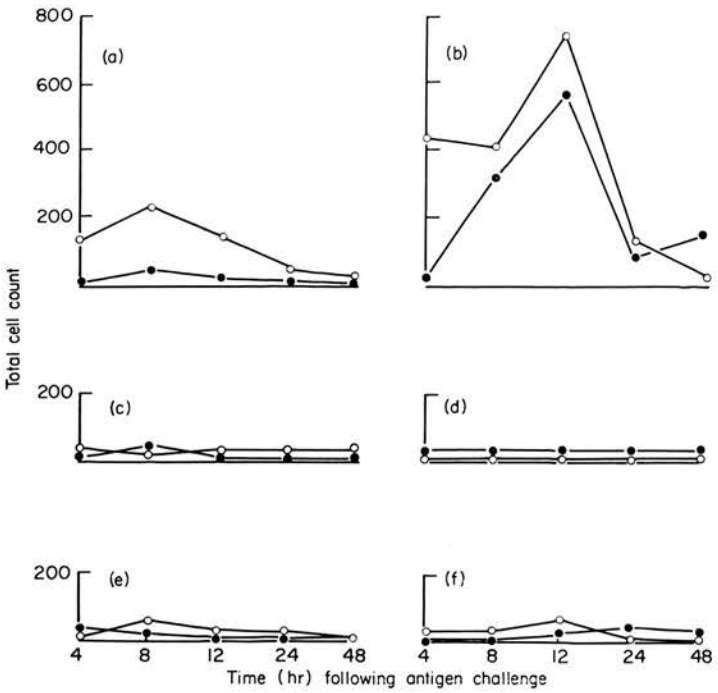

FIG. 4. The time course of eosinophil (●) and neutrophil (○) accumulation into guinea-pig skin following passive cutaneous anaphylaxis and intradermal histamine. Four hours sensitization. (a) IgG_2, (b) IgG_1, (c) saline, (d) uninjected sites, (e) 1 μg histamine, (f) 10 μg histamine.

eosinophil infiltration was associated with IgG_1 containing fractions and not with IgG_2 and so in effect the main observation of this first experiment was confirmed several times.

The time course of eosinophil and neutrophil infiltration into the skin

In initial experiments a time of 24 hr following antigen challenge was arbitrarily chosen for measuring cellular infiltration. Using a dose of 27 μg of antibody protein pairs of animals were treated with Fr 1, Fr 4 and histamine (Fig. 4). Each pair of animals was killed

TABLE 1. Eosinophil and mast cell counts following PCA reactions and intradermal injections of Compound 48/80

	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5	
	E	M	E	M	E	M	E	M	E	M
IgG_1 (Fr 4)	909	0	557	5	258	4	157	0	350	0
IgG_2 (Fr 1)	9	46	2	51	ND	ND	ND	ND	ND	ND
Normal skin	0	39	0	75	24	171	5	99	1	89
Compound 48/80	18	57	75	51	199	2	133	27	40	103

E, Eosinophils; M, mast cells; ND, not done.
Compound 48/80, 10 μg, Experiments 1 and 2; 100 μg, Experiments 3, 4 and 5.

at 4, 8, 12, 24 and 48 hr and the injection sites biopsied as previously described. With IgG_2 (Fr 1) minimal neutrophil infiltration was seen at 8 hr but at this time very few eosinophils were noted. Treatment with IgG_1 (Fr 4) showed that substantial eosinophilia was seen at 8 hr and a considerable eosinophil count was noted at 12 hr. The eosinophil count with IgG_1 roughly paralleled the neutrophil count with the exception that neutrophils appeared earlier than eosinophils. At 48 hr eosinophils were still plentiful but few neutrophils were seen. Areas injected with saline and the uninjected sites were virtually free from eosinophils or neutrophils.

The effect of histamine

Fig. 4 shows that in addition to the study of the cellular events following PCA reactions with IgG_1 and IgG_2 the effect of intradermal histamine was also investigated. The doses of

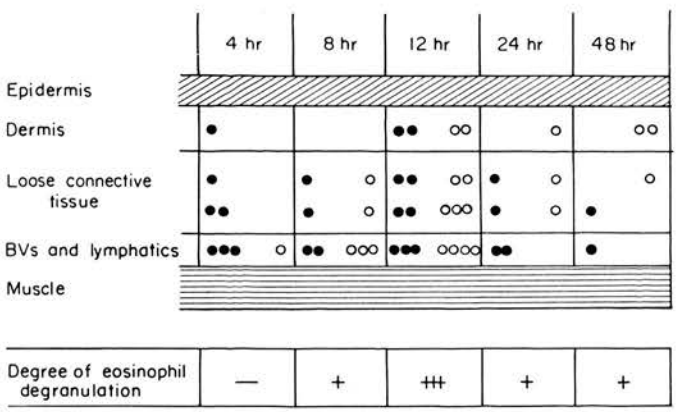

FIG. 5. A diagrammatic representation of the distribution of neutrophils (●) and eosinophils (○) following passive cutaneous anaphylaxis with IgG_1 (Fr 4).

histamine acid phosphate (British Drug Houses) was expressed as the weight of histamine base and was injected in saline at neutral pH. With 1 μg a slight neutrophilia was seen at 8 hr and similarly at 8 and 12 hr when 10 μg of histamine was injected. The number of eosinophils observed following the injection of 1 μg and 10 μg of histamine at intervals over 48 hr was no more than that seen when normal skin was biopsied. The area of blueing noted with 10 μg of histamine was slightly greater than that given by 27 μg of IgG_1.

The morphology and distribution of eosinophils and neutrophils following PCA reactions with IgG_1

At 4 hr a few eosinophils were seen in and around the small blood vessels immediately above the panniculus carnosus (Fig. 5). Considerably more were seen in this area at 8 hr, at which time some had migrated into the loose connective tissue below the dermis. At 12 hr eosinophils were seen in large numbers throughout all layers of the skin. Some were seen gathered in clusters around small vessels or randomly scattered in tissue spaces. Many of the eosinophils, especially those in large accumulations of the cell, showed degranulation with scattering of the granules for some distance around the intact nucleus. The significance

of this observation is discussed later. By 48 hr the eosinophils were confined to the dermis and upper part of the loose connective tissue whilst the remaining neutrophils were seen only in the lower layers of the skin. Neutrophils were seen in fairly large numbers throughout all layers at 4 hr although they were observed in greater numbers in the lower layers of the skin (Fig. 5). At 8 hr the number of neutrophils appeared slightly fewer than at 4 hr but the maximum number were noted at 12 hr when they were observed throughout the area of the skin counted. The neutrophils were more difficult to count, especially when present in large numbers. They often appeared in 'seams' or 'clusters' and many had pyknotic nuclei.

It should be noted that the most numerous 'loose' cells were the tissue macrophages or histiocytes. These cells were far too numerous to count but it appeared that they were more plentiful when the skin was infiltrated with eosinophils and neutrophils.

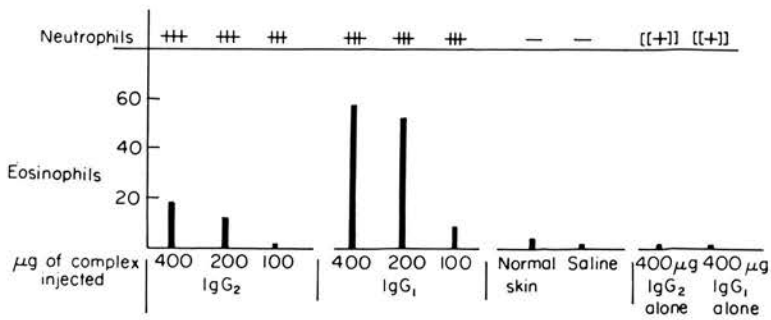

FIG. 6. Eosinophil and neutrophil accumulation into guinea-pig skin 12 hr following intradermal injections of preformed antigen–antibody complexes.

Eosinophil and neutrophil accumulation following the intradermal injection of antigen–antibody complexes

The previous experiments have shown that eosinophilia is associated with PCA reactions mediated by fractions containing IgG_1. Since histamine alone did not appear to generate this eosinophilia other possible mechanisms of eosinophilotaxis were investigated. The products of mast cell degranulation other than histamine may be chemotactic for eosinophils or alternatively IgG_1 and its antigen in a tissue bound or free form may generate such a chemotactic agent. In order to test the latter hypothesis preformed complexes of IgG_1 or IgG_2 and antigen were injected intradermally in amounts indicated in Fig. 6. Biopsies taken 12 hr later showed that with all doses of complexes the amount of neutrophil infiltration was too numerous to count, and has been scored on a plus (+) basis. Eosinophils appeared following treatment with both types of complexes although the numbers were about three times higher with IgG_1–antigen complexes than with IgG_2–antigen complexes. On a weight basis there were far fewer eosinophils with IgG_1–antigen complexes than with PCA reactions with IgG_1. Very few neutrophils were seen following injections of antibody alone, even in a dose of 400 μg. This experiment was repeated and gave a virtually similar result.

The effect of heat on the IgG_1 containing immunoglobulin fraction (Fr 4) which mediated PCA reactions and eosinophila

It is known that the ability of IgG_1 to elicit a homologous PCA reaction depends on a heat stable portion of the antibody molecule. It is conceivable, however, that the eosinophilia

associated with PCA reactions depends on a heat labile part of the molecule or that the eosinophilia is the result of interaction by an as yet unidentified heat labile immunoglobulin residing in the γ_1 region. It is shown in Fig. 7 that the ability of IgG_1 to elicit PCA reactions is partly retained even after prolonged heating and that the associated eosinophilia is proportionately reduced suggesting that the eosinophilia does not in fact depend on a heat labile moeity. Instead of a 4-hr sensitization period between intradermal antibody and antigen challenge the experiment was repeated using a 48-hr sensitization time. This was to confirm that the PCA reaction and associated eosinophilia did in fact depend on tissue bound antibody since presumably non-bound antibody would have diffused away. This experiment gave similar results to the 4 hr sensitization period experiment. Although not recorded in Fig. 7 the neutrophilia associated with IgG_2 is still apparent after a 48-hr sensitization period. There were, however, only about half as many neutrophils as when a 4-hr sensitization period was used.

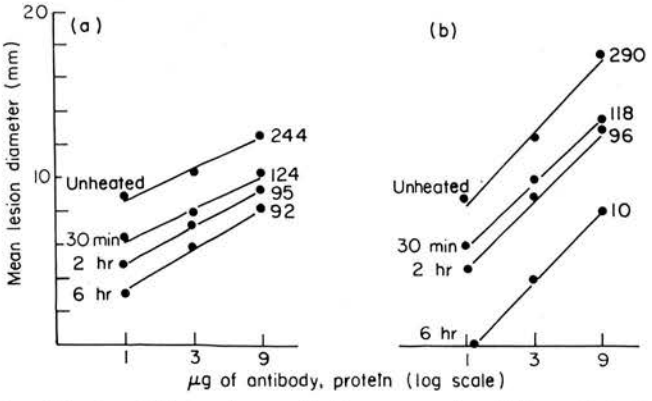

FIG. 7. The effect of heat on PCA reactions and subsequent eosinophilia mediated by IgG_1 (Fr 4). (a) Four hours sensitization, (b) 48 hr sensitization. Figures at end of lines indicate eosinophil counts.

Eosinophil accumulation following injections of Compound 48/80

Since histamine did not appear to be chemotactic for eosinophils and preformed antigen–antibody complexes generated comparatively less eosinophil migration than PCA reactions with IgG_1 the possibility was investigated that a mast cell component other than histamine released eosinophilotactic agents.

Experiments were designed to compare the eosinophil migration following PCA reactions with IgG_1 and injections of Compound 48/80. Preliminary studies showed that it was not possible to get comparative areas of blueing with Compound 48/80 and PCA reactions due to the flat dose–response curve for 48/80. Doses of 10 μg and 100 μg of 48/80 were chosen. In order to show that the effect of 48/80 was in part due to histamine release following mast cell degranulation it was demonstrated that 25 mg of mepyramine maleate inhibited the blueing reaction of 48/80 up to doses of 75 μg. Above this dose the area of blueing was only partially inhibited. Mast cell counts were performed by staining alternate sections with aqueous toluidine blue. It will be seen in Table 1 that with 10 μg of 48/80 eosinophil migration was small (Table 1, Experiments 1 and 2). The fact that there was little alteration in the mast cell count is in agreement with previous observations (Mota & Vugman, 1956a, b) on

the peculiarity of guinea-pigs to treatment with 48/80. In two of the experiments using higher doses of 48/80 eosinophil migration was seen and this was accompanied by low mast cell counts. There was, however, considerable distortion and destruction of the tissue. In one experiment (Experiment 5, Table 1) a small eosinophilia was noted even with 100 μg of 48/80. These experiments do suggest, however, that treatment leading to low mast cell counts, presumably by degranulation, is followed by eosinophil infiltration.

DISCUSSION

Eosinophil infiltration has been observed following PCA reactions mediated by fractions containing IgG_1. This agrees with the work of Parish (1969).

Recent reports have suggested that several classes or subclasses of immunoglobulins may exist in the electrophoretically fast γ_1 region and that PCA reactions may represent an activity in an immunoglobulin as yet unidentified by standard immunoelectrophoretic techniques (Pondman & Van Es, 1969; Van Es & Pondman, personal communication, 1969). In the present study, however, the comparison is made between IgG_1 and the electrophoretically slow IgG_2, the latter being unassociated with PCA activity or eosinophil migration.

Since the eosinophilia is not associated with IgG_2 it would seem unlikely that all antigen–antibody reactions prepare the tissue for a local eosinophilia as implied by Litt (1964). It is improbable that all the IgG_2 would have diffused away during the 4 hr sensitization period since it has been shown that homologous IgG_2 is absorbed onto guinea-pig tissue (Brocklehurst & Colquhoun, 1965) and that IgG_2 contains those antibodies cytophilic for macrophages (Jonas *et al.*, 1965). The neutrophilia associated with IgG_2 could be a result of bacterial contamination or the liberation of complement chemotactic factors (Ward, Cochrane & Müller-Eberhard, 1965). The neutrophil migration observed with IgG_1 (Fr 4) may be the result of contamination with IgG_2 or alternatively substances attracting eosinophils are also chemotactic for neutrophils. The eosinophil degranulation noted when the cells are gathered in large numbers could be artifactual resulting from the fixation process. The eosinophil membrane is, however, fairly resistant and can survive osmotic shock and acetone treatment. The fact that other cells surrounding degranulated eosinophils usually appeared normal indicates that the observation may be significant. Morphological changes accompanying eosinophil chemotaxis *in vitro* have also been noted (Kay, unpublished observations).

Various reports have concluded that histamine has eosinophilotactic properties. The evidence has recently been reviewed (Archer, 1968a). Other studies have been unable to support this view (Cohen & Sapp, 1963; Felarca & Lowell, 1968) and it could be that the situation in horses is exceptional (Archer, 1963). The argument that large local concentrations of histamine are required for eosinophilotaxis is invalid (Archer, 1968b) since in the present study the area of blueing with 10 μg of histamine was greater than that observed following PCA reactions with 27 μg of IgG_1. Both reactions were inhibited by mepyramine maleate but only IgG_1 PCA reactions were associated with an eosinophilia. The failure to produce eosinophil chemotaxis with histamine using the *in vitro* millipore technique (Keller & Sorkin, 1968; Kay, 1969) provides more convincing evidence that histamine is not eosinophilotactic. One explanation of the eosinophilia associated with PCA reactions involving

IgG_1 is that the IgG_1–antigen complex activates a serum or tissue factor, possibly complement, in a different manner to the IgG_2–antigen complexes although earlier reports suggested that IgG_1 was incapable of completing the complement haemolytic sequence (Bloch *et al.*, 1963). This has been recently challenged with the report that preformed IgG_1–antigen complexes can consume certain complement intermediates (Osler *et al.*, 1969). If IgG_1 and its antigen generate eosinophilotactic products intradermal injection of the complex should promote a substantial eosinophila. Both IgG_1 and IgG_2 produce eosinophil migration when injected with antigen as the preformed complex but more eosinophils were noted with IgG_1. However, the numbers on a weight basis were far less than when IgG_1 was introduced as for PCA reactions. Although this makes it unlikely that IgG_1 and its antigen react uniquely with complement to produce eosinophilotactic products it could be that this reaction is only possible when configurational changes have taken place in the antibody molecule as a result of tissue sensitization. Injection of the preformed complex may be an unsatisfactory method of obtaining adequate tissue fixation since the success of this procedure (using human IgE) requires a critical antigen–antibody ratio (Ishizaka & Ishizaka, 1968).

The slight eosinophilia noted with high doses of the IgG_2–antigen complex could be a result of IgG_1 contamination which was not detected by gel-diffusion. However, it has been observed that in *in vitro* studies using the millipore technique of Boyden chemotactic substances for eosinophils and neutrophils have been observed when fresh serum is incubated with IgG_1 and IgG_2 antigen complexes (Kay, unpublished observations). The substantial neutrophilia observed following the injection of preformed complexes may have prevented the recognition of the eosinophilotactic effects.

Although Compound 48/80 is said to be one of the least toxic of the agents affecting mast cells in rats, dogs, cats and man its action in the guinea-pig is thought to be atypical in that it had little effect on mast cell morphology and its toxic action was not prevented by antihistamines (Feinberg & Sternberger, 1955; Mota & Vugman, 1956a, b). It was concluded by Miles & Miles (1952), however, that 48/80 injected intradermally liberates local histamine and that the effect could be blocked by an antihistaminic agent. The present study confirms the findings of Miles and reports an association of a local eosinophilia especially with large doses of 48/80 injected intradermally. These experiments give some support to the view that a mast cell product may be eosinophilotactic but the superadded toxic effects of Compound 48/80 limits the interpretations of these *in vivo* studies. The eosinophilia following PCA reactions, antigen–antibody complexes and Compound 48/80 could represent the effect of several different eosinophilotactic agents generated by distinct mechanisms.

Experiments are in progress using the Boyden millipore technique as a method of quantitating eosinophil chemotaxis *in vitro*. The difficulties of *in vivo* studies have been largely overcome since individual components free from other tissue or serum factors can now be tested directly.

ACKNOWLEDGMENTS

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ANTIGEN–ANTIBODY INDUCED CUTANEOUS EOSINOPHILIA IN COMPLEMENT DEFICIENT GUINEA-PIGS

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ANTIGEN–ANTIBODY INDUCED CUTANEOUS EOSINOPHILIA IN COMPLEMENT DEFICIENT GUINEA-PIGS

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SUMMARY

Decomplementation with cobra venom factor had no effect on eosinophil accumulation into the site of IgG_1-mediated PCA reactions in the guinea-pig. Eosinophil and neutrophil accumulation also followed PCA reactions in animals partially or totally deficient in C4.

The effect of intradermal injections of pre-formed antigen–antibody complexes prepared from guinea-pig IgG_1 or IgG_2 were not affected by decomplementation with cobra venom. The lesions produced with either immunoglobulin complex were similar in appearance in decomplemented and normal animals and were followed by comparable tissue accumulation of eosinophils and neutrophils.

INTRODUCTION

Purified guinea-pig 7S IgG immunoglobulins have been found to mediate the formation of two chemotactic factors, *in vitro*, which selectively attract eosinophils from a mixed cell population. One factor, having similar physicochemical properties to C5a, was generated from whole serum by pre-formed antigen–antibody complexes prepared from either IgG_1 or IgG_2, required the presence of the complement system (Kay, 1970a) and has been designated an eosinophil chemotactic factor derived from complement (ECF-C). The other factor, an eosinophil chemotactic factor of anaphylaxis (ECF-A), was released by antigen challenge of lung fragments passively sensitized with IgG_1, but not IgG_2 (Kay, Stechschulte and Austen, 1971). Complement was not required for the release of ECF-A and on the basis of a molecular size of 500 to 1000 and a different formation mechanism, ECF-A was distinguished from ECF-C which had a molecular weight of approximately 15,000.

An earlier report using cell counts in guinea-pig skin, had shown that both IgG_1 and IgG_2, as pre-formed complexes, prepared the tissue for a subsequent local eosinophilia 12 hr after injection. However, if antibody was first placed in the skin and after a variable

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latent period the animal was challenged with antigen and Evans' blue dye intravenously (as in a usual passive cutaneous anaphylactic reaction), IgG_1 but not IgG_2 elicited a local eosinophil response 8–12 hr after the initial blueing reaction (Kay, 1970b). It was the purpose of the present report to determine the effect of a deficient complement system on the eosinophilia following PCA reactions or intradermal injection of pre-formed complexes.

MATERIALS AND METHODS

Partially purified cobra venom factor was prepared according to a previous report (Ballow & Cochrane, 1969). Guinea-pig serum was obtained by cardiac puncture before and after the intravenous injection of 1 ml of cobra venom factor or Tyrode's solution. The titre of 'late acting' components was measured using EAC $1^{gp}4^{hu}$ cells and purified guinea-pig C2 (Kay *et al.*, 1971). Guinea-pig 7S IgG_1 and IgG_2 anti-ferritin (Kay, 1970a) and anti-ovalbumin (Kay *et al.*, 1971) were the DEAE-cellulose chromatographic fractions previously described. Pre-formed antigen–antibody complexes were prepared as described (Kay, 1970b).

Guinea-pigs, partially or totally deficient in the fourth component of complement (C4), having comparable age and weight were from a strain of animals reported by Ellman *et al.* (1971). The haemolytic titres of C4 were performed by the method of Opferkuch *et al.* (1971).

The preparation of histological sections and the method for counting cells in guinea-pig skin was previously reported (Kay, 1970b). Twelve hours following passive cutaneous anaphylaxis (PCA) reactions or injections of pre-formed complexes, the areas were biopsied to include all layers of the skin including the panniculus carnosus muscle. The specimens were fixed in Susa's medium and following wax embedding and sectioning, were stained with haemotoxylin and chromotrope 2R. Sections were taken to include the injection site and approximately 2 mm on either side. Eosinophils were counted in a total of fifteen random high power fields in each section between the junction of the epidermis and dermis and the upper limit of the panniculus carnosus muscle. The mean cell counts represent the summation of the average count from the three areas examined.

In normal animals and animals decomplemented with cobra venom factor, 0·1 ml of antibody was injected intradermally and after a 4-hr latent period, antigen, 1 mg in 1 ml of 0·25% Evans' blue dye was given intravenously. The diameter of blueing reactions were measured 30 min later. In the C4 deficient animals antibody was administered intradermally and after a 4-hr latent period 0·1 ml of antigen was injected into the same site in order to minimize the hazards of intravenous injection. Fifteen minutes later the area of induration and surrounding erythema were measured.

Pre-formed antigen–antibody complexes and Tyrode's diluent control were injected intradermally immediately following the intravenous administration of 0·25% Evans' blue dye in 1 ml of Tyrode's solution. The lesions were assessed 1 hr later by examining both the diameter and the intensity of the blueing reaction. The lesions were biopsied 12 hr later.

RESULTS

Passive cutaneous anaphylaxis

Animals were injected intradermally with antibody 10 hr after the intravenous administration of 2 units of purified cobra venom factor. At this time less than 5% of the cir-

culating level of terminal complement components was detectable. As shown in Table 1, there was no appreciable difference in the skin eosinophil count between the cobra venom treated animals and those pretreated with intravenous injections of Tyrode's solution. The diameters of the blueing reactions were the same in both groups of animals. Large numbers of neutrophils were also seen in the site of PCA reactions from both groups of animals, but were too numerous to count.

Animals from the C4 deficient strain were comprised of a homozygote, totally deficient in C4, a heterozygote and an animal from the same strain reared under the same laboratory conditions, having a normal C4 level. In the three animals there was no appreciable difference either in the area of induration or erythema following PCA reactions or in the numbers of eosinophils which accumulated in the reaction sites 12 hr after antigen challenge (Table 2). Neutrophil accumulation accompanied the local eosinophilia in numbers too numerous to count accurately.

TABLE 1. The effect of decomplementation by cobra venom factor on eosinophil infiltration following PCA reactions

Animal No.	Treatment	Change in haemolytic titre of terminal complement components	Eosinophil infiltration (Mean Cell count)	Diameter of blueing reaction
1	Cobra venom factor	5120→20	125	25
2	Cobra venom factor	5120→20	120	25
3	Cobra venom factor	5120→20	90	25
4	Tyrode's solution	5120→5120	130	25
5	Tyrode's solution	5120→5120	88	25
6	Tyrode's solution	5120→5120	82	25

Normal skin, and skin injected with Tyrode's buffer alone, had eosinophil counts of less than 5. The antibody was a purified 7S IgG_1 (fraction 6) anti-ferritin (Kay, 1970a) which contained 200 μg of total protein per ml.

Intradermal antigen–antibody complexes

Animals injected with pre-formed antigen–antibody complexes were treated with cobra venom factor in the same manner as described above. The results are shown in Table 3. Animals treated with cobra venom factor showed no appreciable difference in either the diameter or intensity of the blueing reactions from those treated with Tyrode's solution. With IgG_1 or IgG_2 complexes, in both groups of animals the lesions were discrete, intensely blue and all showed small central areas of haemorrhage. The histological reactions were similar in that both groups had eosinophil infiltration in comparable quantities and had vast infiltrations of neutrophils.

DISCUSSION

The capacity of C4 deficient guinea-pigs or guinea-pigs decomplemented with cobra venom factor to react fully in PCA reactions has been previously reported (Maillard & Zarco, 1968; Cochrane, Müller-Eberhard & Aikin, 1970; Ellman *et al.*, 1971). The present study

confirms these observations and demonstrates that this procedure does not affect the subsequent infiltration of eosinophils and neutrophils into the reaction site (Table 1). The experiment with C4 deficient animals (Table 2) indicates that the participation of this early acting complement component is not an essential prerequisite for subsequent eosinophil and neutrophil infiltration following passive cutaneous anaphylaxis. These results are in agreement with *in vitro* data that complement is not a requirement for the antigen-induced release of ECF-A from lung tissue passively sensitized with IgG_1 (Kay *et al.*, 1971). Furthermore, it has been found that there was no difference in the capacity of C4 deficient and normal guinea-pig lung tissue to be passively sensitized for the antigen-induced release of ECF-A (Kay, Stechschulte and Austen, unpublished observations).

The generation of eosinophil chemotactic factors, *in vitro*, by pre-formed complexes prepared from IgG_1 or IgG_2 requires complement (Kay, 1970a) yet when introduced into the skin of decomplemented animals both complexes elicited a subsequent local eosinophilia (Table 3). In the case of IgG_1 this discrepancy could be attributed to the capacity

TABLE 2. Eosinophil accumulation into the site of PCA reactions in guinea-pigs partially or totally deficient in C4

	Haemolytic titre	Eosinophil infiltration	Diameter of induration* (mm)	Diameter of erythema* (mm)
Homozygote	less than 5	97	12	25
Heterozygote	286,000	110	12	20
Normal	777,000	81	10	25

* Mean of two sites in each animal.

Skin injected with antigen or antibody or with Tyrode's solution showed no induration or erythema and had eosinophil counts of less than 5. The antibody was a purified IgG_1 (fraction 5) anti-ovalbumin (Kay *et al.*, 1971) containing 150 mg of total protein per ml. The ovalbumin was administered at a concentration of 300 μg/ml.

of the IgG_1 to release chemical mediators from target cells without prior fixation and in the absence of complement. In this situation the IgG_1 complex may be analogous to rat IgGa complexes which can release histamine from rat mast cells *in vitro* without prior fixation (Bach, Bloch and Austen, 1971). Since IgG_2 is unable to sensitize skin for passive cutaneous anaphylaxis (Ovary, Benacerraf and Bloch, 1963) or lung fragments for histamine (Baker, Bloch and Austen, 1964) and SRS-A release (Stechschulte, Austen and Bloch, 1967) but can fix complement, it was expected that complement may play a part in the cellular infiltration following intradermal injection of IgG_2 complexes. It was found, however, that IgG_2 as a pre-formed complex evoked both a blueing reaction and subsequent eosinophil and neutrophil infiltration as great as the IgG_1 complexes in both normal and cobra venom treated animals. These findings are similar to those of Broder (1969) who demonstrated that soluble complexes prepared from guinea-pig IgG_1 or IgG_2 both have the capacity to liberate histamine from perfused guinea-pig lung. It should be noted, that on a weight basis, IgG_1 is far more efficient in preparing tissue for an eosinophilia when administered in PCA reactions than when injected as pre-formed complexes.

TABLE 3. The effect of decomplementation by cobra venom factor on eosinophil accumulation following intradermal injections of IgG_1 and IgG_2 pre-formed complexes

Animal No.	Treatment	Change in haemolytic titre of terminal complement components	IgG_1 Complexes			IgG_2 Complexes		
			Eosinophil infiltration	Blueing reaction		Eosinophil infiltration	Blueing reaction	
				diameter	intensity		diameter	intensity
1	Cobra venom factor	5120→20	16	6	++	28	5	++
2	Cobra venom factor	5120→20	34	6	++	54	5	++
3	Cobra venom factor	5120→20	11	5	++	11	3	++
4	Tyrode's solution	5120→5120	13	5	++	25	3	++
5	Tyrode's solution	5120→5120	8	4	++	8	3	++
6	Tyrode's solution	5120→5120	20	5	++	25	3	++

Normal skin and skin injected with Tyrode's buffer had eosinophil counts of less than 6. Skin injected with Tyrode's solution gave weak blueing reactions of less than 2 mm. Each complex was injected in 0·1 ml volume and contained 400 μg of total protein.

Recent preliminary experiments have shown that partially purified ECF-A can elicit a local eosinophilia following intraperitoneal injection. These results, and the data presented herein are consistent with the view that ECF-A may play a role in cutaneous eosinophil accumulation *in vivo*.

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STUDIES ON EOSINOPHIL LEUCOCYTE MIGRATION

II. FACTORS SPECIFICALLY CHEMOTACTIC FOR EOSINOPHILS AND NEUTROPHILS GENERATED FROM GUINEA-PIG SERUM BY ANTIGEN–ANTIBODY COMPLEXES

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STUDIES ON EOSINOPHIL LEUCOCYTE MIGRATION

II. FACTORS SPECIFICALLY CHEMOTACTIC FOR EOSINOPHILS AND NEUTROPHILS GENERATED FROM GUINEA-PIG SERUM BY ANTIGEN–ANTIBODY COMPLEXES

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Chemotactic activity for eosinophils and neutrophils has been studied using guinea-pig serum activated by preformed antigen–antibody complexes.

Rabbit complexes or complexes prepared either with guinea-pig IgG_2 or IgG_1 were equally capable of generating a heat *stable* activity from guinea-pig serum that was chemotactic for guinea-pig eosinophils and for neutrophils of both the guinea-pig and the rabbit. This was distinguished from a relatively heat-labile chemotactic activity present in untreated guinea-pig serum.

The heat-stable complex-mediated chemotactic activity was thought to be complement dependent since chemotaxis for eosinophils or neutrophils could not be generated from heated serum, ammonia treated serum or from serum treated with complexes in the presence of 0·01 M EDTA.

Guinea-pig sera, activated either with rabbit, guinea-pig IgG_1 or IgG_2 complexes, was fractionated by sucrose-density gradient ultracentrifugation and by Sephadex chromatography. In all experiments two peaks of cell specific chemotactic activity could be separated. The peak of activity for guinea-pig neutrophils was approximately 4·5S and in the fractionation range of proteins having a molecular weight of between 65,000 and 85,000. The peak of guinea-pig eosinophil chemotactic activity was 1·5S–2S and in the molecular weight range of between 15,000 and 20,000. Those fractions which were chemotactic for guinea-pig neutrophils did not attract rabbit neutrophils. Rabbit neutrophils migrated towards those fractions of guinea-pig serum chemotactic for guinea-pig eosinophils; therefore, the properties associated with guinea-pig eosinophil chemotactic activity were similar to previously published data for a fragment cleaved from the 5th component of complement.

INTRODUCTION

It has been previously demonstrated that eosinophil leucocytes migrate into the site of

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passive cutaneous anaphylactic (PCA) reactions mediated by those fractions of 7S guinea-pig IgG containing IgG_1 whereas passive sensitization with IgG_2 did not evoke a local eosinophil response. Preformed antigen–antibody complexes however, prepared either from IgG_1 or IgG_2 both promoted a local eosinophil and neutrophil migration following injections into guinea-pig skin (Kay, 1970).

Although it is yet to be ascertained that these two methods of treating guinea-pig skin represent more than one mechanism for preparing the tissue for a local eosinophilia the possibility exists that in the case of preformed complexes 'complement mediated' chemotaxis is involved. Furthermore, it has been shown that chemotactic activity for eosinophils is demonstrable *in vitro* following incubation of unheated serum with antigen–antibody complexes (Keller and Sorkin, 1969; Ward, 1969a).

It is the purpose of this report to determine whether, in the guinea-pig, a complement mediated chemotactic activity for eosinophils and neutrophils could be generated *in vitro* and to compare the chemotactic-generating properties of guinea-pig IgG_1 and IgG_2. In addition, guinea-pig serum activated by antigen–antibody complexes prepared from various immunoglobulins has been fractionated in order to determine whether specific chemotactic activity for eosinophils and neutrophils could be separated.

MATERIALS AND METHODS

Preparation of guinea-pig IgG_1 and IgG_2 immunoglobulins

Antiserum to Ferritin (Koch-Light) was raised in guinea-pigs as previously described (Kay, 1970). Guinea-pig anti-sheep red cell serum was prepared according to the schedule described by Jonas *et al.* (1965). 35 ml of guinea-pig anti-ferritin and 5 ml of guinea-pig anti-sheep red cell sera were pooled and precipitated at room temperature with 12·5% sodium sulphate. The precipitate was redissolved in 10 ml of distilled water and dialysed against 0·01 M phosphate buffer at pH 8·0. The sample was applied to a column of DEAE-cellulose (DE 32–Whatman) $3{\cdot}2^2 \times 35$ cm, which had been previously equilibrated with 0·01 M phosphate buffer at pH 8·0. Following the exclusion peak a gradient of increasing phosphate buffer concentrations was applied and the fractions were collected, pooled as shown in Fig. 1, and concentrated to 10 ml volumes using a UM I Amicon–Diaflow Membrane.

Rabbit anti-ovalbumin serum

Antiserum to ovalbumin (Koch-Light) was prepared in rabbits following an injection schedule previously described for preparing rabbit anti-guinea-pig 7S IgG (Kay, 1970).

Preparation of antigen–antibody complexes

Antigen–antibody complexes from unfractionated rabbit anti-ovalbumin or guinea-pig IgG_1 or IgG_2 anti-ferritin were prepared as previously described (Kay, 1970).

Guinea-pig complement reagents

Pooled guinea-pig serum was used as a source of complement (C) and was stored at −20°C for no longer than 12 weeks. Ammonia-treated guinea-pig serum (R4) was prepared as described by Mayer (1961).

Methods used to test the purity of guinea-pig IgG_1 and IgG_2

(a) *Gel-diffusion and immunoelectrophoresis*

Gel-diffusion was performed using a rabbit (R5094) anti-guinea-pig 7S IgG and a rabbit (R5094 absorbed with IgG_2) anti-guinea-pig IgG_1. These reagents were prepared as previously described (Kay, 1970). Immunoelectrophoresis was performed using both the rabbit (R5094) anti-guinea-pig 7S IgG and a rabbit (R1814) antiserum (kindly supplied by Professor R. R. A. Coombs) which had been prepared following a prolonged course of alum-precipitated whole guinea-pig serum.

(b) *Passive cutaneous anaphylaxis (PCA)*

This was performed as previously described (Kay, 1970). The PCA titre of anti-ferritin in each fraction was expressed as the reciprocal of the final dilution which gave a mean lesion diameter of 10 mm.

(c) *Whole complement titre (CH_{50}) following incubation of guinea-pig serum with preformed antigen–antibody complexes*

Samples from fractions 1, 2, 5 and 6 (Fig. 1) were precipitated with ferritin following a standard optimal proportion titration. The precipitates were washed four times with 0·01 M EDTA/saline and finally resuspended in CFT veronal buffer. The protein content of the resuspended precipitate was measured using the Folin method.

0·25 ml volumes of guinea-pig complement were incubated with the precipitates at a concentration of 5 mg of the antigen–antibody complex per 1 ml of complement.

Following incubation for 30 min at 37°C the samples were centrifuged to remove the antigen–antibody precipitates and then titrated in doubling dilutions on microagglutination trays in 0·025 ml volume. 0·025 ml of 1% sensitized sheep cells were added and the mixture was incubated at 37°C for 30 min when the 50% lytic end point was determined visually.

(*d*) *Haemolysin titre of guinea-pig anti-sheep RBC Serum*

Dilutions of the fractions (Fig. 1) in 0·025 ml volumes were incubated with 0·025 ml of a suspension of 1% sheep red blood cells. A 1 in 10 dilution of guinea-pig complement in 0·025 ml volumes was added and following incubating at 37°C for 30 min the 50% lytic end point was determined visually.

(*e*) *Macrophage-cytophilic antibody*

The monolayer test as described by Jonas *et al.* (1965) was used to detect macrophage–cytophilic antibody directed against sheep red blood cells. Dilutions of fractions of antisera were applied to monolayers of guinea-pig peritoneal macrophages. After 1 hr sensitization the monolayers were washed three times and a 0·25% suspension of sheep red blood cells was added and allowed to react for 3 hr. Rosette formation was scored on the + (plus) basis. All dilutions and washings were performed using Hanks's (Oxoid) solution adjusted to pH 7·2.

Chemotactic chambers

Chemotaxis was measured using a modification of the Millipore technique of Boyden

(1962). Millipore filters of 13 mm diameter were attached to sawn-off disposable 2 ml syringe barrels (Johnson and Co., Ltd) using millipore cement (Millipore Corp., Bedford, Mass.). This served as the upper compartment into which was placed 1 ml of the cell suspension containing approximately 1×10^6 cells. The potency of the cell compartment was checked before placing it into a 30 mm × 16 mm plastic pill pack (Camlab (Glass) Ltd) containing 1 ml of the test solution. The syringe barrel was sawn off at the point where the fluid in the cell compartment and the test compartment was level when each contained 1 ml.

The chemotactic chambers were placed in a moist box and incubated in air for 3 hr at 37°C. Following cell fixation, of between 1 and 18 hr using a mixture of 50% ethanol and 50% saturated mercuric chloride, the filters were removed and stained as follows: Carazzi's haematoxylin 5 min, running tap water 2 min, 1% HCL in 70% alcohol 5 sec, running tap water 10 min, 0·5% chromotrope 2R in 1% phenol 30 sec, running tap water 2 min, 20 dips in two changes of 95% absolute alcohol, propanol 2 min, propanol/xylol (equal parts) 2 min, xylol 20 min and mounted in DPX.

Cell counts from five fields taken at random were performed using the high power (×40) objective and a counting grid. Only those cells which had migrated through the thickness of the millipore were counted. The result of each treatment is expressed as the mean cell count. Five high power field counts was considered to be a satisfactory sampling since, although not recorded, the standard deviation was calculated in all experiments (except the fractionation studies) and found, in every treatment, to be less than 15% of the mean cell count.

Rabbit and guinea-pig neutrophils were obtained by the method described by Hirsch & Church (1960). Guinea-pig eosinophils were obtained from the peritoneal cavity following weekly intraperitoneal injections of 2 ml of horse serum. Peritoneal cells were harvested 4–12 weeks following the initial injection and 7 days following the last injection of horse serum and contained between 10% and 40% of eosinophils.

The optimal pore size for each cell type was determined by preliminary experiments using filters of various pore sizes. The pore size selected was that which allowed only minimal background migration when the suspending medium alone was used in the test compartment. For rabbit and guinea-pig neutrophils a 1·2 μ pore size was used whereas guinea-pig eosinophil migration was measured using an 8·0 μ pore size. Hanks's solution containing 0·5% ovalbumin (Koch-Light) and 50 units of penicillin and 5 μg of streptomycin per ml (adjusted to pH 7·2) was used as the suspending medium in both the cell *and* the test compartments.

Sucrose density-gradient ultracentrifugation

A 5 ml linear density gradient was prepared between 8 and 36% sucrose in Hanks's solution with added penicillin and streptomycin but omitting phenol red. Haemoglobin and [^{125}I]thryoglobulin were used as markers. Following application of 0·25 ml of activated serum or normal serum control, the gradients were centrifuged at 35,000 rev/min using a SW39 head in a model L-2 Spinco Ultracentrifuge for 20 hr at 4°C.

Approximately thirty samples of 0·16 ml were collected. Even numbered fractions were tested for guinea-pig neutrophil chemotaxis and odd numbered fractions for guinea-pig eosinophil chemotaxis. The position of the [^{125}I]thyroglobulin marker (19S) was determined using a sodium iodide crystal, well-type scintillation counter. The position of the haemoglobin marker (4·5S) was determined using a Unicam (SP-600) spectrophotometer at 415 mμ.

Gel filtration on Sephadex G-100 and G-50

The columns were equilibrated using phosphate buffered saline (PBS). The Sephadex G-100 column (1·77 $cm^2 \times 85$ cm) and the Sephadex G-50 column (1·77 $cm^2 \times 75$ cm) were standardized by a preliminary fractionation of the molecular markers. With each column a 1 ml sample was applied and 2·5 ml fractions were collected. Odd numbered fractions were tested for guinea-pig eosinophil chemotaxis and even numbered fractions were tested for guinea-pig neutrophil chemotaxis. In some experiments even numbered fractions were also tested against rabbit neutrophils. Volumes of 0·9 ml of the fractions were placed in the chemotaxis chambers to which were added 0·1 ml of 5% ovalbumin in order to give a final concentration of 0·5% ovalbumin in isotonic Hanks's balanced salt solution.

RESULTS

The purity of fractions of guinea-pig IgG_1 and IgG_2

The purity of the fractions obtained by DEAE-cellulose chromatography (Fig. 1) was assessed both by gel-diffusion and immunoelectrophoresis in addition to the estimation of various biological activities known to reside in either the IgG_1 or IgG_2 containing fractions (Table 1).

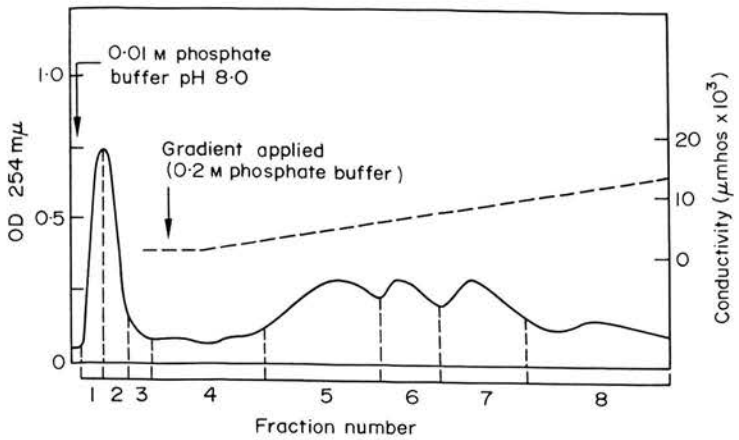

FIG. 1. The separation of guinea-pig IgG_1 and IgG_2 by DEAE(DE-32)-cellulose chromatography of a pooled anti-ferritin and anti-sheep red-blood cell serum.

TABLE 1. The purity of fractions of guinea-pig IgG_1 and IgG_2 determined by estimations of their known biological activities

Fraction number	1	2	3	4	5	6	7	8
PCA titre	<10	<10	<10	40	640	1280	80	40
Haemolysin titre	48	48	4	2	4	0	0	4
Macrophage–cytophilic antibody	w	++	+	+	w	w	w	w
CH_{50} following incubation with preformed complexes	<1	<1	NT	NT	>256	>256	NT	NT
Protein concentration in mg (Folin)	1·5	1·4	0·3	0·32	1·0	1·0	0·75	0·55

w = Weak, NT = not tested.

By gel-diffusion it was shown that IgG_1 was detectable only in fractions 5 and 6 (Fig. 2). Similar results were obtained using immunoelectrophoresis since the precipitation arcs produced by fractions 5 and 6 corresponded to the IgG_1 spur noted with the unfractionated antiserum (Fig. 3).

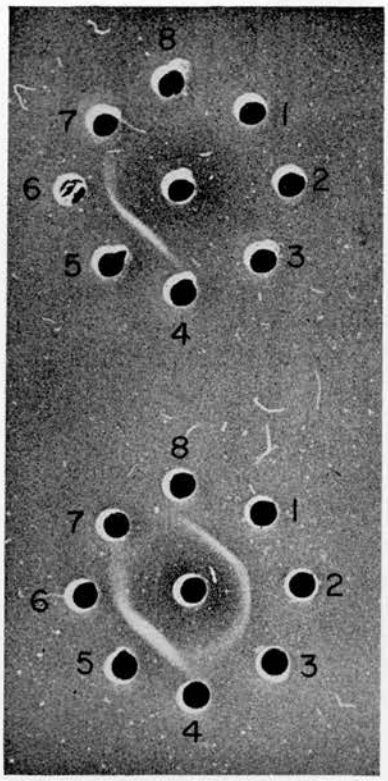

FIG. 2. The location of the fractions containing IgG_1 and IgG_2 as shown by gel-diffusion. The fractions obtained by DEAE-cellulose chromatography were placed in the outer wells as indicated and correspond to the fractions shown in Fig. 1. The centre well in the lower pattern contains a rabbit (R5094) anti-guinea-pig 7S IgG. A specific anti-IgG_1 (R5094 absorbed with IgG_2) was placed in the centre well illustrated in the upper pattern.

High PCA titres were obtained only with the IgG_1 containing fraction but not with IgG_2 eluted with the lower molarity buffer (Table 1).

The relative inefficiency of IgG_1 to fix complement has been confirmed. This was demonstrated both by estimation of the haemolysin titre and also by measuring whole complement (CH_{50}) following incubation of guinea-pig serum with preformed complexes prepared from either IgG_1 or IgG_2 containing fractions (Table 1). Only IgG_2 containing fractions showed fixation of whole guinea-pig complement.

The fractionation of pooled, anti-ferritin and anti-sheep RBC sera was convenient for identifying macrophage–cytophilic antibody in addition to the measurement of the haemolysin titre. A previous report (Jonas *et al.*, 1965) that macrophage–cytophilic antibody resided in the electrophoretically fasterIgG_2 containing region was confirmed (Table 1) since only fraction 2 showed a significant rosette-forming reaction.

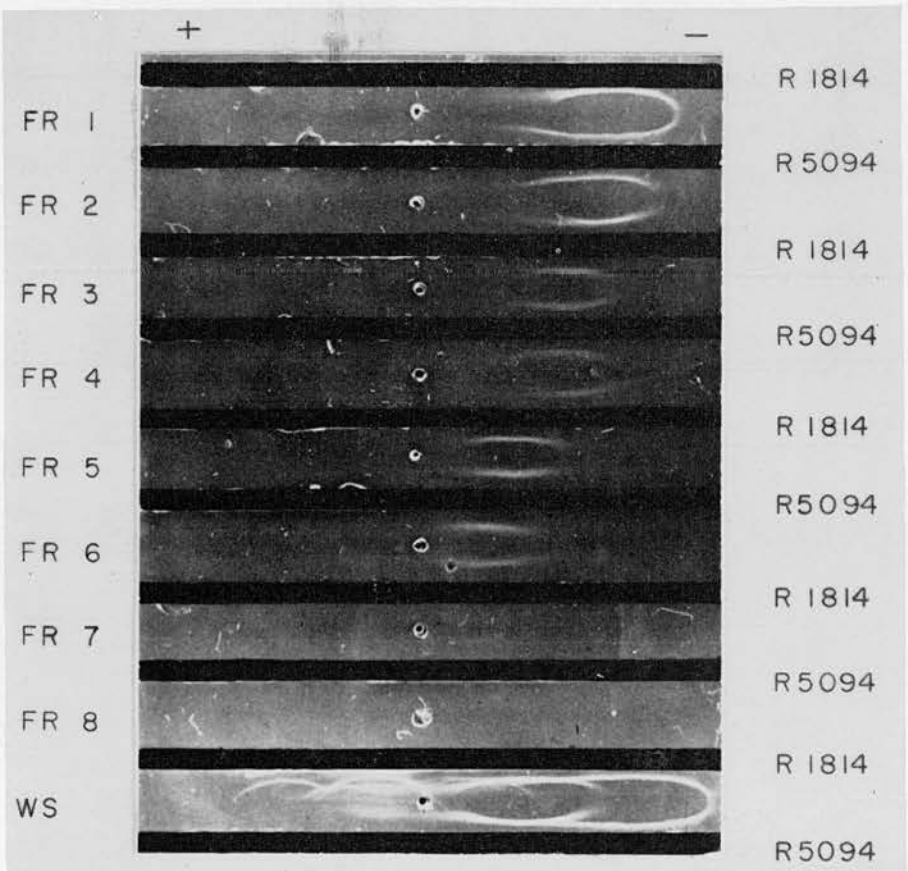

FIG. 3. The purity of the fractions obtained by DEAE-cellulose chromatography (Fig. 1) as shown by immunoelectrophoresis. The fractions and the unfractioned whole serum (ws) were placed in the wells as indicated on the left and antisera was applied to the troughs as shown on the right.

As a result of the gel-diffusion and immunoelectrophoretic studies Fractions 1 and 6 were chosen as the most pure fractions of IgG_2 and IgG_1 respectively.

Chemotaxis generated from guinea-pig serum by preformed antigen–antibody complexes

In initial experiments it was found that normal, untreated guinea-pig serum had chemotactic activity (Table 2). Using guinea-pig eosinophils and neutrophils from the guinea-pig and rabbit it was shown that this chemotactic activity was partially destroyed by heating the serum at 56°C for 30 min.

Following activation of normal guinea-pig serum by preformed antigen–antibody complexes prepared from rabbit antiserum and then subsequently heating at 56°C for 30 min chemotactic activity could be demonstrated (Table 3). Therefore, by heating activated serum in addition to the normal serum controls the chemotactic activity of normal unheated serum could be distinguished from the chemotactic activity generated by antigen–antibody complexes. Consequently, following incubation of guinea-pig serum with the antigen–antibody complexes for 15 min at 37°C, the complexes were removed by centrifugation and

TABLE 2. The effect of heat on the chemotactic activity of normal guinea-pig serum. The figures are mean cell counts and represent the pooled results of three experiments

	Neutrophils		Eosinophils
	Guinea-pig	Rabbit	Guinea-pig
Serum, unheated	29·2	27·2	12·6
Serum, heated (56°C for 30 min)	11·4	8·7	2·9

Each test compartment contained 0·1 ml of serum (heated or unheated) and 0·9 ml of 0·5% ovalbum in inHanks's solution.

TABLE 3. The property of rabbit antigen–antibody complexes to generate chemotactic activity from guinea-pig serum. The figures are mean cell counts and represent the pooled results of three experiments

	Neutrophils		Eosinophils
	Guinea-pig	Rabbit	Guinea-pig
1. Serum	3·5	5·8	0·7
2. Serum + rabbit complexes	23·6	37·4	36·2
3. Diluent alone	0	0	0

1. Serum, 0·1 ml (incubated 37°C for 15 min, 56°C for 30 min) + 0·9 ml of 0·5% ovalbumin in Hanks's solution.

2. Serum, 0·1 ml (incubated at 37°C for 15 min, with 5mg of complex per ml of serum, complexes removed by centrifugation, 56°C for 30 min) + 0·9 ml of 0·5% ovalbumin in Hanks's solution.

3. Diluent, 1 ml of 0·5% ovalbumin in Hanks's solution.

TABLE 4. The property of antigen–antibody complexes prepared from guinea-pig IgG_1 or IgG_2 to generate chemotactic activity from guinea-pig serum. The figures are mean cell counts and represent the pooled results of three experiments

	Neutrophils		Eosinophils
	Guinea-pig	Rabbit	Guinea-pig
Serum	8·6	5·9	0·5
Serum + IgG_2 (Fr 1)	49·2	30·1	16·0
Serum + IgG_1 (Fr 6)	62·3	43·3	17·2
Diluent alone	0	0	0

Test compartments prepared as in Table 3.

the sera, including the unheated controls, were heated for 30 min at 56°C and 0·1 ml volumes tested for chemotactic activity.

The chemotactic generating ability of complexes prepared either from guinea-pig IGg_1 or IgG_2 is shown in Table 4. It was established in preliminary experiments that the various antigen–antibody complexes were not chemotactic *per se* but both IgG_1 or IgG_2, as preformed complexes, were equally capable of generating chemotactic activity for guinea-pig neutrophils, rabbit neutrophils and guinea-pig eosinophils following incubation with guinea-pig serum.

Although little difference was observed in the chemotactic generating properties of guinea-pig IgG_1 and IgG_2 complexes, measurements of the CH_{50} levels following activation of serum but prior to heating at 56°C was <1 for complexes prepared from Fr 1 (IgG_2) and Fr 2 (IgG_2) and >256 for complexes prepared from Fr 5 (IgG_1), Fr 6 (IgG_1) and for untreated guinea-pig serum, as shown in Table 1.

Antigen–antibody complexes prepared from guinea-pig IgG_1 and IgG_2 and from unfractioned rabbit antiserum were unable to generate chemotactic activity for guinea-pig eosinophils and neutrophils and for rabbit neutrophils in the presence of 0·01 M EDTA (Table 5). The serum was incubated with antigen–antibody complexes in the presence of

TABLE 5. The inability of antigen–antibody complexes to generate chemotactic activity from guinea-pig serum in the presence of 0·01 EDTA

		Neutrophils		Eosinophils
		Guinea-pig	Rabbit	Guinea-pig
	Serum	8	4·6	4·4
	Serum + IgG_1 (Fr 6)	21·5	46·4	15·8
EDTA	Serum + IgG_1 (Fr 6)	0	8	2·6
	Serum + IgG_2 (Fr 1)	19·5	36·1	11·1
EDTA	Serum + IgG_2 (Fr 1)	0	4	4·2
	Serum + Rabbit complexes	14·7	37·7	21·4
EDTA	Serum + Rabbit complexes	1	11·4	3·7

The figures are mean cell counts and represent the pooled results of two experiments.

Test compartments prepared as in Table 3. Treatment with 0·01 EDTA prepared as described in text.

0·01 M EDTA and dialysed overnight at 4°C. Serum incubated with antigen–antibody complexes in the absence of EDTA and subsequently dialysed served as a control. The experiments were repeated omitting dialysis but by replacing the divalent cations before testing for chemotaxis. Essentially similar results were obtained.

It was also shown that neither guinea-pig nor rabbit antigen–antibody complexes could release chemotactic activity for neutrophils or eosinophils from ammonia-treated (R4) serum or from serum previously treated at 56°C for 30 min.

Fractionation of guinea-pig serum activated by antigen–antibody complexes

(A) *Sucrose density-gradient ultracentrifugation.* Since antigen–antibody complexes generated chemotactic activity for guinea-pig neutrophils and eosinophils, attempts were

made, using fractionation procedures, to determine whether the same chemotactic factors were active for both cell types.

Using sucrose density-gradient ultracentrifugation, it was determined in preliminary experiments that neither high concentrations of sucrose nor the [^{125}I]thyroglobulin or haemoglobin markers had any chemotactic effect *per se* or that they influenced the chemotactic properties of serum activated by antigen-antibody complexes.

Following ultracentrifugation of guinea-pig serum activated by rabbit antigen–antibody complexes, the chemotactic activity for both cell types was observed in the slowly sedimenting region (Fig. 4). The peak activity for guinea-pig eosinophils was in the range of proteins

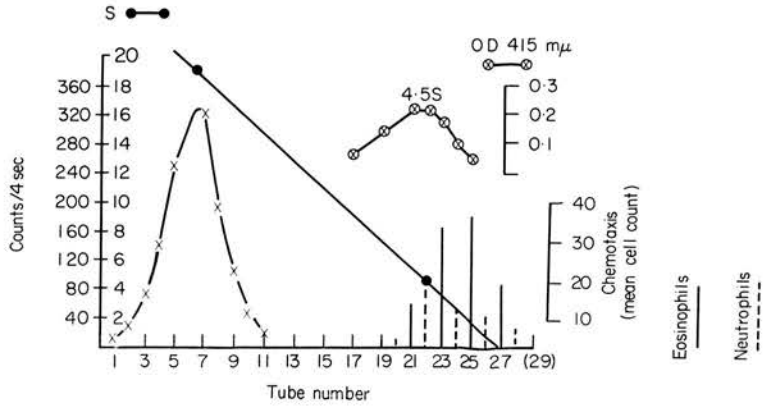

FIG. 4. The ultracentrifugal analysis of guinea-pig serum activated by rabbit antigen–antibody complexes. The position of the two molecular markers [^{125}I]thyroglobulin (19S), measured as counts per 4 sec, and haemoglobulin (4·5S), measured at OD 415 mμ is illustrated and from this was drawn a linear relationship for S valves.

The peak of eosinophil and neutrophil chemotactic activity was found in the slowly sedimenting region. Eosinophil chemotactic activity was between 1·5S and 2S whereas the peak of neutrophil chemotactic activity was less well defined but sedimented slightly faster and was approximately 4·5S.

having a sedimentation constant (SW_{20}) of approximately 1·5S–2·0S whilst the peak activity for guinea-pig neutrophils, although less well defined, was slightly faster sedimenting and was approximately 4·5S. Rabbit neutrophil chemotaxis was not determined in the ultracentrifugation fractionation procedures.

Following fractionation of serum incubated with guinea-pig IgG_1 and IgG_2 virtually identical results were obtained as with incubation with rabbit antigen-antibody complexes except that the mean cell counts for chemotaxis were slightly less.

(B) *Gel-filtration on Sephadex G-100 and G-50.* In an attempt to differentiate further the eosinophil and neutrophil chemotactic activity demonstrated on sucrose density-gradient ultracentrifugation and to obtain preliminary data on the molecular weight of the chemotactic factors, the activated serum was fractionated using gel-filtration.

Guinea-pig serum activated with rabbit antigen–antibody complexes was passed through a column of Sephadex G-100, the void volume of the molecular markers blue dextran, haemoglobin and cytochrome C having been previously determined. Two peaks of cell-specific chemotactic activity were noted (Fig. 5). The guinea-pig eosinophil chemotactic

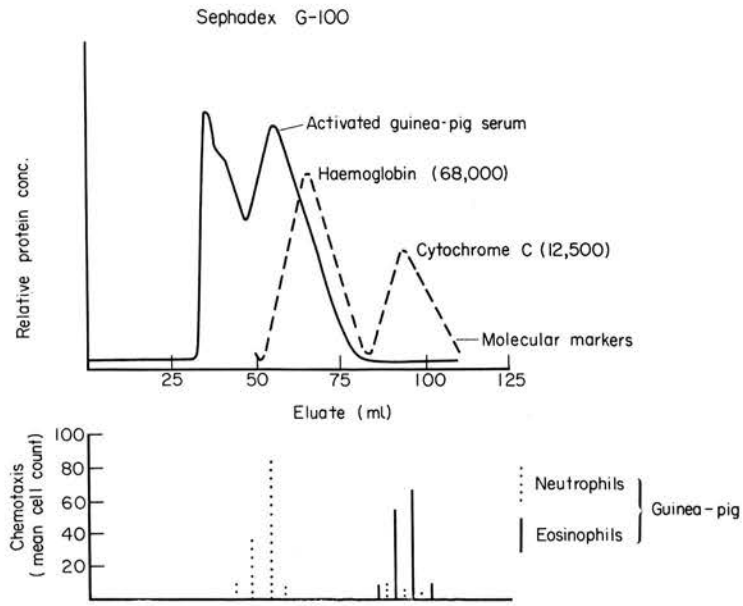

FIG. 5. The fractionation on Sephadex G-100 of guinea-pig serum activated by rabbit antigen–antibody complexes. Alternate fractions were tested for guinea-pig eosinophil or neutrophil chemotactic activity and could be separated and were found to correspond to the cytochrome C and the haemoglobulin markers respectively.

peak was in the fractionation range for molecules having a range of molecular weight of between 15,000 and 20,000 whilst guinea-pig neutrophil chemotactic activity was in the range of 65,000–85,000 molecular weight. In a separate experiment no chemotactic activity for guinea-pig eosinophils and neutrophils (and rabbit neutrophils) was found when heated serum, without prior activation, was fractionated on Sephadex G-100.

Two further fractionation studies were performed using serum activated with guinea-pig IgG_1 and IgG_2 and their antigens respectively. Essentially similar results were obtained as with serum activated with rabbit antigen–antibody complexes with the exception that, as with the ultracentrifugation studies, the values of the mean cell counts for eosinophils and neutrophils were less than half that shown in Fig. 4 although the peaks of activity were virtually identical.

In order to determine more precisely the molecular weight range of the more slowly sedimenting eosinophil chemotactic activity, fractionation of serum activated with rabbit antigen–antibody complexes was performed on Sephadex G-50, since it was considered that the fractionation range of G-50 was such that a more accurate determination could be made of this molecular weight. In addition, alternate fractions were also examined for rabbit neutrophil chemotactic activity since a low molecular weight factor chemotactic for rabbit neutrophils generated from guinea-pig serum has also been described (Snyderman, Gewurz & Mergenhagen, 1968). The results are shown in Fig. 6. Similar findings were obtained with guinea-pig neutrophils and eosinophils as in the fractionation with Sephadex G-100, although the peaks of activity were not so widely separated. The eosinophil chemotactic activity was again in the range of molecular weight between 10,000 and 15,000 whilst the

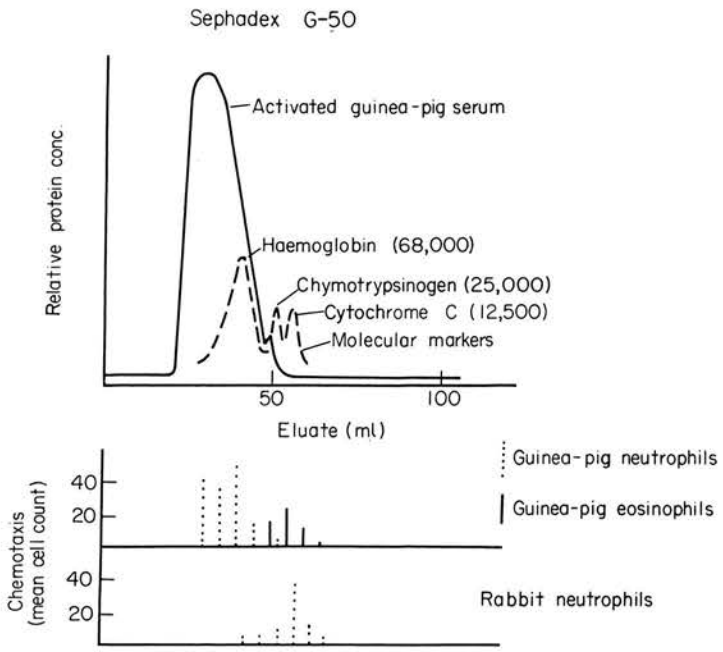

FIG. 6. Sephadex G-50 fraction of guinea-pig serum activated rabbit antigen–antibody complexes. In addition to guinea-pig neutrophils and eosinophils rabbit neutrophils were also tested for chemotaxis and found to migrate towards those fractions chemotactic for guinea-pig eosinophils.

guinea-pig neutrophil peak was in the position of the haemoglobin marker. The peak of activity for rabbit neutrophils' chemotaxis was similar to that for guinea-pig eosinophils, an observation that was repeated by fractionation on Sephadex G-100 and on Sephadex G-50 using serum activated by guinea-pig IgG_1 and IgG_2 antigen–antibody complexes. Fractionation on Sephadex G-50, therefore, did not allow a more precise determination of the molecular weights of the specific guinea-pig and neutrophil chemotactic factors but confirmed the observations obtained with Sephadex G-100 and showed that guinea-pig and rabbit neutrophils migrated towards separate fractions whilst guinea-pig eosinophil and rabbit neutrophil chemotaxis had a virtually identical chromatographic pattern.

The eosinophil and neutrophil chemotactic activity was specific as indicated in Figs 5 and 6. When the neutrophil rich suspension (which normally contained about 5% of eosinophils) was tested with the fraction having peak chemotactic activity for eosinophils, migration of eosinophils alone was observed although the pore size was only 1·2 μ. Most of the neutrophils, on the other hand, remained on the upper surface of the millipore, very little migration having occurred.

DISCUSSION

The main findings of this report are that eosinophils and neutrophlis migrate towards serum activated with antigen–antibody complexes prepared either with guinea-pig IgG_1 or with

IgG_2 and that, irrespective of the manner in which the serum was activated, chemotactic activity for these two cell types could be separated by fractionation procedures.

The technique used to separate guinea-pig IgG_1 and IgG_2 has enabled these immunoglobulins to be obtained in a reasonably pure state. This was demonstrated both by gel diffusion and immuno-electrophoresis and also by the confirmation of previous reports that haemolysin and macrophage–cytophilic antibody are detectable only in the IgG_2 region (Bloch *et al.*, 1963; Jonas *et al.*, 1965) and that PCA activity is confined to the IgG_1 containing fractions (Ovary, Benacerraf & Bloch, 1963).

The ability of IgG_1 and its antigen to generate a heat labile chemotactic agent from guinea-pig serum contrasts with the findings of Keller, Nussenzweig & Sorkin (1968). Using an IgG_1 preparation containing approximately 10% IgG_2, an experiment was reported in which the cell counts per field were 93 and 288 for IgG_1 and IgG_2 respectively compared with a count of 39 for the untreated serum control. They concluded that the moderate count with IgG_1 was a reflection of contaminating IgG_2. In the present study, however, the purified IgG_1 preparation gave, in some cases, a slightly higher count than treatment with complexes prepared from IgG_2 (Table 2).

It has been claimed recently that preformed antigen–antibody complexes prepared from IgG_1 can fix total complement (Osler *et al.*, 1969). The present report was unable to confirm these observations (Table 1). It would appear, however, that the chemotactic activity generated either by IgG_2 or IgG_1 is in fact complement mediated since it was shown that activity could not be generated in the presence of 0·01 M EDTA or from ammonia-treated serum or from serum previously heated at 56°C for 30 min. A possible explanation as to why antigen–antibody complexes prepared from IgG_1 liberated chemotactic activity but did not affect total complement levels as measured by the CH_{50} was that in the experiments described here a 'limited' complement system was employed. For instance, it can be shown that under certain conditions of testing, sheep IgG_2, like guinea-pig IgG_1, is also 'non-complement fixing' but when increasing amounts of complement are used, sheep IgG_1 and IgG_2 were shown to have equal haemolytic activity (Feinstein & Hobart, 1969). Furthermore, both immunoglobulins as preformed complexes could generate chemotactic activity from rabbit serum (Kay & Hobart, unpublished observation).

It has been shown that specific chemotactic activity for rabbit neutrophils and rabbit macrophages can be separated when rabbit serum, heated at 56°C for 30 min, is fractionated on Sephadex G-200 (Wilkinson, Borel, Stecher-Levin & Sorkin, 1969). The present study is another example of how chemotactic activity for more than one cell type can be separated following chromatography, but here the activities were generated by antigen–antibody complexes and were specific for eosinophils and neutrophils. In contrast to the findings of Wilkinson *et al.* (1969), no chemotaxis was noted following fractionation of heated guinea-pig serum, although unheated serum had a relatively heat labile chemotactic activity.

The small molecular weight factor reported in this study which had chemotactic activity for guinea-pig eosinophils was similar in molecular weight and sedimentation constant to a factor previously described which had chemotactic activity for *rabbit neutrophils* but was generated from *guinea-pig serum* by endotoxic lipopolysaccharide and cobra factor (Snyderman *et al.*, 1968; Shin, Gewurz & Snyderman, 1969) and from purified guinea-pig C5 by EAC1423 (Shin *et al.*, 1968). The present experiments showed that there was a different profile of chemotactic activity for *rabbit* and *guinea-pig* neutrophils when activated guinea-pig serum was fractionated. The use of *rabbit* neutrophils, therefore, to detect activity

generated in *guinea-pig* serum must limit the *in vivo* significance of such experiments. It would seem likely, however, that the factor described here which was chemotactic for guinea-pig eosinophils is similar in molecular weight and sedimentation constant to the small molecular weight factor described by Shin *et al.* (1968). These workers concluded this to be a fragment cleaved from the 5th component of complement and showed that the factor also had anaphylatoxic properties (Shin *et al.*, 1968).

The factor chemotactic for guinea-pig neutrophils which was observed in the molecular weight range of 68,000 has not been further investigated but it would seem unlikely that this represents the chemotactic C $\overline{567}$ macromolecular complex described by Ward, Cochrane & Müller-Eberhard (1966). It has been confirmed, nevertheless, that human C $\overline{567}$ generated in free solution is chemotactic for human neutrophils and eosinophils (Lachmann, Kay & Thompson, 1970) suggesting that for eosinophils, as neutrophils, there is also a heterogeneity of chemotactic factors generated from the complement system (Ward, 1969b). It is emphasized that in the present study a macromolecular chemotactic factor was not detectable when whole guinea-pig serum was activated with preformed complexes. Either there is a species difference in the ability to form chemotactic C$\overline{567}$ or the conditions required for its generation from whole serum need to be more clearly defined.

A specific eosinophilotactic factor, therefore, has been generated from guinea-pig serum *in vitro* but the *in vivo* significance of this observation is yet to be determined. Experiments currently in progress are attempting to ascertain whether this factor is associated with the moderate accumulation of eosinophils observed in Arthus (Type III) reactions.

It was previously shown that in guinea-pig skin a local eosinophilia followed PCA reactions with IgG_1, but not after the passive administration of IgG_2 and subsequent antigen challenge. Injections of compound 48/80 also evoked a local eosinophil response (Kay, 1970); therefore, the possibility exists that the eosinophilia associated with Type I reactions is produced independently of the complement system. This possibility is also being investigated.

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The Chemotactic Activity for Neutrophil and Eosinophil Leucocytes of the Trimolecular Complex of the Fifth, Sixth and Seventh Components of Human Complement ($C\overline{567}$) Prepared in Free Solution by the 'Reactive Lysis' Procedure

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The Chemotactic Activity for Neutrophil and Eosinophil Leucocytes of the Trimolecular Complex of the Fifth, Sixth and Seventh Components of Human Complement ($C\overline{567}$) Prepared in Free Solution by the 'Reactive Lysis' Procedure

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Summary. $C\overline{567}$, prepared by the interaction of purified $C\overline{56}$ with purified C7 in solution, has been shown to be chemotactic for neutrophils and eosinophils. This activity was not significantly affected by the presence of C8 and C9, even in substantial excess. However, if the interaction of $C\overline{56}$ with C7 took place in the presence of an excess of erythrocytes, these cells bound the $C\overline{567}$ and were converted to EC567; and no chemotactic factor was found.

INTRODUCTION

In human acute phase sera treated with zymosan it is possible to generate a stable, activated complex of the fifth and sixth components of complement—$C\overline{56}$. $C\overline{56}$ has been substantially purified and on interaction with purified C7 gives arise to an evanescent complex, $C\overline{567}$*†, which briefly has the capacity to attach to red cell membranes, converting them to EC567 and enabling them to be lysed by C8 and C9 (Reactive Lysis). If binding is not achieved the binding site decays and the resulting complex, $C\overline{567}$, while haemolytically inactive, retains its capacity to react with C8 and C9 (Thompson and Lachmann, 1970; Lachmann and Thompson, 1970). The chemotactic activity of this $C\overline{567}$ prepared in free solution from functionally purified $C\overline{56}$ and C7 is reported in this communication.

The chemotactic activity of the $C\overline{567}$ complex of human or rabbit complement liberated during the conventional complement fixation reaction was described by Ward, Cochrane and Müller-Eberhard (1966). Their findings have however been disputed by Stecher and Sorkin (1969) on the grounds that they were unable to detect any difference between the chemotactic activity of normal and C6-deficient rabbit serum after treatment with antigen–antibody complexes.

MATERIALS AND METHODS

$C\overline{56}$ was purified from acute phase serum activated with zymosan as described by Lachmann and Thompson (1970). The preparation used had a protein concentration of

† The overbar refers to activation of the site allowing reaction with subsequent complement components; the asterisk to activation of the binding site for the cell membrane.

600 μg/ml and its titre for giving reactive lysis of sheep erythrocytes was 512. 0·1 ml of 1/10 dilution was used in the chemotactic test. A less purified preparation, still containing C9—referred to as '$\overline{C56}$+9'—was used at an approximately equivalent titre.

C7 was purified from normal human serum as described by Thompson and Lachmann (1970). The preparation used had a protein concentration of 240 μg/ml and a haemolytic titre of 1000. 0·1 ml of 1/5 dilution was used in the chemotactic system to ensure C7 excess. A less purified preparation still containing C8—referred to as 'C7+8'—was used at an approximately equivalent titre.

C8+9 was prepared from a rivanol precipitate of normal human serum by C-M cellulose chromatography as described by Manni and Müller-Eberhard (1969). The haemolytic titre for sheep EC567 was in the region of 5000 and the protein concentration around 17 mg/ml.

Activated serum was prepared by absorbing normal human serum with zymosan (2 mg/ml) for 30 minutes at 37°. In some experiments unactivated normal serum was used.

The technique used for testing for chemotactic activity was modified from that originally described by Boyden (1962) and is to be described more fully elsewhere (Kay, 1970.)

Human leucocytes were obtained from a single donor and extracted by dextran sedimentation. Counts of migrating leucocytes were made in the body of a millipore filter 0·65 μ pore size (Millipore filter Corporation, Bedford, Mass.) after 3 hours incubation at 37°. The diluent in both compartments was a 0·5 per cent solution of crystalline ovalbumin (Koch Light & Co. Ltd., Colnbrook, Bucks.) in Hanks's buffered salt solution, pH 7·2, containing 50 units penicillin and 5 μg streptomycin/ml.

RESULTS

Six separate experiments were performed and in three of these eosinophils were counted separately. The data are set out in Table 1. The data pooled into suitable groups and their statistical analysis are shown in Table 2.

THE CHEMOTACTIC ACTIVITY OF $\overline{C567}$

In Table 2 the data have been pooled in the first place, into three groups: normal, unactivated complement components; $\overline{C56}$ in the absence of C7; and $\overline{C567}$ (89) except those where the $\overline{C567}$ was made in the presence of E.

It is clear that $\overline{C567}$ shows strong and highly significant chemotactic activity for both neutrophils and eosinophils. The effect is substantially larger for neutrophils, this being true also for activated serum.

$\overline{C56}$ shows a minor degree of chemotactic activity for polymorphs which is not significant at the 5 per cent level. This is not seen with the eosinophils.

THE EFFECT OF C8 AND C9 ON THE CHEMOTACTIC ACTIVITY OF $\overline{C567}$

In one experiment (D) a sharp fall in chemotactic activity was observed in the presence of C8 and C9. However, more extensive testing with a number of dilutions of C8 and C9 failed to repeat this one observation and taking all the observations together there is no statistically significant effect for either neutrophils or eosinophils.

TABLE 1

Experiment:	Neutrophils A	B	C	D	E	F	Eosinophils C	D	E
Ovalbumin 0·5%	10·4	3·8	20·8	14·8	4·0	41·8	0·0	3·8	0·6
Mixtures containing only normal complement components									
C7		4·0	−11·6	6·6	15·4	−6·6	6·0	3·8	0·6
C7+8	−3·8	1·2	−9·6				2·0		
C7→C8+9				1·8				−1·6	
C8+9 1/50					10·0	−11·8			3·2
Mixtures containing $C\overline{56}$ without C7									
$C\overline{56}$	−6·4	13·8	5·2	10·8	4·6	8·0	4·0	3·2	1·8
$C\overline{56}$+9			12·4				1·4		
Mixtures containing $C\overline{567}$									
(i) Without C8									
$C\overline{56}$→C7		26·8	21·0	34·2	35·4	26·0	8·0	8·8	6·6
$C\overline{56}$+9→C7			20·6				1·2		
(ii) With C8 but without C9									
$C\overline{56}$→C7+8	49·6	28·0	16·6				10·0		
(iii) With C8+C9									
$C\overline{56}$+9→C7+8			18·2				11·0		
$C\overline{56}$→C7→C8+9 1/20						29·0			
$C\overline{56}$→C7→C8+9 1/40						23·4			
$C\overline{56}$→C7→C8+9 1/50					42·6				7·2
$C\overline{56}$→C7→C8+9 1/100				5·0	45·4	27·2			6·8
$C\overline{56}$→C7→C8+9 1/1000					43·0				6·4
$C\overline{56}$→C7→C8+9 1/10,000					21·6				7·8
(iv) $C\overline{567}$ made in presence 30 μl packed sheep erythrocytes									
$C\overline{56}$→E→C7		7·2		2·4				3·6	
$C\overline{56}$→E→C7+8	−3·6	6·8							
Activated serum	22·8	20·6	>30	41·6	34·8	>60	8·4	1·2	6·6

Each figure in this table is based on the mean of five readings taken on one chamber. The 95 per cent confidence limits for these means are ±5·8 for the neutrophils and ±1·9 for the eosinophils.

The figures for the ovalbumin controls are given as counted. In all other cases the ovalbumin blank for that experiment has been subtracted from the count. The substantial variation in the blank appears to depend on the particular batch of millipore membranes used.

THE EFFECT OF MAKING $C\overline{567}$ IN THE PRESENCE OF EXCESS ERYTHROCYTES

There were four paired observations for neutrophils where in one, $C\overline{56}$ was mixed with excess E before the addition of the C7-containing reagent, thereby allowing the $C\overline{567}$* to bind to red cell membranes. The mean chemotactic activity of the $C\overline{56}$-E-C7 was not significantly different from that of the normal complement components. The formation of $C\overline{567}$ in the presence of E thus abolishes its chemotactic effect.

The single paired observation for eosinophils shows the same result.

The red cells taken from the chambers could be lysed by C8+9, showing that they had been converted to EC567.

DISCUSSION

The data confirm the findings of Ward *et al.* (1966) that the trimolecular complex

TABLE 2

Groups	Neutrophils			Eosinophils		
	No. of results	Mean	95 per cent confidence interval	No. of results	Mean	95 per cent confidence interval
'C7, etc' Normal, unactivated components, i.e. C7; C7+8; C8+9	11	−0·4	±6·1	6	2·3	±2·7
'$C\overline{56}$' without C7	7	6·9	±6·3	4	2·6	±1·9
'$C\overline{567}$(89)' All, except those made in presence of E	18	28·5	±5·7	10	7·4	±1·9
$C\overline{56}\rightarrow$C7	6	28·7	±7·5	3	7·8	±0·9
$C\overline{56}\rightarrow$C7$\rightarrow$C8+9	9	28·4	±10·3	5	7·8	±0·7
'$C\overline{56}\rightarrow$E$\rightarrow$C7'	4	3·2	±8·0	1	3·6	–
(Activated) serum	6	>35	—	3	5·4	±9·3

Comparison between groups	Neutrophils		Eosinophils	
	Δ Means	Significance	Δ Means	Significance
$C\overline{567}$(89) *v.* C7, etc.	28·9	$P \ll 0{\cdot}001$	5·1	$P < 0{\cdot}01$
$C\overline{567}$(89) *v.* $C\overline{56}$	21·6	$P < 0{\cdot}001$	4·8	$P < 0{\cdot}01$
$C\overline{567}$(89) *v.* $C\overline{56}$ and C7, etc.	26·1	$P \ll 0{\cdot}001$	5·0	$P < 0{\cdot}001$
$C\overline{56}$ *v.* C7, etc.	7·3	$0{\cdot}05 < P < 0{\cdot}1$	0·3	$0{\cdot}8 < P < 0{\cdot}9$
$C\overline{56}\rightarrow$7 *v.* $C\overline{56}\rightarrow$C7$\rightarrow$C8+9	0·3	$0{\cdot}8 < P < 0{\cdot}9$	0	
$C\overline{56}\rightarrow$C7 *v.* $C\overline{56}\rightarrow$E$\rightarrow$C7 (paired variates)	31·5	$P \ll 0{\cdot}001$	5·2	(One result only)
$C\overline{56}\rightarrow$E$\rightarrow$C7 *v.* C7, etc.	3·6	$0{\cdot}4 < P < 0{\cdot}5$	1·3	(One result only)

$C\overline{567}$ is chemotactic for neutrophils. A similar effect has been found for eosinophils.

The complex used was generated from two reagents—$C\overline{56}$ and C7.

The $C\overline{56}$ was fractionated from serum initially treated with zymosan and therefore presumably highly chemotactic. The $C\overline{56}$ preparations themselves, however, showed only trivial—and dubiously significant—chemotactic activity. While this activity may represent a property of $C\overline{56}$ itself there are at least two other possible explanations. Firstly, it is possible that the preparation is contaminated with other chemotactic factors. The fractionation procedure which involves euglobulin precipitation, ion-exchange chromatography and G-200 sephadex filtration makes it most unlikely that any low molecular weight factors (e.g. C5a) are present but a trivial contamination with preformed $C\overline{567}$ (89) is difficult to exclude. Secondly it is possible that a little $C\overline{567}$ is generated in the diffusion chambers from C7 present in the leucocyte preparation. This is thought unlikely since the effect is much the same if the leucocytes are washed twice or three times.

The C7 preparation, and the C8 and 9 preparations subsequently used, were free of chemotactic activity.

The chemotactic activity generated by mixing $C\overline{56}$ with C7 is not found if the $C\overline{56}$ is first mixed with excess sheep cells; thus allowing fixation of $C\overline{567}$* to the cell membrane. This control provides good evidence that the chemotactic activity is indeed a property of the material that can be bound to cells (i.e. the $C\overline{567}$ itself) and not of some factor pro-

duced in the interaction. The availability of cell membranes or other hydrophobic surfaces to bind $\overline{C567}$* as it is formed may thus be important in determining the amount of $\overline{C567}$ that appears free in solution to act as a chemotactic factor; and may help to explain the discrepancy between the findings of Ward *et al.* (1966) on the one hand and of Sorkin and his colleagues (Stecher and Sorkin, 1969; Wilkinson, Borel, Stecher-Levin and Sorkin, 1969) and of Snyderman, Shin, Phillips, Gewurz and Mergenhagen (1969) on the other as to the role of $\overline{C567}$ in the chemotactic activity generated in serum by complement activation.

C8+9 even in great excess were without significant effect on the chemotactic activity of $\overline{C567}$ although it is known that these components do react with $\overline{C567}$ in solution (Lachmann and Thompson, 1970). This lack of effect is at variance with a result of Ward *et al.* (1966) who found that the terminal components reduced chemotactic activity. Such inhibition also occurred in just one of our experiments but could not be repeated even with the same reagents.

Although the data presented all deal with an entirely human system, one experiment using rabbit peritoneal leucocytes gave essentially similar results.

Since a number of chemotactic factors are now known to be generated by antigen–antibody interaction in serum, there would seem to be no essential contradiction between finding chemotactic activity both in $\overline{C567}$ and in C6-deficient rabbit serum.

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AN EOSINOPHIL LEUKOCYTE CHEMOTACTIC FACTOR OF ANAPHYLAXIS*

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The mechanism by which eosinophil leukocytes migrate into the site of immediate-type hypersensitivity reactions has been difficult to study in vivo because of the large numbers of variables in any experimental design. Certain of these difficulties have been overcome by the use of a modification of the in vitro Millipore technique of Boyden by which the eosinophilotactic properties of substances can be directly measured. Using this technique, it was shown that guinea pig IgG_1 or IgG_2, as preformed antigen–antibody complexes, could both generate from whole serum a factor which was specifically chemotactic for eosinophils (ECF-C)[1] and was apparently identical to C5a (1).

An earlier report (2), using cell counts in guinea pig skin, had shown that both IgG_1 and IgG_2, as preformed complexes, also prepared the tissue for a subsequent local eosinophilia 12 hr after injection. However, if antibody was first placed in the skin and after a variable latent period the animal was challenged with antigen and Evans blue dye intravenously (as in a usual passive cutaneous anaphylactic reaction), IgG_1 but not IgG_2 elicited a local eosinophil response 8–12 hr after the initial blueing reaction (2).

It was the purpose of the present study to determine whether tissue, actively sensitized or passively sensitized with IgG_1, but in the absence of serum, can

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[1] In this presentation, an eosinophil chemotactic factor (ECF) derived from whole guinea pig serum by treatment either with antigen–antibody complexes (1) or with zymosan,[2] which appears to be identical with C5a and therefore a product of the interaction of complement proteins, is termed ECF-C. Material selectively attracting neutrophils, also of complement origin (1) but having a larger molecular size, is termed NCF-C. A factor with eosinophilotactic activity appearing in association with immediate-type hypersensitivity reactions and generated in the apparent absence of a complement requirement is designated ECF-A. *Other abbreviations used in this paper:* DNP-BGG, dinitrophenol conjugates of bovine gamma globulin; DNP-BSA, dinitrophenol conjugates of bovine serum albumin; SRS-A, slow reacting substance of anaphylaxis; zym G/P, zymosan-treated guinea pig.

release an eosinophilotactic agent after the addition of specific antigen and whether such an agent is the same or different from that produced from the interaction of serum with preformed antigen–antibody complexes (1) or zymosan.[2] For this purpose, an in vitro model involving the release of chemical mediators from guinea pig lung was used, which has been employed by numerous workers as a model of immediate-type hypersensitivity in the guinea pig (4–7). It has enabled the definition of an immunoglobulin mediating the release of pharmacological agents (6, 7) and allowed the mediators themselves to be quantitated (4, 7). This report, therefore, combines two in vitro systems, namely the Millipore technique for measuring chemotaxis and an in vitro model of anaphylaxis.

Materials and Methods

Materials were obtained as follows: histamine acid phosphate, zymosan, serotonin creatinine sulphate, and succinic acid (Mann Research Labs. Inc., New York); bradykinin triacetate (Sandoz Pharmaceuticals, Basel, Switzerland); prostaglandins PGE_1, PGE_2, and PGF_{2a} (generously supplied by Dr. John Pike of the Upjohn Co., Kalamazoo, Mich.); maleic acid (Hopkin & Williams, Ltd., Chadwell Heath, Essex, England); blue dextran (Pharmacia Fine Chemicals Inc., New Market, N. J.); glucagon (Nutritional Biochemicals Corporation, Cleveland, Ohio); vitamin B_{12} (Wyeth Laboratories, Philadelphia, Pa.); glycogen (J. T. Baker Chemical Co., Phillipsburg, N. J.); horse serum (Grand Island Biological Co., Grand Island, N. Y.); ovalbumin, five times crystallized (Pentex Biochemical, Kankakee, Ill.); and guinea pig serum (Pel-Freez Biologicals, Inc., Rogers, Ark.).

Dinitrophenol conjugates of bovine gamma globulin (DNP-BGG) and bovine serum albumin (DNP-BSA) were prepared according to the method of Benacerraf and Levine (8) and contained 40 and 20 haptenic groups per molecule, respectively. Partially purified cobra venom was prepared as previously described (9). The titer of "late acting" complement components was estimated in microtiter plates using equal volumes of 1×10^8 sensitized sheep erythrocytes prepared with the first component of guinea pig complement and the fourth component of human complement ($EAC1^{gp}4^{hu}$), 50 effective molecules of guinea pig second component (C2), and doubling dilutions of guinea pig serum as a source of terminal components. The plates were incubated for 30 min each at 30° and 37°C and centrifuged at 100 rpm for 5 min at 4°C. The 50% lytic endpoint was determined by inspection. The guinea pig serum was obtained by cardiac puncture before and after the intravenous injection of 1 ml of cobra venom factor or Tyrode's solution.

Guinea pig IgG_1 and IgG_2 7S immunoglobulin fractions were prepared as follows. Antisera to ovalbumin were prepared in guinea pigs following the same injection schedule as previously described for raising guinea pig antiserum to ferritin (2). The antiserum was fractionated by diethylaminoethyl (DEAE)-cellulose chromatography (1). The functional purity of the IgG_1- and IgG_2-containing fractions was tested by passive cutaneous anaphylaxis and by passive hemolysis as described by Bloch et al. (10) and by immunoelectrophoresis and gel diffusion using rabbit anti-7S IgG and specific anti-guinea pig IgG_1 (2). Passive hemagglutination was performed as previously described (10).

Preparation of Lung Tissue for the In Vitro Release of Mediators.—Whole lung: The heart and lungs from guinea pigs were excised and perfused free of visible blood with Tyrode's solution as previously described (4). Animals were actively sensitized with DNP-BSA by the

[2] A. B. Kay. Unpublished observations.

method of Benacerraf et al. (11), or with ovalbumin in phenol (5), or by a single injection of 200 μg of ovalbumin in complete Freund's adjuvant into each hind footpad. The lungs of actively sensitized animals were challenged with 4 mg of antigen (ovalbumin or DNP-BGG) via the pulmonary artery and the perfusate was collected for 30 min after antigen challenge. The perfusates were placed on ice and assayed the same day for histamine and slow reacting substance of anaphylaxis (SRS-A) according to the method of Brocklehurst (4), and for chemotaxis as described below. Some perfusates were tested after a single freezing at −70°C and rapid thawing.

Lung fragments: In experiments using lung fragments the perfused tissue was sliced with fine scissors, washed in Tyrode's solution, and divided into 300 mg portions using an overhead weighing balance. With actively sensitized lung, the portions were suspended in 2.5 ml of Tyrode's solution to which was added the antigen suspended in 0.5 ml of Tyrode's solution. The samples were gently agitated in a water bath at 37°C. For passive sensitization, the lung fragments were suspended in 1.8 ml volumes of fractions containing antibody at 37°C. After the incubation period the fragments were washed twice and resuspended in 2.5 ml of Tyrode's solution. Antigen was then added as for the actively sensitized lung. After incubation with antigen the diffusates were removed with a Pasteur pipette and assayed for histamine, SRS-A, and chemotaxis. In all studies using lung fragments the values for chemotaxis, histamine release, and SRS-A release represent the mean of duplicate samples. For all three measurements the values obtained with duplicate samples varied by less than 15%.

Some experiments with lung fragments were modified as follows. In experiments with ethylenediaminetetraacetate (EDTA), the samples were washed once either with Ca^{++}- and Mg^{++}-free Tyrode's, containing 5 mM EDTA, or in the case of the control samples, with Ca^{++}- and Mg^{++}-free Tyrode's containing EDTA in which Ca^{++} and Mg^{++} had been replaced before the addition of antigen. The reconstituted Tyrode's solutions were adjusted by adding 6.8 mM of Ca^{++} and 1 mM of Mg^{++}. The effect of succinate or maleate was studied using 5 mM concentrations in Tyrode's solution adjusted to pH 7.4 with 0.1 N NaOH. Antigen was added to actively or passively sensitized fragments 1 min after incubation of the lung samples with maleate or succinate. The diffusate from the samples challenged with antigen alone was tested for chemotaxis in the presence of 5 mM of succinate or maleate.

Measurement and Fractionation of Eosinophil Chemotactic Activity of the Anaphylactic Reaction Mixture.—

Measurement of chemotaxis: A modification of the Millipore technique of Boyden was used as previously described (1). Guinea pig eosinophils were obtained by peritoneal lavage from animals which had received multiple injections of horse serum (1). Neutrophils were harvested from the peritoneal cavity of animals injected with glycogen 3–6 hr previously. For measuring eosinophil migration an 8.0 μ pore size was used and for neutrophils 1.2 μ, 3.0 μ, and 8.0 μ pore sizes were employed.

In the assay for chemotaxis, Tyrode's solution containing 0.5% ovalbumin was used in both the cell and the test compartments. Zymosan-treated guinea pig (zym G/P) serum served as a reference pool of material having both eosinophil and neutrophil chemotactic activity. Zymosan which had been boiled and washed twice in distilled water was incubated for 30 min at 37°C with guinea pig serum using 20 mg of zymosan/ml of serum. The zymosan was removed by centrifugation and the serum subsequently heated at 56°C for 30 min. Samples were stored at −70°C until used.

In experiments using the perfusate from whole lung, a value for the total chemotactic activity of the perfusate was expressed as V × C. V represented the volume of the perfusate and C the percentage chemotaxis of 1 ml of perfusate as compared with 0.1 ml of a zymosan-treated reference serum when both were tested against the same suspension of eosinophils.

The chemotactic activity of the diffusate from lung fragments was usually determined using

0.6 ml volumes to which were added 0.1 ml of 5% ovalbumin and 0.3 ml of Tyrode's solution. When the release of SRS-A and histamine was low, 0.9 ml volumes of the diffusate was used. The chemotactic counts were expressed as the mean cell count of five high power fields (1).

Fractionation of chemotactic activity by gel filtration chromatography: A Sephadex G-25 column (40 × 1 cm) was equilibrated using Tyrode's solution as a buffer. In each experiment, 1 ml of lung supernate was applied and 1 ml fractions were collected. The column was standardized by preliminary fractionation of various substances of known molecular weight. The tubes containing the highest concentration of blue dextran and vitamin B_{12} were read visually. The peak of glucagon was recorded at 280 mμ using a DU-2 Beckman spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.).

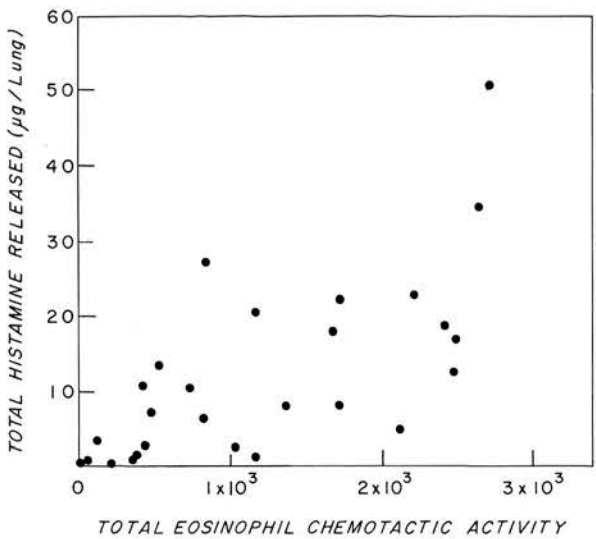


FIG. 1. Relation between the total amount of lung histamine released and the total chemotactic activity, V × C (see Methods), of the perfusate from sensitized lung following challenge with antigen. The animals were killed at various time intervals following an initial sensitizing injection. The data on individual animals sensitized with ovalbumin either in complete Freund's adjuvant or phenol are depicted. Correlation coefficient = 0.48; number of samples = 27; $0.02 > P > 0.01$.

RESULTS

The Antigen-Induced Release In Vitro of an Eosinophil Chemotactic Factor from Actively Sensitized Guinea Pig Lung.—When guinea pig lungs from actively sensitized animals were perfused free of visible blood via the pulmonary artery and subsequently challenged with specific antigen, the perfusate contained, in addition to histamine and SRS-A, a factor specifically chemotactic for guinea pig eosinophil leukocytes. This eosinophil chemotactic factor of anaphylaxis has been designated ECF-A. ECF-A was measured in the perfusate from animals sensitized with either ovalbumin or DNP-BGG and challenged with ovalbumin or DNP-BSA, respectively. A single injection of ovalbumin given in

complete Freund's adjuvant was slightly better than ovalbumin in phenol for preparing guinea pigs for the specific antigen-induced release of histamine, SRS-A, and ECF-A. Sensitization for the release of all these mediators was observed 10 days after the administration of ovalbumin in phenol or complete Freund's adjuvant but was not pronounced until 3 wk or later and was satisfactory up to 8 wk, at which time the study was terminated. The relationship between the total quantity of specific antigen-induced release of histamine and ECF-A from the lungs of each animal is shown in Fig. 1. A similar direct relationship was also demonstrated between the total amount of SRS-A released and the total ECF-A activity.

Studies were next carried out to determine whether ECF-A could be released from fragments of actively sensitized lung, and the results are shown in Table I. Addition of specific antigen to the lung fragments induced the release of histamine, SRS-A, and ECF-A, whereas no activities were detected in the absence of antigen. A linear dose-response curve was obtained when dilutions of the

TABLE I

Antigen-Induced Release of ECF-A, Histamine, and SRS-A from Fragments of Actively Sensitized Guinea Pig Lung

	Eosinophil chemotaxis	Histamine release	SRS-A
	mean cell count	*μg/g*	*units/g*
Lung + antigen	21.5	4	450
Lung + diluent	1.6	0	0
Zym G/P serum	18.6		

anaphylactic diffusate were tested for chemotaxis (Fig. 2). Replicate samples of sensitized lung fragments therefore provided a convenient test system for investigating both the antibody involved and the mechanism of release of ECF-A.

Selective Chemotactic Activity of the Anaphylactic Diffusate for Eosinophils.— Anaphylactic diffusates were examined for eosinophil and neutrophil chemotactic activity using a suspension of cells rich in neutrophils but containing 12% eosinophils. Guinea pig serum treated with zymosan has both neutrophil and eosinophil chemotactic activity (2) but attracted only neutrophils when millipores with a 1.2 μ or 3.0 μ pore size were used. With an 8.0 μ pore size both eosinophils and neutrophils migrate towards zym G/P serum, the ratio of migrating eosinophils to neutrophils being almost the same as in the cell compartment before migration commenced. Chemotactic activity of the lung diffusate was detectable only with an 8.0 μ pore size; 84% of the cells migrating were eosinophils although only 12% were present in the cell compartment (Table II). The experiment was repeated using diffusates from actively or passively sensitized lung challenged with antigen. On these occasions, using a

neutrophil-rich suspension and an 8.0 μ millipore size, 100% of the cells migrating towards the anaphylactic diffusate were eosinophils. Taken together these experiments demonstrated a selective chemotactic property for eosinophils of the anaphylactic lung diffusate.

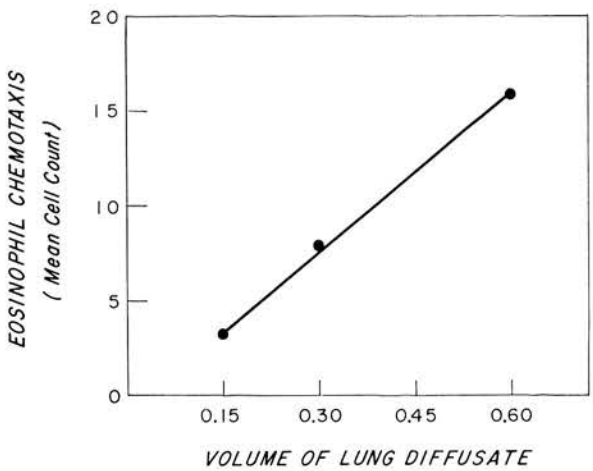


FIG. 2. Chemotactic dose-response curve following dilutions of lung diffusate containing ECF-A.

TABLE II

Selective Chemotactic Property of an Anaphylactic Lung Diffusate for Eosinophil Leukocytes

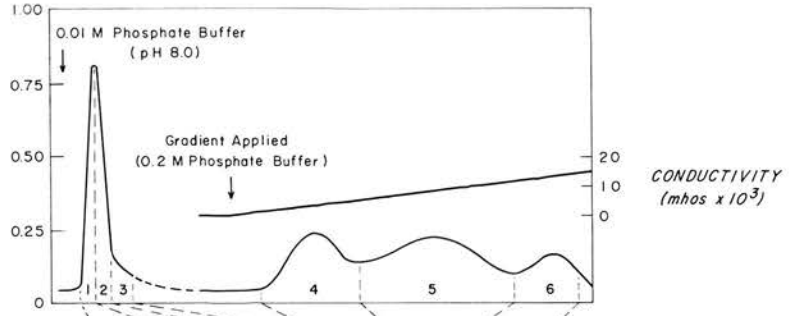
Millipore size	Eosinophil and neutrophil chemotaxis (*mean cell count*)*		
	1.2 μ	3.0 μ	8.0 μ
Anaphylactic lung diffusate	$\frac{1.2 + 1.4}{2.8}$	$\frac{2.2 + 0.6}{2.8}$	$\frac{14.2 + 4.6}{18.8}$
Zym G/P serum	$\frac{0 + 12}{12}$	$\frac{0 + 13}{13}$	$\frac{6.4 + 26.4}{32.8}$
Diluent	0	0	0

* Mean cell counts are expressed as $(E + N)/T$ where E is the eosinophil count, N the neutrophil count, and T the total mean cell count. The cell suspension contained 80% neutrophils, 12% eosinophils, and 8% mononuclear cells.

Identification of the Antibody Mediating the Release of ECF-A from Guinea Pig Lung.—Analysis of the sera obtained from the actively sensitized animals shown in Fig. 1 revealed a relationship between the passive cutaneous anaphylaxis (PCA) titer of the sera and the histamine release from the donor animals after antigen challenge. The sera of animals whose lungs released large amounts

of histamine and ECF-A had circulating antibody characteristic of IgG_1. The antibody was heat stable and gave a high 4 hr PCA titer which at 48 hr showed a falling titer of one or two doubling dilutions. No evidence was found of an IgE-type antibody.

Direct evidence that IgG_1 mediated the release of ECF-A was sought by experiments in which lung fragments were passively sensitized with fractions



	1	2	3	4	5	6
PASSIVE HEMAGGLUTINATION	5120	5120	2560	5120	320	20
PASSIVE HEMOLYSIS	5120	2560	1280	<20	<20	<20
PCA TITER (4 hr)	<40	<40	<40	1920	960	80
EOSINOPHIL CHEMOTAXIS (Mean Cell Count)	0	0	0	9.6	7.3	2
HISTAMINE RELEASE (μg/g)	0	0	0	2.0	1.40	1.10
SRS-A RELEASE (units/g)	0	0	0	330	150	150

FIG. 3. Ability of fractions of guinea pig IgG_1 and IgG_2, obtained by DEAE-cellulose chromatography, to passively sensitize lung fragments for the antigen-induced release of ECF-A, histamine, and SRS-A. Antibody fractions 1–5 were diluted 1:50, fraction 6 was diluted 1:5.

(Fr) of guinea pig 7S IgG. Biologic evidence for separation of IgG_1 and IgG_2 is shown in Fig. 3. Fractions 1–5 all agglutinated red cells which were passively sensitized with ovalbumin, but only those cells sensitized with the IgG_2 containing fractions (Fr 1, 2, and 3) hemolyzed on the addition of guinea pig complement. PCA titers were given only with the IgG_1 containing fractions (Fr 4, 5, and 6). Immunoelectrophoretic and gel diffusion studies with specific antisera to guinea pig 7S IgG_1 demonstrated IgG_1 only in fractions 4, 5, and 6. Only those fractions which contained IgG_1 (Fr 4, 5, and 6) prepared lung fragments for the antigen-induced release of ECF-A, histamine, and SRS-A (Fig. 3). In

these experiments, the dose of antigen, the time of sensitization with antibody, and the time of incubation with antigen were arbitrarily chosen on the basis of a previous study (6).

The heat stability of the antibody mediating the release of ECF-A was also determined and provided further evidence that IgG_1 and not an IgE-type antibody mediated its release. Fractions 4 and 5 were heated for 4 hr at 56°C and when compared with the unheated fractions showed no difference in their ability to mediate the release of histamine, SRS-A or ECF-A (Table III).

The Mechanism of Release of ECF-A.—

Optimal conditions for the release of ECF-A after passive sensitization of lung fragments: The optimal conditions required for the release of ECF-A after

TABLE III

*Effect of Heat on the Ability of IgG_1 to Prepare Guinea Pig Lung Fragments for the Antigen-Induced Release of ECF-A, Histamine, and SRS-A**

Sensitization	Eosinophil chemotaxis	Histamine release	SRS-A
	mean cell count	*μg/g*	*units/g*
Fraction 4			
Unheated	12.7	1.5	50
Heated	14.7	1.5	80
Fraction 5			
Unheated	14.1	1.3	80
Heated	14.4	1.3	70

* Fractions were heated for 4 hr at 56°C and diluted 1:50. 0.9 ml volumes of lung effluents were tested for chemotaxis to which was added 0.1 ml of 5% ovalbumin. Control samples sensitized with fraction 4 or 5 but not challenged with antigen gave a background chemotactic count of less than 1.5.

passive sensitization of normal lung were determined as follows. Using a 2½ hr sensitization period and 200 μg of ovalbumin/300 mg of lung tissue, a 1:50 dilution of an IgG_1-containing fraction (Fr 4) was found to release more ECF-A than a 1:5 or a 1:500 dilution. The time course of sensitization of tissue with antibody is shown in Fig. 4. The release of ECF-A, histamine, and SRS-A was detectable after 10 min of sensitization, reached a peak at 1 hr, and was maintained for at least 2½ hr. In further experiments either a 1 hr or a 2½ hr sensitization time was used. With regard to the time course of release, incubation with antigen beyond 15 min did not result in greater release of ECF-A. With a 1 hr sensitization period and a 1:50 dilution of fraction 4 (IgG_1) 20 and 200 μg of ovalbumin yielded greater release of ECF-A, histamine, and SRS-A than 2 or 2,000 μg of ovalbumin/300 mg of lung (Fig. 5).

The effect of decomplementation with cobra venom factor: Animals were depleted of circulating complement by an intravenous injection of a purified factor from

cobra venom. Less than 5% of the circulating complement level was detectable 10 hr after the administration of 2 units of purified cobra venom factor. Accordingly, actively sensitized animals were sacrificed 10 hr after injection, their lungs removed, perfused, and sliced as described above and portions challenged

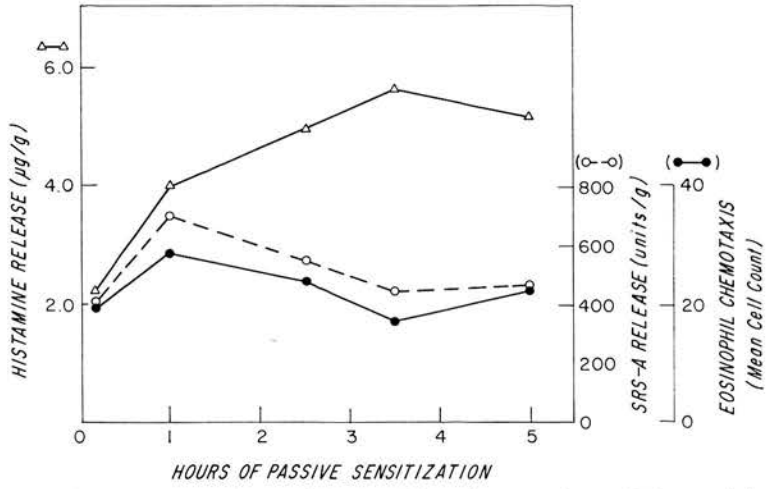


FIG. 4. Time course of passive sensitization of lung fragments by an IgG_1-containing fraction (fraction 4 diluted 1:50) for the antigen-induced release of ECF-A, histamine, and SRS-A.

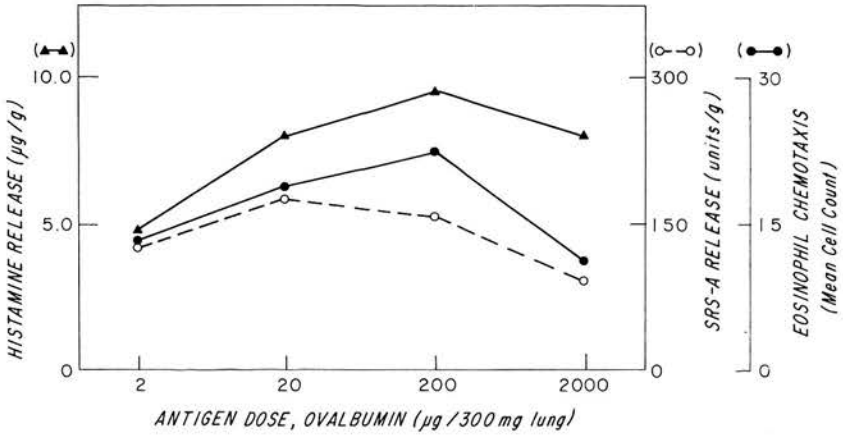


FIG. 5. Effect of antigen concentration on the release of ECF-A, SRS-A, and histamine from guinea pig lung passively sensitized with IgG_1 (fraction 4 diluted 1:50).

with specific antigen. Table IV shows that there was no difference in the amounts of ECF-A released from lungs of cobra venom-treated animals and untreated animals. Although absolute histamine release in the actively sensitized animals appeared to be somewhat affected by venom treatment, the per

cent release was similar in the two groups, being in animals one to six 31.0, 37.5, 31.0, 43.2, 36.2, and 35.2 respectively.

An experiment was also performed using normal animals whose circulating complement levels were depleted with cobra venom factor and whose lungs in the same manner as described were then passively sensitized with a 1:50 dilution of fraction 4 (IgG_1). Virtually identical results were obtained as with lungs from actively sensitized animals (Table IV).

TABLE IV

*Effect of Complement Depletion In Vivo on the In Vitro Release of ECF-A, Histamine, and SRS-A from Guinea Pig Lung Fragments**

Animal No.	Treatment	Change in hemolytic titer of the terminal complement components	Eosinophil chemotaxis	Histamine release	SRS-A
			mean cell count	*μg/g*	*units/g*
		Actively sensitized lung			
1	Cobra venom factor	5120 → 160	26.1	5.5	400
2	" " "	5120 → 80	34.4	7.0	520
3	" " "	5120 → 160	30.3	5.5	550
4	Tyrode's solution	5120 → 5120	35.1	9.5	1000
5	" "	5120 → 5120	29.7	7.1	620
6	" "	5120 → 5120	34.2	9.5	1100
		Passively sensitized lung			
7	Cobra venom factor	10,240 → 640	17.4	4.0	500
8	" " "	10,240 → 640	16.1	3.6	400
9	" " "	10,240 → 640	15.5	3.5	400
10	Tyrode's solution	10,240 → 10,240	16.7	3.3	1000
11	" "	10,240 → 10,240	15.0	3.6	750

* With animals 1–6, 0.9 ml volumes of lung effluent were tested for chemotaxis and with animals 7–11, 0.6 ml volumes were used. Lung fragments suspended in Tyrode's alone gave background counts of less than 3.4 and 0 in the two experiments, respectively. Lungs were passively sensitized for 2½ hr with a 1:50 dilution of fraction 4.

Inhibition and enhancement: A requirement of divalent cations for the release of ECF-A was determined by experiments in which actively sensitized lung was challenged with antigen in the presence of 5 mM of EDTA (Table V). In the absence of calcium and magnesium ions, there was no release of ECF-A, histamine, or SRS-A. Before testing for chemotaxis, calcium and magnesium ions were added to the diffusates containing EDTA. Tyrode's EDTA in which the divalent cations had been replaced served as a control in that this solution, together with antigen, was used to challenge sensitized lung for ECF-A release and the same solution was used in the chemotactic chamber. Replacement of

cations fully restored antigen-induced release of ECF-A, histamine, and SRS-A. The experiment was repeated with passively sensitized lung and gave the same result.

The capacity of succinate and maleate to enhance the release of histamine and SRS-A (12) was also sought in terms of ECF-A release. When actively sensitized lung was used, the enhancement of ECF-A release in the presence of 5

TABLE V

Effect of EDTA on the Antigen-Induced Release of ECF-A, Histamine, and SRS-A from Actively Sensitized Guinea Pig Lung Fragments

Suspending medium for lung fragments	Challenge	Eosinophil chemotaxis	Histamine release	SRS-A
		mean cell count	*μg/g*	*units/g*
5 mM EDTA in Tyrode's	Antigen	0.4	0	0
5 mM EDTA in Tyrode's with Ca^{++}, Mg^{++} replaced	Antigen	20.4	9	100
Normal Tyrode's solution	Antigen	24.6	8	110
Normal Tyrode's solution	Tyrode's	3.6	0	0

TABLE VI

Effect of Succinate and Maleate on the Antigen-Induced Release of ECF-A and Histamine from Actively Sensitized Guinea Pig Lung Fragments

Suspending medium for lung fragments	Eosinophil chemotaxis (*mean cell count*)			Histamine release
	Volume of lung diffusate assayed			
	0.6 ml	0.3 ml	0.15 ml	
				μg/g
Antigen with 5 mM succinate in Tyrode's	30.8	30.2	16.2	3.0
Antigen with 5 mM maleate in Tyrode's	31.8	26.2	18.2	3.5
Antigen in Tyrode's (succinate)*	26.2	17.0	7.8	1.6
Antigen in Tyrode's (maleate)‡	25.0	16.2	8.0	1.6
Tyrode's solution	1.2	0.0	0.0	0.0

* Succinate (5 mM) added to chemotactic chamber.

‡ Maleate (5 mM) added to chemotactic chamber.

mM of succinate or maleate approximated the augmentation of histamine release (Table VI). When the usual volume of 0.6 ml of lung diffusate was used, the enhancement was less apparent than when smaller volumes were employed. The experiment was repeated using lung fragments passively sensitized for 2½ hr with a 1:50 dilution of IgG_1 (Fr 4); 5 mM succinate or maleate again strikingly enhanced the release of ECF-A and histamine. SRS-A was not measured in these enhancement experiments.

Differentiation of ECF-A from the Other Pharmacological Mediators of Ana-

TABLE VII

Experiments to Show that Histamine, Bradykinin, Serotonin, Prostaglandins E_1, E_2, and $F_{2\alpha}$ are not Chemotactic for Eosinophils Per Se and are not Responsible for the Release of these Agents

	Eosinophil chemotaxis
	mean cell count
Experiment 1	
Lung + antigen	27.1
Lung + Tyrode's	2.6
Lung + histamine (0.5 μg/ml)	3.0
Lung + histamine (5 μg/ml)	4.4
Histamine (0.5 μg/ml)	0.0
Histamine (5 μg/ml)	0.0
Experiment 2	
Lung + antigen	20.4
Lung + Tyrode's	0.0
Lung + bradykinin (0.01 μg/ml)	0.0
Lung + bradykinin (0.1 μg/ml)	0.0
Lung + bradykinin (1.0 μg/ml)	0.0
Bradykinin (1.0 μg/ml)	0.0
Lung + serotonin (0.01 μg/ml)	0.0
Lung + serotonin (0.1 μg/ml)	0.0
Lung + serotonin (1.0 μg/ml)	0.0
Serotonin (1.0 μg/ml)	0.0
Experiment 3	
Lung + antigen	17.0
Lung + Tyrode's	3.2
Lung + PGE_1 (0.01 μg/ml)	1.0
Lung + PGE_1 (0.1 μg/ml)	2.0
Lung + PGE_1 (1.0 μg/ml)	0.6
PGE_1 (1.0 μg/ml)	0.0
Lung + PGE_2 (0.01 μg/ml)	1.0
Lung + PGE_2 (0.1 μg/ml)	1.0
Lung + PGE_2 (1.0 μg/ml)	1.4
PGE_2 (1.0 μg/ml)	0.0
Lung + $PGF_{2\alpha}$ (0.01 μg/ml)	1.0
Lung + $PGF_{2\alpha}$ (0.1 μg/ml)	1.0
Lung + $PGF_{2\alpha}$ (1.0 μg/ml)	3.4
$PGF_{2\alpha}$ (1.0 μg/ml)	0.0
Experiment 4	
Lung + antigen	16.6
Lung + Tyrode's	0.0
Lung + SRS-A* (15 units/ml)	2.0
Lung + SRS-A (30 units/ml)	0.6
SRS-A (50 units/ml)	4.0

* The SRS-A was prepared by boiling an SRS-A ethanol extracted preparation in 0.05 N NaOH for 10 min. Before boiling in alkali the preparation had 50 units of SRS-A/ml and an eosinophil chemotactic count of 61.0.

The histamine release in micrograms per milliliter of lung diffusate was 0.5, 0.25, 0.35, and 0.13 in experiments 1, 2, 3, and 4, respectively.

phylaxis.—In order to determine whether ECF-A was in fact an already recognized pharmacological agent, various chemical mediators of anaphylaxis were tested directly for their ability to evoke the migration of eosinophils (Table VII). It was found that histamine, bradykinin, serotonin, and prostaglandins PGE_1, PGE_2, and $PGF_{2\alpha}$ were not chemotactic for eosinophils per se; and, furthermore, when incubated with sensitized lung in the absence of antigen, these agents did not secondarily affect the release of ECF-A. The 0.5 μg dose of histamine used in experiment 1 was comparable to the amount of histamine released from the same lung by specific antigen.

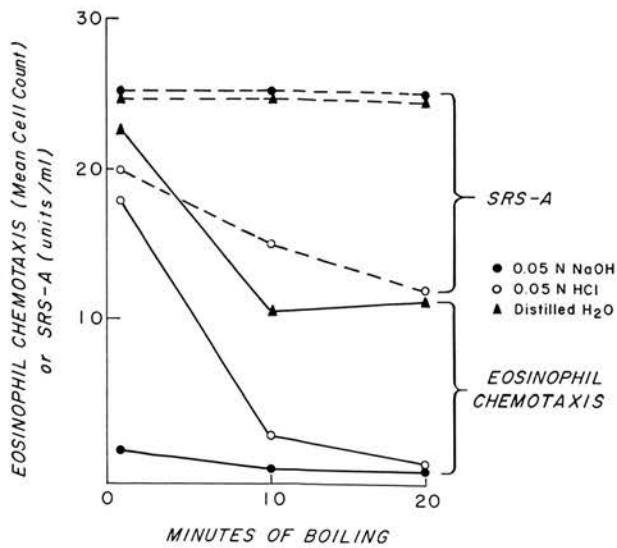


FIG. 6. Effect on ECF-A and SRS-A activity of boiling in acid or alkaline solutions.

Some crude preparations of SRS-A were chemotactic for eosinophils. These were obtained by extraction of lung diffusates in 80% ethanol, removal of the precipitate by centrifugation, and evaporation to dryness of the supernate under vacuum using a rotary evaporator. The extracted material was reconstituted in distilled water. It was possible, however, to differentiate ECF-A and SRS-A activity by boiling the crude material in alkaline solution. Since it had been previously reported that SRS-A survives boiling in 0.05 N NaOH but not in 0.05 N HCl (13), the behavior of ECF-A under these conditions was investigated. As seen in Fig. 6, ECF-A activity rapidly disappeared after boiling in acid or neutral solution and even more rapidly when boiled in alkali. In these experiments, the preparations were immediately adjusted to neutral pH before testing for SRS-A and chemotaxis. Thus by boiling in alkali, it was possible to destroy all ECF-A without influencing SRS-A activity (Table VII). It was also

possible to separate ECF-A from SRS-A by Sephadex G-25 chromatography (Fig. 7, *vide infra*). The peak of SRS-A activity was usually ill defined but appeared after ECF-A.

Differentiation of ECF-A from ECF-C.—ECF-A partially survived ethanol ex-

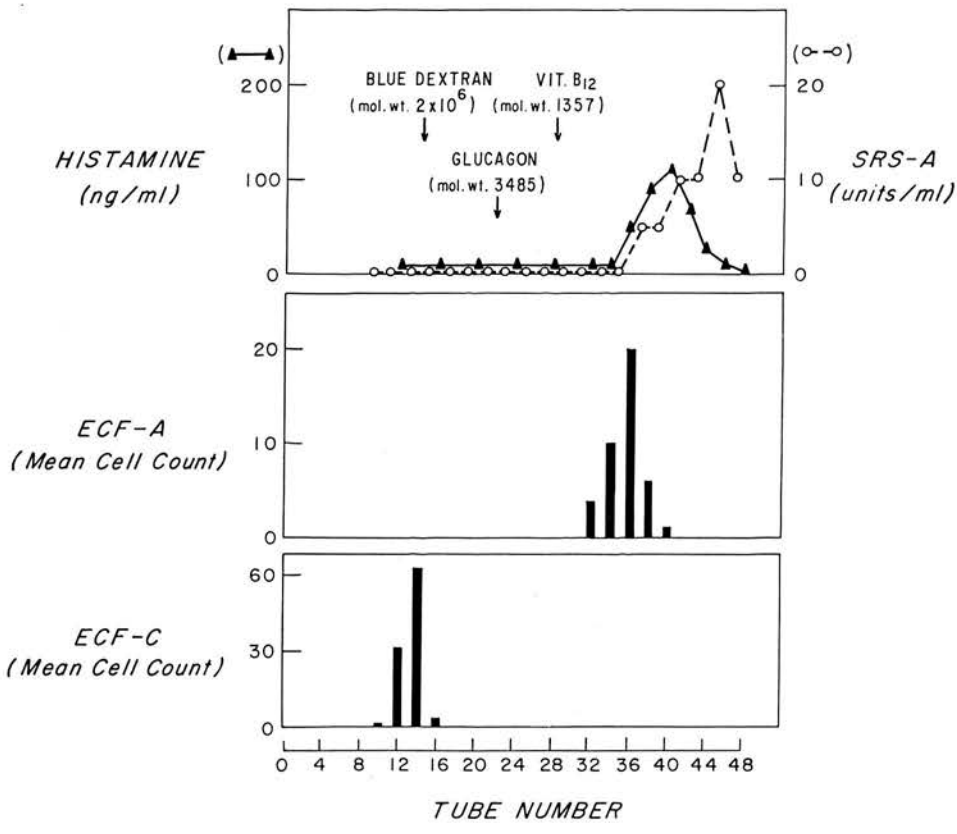


FIG. 7. Sephadex G-25 chromatography of anaphylactic lung diffusate and zymosan-treated guinea pig serum. Even numbered fractions from tubes 10–48 were tested for chemotaxis.

traction suggesting that its biological activity is not associated with a large molecule; for this reason, a lung diffusate having high chemotactic activity was applied to a column of Sephadex G-25. In each of six experiments, five using material from actively sensitized lung and one from a passively sensitized lung, the peak of eosinophil chemotactic activity eluted after the fractions containing the molecular marker glucagon (mol. wt. 3485) and vitamin B_{12} (mol. wt. 1357) but before histamine acid phosphate (mol. wt. 310) (Fig. 7). Thus, the molecular weight of ECF-A is thought to be approximately 500–1000.

In order to calculate the per cent recovery of ECF-A following passage through a Sephadex column, advantage was taken of the linear dose response for chemotaxis when dilutions are made of material containing ECF-A activity (Fig. 2). In this way, it was possible to obtain an arbitrary chemotactic value for an ECF-A preparation in relation to one preparation of target cells. When the same cells were tested against fractions of the ECF-A preparation, it was possible to demonstrate a 100% recovery of ECF-A activity after passage through a column of Sephadex G-25.

It has been shown that ECF-C, produced either by treating whole serum with antigen–antibody complexes (1) or with zymosan,[2] has a molecular weight of approximately 15,000. As shown in Fig. 7, ECF-C activity from zymosan-treated serum was excluded by Sephadex G-25.

DISCUSSION

The capacity of actively (Table I) or passively (Fig. 3) sensitized guinea pig lung to react with antigen to release a factor chemotactic for eosinophil leukocytes (ECF-A) has been demonstrated. In all experiments, the release of ECF-A was also accompanied by the release of both histamine and SRS-A. Indeed, the release of all these mediators exhibited a similar reponse in terms of the time course of passive sensitization (Fig. 4), the effect of antigen dose (Fig. 5), and the time course of release. Furthermore, the release of ECF-A was dependent on divalent cations (Table V) and was strikingly enhanced by the presence of succinate or maleate (Table VI). These features are again characteristic of the release of histamine and SRS-A (12, 14).

It has previously been demonstrated that guinea pig IgG_1 can passively sensitize guinea pig lung slices for the antigen-induced release of histamine (6) and SRS-A (7), and this same immunoglobulin has been shown to mediate the release of ECF-A (Fig. 3). The fractions which prepared tissue for ECF-A release (Fr 4 and 5) lost no activity after heating at 56°C for 4 hr (Table III), a property characteristic of IgG_1 but not of an IgE-like immunoglobulin. The IgG_2-containing fractions, having hemagglutinating activity virtually equal to that of IgG_1 fractions, failed to prepare lung fragments for the antigen-induced release of histamine, SRS-A, or ECF-A (Fig. 3).

Since guinea pig IgG_1 appears capable of mediating the release of several pharmacologic agents, it was necessary to exclude the possibility that ECF-A was an already recognized chemical mediator or was released secondary to the presence of other reaction products recognized herein. Histamine, SRS-A, and the prostaglandins PGE_1, PGE_2, and $PGF_{2\alpha}$ were not eosinophilotactic per se; neither was ECF-A detected after the incubation of these agents with sensitized lung in the absence of antigen (Table VII). Studies with sensitized guinea pig lung (15, 16) have shown the release of kallikrein after antigen challenge; this enzyme was not studied herein, but it was demonstrated that the product of its

reaction with kininogen, namely bradykinin, was not itself chemotactic for eosinophils and did not secondarily affect the release of ECF-A (Table VII). Similar findings were observed with serotonin (Table VII).

Since eosinophilotactic activity could still be detected after extraction of anaphylactic lung diffusate in 80% ethanol and evaporation to dryness, the possibility was considered that SRS-A contained chemotactic activity. Reasons for considering ECF-A as separate from SRS-A are that SRS-A survived boiling in alkaline solution for 20 min whereas ECF-A activity was abolished by this procedure (Fig. 6); and in addition SRS-A and ECF-A could be separated by gel filtration (Fig. 7).

The finding that ECF-A generation is a consequence of antigen–antibody interaction and dependent upon the presence of divalent cations raises the question of whether it is identical with the eosinophilotactic factor formed in guinea pig serum following treatment with immune complexes (1) or zymosan.[2] ECF-C produced in vitro by antigen–antibody complexes, prepared either with IgG_1 or IgG_2 and antigen, requires the participation of complement since ECF-C is not generated from serum heated at 56°C for 30 min or from ammonia-treated serum or from serum activated in the presence of 0.01 M EDTA (1). No evidence was found, however, that ECF-A generation was dependent on an intact complement system. Guinea pig lung, perfused free of visible blood and then cut in slices and thoroughly washed was capable of being passively sensitized with an IgG_1-containing fraction (even after heating for 4 hr at 56°C) for the subsequent antigen-induced release of ECF-A (Table III, Fig. 3). Furthermore, depleting the donor animals of 95% of the circulating complement level had no effect on the capacity of their isolated lung fragments to yield ECF-A after passve sensitization and antigen challenge (Table IV). Decomplementation was accomplished by the administration of purified cobra venom factor, a procedure which depletes C3 and to a lesser extent the complement components acting thereafter (17, 18). This maneuver also had no effect on the antigen-induced release of ECF-A from lung fragments from actively sensitized animals (Table IV).

ECF-A and ECF-C can be distinguished not only in terms of the complement requirement for their generation but also by their apparent size differences as determined by gel filtration. On Sephadex G-25, ECF-C is excluded as shown by the molecular marker blue dextran (Fig. 7), consistent with its previously reported molecular weight of approximately 15,000 (1). In contrast, ECF-A appeared after the molecular marker vitamin B_{12} (mol. wt. 1357) but before histamine acid phosphate (mol. wt. 310), suggesting a molecular weight of approximately 500–1000 (Fig. 7). It should further be noted that the complete recovery of ECF-A in the diffusate introduced onto the Sephadex column indicated that the eosinophilotactic activity of the lung diffusate is unlikely to be a mixture of molecules having appreciable size differences.

In a cell suspension containing predominantly neutrophils, only eosinophils migrated towards ECF-A whereas zymosan-treated serum, which contains both ECF-C and NCF-C (1),[2] attracted both neutrophils and eosinophils in the same proportion as was present in the original cell suspension (Table II). Thus, ECF-A represents a hitherto undescribed agent which, like ECF-C, selectively attracts eosinophil leukocytes.

SUMMARY

The capacity of actively or passively sensitized guinea pig lung to react with antigen to release a factor specifically chemotactic for eosinophil leukocytes (ECF-A) has been demonstrated. The release of ECF-A was also accompanied by the elaboration of both histamine and SRS-A and the appearance of all these mediators exhibited a similar response in terms of the time course of passve sensitization, the effect of antigen dose, the time course of release, divalent cation dependence and enhancement by the presence of succinate or maleate. Decomplementation by the administration of purified cobra venom factor had no effect on the antigen-induced release of ECF-A from actively or passively sensitized lung fragments.

When fragments of guinea pig lung were passively sensitized with fractions of guinea pig 7S IgG, only the IgG_1-containing fractions prepared tissue for the antigen-induced release of ECF-A. Histamine, SRS-A, bradykinin, serotonin, and the prostaglandins PGE_1, PGE_2, and $PGF_{2\alpha}$ were not eosinophilotactic per se; neither was ECF-A detected following the incubation of these agents with sensitized lung in the absence of antigen.

Both eosinophilotactic activity and SRS-A survived extraction in 80% ethanol and evaporation to dryness. SRS-A, however, withstood boiling in alkaline solution for 20 min, whereas ECF-A activity was abolished by this procedure. SRS-A and ECF-A could also be separated by gel filtration. ECF-A activity was completely recovered following its passage through a column of Sephadex G-25 and had an estimated molecular weight of between 500 and 1000. On the basis of size and a formation mechanism independent of the complement system, ECF-A is distinguishable from a previously described complement-dependent eosinophilotactic factor (ECF-C). Thus, ECF-A represents a hitherto undescribed agent which selectively attracts eosinophil leukocytes.

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The IgE-Mediated Release of an Eosinophil Leukocyte Chemotactic Factor from Human Lung[1]

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We have recently demonstrated the capacity of actively or passively sensitized guinea-pig lung to react with antigen to release a factor specifically chemotactic for guinea-pig eosinophil leukocytes (ECF-A) (1). The antibody mediating the release of ECF-A was shown by fractionation studies to be of the immunoglobulin class IgG_1, which also prepares guinea-pig tissue for passive cutaneous anaphylaxis (PCA) and the release of histamine and slow reacting substance of anaphylaxis (SRS-A) from lung fragments. Histamine, SRS-A and other chemical mediators of anaphylaxis failed to attract eosinophils. ECF-A was released from lung tissue by a mechanism independent of the complement system, had an estimated molecular weight of between 500 and 1000, and therefore differed from a previously described complement-dependent eosinophilotactic factor (ECF-C) (2).

The present report describes the recognition of a comparable material from human lung, the release of which is shown to be mediated by IgE.

MATERIALS AND METHODS

Histamine acid phosphate, serotonin creatinine sulphate, bradykinin triacetate, prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ and ovalbumin were obtained as previously described (1), as was the source of ragweed antigen E and the serum from the ragweed-sensitive patient EW (3). Heparin (1000 units/ml) was purchased from Organon, Inc., West Orange, N. J. Serum from a ragweed-sensitive individual AR, serum AR absorbed with rabbit anti-IgE and monospecific rabbit anti-IgE and anti-IgG were generously supplied by Dr. K. Ishizaka. Serum AR was absorbed with the γ globulin fraction of monospecific rabbit anti-IgE coupled to Sepharose 2B (Mann Research Laboratories, New York, N. Y.) according to a previous report (4). PCA reactions in monkey skin and the measurement of IgE were carried out as previously described (5, 6). Human plasma kallikrein was prepared by diethylaminoethyl (DEAE) cellulose chromatography (7).

Human lung, obtained at pneumonectomy, was dissected free of pleura, bronchi and large blood vessels, sliced into 50- to 100-mg fragments by hand, and washed with Tyrode's solution until the surrounding fluid was free of blood. Fragments were divided into 300-mg portions using an overhead balance, suspended in 2-ml volumes of diluted serum or Tyrode's buffer, and passively sensitized for 18 hr at room temperature. The sera from the ragweed-sensitive individuals EW and AR were diluted 1:4 and 1:5, respectively. A 1:4.5 dilution of serum AR absorbed with anti-IgE was used to approximate the equivalent IgG concentration of the unabsorbed serum. Following the sensitization period, the lung fragments were washed three times with Tyrode's solution and resuspended in 3.0 ml Tyrode's buffer containing 0.2 μg ragweed antigen E/ml. Lung diffusates were harvested 45 min after antigen challenge, placed on ice, and assayed the same day for histamine and SRS-A as previously described (8) and for chemotaxis as indicated below. Some diffusates were tested after a single freezing at $-70°C$ and rapid thawing. Sephadex G-25 chromatography of anaphylactic diffusate was performed as described (1).

Chemotaxis was measured by a modification of the Millipore technique of Boyden (2). Human peripheral blood, rich in eosinophils, was drawn into a syringe containing 50 units of heparin/ml and transferred to plastic test tubes. Red blood cells were allowed to settle for 90 min at 37°C, after which the leukocyte-rich supernatant was removed and centrifuged for 5 min at $100 \times G$. The cells were washed once, resuspended in Tyrode's solution containing 0.5% ovalbumin, and adjusted to a final concentration of 1.5×10^6

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[2] T. K. Stubbins Research Fellow, Royal College of Physicians of London, and recipient of a Wellcome Research Travel Grant and a McCunn (Medical Research Traveling) Scholarship.

TABLE I

The selective chemotactic activity of human lung anaphylactic diffusate for human peripheral blood eosinophils

Donor	Diagnosis	Cells in Test Compartment Prior to Migration %		Chemotactic Response to Lung Diffusate (Mean Cell Count)		Chemotactic Response to Kallikrein (Mean Cell Count)	
		Eosinophils	Neutrophils	Eosinophils	Neutrophils	Eosinophils	Neutrophils
G. R.	Rheumatoid arthritis	73	19	28	1	2	38
A. K.	Hay fever	8	40	23	10	0	35
J. N.	Drug hypersensitivity	20	52	20	2	2	>50
F. F.	Carcinomatosis and possible drug hypersensitivity	14	68	31	2	2	36
D. P.	Extrinsic asthma	14	48	20	17	0	>50
K. T.	Extrinsic asthma	11	52	10	2	0	>50

leukocytes/ml. Chemotactic experiments were performed using a 3.0 μ Millipore filter. In most experiments cells from the patient G.R. were used (Table I). Unless stated otherwise, 0.9 ml volumes of lung diffusate to which were added 0.1 ml of 5% ovalbumin were tested for chemotaxis. Three-tenths-milliliter volumes of the kallikrein preparation were used to which were added 0.6 ml of Tyrode's solution and 0.1 ml of 5% ovalbumin. The chemotactic counts were expressed as the mean cell count of five high-power fields (1, 2).

RESULTS

The anaphylactic diffusate from human lung, passively sensitized with sera from ragweed-sensitive individuals and subsequently challenged with ragweed antigen E, contained histamine, SRS-A and a factor that selectively attracted eosinophil leukocytes (ECF-A). Peak release of ECF-A was observed 15 to 45 min after antigen challenge. A linear dose response curve was obtained when dilutions of the anaphylactic diffusate were tested for chemotaxis (Fig. 1). No chemotactic activity was detected in the diffusates of passively sensitized lung challenged with Tyrode's solution or of unsensitized lung incubated with ragweed antigen E.

The selectivity of ECF-A for peripheral blood eosinophils obtained from patients with an eosinophilia due to a variety of causes is shown in Table I. In all of the cases investigated the cells migrating towards the anaphylactic diffusate were predominantly eosinophils, whereas when the same cell suspensions were tested against kallikrein only neutrophil chemotaxis was observed (Table I).

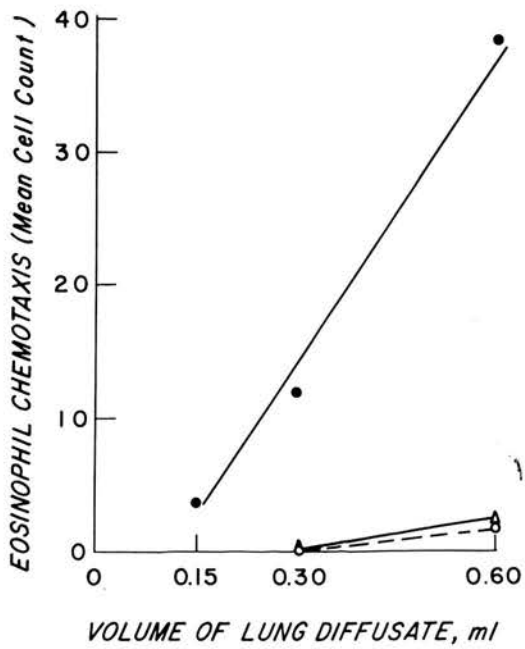

Figure 1. The specific antigen-induced release of an eosinophil chemotactic factor from passively sensitized human lung. The lung diffusate showed a chemotactic dose-response curve. The perfusate from lung sensitized with antibody and challenged with antigen (●) also contained 600 ng/ml of histamine and 200 units/ml of SRS-A. No mediator release was detected with unsensitized lung incubated with antigen (○) or sensitized lung incubated with Tyrode's solution (△).

ECF-A was distinguished from histamine, serotonin, bradykinin and the prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ since these agents alone, in concentrations of 0.01, 0.1 and 1.00 μg/ml, were unable to affect eosinophil migration

in vitro. Because of their similar size, human ECF-A and SRS-A could not be fully separated by passage through a column of Sephadex G-25. The stability of ECF-A and SRS-A differed (Table II). ECF-A was preserved following lyophilization and boiling in acid solution, whereas 50% of SRS-A activity was lost. In contrast, ECF-A did not survive boiling in alkaline solution whereas SRS-A activity was fully retained.

The evidence that IgE was the immunoglobulin class which prepared human lung for the antigen-induced release of ECF-A included heat lability, loss of effect with specific absorption and response of the lung tissue to anti-IgE. Serum EW, heated for 4 hr at 56°C, was compared with unheated serum for its ability to prepare lung fragments for antigen-induced mediator release. Unheated serum gave an eosinophil chemotactic count of 38 and released 3.5 μg/g of histamine and 500 units/g of SRS-A. The corresponding figures for heated serum were 3, 0.03 and 0, respectively. When serum AR was selectively absorbed with a rabbit antiserum to IgE, it no longer passively sensitized lung fragments for the subsequent antigen-induced release of ECF-A, histamine and SRS-A (Table III). Absorption removed more than 99% of the total IgE and the serum no longer sensitized monkey skin for passive cutaneous anaphylaxis; the levels of IgG and IgA were virtually unchanged. The release of ECF-A, histamine and SRS-A was also affected by the reversed-type reaction employing a specific antibody to IgE prepared in the rabbit. When lung was incubated with anti-IgE at a concentration of 0.1 μg protein/ml, the diffusate gave an eosinophil chemotactic count of 13.8 and contained 1.85 μg of histamine/g of lung and 800 units/g of SRS-A. Anti-IgE alone was not chemotactic. Lung incubated with a specific anti-IgG, even at a protein concentration 10 times that of anti-IgE, did not affect mediator release. With human lung the release of chemical mediators by anti-IgE did not require prior passive sensitization of tissue with reaginic serum, presumably because IgE was present on the tissue.

TABLE II

The effect of ECF-A and SRS-A in human lung anaphylactic lung diffusate of lyophilization or boiling in acid or alkaline solution

	Eosinophil Chemotaxis (Mean Cell Count)	SRS-A Activity
		units/ml
Starting material	19	100
Lyophilization	23	50
Boiling in 0.05 N HCl (10 min)	20	40
Boiling in 0.05 N NaOH (10 min)	2	100

DISCUSSION

The ability of passively sensitized human lung to release a factor specifically chemotactic for human eosinophils (ECF-A) has been demonstrated following challenge with specific antigen (Fig. 1.). A linear dose-response curve was obtained when dilutions of the anaphylactic diffusate were tested for chemotaxis. Peripheral blood eosinophilia in relation to a variety of clinical problems reacted similarly to human lung anaphylactic diffusates (Table I). In contrast, only the neutrophils from these patients migrated towards a partially purified plasma kallikrein preparation, as previously described (9).

Human ECF-A was distinguished from histamine, bradykinin, serotonin and the prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ since these agents were not chemotactic *per se*. After treatment of the lung diffusate with 80% ethanol and evaporation to dryness, the reconstituted material contained SRS-A and ECF-A activity

TABLE III

The capacity of IgE to mediate the release of ECF-A, histamine and SRS-A from human lung

Serum Used for Sensitization	Immunoglobulin Concentration			PCA Titer (Monkey Skin)	Eosinophil Chemotaxis (Mean Cell Count)	Histamine Release	SRS-A Release
	IgG	IgA	IgE				
	mg/ml	*mg/ml*	*μg/ml*			*μg/g*	*units/g*
Unabsorbed	15.50	2.12	7.40	128	15.2	3.25	750
Absorbed	13.50	1.80	0.05	0	0	0	0
None					0	0	0

which chromatographed together on Sephadex G-25 in the same region as guinea-pig SRS-A (1). Human SRS-A and ECF-A could, however, be separated functionally since the former was partially destroyed by lyophilization and boiling in acid solution and the latter by boiling for 10 min in alkaline solution (Table II).

The antigen-induced release of ECF-A from human lung was mediated by IgE. Serum absorbed with anti-IgE no longer passively sensitized lung for the antigen-induced release of ECF-A, histamine or SRS-A (Table III), and anti-IgE but not anti-IgG released ECF-A, histamine and SRS-A from normal lung.

SUMMARY

Following antigenic challenge of human lung passively sensitized with serum from a ragweed-sensitive donor, the anaphylactic diffusate contained, in addition to histamine and slow reacting substance of anaphylaxis (SRS-A), a factor selectively chemotactic for human eosinophils (ECF-A). ECF-A evoked the migration of eosinophils obtained from the blood of a number of donors with eosinophilia. ECF-A was distinguished from histamine, bradykinin, serotonin, the prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ and from SRS-A. The immunoglobulin mediating the release of ECF-A was IgE. Heating or absorption of ragweed-sensitive serum with a rabbit antibody specific for IgE removed its ability to passively sensitize lung for mediator release, and anti-IgE released ECF-A from normal lung. ECF-A, a unique mediator of the anaphylactic reaction, has been identified in human lung tissue.

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Selective Attraction of Eosinophils and Synergism between Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A) and a Fragment Cleaved from the Fifth Component of Complement (C5a)

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Summary. ECF-A and C5a were chemotactic both for eosinophils and neutrophils. However, when eosinophils comprised approximately 10 per cent or more of a mixed leucocyte population, they were preferentially attracted by both of these agents.

Marked synergism was observed between ECF-A and C5a in their ability to attract eosinophil leucocytes.

INTRODUCTION

In previous reports, two chemotactic factors have been described which selectively attract eosinophil leucocytes from a mixed cell population. The first factor (ECF-C) required the presence of an intact complement system and was generated by incubating fresh guinea-pig serum with antigen–antibody complexes prepared from homologous IgG_1 or IgG_2 (Kay, 1970). By Sephadex G-100 chromatography, it was shown that ECF-C had an estimated molecular size of approximately 15,000 and was thought therefore to be identical to a fragment cleaved from the fifth component of complement (C5a). The second factor, an eosinophil chemotactic factor of anaphylaxis (ECF-A) was released by antigen challenge of actively sensitized lung or lung passively sensitized with IgG_1 (Kay, Stechschulte and Austen, 1971). The release of ECF-A did not require the presence of complement and, on the basis of a molecular size of between 500 to 1000, it was shown to be distinct from ECF-C.

C5a is also known to attract neutrophils (Shin, Snyderman, Friedman, Mellors and Mayer, 1968; Ward and Newman, 1969) and monocytes (Snyderman, Shin and Hausman, 1971; Hausman, Snyderman and Mergenhagen, 1972), and a purpose of the present report is to extend studies on the chemotaxis of eosinophils using C5a derived from highly purified C5 and to examine further selective chemotaxis of eosinophils by ECF-A. In addition, studies have been conducted on the relationship between ECF-A and C5a in terms of their ability to act synergistically in eosinophil chemotaxis.

MATERIALS AND METHODS

Materials were obtained as follows. Histamine acid phosphate, cytochrome C (BDH Chemicals Ltd, Poole, England); blue dextran (Pharmacia Fine Chemicals, Uppsala, Sweden); vitamin B_{12} (Glaxo Laboratories, Ltd, Greenford, England); glycogen, ovalbumin five times crystallized (Koch-Light Laboratories, Colnbrook, England); and horse serum (Wellcome Reagents Ltd, Beckenham, England).

Preparation of ECF-A

Guinea-pig anaphylactic lung diffusate was obtained from actively sensitized guinea-pig lung perfused free of blood and challenged with ovalbumin as previously described (Kay *et al.*, 1971). The cell free diffusate was extracted in 80 per cent ethanol, freed of precipitate by centrifugation, evaporated to dryness under vacuum using a rotary evaporator, reconstituted to one-tenth of the volume of the starting material in distilled water and centrifuged again at 15,000 *g* for 30 minutes. One millilitre of the concentrated lung diffusate was applied to a column of Sephadex G-25 (95 × 3·5 cm) and alternate 2-ml fractions tested for eosinophil and neutrophil chemotaxis using 0·2-ml volumes. For calibrating the column, the tubes containing the highest concentration of blue dextran and vitamin B_{12} were read visually. Histamine and slow reacting substance of anaphylaxis (SRS-A) were assayed as previously described (Kay *et al.*, 1971).

Preparation of C5a

Guinea-pig C5 was prepared by isoelectric precipitation, diethylaminoethyl (DEAE) and carboxylmethyl (CM)—cellulose chromatography and hydroxylapatite chromatography as previously described (Cook, Shin, Mayer and Laudenslayer, 1971). The C5 preparation contained approximately 400 μg of protein per ml and was treated with 4 μg of trypsin for 30 minutes at 25°. The reaction was stopped with 8 μg of soybean trypsin inhibitor (SBTI). The total volume of trypsin and SBTI added was 1/50 of the volume of C5. This mixture was used as the source of C5a. In experiments designed to show that C5a was chemotactic for both eosinophils and neutrophils, 0·3 ml of trypsinized C5 was applied to a column of Sephadex G-100 (40 × 1·0 cm) and 1-ml fractions collected.

Measurement of chemotaxis

A modification of the Millipore technique of Boyden was used as previously described (Kay, 1970). Guinea-pig eosinophils were obtained by peritoneal lavage from animals which had received multiple injections of horse serum. Neutrophils were harvested from peritoneal cavity of animals injected with glycogen 3–6 hours previously. Eosinophil and neutrophil migration were both measured using an 8·0-μ pore size, since previous studies with guinea-pig peritoneal cells had shown that this pore size gave full expression of eosinophil and neutrophil chemotaxis towards ECF-A and 'activated guinea-pig serum'. Under these conditions background counts with diluent alone were zero. Neutrophils or eosinophils were used at a concentration of 2×10^6 cells/ml. Tyrode solution containing 0·5 per cent ovalbumin was used as the diluent throughout.

RESULTS

EOSINOPHIL AND NEUTROPHIL CHEMOTACTIC ACTIVITY OF ECF-A AND C5a

In previous studies we have shown that compared with eosinophils, there was minimal

chemotaxis of neutrophils towards an anaphylactic lung diffusate (Kay *et al.*, 1971). When the concentrated anaphylactic diffusate was applied to a column of Sephadex G-25, neutrophil and eosinophil chemotactic activity eluted in the same position thus providing some evidence that both activities were a function of ECF-A (Fig. 1).

When trypsinized C5 was applied to a column of Sephadex G-100, eosinophil and neutrophil chemotactic activity eluted together (Fig. 2). The peak of activity for both cell types eluted at approximately the same position as the cytochrome C marker (mol. wt 12,270), a molecular size which closely corresponds to C5a (mol. wt approximately 15,000)

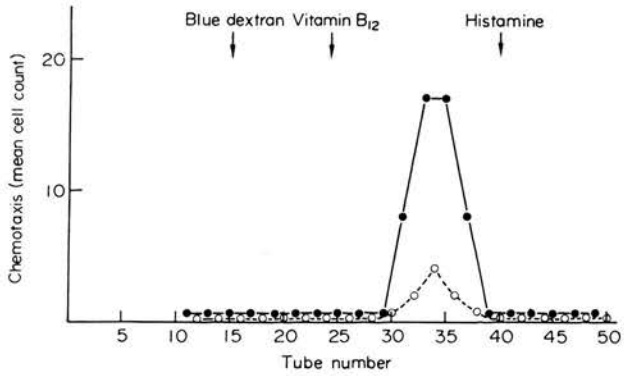

FIG. 1. Sephadex G-25 chromatography of an anaphylactic diffusate. Even numbered tubes were tested for (○) neutrophil chemotaxis and odd numbered tubes tested for (●) eosinophil chemotaxis. Although neutrophil response was minimal to ECF-A, the cell preparation gave a count of 80 towards 10 μg of trypsinized C5.

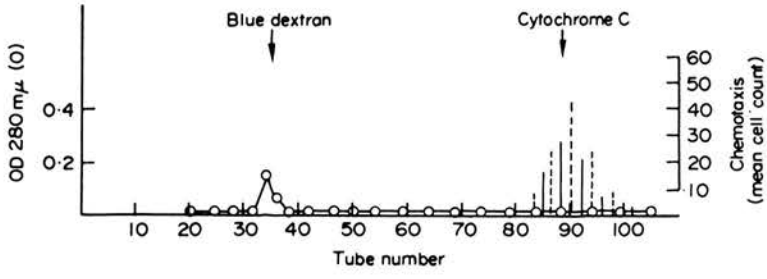

FIG. 2. Sephadex G-100 chromatography of trypsinized C5 showing the chemotactic response of fractions towards (——) eosinophils and (– – –) neutrophils.

ECF-A free of antigen, histamine, and SRS-A after Sephadex G-25 gel-filtration and C5a derived from highly purified C5 were then tested for their ability to attract eosinophils and neutrophils. The neutrophil suspension contained less than 1 per cent of eosinophils. The eosinophil preparation contained 52 per cent eosinophils, the remainder being only mononuclear cells. The cell counts were adjusted so that each preparation had the same number of eosinophils and neutrophils respectively. The total white cell count of the eosinophil preparation was therefore approximately twice that of the neutrophil suspension. The ability of these two cell preparations to migrate towards C5a is shown in Fig. 3. About ten times as many neutrophils migrated compared to eosinophils. With ECF-A,

however, eosinophil chemotaxis was predominant; very little neutrophil chemotaxis being noted even with the highest concentration of ECF-A (Fig. 3).

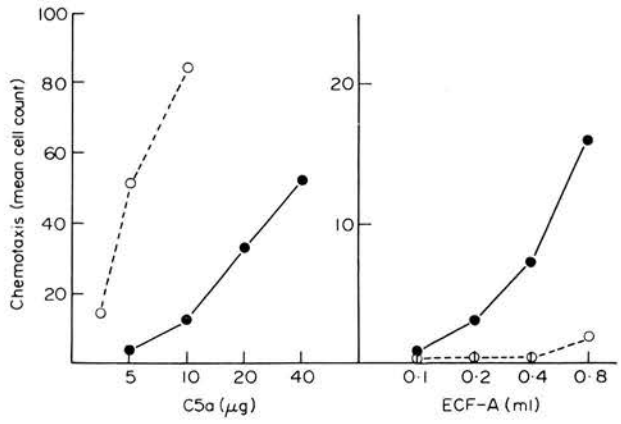

FIG. 3. The capacity of (○) neutrophils and (●) eosinophils to respond in chemotaxis to increasing doses of C5a and ECF-A. No chemotaxis was observed with untreated C5 or with the trypsin/SBTI mixture. The weight of C5a is expressed as that of the untreated C5.

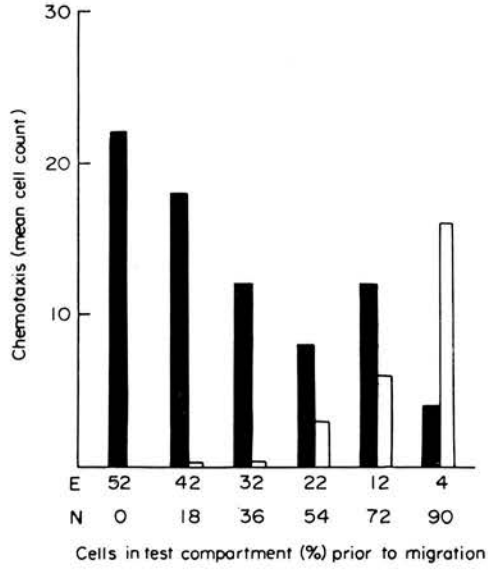

FIG. 4. The effect of alterations in the percentage of (■) eosinophils (E) and (□) neutrophils (N) in the test compartment on the chemotactic response to 0·8 ml of ECF-A.

THE EFFECT OF VARIATIONS IN PERCENTAGE OF EOSINOPHILS AND NEUTROPHILS

An eosinophil preparation containing no neutrophils was mixed in varying proportions with a suspension of neutrophils having only 4 per cent of eosinophils. When these cell preparations and their mixtures were tested against ECF-A, it was found that as the percentage of eosinophils increased this cell type was selectively attracted (Fig. 4). The

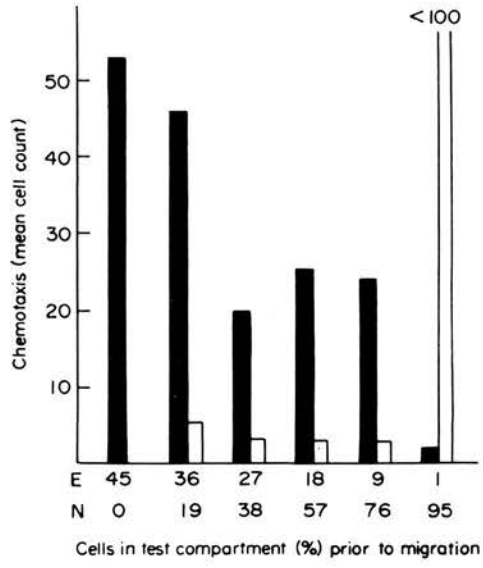

FIG. 5. The effect of alterations in the percentage of (■) eosinophils (E) and (□) neutrophils (N) in the test compartment on the chemotactic response to C5a derived from 40 μg of C5.

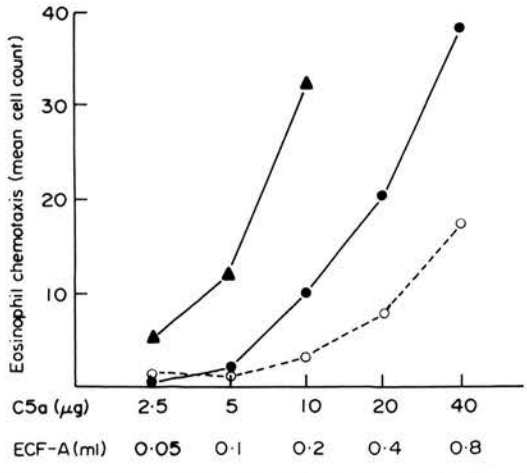

FIG. 6. Synergism between (▲) ECF-A and C5a. A dose response for (●) C5a, (○) ECF-A and a mixture of the two. The concentration of C5a is defined as in Fig. 3.

threshold for predominant eosinophil attraction was as low as 12 per cent. A similar effect was found with C5a (Fig. 5) with the threshold being 9 per cent in the experiment depicted. When there was no competition from eosinophils, neutrophils migrated toward both C5a and ECF-A.

SYNERGISM BETWEEN ECF-A AND C5a

Since ECF-A and C5a are distinct both in their molecular size and formation mechanism, it was of interest to determine the effect of combining the two agents in eosinophilotaxis. As seen in Fig. 6, the cell counts obtained after mixing were three times or greater

than that which would have been expected by summation of counts when the agents were assayed alone. This suggested that ECF-A and C5a acted synergistically in their ability to attract eosinophils.

More evidence of synergism was obtained in the experimental designs shown in Fig. 7. Chemotaxis towards ECF-A was examined in a dose-response fashion with and without the presence of a dose of C5a which gave a low chemotactic count. The counts obtained with the mixtures were far higher than would have been expected by summation (Fig. 7a). A dose-response study of C5a with a low concentration of ECF-A again revealed an increase in chemotactic counts far greater than would have been expected by addition. Since the preparation of C5a contained a trace of trypsin and SBTI, it was necessary to determine that this enzyme and inhibitor were not contributing to the synergism observed with ECF-A. Trypsin and SBTI in equivalent concentrations had no effect on the dose-response obtained with increasing dilutions of ECF-A.

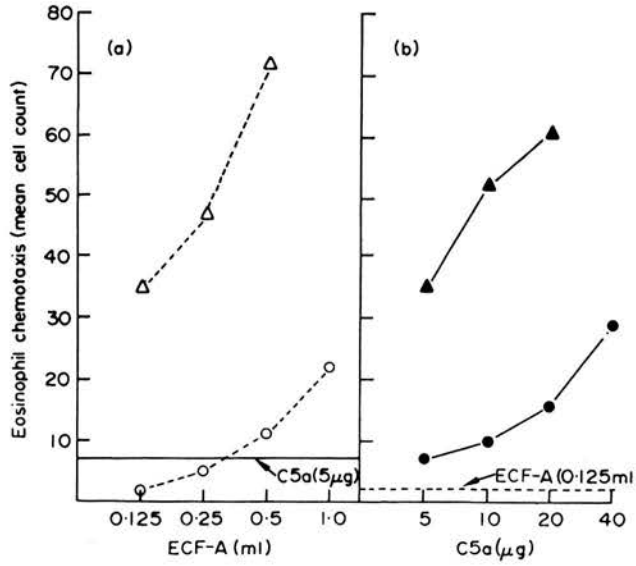

FIG. 7. Synergism between ECF-A and C5a. (a) Dose response for ECF-A (○) is compared with the same volumes of ECF-A to which 5 μg of C5a (△) giving a chemotactic count of 8 was added. (b) A dose response of C5a (●) is compared with the same volumes of C5a to which 0·125 ml of ECF-A (▲) giving a chemotactic count of 2, was added. The concentrations of C5a are defined as in Fig. 3.

DISCUSSION

Chromatographic separation of ECF-A and C5a (Figs 1 and 2) demonstrated that both eosinophil and neutrophil chemotactic activity eluted at the same bed volume characteristic for the size of these molecules (mol. wt approximately 1000 and 15,000 respectively).

When ECF-A and C5a were examined in a dose-response fashion against a suspension of cells which contained greater than 95 per cent of neutrophils this cell type migrated towards C5a whereas neutrophils migrated only in small numbers to ECF-A (Fig. 3). When eosinophils were examined in the same manner using a suspension of cells containing 52 per cent eosinophils and 48 per cent mononuclear cells, migration was demonstrated in a dose-response fashion towards both factors (Fig. 3). Under these conditions

the neutrophil was attracted in larger numbers, however, attraction of eosinophils to C5a may have been impaired, in part, by the presence of the mononuclear cells.

The highest doses of C5a and ECF-A shown in Fig. 3 were then examined for their effect on various mixtures of eosinophils and neutrophils. When eosinophils comprised only about 10 per cent of the target-cell population they were selectively attracted by either ECF-A (Fig. 4) or C5a (Fig. 5). The preferential attraction of eosinophils by ECF-A and C5a was particularly striking when eosinophils and neutrophils were present in the cell compartment in approximately equal numbers. In both situations there was marked eosinophil migration whereas neutrophil chemotaxis was virtually absent. The experimental design illustrated in these studies involved dilution of either eosinophils or neutrophils by the other cell types. Dilution is not considered to account for the selective eosinophil migration since previous studies (Kay, unpublished observations), have shown that each cell type would respond maximally at the lowest concentration used in these studies. It should be noted that the summation of eosinophil and neutrophil responses is less for mixtures than for the original suspensions in which one or the other of the cell types predominates.

A broad specificity of chemotactic factors can be demonstrated when conditions for migration are optimal and when other cells which may be preferentially attracted are absent. Thus, in unusual cases of basophilia in association with chronic leukaemia, basophils migrated in small numbers towards C5a and an anaphylactic diffusate containing ECF-A (Kay and Austen, 1972). In this situation, the conditions for basophil chemotaxis were optimal since there was little competition from other cell types. In addition, mononuclear cell chemotaxis has been demonstrated with C5a when this cell type predominated in the cell chamber (Snyderman *et al.*, 1971; Hausman *et al.*, 1972). The present studies confirm the report of Shin *et al.* (1968) and Ward and Newman (1969), who described neutrophil chemotaxis to C5a. In addition, conditions have been demonstrated under which eosinophil chemotaxis can predominate.

Synergism between ECF-A and C5a was shown in three sets of experiments. When C5a and ECF-A, which when tested alone gave low cell counts, were mixed together, the resultant chemotactic counts were three times or more than would have been expected by summation alone (Fig. 6). When a low dose of C5a was added to increasing doses of ECF-A, the resultant increase in chemotaxis was also three times or more than would have been expected by addition of the counts (Fig. 7a). Virtually identical results were obtained when ECF-A was added to increasing doses of C5a (Fig. 7b). It is possible that eosinophils have more than one receptor for chemotaxis and that, if different types of receptors are stimulated at a low threshold, this produces an increased chemotactic response. These observations on synergism may be of significance in parasitic infestations, many of which are associated with a pronounced eosinophilia. Homocytotropic antibody and complement-fixing antibody occur together in a variety of parasitic diseases, situations in which ECF-A and C5a might act together.

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The Effect of Enzyme Digestions on the Activity of Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A)

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Summary. Preparations of ECF-A derived from anaphylactic guinea-pig lung diffusates were subjected to a variety of enzymatic degradations. The enzymes employed had specificity only for their appropriate substrates. No effect was found following treatment with relatively high doses of trypsin, α-chymotrypsin, pronase, alkaline phosphatase and sialidase. In contrast a loss of activity was demonstrated in a dose-dependent fashion following incubation with tyrosinase, aryl sulphatase and leucine aminopeptidase, suggesting that ECF-A contains a phenolic hydroxyl group, a sulphate ester and a peptide linkage with a free α-amino group.

INTRODUCTION

The antigen challenge of sensitized lung leads to the release of histamine, slow-reacting substance of anaphylaxis and an eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay, Stechschulte and Austen, 1971). ECF-A selectively attracts eosinophils from a mixed leucocyte population and has an estimated molecular size of between 500 and 1000 (Kay, Shin and Austen, 1973). Preliminary data suggested that ECF-A has the characteristics of a peptide (Kay, Stechschulte, Kaplan and Austen, 1971). In the present study we have attempted to gain further insight into the chemical nature of ECF-A by incubating a partially purified preparation with a variety of enzymes of known specificity. With each enzyme preparation particular care was taken to estimate the presence of other enzymatic activities.

MATERIALS AND METHODS

Materials for the chemotactic assays were obtained as previously described (Kay, Shin and Austen, 1973). The source of enzymes is shown in Table 1. The enzyme substrates were obtained from Sigma Chemical Company (St Louis, Missouri), with the exception of *N*-acetyl-neuraminyl-lactose which was obtained from Calbiochem, (La Jolla, California), as was the buffer 2-(*N*,-morpholino)-ethanesulphonic acid (MES).

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TABLE 1

SOURCES OF ENZYMES AND METHODS FOR THEIR ASSAY

Name	E.C. number	Source and grade	Assay method*
Trypsin	3.4.4.4	Calbiochem, A Grade	Formation of TCA-soluble material from casein (Kunitz) absorbing at 280 nm
α-Chymotrypsin	3.4.4.5	Worthington Type CDS	
Pronase	3.4.4.–	Calbiochem, autodigested†	
Leucine aminopeptidase	3.4.1.1	Sigma, Type III CP	Rate of hydrolysis of L-leucine-*p*-nitro-anilide‡
Carboxypeptidase A	3.4.2.1	Sigma, crystalline, DFP-treated	Rate of hydrolysis of hippuryl-L-phenylalanine
Carboxypeptidase B	3.4.2.2	Sigma, type 'partially purified', DFP-treated	Rate of hydrolysis of hippuryl-L-arginine
Aryl sulphatase	3.1.6.1	Sigma, Type III	Cleavage of *p*-nitrocatechol sulphate, pH 5·5§
Tyrosinase	1.10.3.1	Worthington	Rate of increased absorption at 280 nm
Sialidase	3.2.1.18	Sigma, affinity purified—see text	Formation of NANA from NANA-lactose
Alkaline phosphatase	3.1.3.1	Sigma, Type III	Rate of hydrolysis of *p*-nitrophenyl phosphate

* Assays involving a 'rate' were carried out in Gilford multiple sample absorbance recorder thermostated at 37°, using the linear portion of the rate curve. All others were fixed time incubations using multiple enzyme concentrations.

† Digested in 0·1 per cent sodium dodecyl sulphate (Johnson and Bach, 1968).

‡ Leucine aminopeptidase was first activated by incubation at pH 8·5 in 12·5 μg/ml of $MnCl_2$ solution.

§ Carried out in Tyrode's solution plus 40 μl/ml of 1 M MES.

Chemical preparations

Tyrosine-*O*-sulphate was prepared as described by Tallan, Bella, Stein and Moore (1955). Sialidase was isolated from *Clostridium perfringens* and purified by affinity chromatography as described by Cuatrecasas and Illiano (1971). The purified enzyme had no demonstrable phospholipase C activity (Bach and Brashler, 1973).

Preparation of ECF-A

Partially purified guinea-pig ECF-A was prepared from anaphylactic lung diffusates by ethanol extraction as described (Kay *et al.*, 1973). A control preparation was prepared by subjecting an equivalent amount of ovalbumin antigen and histamine to the same isolation procedure. In experiments testing for the production of inhibitory substances by enzymatic digestion the control preparation was an ethanol-extracted diffusate from normal lung in the presence of antigen. Tyrode's buffer was used throughout.

Measurement of chemotaxis

Guinea-pig eosinophils were obtained by peritoneal lavage from animals which had received multiple injections of horse serum. Chemotaxis was measured by a modification of the Millipore technique of Boyden using a pore size of 8·0 μm as previously described (Kay, 1970). For purposes of comparison, the migration induced by untreated ECF-A preparations was normalized to 100 per cent, and migration induced by treated samples was expressed relative to this value.

Enzyme incubations

Aliquots of 0·9 ml of ECF-A-containing pools were incubated in quadruplicate at 37° for 30 minutes with 0·1-ml additions containing the enzymes to be tested. At the end of this incubation, the samples were heated to 56° for 1 hour to inactivate those enzymes which are heat-sensitive. Control incubations of ECF-A with heat-inactivated enzymes were included wherever appropriate. In addition, enzymes were incubated with control preparations to exclude chemotactic activity in the enzyme preparations themselves or the generation of chemotactic activity from contaminants in the ECF-A preparation.

Aryl sulphatase has a pH optimum which is more acidic than the pH of Tyrode's solution. To study the effects of this enzyme 40 μl of 1 M MES was added to the incubations prior to addition of the enzymes. After incubation and before heat inactivation, 40 μl of 1 M NaOH were then added to restore the original pH. Controls had shown that these modifications did not affect the chemotactic activity of ECF-A, nor did they evoke chemotactic activity in the control preparations.

Enzyme calibrations

The enzyme and the methods used for estimating their activity under the conditions of incubations are shown in Table 1.

RESULTS

ENZYME CALIBRATIONS

The activities of the enzymes used on their usual assay substrates are shown in Table 2. In addition, an estimate of the levels of contaminating activities in the enzyme preparations is also recorded. Values denoted as '<' indicate that there was no detectable activity found at the doses tested.

TABLE 2

ACTIVITIES AND HEAT STABILITIES (IN PARENTHESES)* OF ENZYMES EMPLOYED

Enzyme used	Activities tested in units/mg (or μl) on substrates of enzymes indicated							
	Protease†	Carboxy-peptidase A	Carboxy-peptidase B	Leucine amino-peptidase	Aryl sulphatase	β-Glucuronidase	Tyrosinase†	Others‡
Trypsin	1000 (0)	n.t.	0·033	< 0·001	< 2	< 0·15	n.t.	n.t.
α-Chymotrypsin	3700 (0·5)	n.t.	0·024	< 0·001	< 2	< 0·15	n.t.	n.t.
Pronase	375 (100)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Leucine aminopeptidase	n.t.	< 0·4	10	0·90 (92)	< 200	< 0·5	< 500	
Carboxypeptidase A	n.t.	21 (0)	0·57	< 0·001	6·7	< 0·75	< 5	n.t.
Carboxypeptidase B	n.t.	4·6	74 (25)	< 0·001	31	< 0·75	< 5	n.t.
Aryl sulphatase	n.t.	< 1	0·27	< 0·001	2000 (24)	< 0·1	< 5)	n.t.
Tyrosinase	n.t.	< 1	n.t.	0·001	< 20	< 1·0	2600 (0·1)	n.t.
Alkaline phosphatase	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	100 (37)
Sialidase (μl)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0·00017 (0)

n.t. = Not tested.

* Heat stability is given as percentage retention of activity after heating at 56° for 1 hour.
† A unit is defined as a change in absorption of 0·001 at 280 nm/minute. For proteases results are extrapolated to infinite enzyme dilution.
‡ A unit is defined as hydrolysis of 1 μmole of substrate/minute, and refers to the assay of the main activity of the respective enzyme.

EFFECT OF ENZYME TREATMENTS ON THE CHEMOTACTIC ACTIVITY OF ECF-A

Enzymes causing inhibition of chemotactic activity are shown in Fig. 1. Aryl sulphatase, tyrosinase and leucine aminopeptidase each produced a dose-dependent loss of activity. The heated preparations of the thermolabile enzymes (aryl sulphatase and tyrosinase) had no effect, suggesting that the loss of activity was not a result of non-specific binding or adsorption of ECF-A. No chemotactic activity was generated in control preparations incubated with these enzymes. Mixtures of control preparations which were incubated with tyrosinase and aryl sulphatase and subsequently heat inactivated did not reduce the chemotactic response of untreated ECF-A.

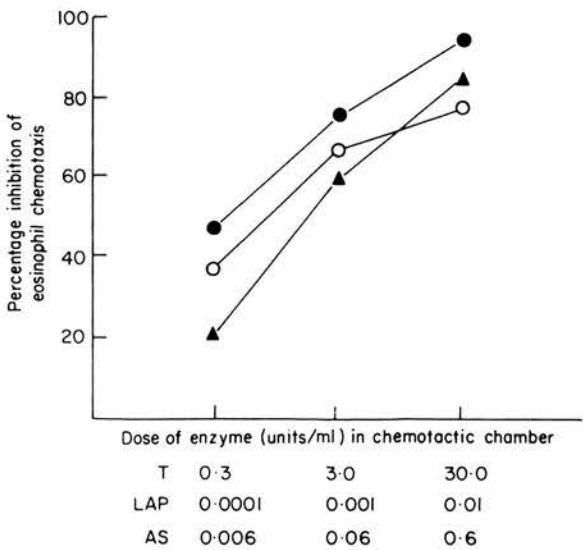

FIG. 1. Inhibition of the chemotactic activity of ECF-A following treatment with tyrosinase (T) (●), leucine aminopeptidase (LAP) (○) and aryl sulphatase (AS) (▲). The points represent the pooled results from three experiments. The standard error of the assay was $< \pm 15$ per cent.

Trypsin, α-chymotrypsin, pronase, alkaline phosphatase and sialidase did not inhibit ECF-A activity in doses of 1000, 3700, 375, 100 and $1{\cdot}7 \times 10^{-2}$ units respectively.

Preparations of carboxypeptidase A and B inhibited ECF-A activity but these preparations contained amounts of aryl sulphatase activity which could account for the observed effect (Table 2, Fig. 1).

DISCUSSION

For the purposes of this study enzymes that inactivated the preparation of ECF-A were defined as those which caused a dose-dependent loss of activity with the maximum inhibition being at least 75 per cent.

Although these experiments indicate that ECF-A is susceptible to treatment by several enzymes, there are a number of aspects to be considered in the interpretation of the results. Thus the enzymes employed may have had a direct effect on target cells. This possibility

was largely excluded with heat-sensitive enzymes, since preheating did not affect ECF-A activity. The same control was not possible with leucine aminopeptidase; the only enzyme that affected ECF-A activity but was heat stable. Further, enzymes that had an apparent effect on ECF-A may have generated inhibitors of chemotaxis. This was largely excluded by taking control preparations and incubating in the presence of enzymes, subsequently heating, and adding the mixture to ECF-A and demonstrating no loss in activity. However, the generation of heat-sensitive inhibitors remains a possibility.

It should be stated that leucine aminopeptidase was activated by preincubation with manganese chloride and that the incubation with aryl sulphatase was in the presence of MES. Control experiments have shown that neither manganese chloride nor MES alone, at the maximal concentration used, influenced ECF-A activity.

A number of enzymes had no effect on ECF-A activity, even at doses greater than twice those at which the same enzymes are active in other biological systems (Codington, Sanford and Jeanloz, 1970; Bach, Brashler, Bloch and Austen, 1971; Bach and Brashler, 1973).

The failure of pronase to inactivate ECF-A in these studies is at variance with the work of Wasserman, Goetzl, Ellman and Austen (1974) using ECF-A of human origin. This discrepancy may be due to a difference in species or in the preparation of the enzyme.

Contaminating enzymatic activities could not account for the loss of chemotactic activity in preparation treated with leucine aminopeptidase, tyrosinase and aryl sulphatase (Table 2). Assuming that the loss of activity with these enzymes was due to their recognized biochemical activity the following conclusions can be drawn. The effect of leucine aminopeptidase suggests that ECF-A contains a free α-amino group belonging to a relatively hydrophobic amino acid in peptide linkage. Inactivation by tyrosinase indicates the presence of a phenolic hydroxyl group which is not necessarily present as tyrosine. The action of aryl sulphatase suggests the presence of a sulphate ester. Model experiments have excluded tyrosine-*O*-sulphate as a substrate of the aryl sulphatase employed in these experiments (Dodgson and Rose, 1970). Therefore the presence of a sulphate residue indicates that ECF-A contains in its structure a component which is not usually found in peptides.

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Inhibition of eosinophil chemotaxis by the antagonist of slow reacting substance of anaphylaxis — compound FPL 55712*

The antigen challenge of sensitized lung and skin leads to the release of a number of pharmacological mediators which include histamine, a slow reacting substance of anaphylaxis (SRS-A) and an eosinophil chemotactic factor of anaphylaxis (ECF-A) (Brocklehurst, 1960; Kay, Stechschulte & Austen, 1971; Jones & Kay, 1974). ECF-A selectively attracts eosinophils from a mixed leucocyte population (Kay, Shin & Austen, 1973) has an estimated molecular size of between 500 and 1000 and probably has a peptide-like structure (Kay, Stechschulte & others, 1971).

In a recent report it was shown that the compound FPL 55712* is a potent inhibitor of SRS-A whereas it had relatively little antagonistic effect on the ileum-contracting activity of histamine and other pharmacological agents (Augstein, Farmer & others, 1973). In the present study we have tested the capacity of FPL 55712 to inhibit ECF-A activity. In addition the antiallergic compounds hydrocortisone and disodium cromoglycate have been tested for inhibition of ECF-A-induced eosinophil chemotaxis, since both these agents are associated with a reduction in the number of circulating eosinophils when administered *in vivo* (Thorn, Forsham & others, 1948; Easton, 1973).

A partially purified preparation of guinea-pig ECF-A was prepared as previously described (Kay & others, 1973). Chemotaxis was measured by a modification of the Millipore technique of Boyden using guinea-pig eosinophils prepared from the peritoneal cavity of animals which had received multiple injections of horse serum (Kay, 1970).

Solutions of FPL 55712, disodium cromoglycate and hydrocortisone were freshly prepared by dissolving the compounds in distilled water. One part of increasing

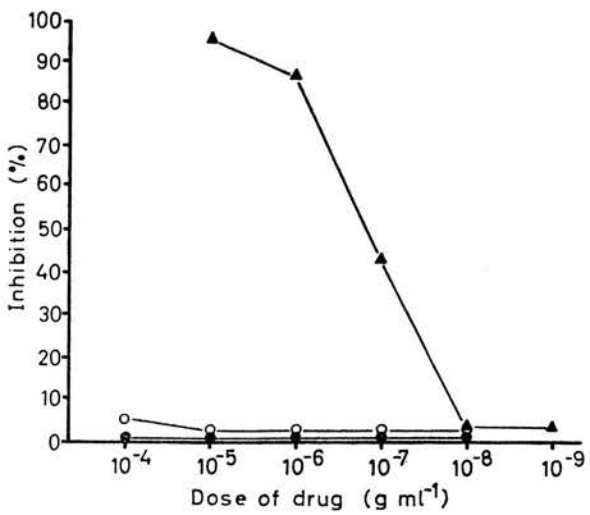

FIG. 1. The graph shows the percent inhibition of the chemotactic response of guinea-pig eosinophils towards partially purified guinea-pig ECF-A by increasing concentrations of FPL 55712 (▲ — ▲), disodium cromoglycate (○ — ○), and hydrocortisone (● — ●).

* Sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4*H*-chromene-2-carboxylate.

dilutions of the compound were added to nine parts of ECF-A in Tyrode solution. The results were expressed as the percent inhibition of eosinophil chemotaxis as compared with the same volume of ECF-A containing one part of distilled water. As shown in Fig. 1, FPL 55712 inhibited ECF-A in a dose-dependent fashion and had an IC50 of 0·2 μg ml^{-1} (3·8 $\times$ 10^{-7}M). Neither hydrocortisone nor disodium cromoglycate inhibited the activity of ECF-A at concentrations of up to 100 μg ml^{-1} (2·8 $\times$ 10^{-4} and 1·9 $\times$ 10^{-4}M respectively). These experiments were performed three times and gave similar results.

We were also able to confirm both the antagonistic effect of FPL 55712 on the ileum-contracting activity of guinea-pig SRS-A, and its negligible effect on histamine.

Thus the present report suggests that the reduction in the number of circulating eosinophils *in vivo* associated with the administration of corticosteroids and disodium cromoglycate is unlikely to be due to their direct effect on this cell. The inhibitory effect of FPL 55712 on ECF-A-induced chemotaxis of eosinophils could be a result of an alteration of the ECF-A molecule itself or on the recognition by the eosinophil of the chemotactic stimulus. Thus the unique action of FPL 55712 may serve as a useful adjunct for further study on the mechanism of ECF-A-induced chemotaxis of eosinophils.

Hydrocortisone sodium succinate was obtained from Organon Laboratories Ltd., Morden, Surrey. FPL 55712 and disodium cromoglycate were a gift from Fisons Research Laboratories. We are grateful to Miss Doreen Spence for her technical assistance. This work was supported by the Medical Research Council.

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Eosinophil chemotaxis of supernatants from cultured Hodgkin's lymph node cells

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Eosinophil chemotaxis of supernatants from cultured Hodgkin's lymph node cells

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SYNOPSIS Cultured lymph node cell supernatants from five out of six cases of Hodgkin's disease were relatively more chemotactic for peripheral blood eosinophils than neutrophils. In contrast, supernatants from two cases of lymphocytic lymphoma and four nodes showing reactive hyperplasia were chemotactic for neutrophils but had little eosinophil chemotactic activity. In most instances the degree of eosinophil infiltration observed histologically in the Hodgkin's lymph node correlated with elaboration of eosinophil chemotactic activity from the cultured cells. Following gel-filtration of three of the Hodgkin's lymph node supernatants, four peaks of eosinophil chemotactic activity were demonstrated in each case. One of these corresponded in molecular size to the previously described eosinophil chemotactic factor of anaphylaxis (ECF-A). It is suggested that the eosinophil chemotactic activity of cultured lymph node cell supernatants may be of value in the diagnosis and classification of Hodgkin's disease.

The histological diagnosis of Hodgkin's disease is made by the recognition of the characteristic Reed-Sternberg cell which is a large multinucleate cell containing two or more nuclei with prominent nucleoli (Butler, 1970). Other morphological features of the lymph nodes are variable, and this has led to several attempts at classification with the object of relating the histology to clinical staging and prognosis (Jackson and Parker, 1944a; Jackson and Parker, 1944b; Lukes and Butler, 1966; Smithers, 1967; Smithers, 1970; Dorfman, 1972). One of the characteristics of the Hodgkin's lymph node is the presence of eosinophil leucocytes (Dreschfeld, 1892), and it has been suggested that this may represent part of the inflammatory infiltrate (Stuart, 1970), the eosinophil accumulation in the nodes being a result of chemotaxis (Vianna *et al*, 1971). In the present study we provide evidence to support this concept by showing that supernatants from cultured Hodgkin's lymph nodes attract a greater proportion of eosinophils from mixed leucocyte populations than do supernatants from cultures of non-Hodgkin's lymph nodes.

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Material and Methods

PREPARATION OF LYMPH NODE CELL CULTURE SUPERNATANTS

Lymph nodes obtained at surgery were bisected. One-half was taken for routine histology and the other for cell culture as described (Habeshaw, 1972). Cells for culture were obtained by carefully dividing the tissue into small fragments with scalpel blades, gently homogenizing and passing through a sieve to remove fibrous debris. Cell counts were adjusted to 5×10^6 cells/ml and the viability was assessed by Trypan blue exclusion and phase contrast microscopy. Only those preparations which contained greater than 90% of viable cells were subsequently cultured. Cultures were set up in 'flying cover slips' in glass test tubes, and the cells were contained in 1 ml volumes of medium 199 with 0·5% lactalbumin, 50 units of penicillin, and 5 μg of streptomycin. The tubes were incubated at 37°C and the supernatants removed at day 3, replaced with culture medium, and removed again at day 5. Supernatants thus obtained were centrifuged to remove particulate material, pooled and dialysed for 24 hours against phosphate buffered saline (PBS), and lyophilized. Before chemotaxis the material

was reconstituted in distilled water and the protein concentrations were measured by the Folin-Ciolcalteau method.

MEASUREMENT OF CHEMOTAXIS

Chemotaxis of human peripheral blood eosinophils and neutrophils was assayed by a modification of the Millipore technique of Boyden as previously described (Kay, 1970). Cell counts were performed in duplicate or triplicate. A chemotactic index to show the relative proportion of eosinophils migrating was calculated from the equation:

$$\frac{\text{Eosinophil chemotactic count/ Neutrophil chemotactic count}}{\text{\% eosinophils in cell suspension before migration/ \% neutrophils in cell suspension before migration}}$$

GEL FILTRATION

Hodgkin's lymph node cell supernatants were concentrated by rotary evaporation at 37°C under vacuum. Samples of 1 ml containing approximately 11·5 mg/ml of protein were applied to a column of Sephadex G-50 (104 × 2·5 cm) in PBS. From alternate 2 ml fractions 0·5 ml volumes were taken and tested in duplicate for neutrophil and eosinophil chemotaxis.

Results

Chemotactic activity from cultured lymph node cell supernatants was demonstrable in a dose-dependent fashion as shown in fig 1 for cultures from a Hodgkin's and a reactive lymph node cell supernatant.

The chemotactic activity of lymph node cell supernatants from six patients with Hodgkin's disease, two with lymphocytic lymphoma, and four nodes showing reactive hyperplasia are shown (fig 2). Neutrophil and eosinophil chemotaxis was demonstrable with all supernatants but five out of the six Hodgkin's supernatants gave greater eosinophil chemotactic indices than the highest index obtained with non-Hodgkin's supernatants.

The degree of eosinophil infiltration in the lymph nodes, arbitrarily assessed 0 to + +, to some extent correlated with the eosinophil chemotactic activity from the cell culture supernatants (fig 2). However, one Hodgkin's node had no eosinophils, and eosinophil infiltration was present in one of the non-Hodgkin's nodes.

When the chemotactic gradient was eliminated by placing supernatants from either Hodgkin's or non-Hodgkin's lymph nodes in both the test *and* cell compartments of the chemotactic chambers little or no migration was observed for either cell type. This suggests that the leucotaxis was a result of directional, rather than random, migration.

When concentrated cell supernatants from three Hodgkin's lymph nodes were applied to a column of Sephadex G-50 four peaks of eosinophil chemotactic

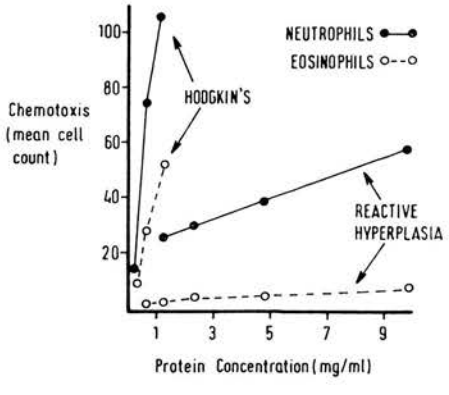

Fig 1 *Neutrophil and eosinophil chemotactic activity of a Hodgkin's and reactive hyperplasia cultured lymph node cell supernatant.*

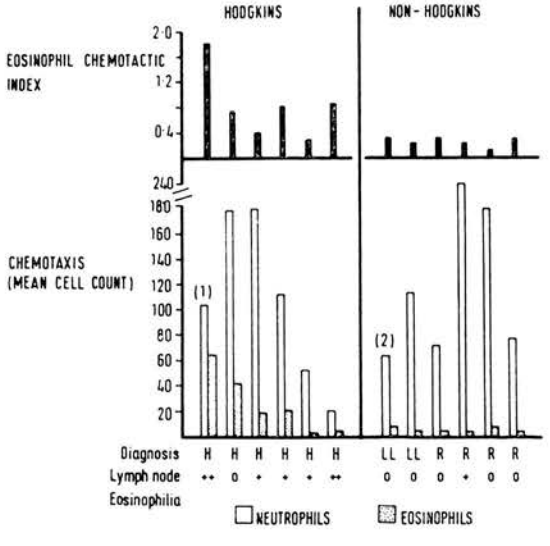

Fig 2 *Neutrophil and eosinophil chemotaxis and the eosinophil chemotactic index from Hodgkin's and non-Hodgkin's cultured lymph node cell supernatants. The degree of lymph node eosinophil infiltration is also indicated: H = Hodgkin's disease, LL = lymphocytic lymphoma, R = reactive hyperplasia. The measurements represent the pooled results from three experiments except in (1) and (2) where there was sufficient sample for only one and two estimations respectively. All samples were tested at a protein concentration of 0·6 mg/ml.*

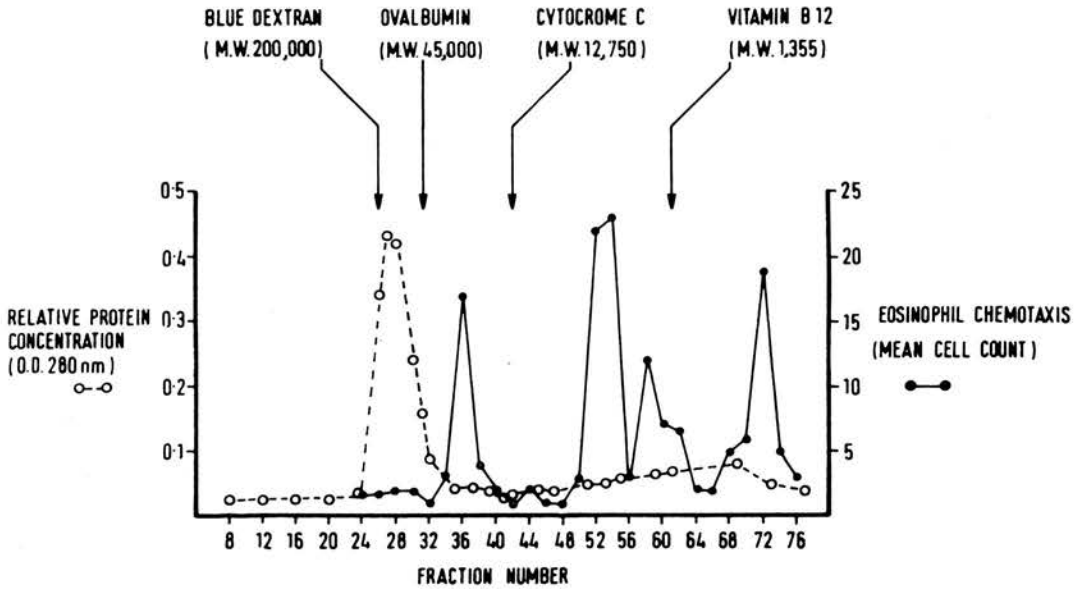

Fig 3 *Sephadex G-50 chromatography of a cultured lymph node cell supernatant from Hodgkin's disease. The column was previously calibrated with markers having the molecular sizes indicated.*

activity were consistently demonstrable (fig 3). The molecular markers indicated that these four activities were associated with molecules having an approximate molecular size of 30 000, 6000, 2000, and 500 daltons respectively (fig 3).

Discussion

The capacity of human peripheral blood lymphocytes to release chemotactic factors for neutrophils and monocytes following stimulation by mitogens and specific antigen has been reported by a number of workers (Ward *et al*, 1969; Altman *et al*, 1973). In addition, an eosinophil chemotactic factor from lymphocyte supernatants has also been reported, but in this system there was an additional requirement for antigen-antibody complexes (Cohen and Ward, 1971). In the present study we have demonstrated the elaboration of neutrophil and eosinophil chemotactic agents from cultured lymph node cell supernatants but in addition have shown that Hodgkin's lymph node preparations attract relatively more eosinophils than the non-Hodgkin's supernatants (figs 1 and 2).

Care was taken to use the same protein concentrations when making comparisons between the activity of the cell supernatants. A dose-response effect was obtained with each supernatant as well as the ones depicted in figure 1.

Although relatively more eosinophil than neutrophil chemotactic activity was observed in a node in which histologically there were no demonstrable eosinophils, there was in general an association between lymph node eosinophilia and the elaboration of eosinophil chemotactic activity. More direct evidence would be possible by performing these measurements on several nodes from individual patients.

Several peaks of activity were obtained following chromatography on Sephadex G-50, suggesting that there is a heterogeneity of eosinophil chemotactic factors from Hodgkin's lymph node cell supernatants (fig 3). One peak corresponded to a molecular size of the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay and Austen, 1971; Kay *et al*, 1971). In a previous report a substance of compatible molecular size was extracted from an undifferentiated carcinoma of lung associated with a peripheral blood eosinophilia (Wasserman *et al*, 1974).

The finding that Hodgkin's lymph node cell supernatants attract relatively more eosinophils than non-Hodgkin's cultures suggests that this may be of value both in diagnosis and in the classification of various forms of the disease.

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Eosinophils and mediators of anaphylaxis

HISTAMINE AND IMIDAZOLE ACETIC ACID AS CHEMOTACTIC AGENTS FOR HUMAN EOSINOPHIL LEUCOCYTES

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Summary. Histamine and one of its major catabolites, imidazole acetic acid (ImAA), were selectively chemotactic for human eosinophils, whereas L-histidine and other histamine catabolites including 1,4-methylhistamine, 1-methyl-4-imidazole acetic acid and *N*-acetylhistamine were inactive in eosinophilotaxis over a large dose range. The dose response for histamine was dependent on the chemotaxis incubation time and the source of eosinophils, although the latter was not clearly associated with particular disease states. When histamine and ImAA were combined the chemotactic response was similar to that obtained when one agent was assayed alone, no additive or synergistic effects being observed. There was cross-deactivation between histamine and ImAA. These experiments suggest that histamine and ImAA activate the same chemotactic recognition mechanism for eosinophils. Thus ImAA joins histamine and the tetrapeptides (ECF-A) as anaphylaxis-associated selective chemoattractants for human eosinophils.

INTRODUCTION

Human lung can be passively sensitized by IgE for the antigen-induced release of a number of pharmacological mediators of anaphylaxis. These include histamine, a slow reacting substance (SRS-A) and an eosinophil chemotactic factor (ECF-A). ECF-A is now recognized as two associated acidic tetrapeptides, Val–Gly–Ser–Glu and Ala–Gly–Ser–Glu (Goetzl & Austen, 1975). An analogue Val–Gly–Asp–Glu also selectively attracts human eosinophils from a mixed cell population (Kay, 1976).

The role of histamine in the recruitment of eosinophils to the site of allergic tissue reactions has been the subject of some controversy (Parish, 1974). Whereas subcutaneous injections of histamine into the horse resulted in eosinophil accumulation (Archer, 1956) these observations have not been confirmed in the guinea-pig (Kay, 1970a) or man (Felarca & Lowell, 1968). Similarly *in vitro* experiments failed to show chemoattractant properties for histamine when eosinophils from guinea-pig and man were used as target cells (Kay, Stechschulte & Austen, 1971; Kay & Austen, 1971). However, Clark, Gallin & Kaplan (1975) were able to show human eosinophilotaxis by histamine although the activity was only demonstrable at concentrations of approximately 5×10^{-5} mol/l, there being inhibition with higher doses. It now appears that these apparent discrepancies in the chemotactic property of histamine can be explained largely on the type of micropore filters used in the assays. Thus with the more traditional Millipore filter, chemotaxis by

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histamine cannot be demonstrated whereas these membranes were suitable for the identification and characterisation of ECF-A (Kay & Austen, 1971). In contrast, cellulose nitrate filters were satisfactory for the assay of both the ECF-A peptides and histamine (Kay, 1976).

In the present report we have confirmed and extended the observation on eosinophil chemotaxis by histamine and in addition describe a similar eosinophilotactic property for a major histamine metabolite, imidazole acetic acid (ImAA). The interactions between histamine and ImAA in terms of chemotaxis and chemotactic deactivation are also described.

MATERIALS AND METHODS

Materials

The materials were obtained as follows. Histamine acid phosphate (BDH Chemicals Ltd, Poole); imidazole acetic acid hydrochloride (ImAA) chromatographically free of histidine, histamine and isopropylester, 98·8 per cent pure; 1-methyl-4-imidazole acetic acid hydrochloride (1,4-MeImAA), L-histidine, free base, 1,4-methylhistamine dihydrochloride (1,4-MeHm), *N*-acetylhistamine (N-AcHm), all chromatographically homogeneous (Calbiochem Ltd, San Diego, California); ovalbumin five times crystallised (Koch-Light Laboratories, Colnbrook, Bucks.); cellulose nitrate filters, 8·0 μm (Sartorius-membrane filters, 34 Göttingen, W. Germany).

Eosinophil chemotaxis

Peripheral blood was withdrawn from patients with an eosinophilia of between 10 and 25 per cent. Leucocyte rich plasma, obtained by dextran sedimentation of heparinised blood (Kay, 1970b) was applied to a density gradient of 9 per cent Ficoll solution and sodium diatrizoate (density 1·140) in the proportions 2·4:1 (English & Andersen, 1974). Following centrifugation, at 400 *g* for 40 min at 20°, the eosinophil-rich pellet was retained, washed in Tyrode's buffer and resuspended in Tyrode's containing 0·5 per cent ovalbumin. This procedure increased the eosinophil concentration two- to three-fold, the contaminating cells being mostly neutrophils. The cell counts were then adjusted to 2×10^6 cells/ml, with a final eosinophil concentration of 20–30 per cent. Blood with an initial eosinophil concentration in this range was prepared by dextran sedimentation alone. The chemotactic assay procedure was as previously described (Kay, 1970b) but using 8·0 μm Sartorius micropores. All agents tested for chemotaxis were dissolved in Tyrode's solution and the pH adjusted to 7·24.

Statistics

Measurements from duplicate filters from three separate experiments were pooled, the mean and standard error determined and observations compared using the Student's *t*-test. Such pooling was considered to be statistically valid since in the present study the variation between duplicate filters was similar to variations between individual experiments as previously observed (Clark *et al.*, 1975).

RESULTS

(1) Eosinophil chemotactic activity of histamine and histamine metabolites

Histamine, L-histidine, ImAA, 1,4-MeHm, 1,4-MeImAA and *N*-AcHm were assayed for chemotactic activity at the concentrations shown in Fig. 1. With a 135-min incubation period eosinophil chemotaxis by histamine was demonstrable at concentrations of 10^{-5} and 10^{-6} mol/l with apparent inhibition of migration at 10^{-4} mol/l. In contrast, chemotaxis by ImAA gave a linear-dose response from 10^{-3} to 10^{-5} mol/l. No chemotactic activity was found with histidine or the other histamine catabolites. Within the dose-ranges tested in Fig. 1,

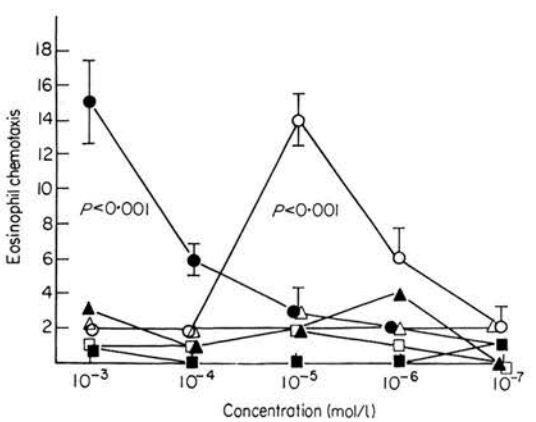

Figure 1. Chemotaxis for human eosinophils by histamine (○), ImAA (●), 1,4-MeImAA (▲), 1,4-MeHm (△), N-AcHm (■) and L-histidine (□). Incubation period 135 min. The bars for Figs 1–5 represent ± s.e.m. The *P* values refer to the points immediately above or below.

Table 1. Pattern of chemotactic response to histamine by human eosinophils from various patients

Patient	Associated disease	No. of experiments	Pattern of response
H.W.	Microfilariasis (D. perstans)	4	Linear dose–response with maximal density at 10^{-3} mol/l
J.P.	Microfilariasis (D. perstans)	1	Linear dose–response with maximal density at 10^{-3} mol/l
G.M.	Drug reaction	2	Linear dose–response with maximal density at 10^{-3} mol/l
E.S.	Allergic asthma	1	Peak activity at 5×10^{-6} mol/l with inhibition at higher dose
R.G.	Drug reaction	2	Peak activity at 5×10^{-6} mol/l with inhibition at higher dose
F.F.	Hypereosinophilic syndrome	2	Peak activity at 5×10^{-6} mol/l with inhibition at higher dose

neutrophil migration was not observed with histamine, ImAA or the other compounds.

(2) Time-course studies

Using a 180-min incubation time eosinophil chemotactic activity by histamine was found to give a linear-dose response between 10^{-3} and 10^{-7} mol/l (Fig. 2). However at 135 min there was an inhibition of chemotaxis with a dose of 10^{-3} mol/l. Negligible activity was found at all doses with a 90-min incubation time. Therefore by increasing the incubation period the apparent inhibition of migration by higher concentrations of histamine could be abolished. However eosinophils from certain individuals gave a peak of activity at approximately 5×10^{-6} mol/l with inhibition at higher doses even with a 180-min incubation period (Table 1). The two types of chemotactic profile, i.e. maximal activity at 10^{-3} or at 5×10^{-6} mol/l were not clearly associated with the disease state. In subsequent studies only the eosinophils from patients giving the linear dose-response pattern of activity shown in Fig. 2 (180 min) were used.

Similar experiments were carried out with ImAA. The latter was selectively chemotactic for human eosinophils between 10^{-3} and 10^{-4} mol/l using either 135- or 180-min incubations and invariably gave a linear-dose response irrespective of the source of eosinophils. As with histamine little activity was seen at 90 min. In all further experiments a 180-min incubation time was used.

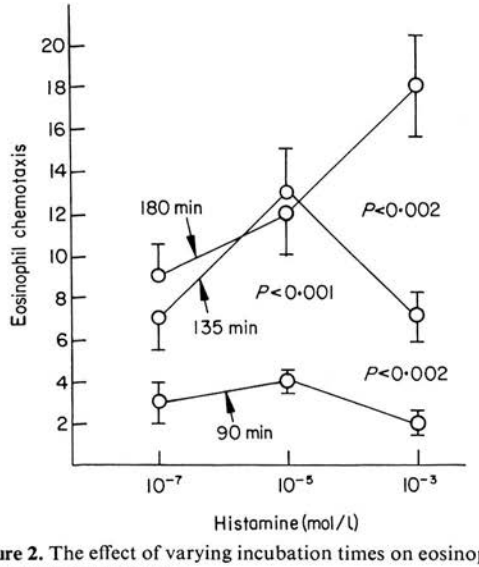

Figure 2. The effect of varying incubation times on eosinophil chemotaxis by histamine.

(3) The effect of combining histamine and ImAA

When histamine was combined with ImAA (10^{-3} mol/l) there was virtually no difference in the eosinophil chemotactic response when compared to hista-

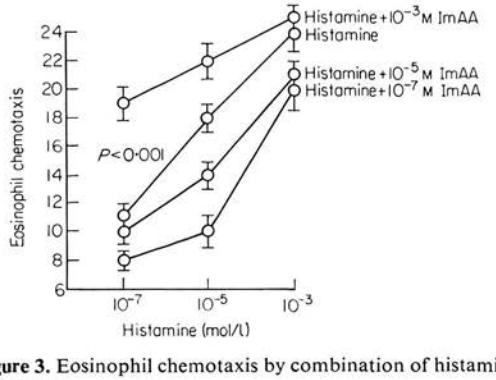

Figure 3. Eosinophil chemotaxis by combination of histamine and ImAA.

mine alone, with the exception of the lowest dose of histamine (10^{-7} mol/l) which when added to ImAA resulted in higher counts (Fig. 3). However histamine (10^{-5} mol/l) and ImAA (10^{-7} mol/l) in combination gave less chemotaxis than histamine alone at 10^{-5} mol/l. Thus although there was some augmentation or depression of the chemotactic response when these agents were combined at various concentrations there were, in general, no marked additive or synergistic effects.

(4) Deactivation of eosinophil chemotaxis by histamine and ImAA

Cells preincubated with histamine (5×10^{-6} mol/l) or ImAA (10^{-3} mol/l) for 20 min at 37°, washed in

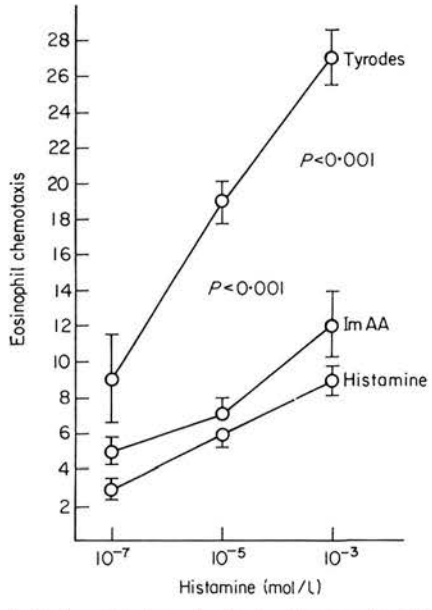

Figure 4. Eosinophil chemotactic deactivation for histamine by histamine and ImAA.

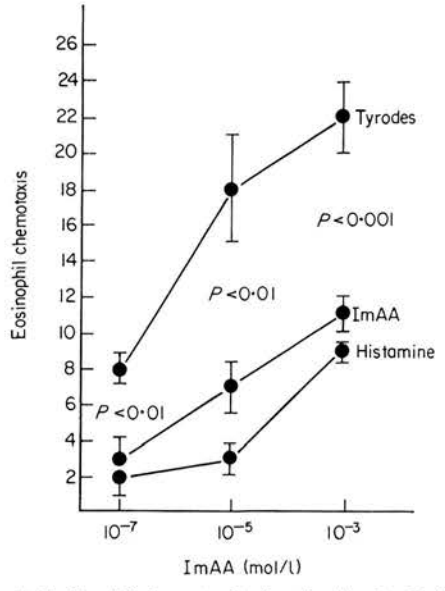

Figure 5. Eosinophil chemotactic deactivation for ImAA by ImAA and histamine.

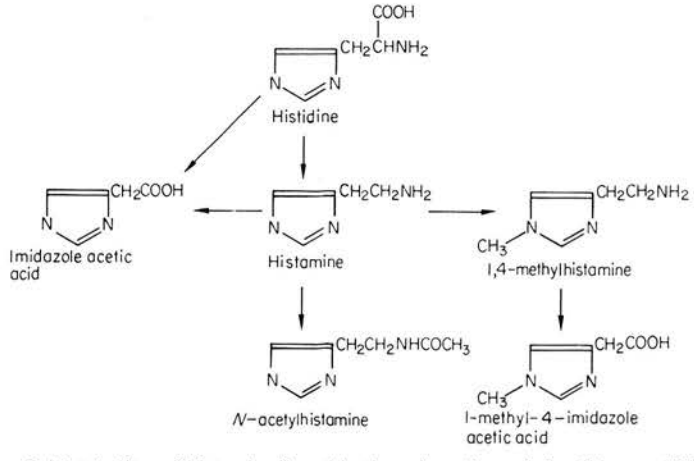

Figure 6. Metabolites of histamine found in the urine of man (after Schayer, 1959).

Tyrode's solution and resuspended in buffered ovalbumin/Tyrode's solution, pH 7·24, were set up in chemotaxis against increasing doses of histamine (Fig. 4). When compared to preincubation in Tyrode's there was a significant inhibition of eosinophil migration in both cases. Similar experiments performed using ImAA as the chemoattractant (Fig. 5) gave comparable results indicating a cross-deactivation between histamine and ImAA. Histidine and the histamine catabolites, 1,4-MeHm, 1,4-MeImAA and *N*-AcHm did not deactivate eosinophils in chemotaxis to either histamine or ImAA.

DISCUSSION

A number of agents associated with the anaphylactic response have been shown to attract selectively human eosinophils *in vitro*. These include the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay & Austen, 1971), now recognized as two related acidic tetrapeptides, Val–Gly–Ser–Glu and Ala–Gly–Ser–Glu (Goetzl & Austen, 1975), an analogue Val–Gly–Asp–Glu (Kay, 1976) and histamine (Clark *et al.*, 1975). The recognition of histamine as an eosinophilotactic agent was not possible using the traditional Millipore filters and was only apparent using cellulose-nitrate filters. This may be related to variations in thickness and charge between micropores from various commercial sources. We have extended this observation on human eosinophil chemotaxis by histamine, to include its precursor histidine, and its major metabolites.

ImAA was found to give a linear dose–response, irrespective of incubation times. In contrast the apparent inhibition of chemotaxis by higher dose of histamine was abrogated using increased incubation times in three subjects. However, other individuals gave peak activity at 5×10^{-6} mol/l with inhibition at high doses even with the longer period of incubation (Table 1). This was not clearly related to the associated clinical conditions and the reason for the altered response is yet to be determined. It may be related to the content of leucocyte histaminase, and this possibility is being explored.

The ImAA used in these experiments was free of histamine and histidine as assessed by gas chromatography and had 10,000 times less ileal-contracting activity than histamine (Turnbull, L.W., unpublished observation).

The major metabolites of histamine found in the urine of man are shown in Fig. 6. These include ImAA, 1,4-MeHm, 1,4-MeImAA and *N*-AcHm and account for 25, 8, 60 and < 1 per cent respectively of the urinary histamine breakdown products following a loading dose of labelled histamine (Nilsson, Lindell, Schayer & Westling, 1959). It is not known which metabolic pathway predominates following various types of anaphylactic response but it should be noted that ImAA can also be formed directly from histidine so raising the possibility that there is a relationship between eosinophil recruitment and this histidine-independent pathway.

There is no obvious explanation, from the chemical structures of these agents, as to why histamine and ImAA but not histidine and the other histamine catabolites are chemotactic for eosinophils. There is presumably some form of steric hindrance by the compounds inactive in eosinophil chemotaxis.

When ImAA was added to histamine the resultant chemotaxis was in general no more than found with each agent alone. There was however some augmentation or depression depending on the relative doses of the two agents. These observations suggest therefore that both compounds are competing for the same recognition mechanism. Further evidence was provided by the cross-deactivation exhibited between the two substances (Figs 4 and 5).

These experiments provide further evidence that a breakdown product of histamine is not inert but has biological activity. Other activities for ImAA have been reported and include effects on the central nervous system such as a depressant action on the neurons of the cerebral cortex of the cat (Hösli and Haas, 1971). In these experiments ImAA was more active than histamine but in the present study using a 180-min incubation period both agents had comparable activities at 10^{-3} mol/l. However histamine, under the experimental conditions cited (Figs 1 and 2) was far more active at lower doses.

Thus ImAA joins histamine and the acidic tetrapeptides (ECF-A) as selective anaphylaxis-associated chemotactic agents for eosinophils.

ACKNOWLEDGMENTS

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Chemotactic Activity of Guinea Pig Eosinophils for the ECF-A Acidic Tetrapeptides, Histamine, Histamine Metabolites, and the Effect of H1- and H2-Receptor Antagonists

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Abstract. Histamine and one of its major metabolites, imidazoleacetic acid, were selectively chemotactic for guinea pig eosinophils, whereas *L*-histidine, 1,4-methylimidazoleacetic acid, 1,4-methylhistamine and N-acetylhistamine were inactive. The response to histamine was unaffected by concentrations of eosinophils of between 30 and $> 90\%$ but it was abrogated by preincubation of the cells with histamine prior to assay (self-deactivation). Eosinophilotaxis was also inhibited by H1-(mepyramine-) and H2-(burimamide-)receptor antagonists at high doses (10^{-3} *M*), although at lower concentrations (10^{-5} *M*) inhibition was principally associated with burimamide. The human tetrapeptide, alanine-glycine-serine-glutamic acid, and the analogue, valine-glycine-aspartic acid-glutamic acid, were inactive whereas alanine-glycine-serine-glutamic acid was chemotactic for the guinea pig eosinophil. These results support the concept that the tissue accumulation of eosinophils following anaphylaxis depends on a complex interaction of factors, which in part may be mediated by H2 receptors on the target cells. There may be species differences in the composition of ECF-A.

Introduction

The accumulation of eosinophil leucocytes in the tissues is a feature of immediate-type 'allergic' hypersensitivity reactions in several species including guinea pig and man [8]. In both species, eosinophil recruitment was initially attributed to the release of a preformed mast-cell-associated mediator released following anaphylaxis *in vitro* [5, 6, 9]. This principle was designated the eosinophil chemotactic factor of anaphylaxis (ECF-A) and in man has now been identified as a family of closely related acidic tetrapeptides including valine-glycine-serine-glutamic acid (VGSG) and alanine-glycine-serine-glutamic acid (AGSG) [4]. The presence of other amino acids, particularly aspartic acid, in purified ECF-A preparations raised the possibility of other affiliated peptides contributing towards its activity and it has recently been shown that an analogue, valine-glycine-aspartic acid-glutamic acid ($VGAG_a$), inaddition to syn-

thetic VGSG and AGSG, possessed selective chemotactic activity for human eosinophils [12].

Histamine has been variously ascribed roles in anaphylaxis-associated eosinophil accumulation [11]. However, two recent reports [2, 13] have confirmed that histamine selectively attracts eosinophils from mixed human leucocyte populations *in vitro*. In the latter study, eosinophilotactic activity was also shown to be a property of a major histamine catabolite – imidazoleacetic acid (ImAA).

In the present study, we have assessed the ability of histamine and its major metabolites including *L*-histidine, 1,4-methylhistamine (1,4-MeHm), 1-methyl-4-imidazoleacetic acid (1,4-MeImAA) and N-acetylhistamine (N-AcHm) to induce the migration of guinea pig peritoneal eosinophils and the modulation of this response by H1- and H2-receptor antagonists and by self-deactivation. In addition, we have similarly tested synthetic VGSG, AGSG and $VGAG_a$ for chemotactic activity towards guinea pig cells.

Materials and Methods

Materials

Dunkin-Hartley strain guinea pigs, weighing 300–400 g, of either sex, were used as a source of target leucocytes throughout.

Materials were obtained as follows: histamine acid phosphate (BDH Chemicals Ltd, Poole, England); imidazoleacetic acid hydrochloride, chromatographically free of histidine, histamine and isopropyl ester 98.8% pure; *L*-histidine, free base, 1,4-methylhistamine dihydrochloride, 1-methyl-4-imidazoleacetic acid hydrochloride, N-acetylhistamine, all chromatographically homogeneous (Calbiochem Ltd, San Diego, Calif., USA); mepyramine maleate (May & Baker Ltd, Dagenham, England); ovalbumin, 5 times crystallised (Koch-Light Laboratories, Colnbrook, England); horse serum (Wellcome Research Laboratories, Beckenham, England); sodium diatrizoate (Winthrop Laboratories, Surbiton-on-Thames, England); cellulose nitrate filters, 8.0 μm pore size (Sartorius-Membrane Filters, 34 Göttingen, FRG). (HCl)-VGSG (molec. wt 427), (HCl)-AGSG (molec. wt 497) and (HCl)-$VGAG_a$ (molec. wt 455) were a generous gift from Dr. *R. Camble,* ICI Ltd, Pharmaceuticals Division, Alderley Park, Macclesfield, England, and burimamide was kindly donated by S. K. & F. Laboratories Ltd, Welwyn Garden City, England.

Eosinophil Chemotaxis

Eosinophils were obtained from the peritoneal cavities of animals which had received 4–6 weekly intraperitoneal injections of horse serum [7]. Where necessary, these preparations were further purified by centrifugation on cushions of sodium diatrizoate (d = 1.140 at 20 °C) [3]. Neutrophils were similarly obtained from guinea pigs 3–4 h after the administration of glycogen suspensions [7]. Eosinophil chemotactic assays were performed in modified Boyden micropore chambers [7] using 8.0-μm pore size cellulose nitrate filters and a final total cell concentration of 2×10^6 ml^{-1} in Tyrode's buffer 0.5% ovalbumin, pH 7.2. Following a 3-hour incubation period at 37 °C, the filters were removed, fixed and stained as previously described [7]. Cell counts were expressed as the mean total of 5 high-power (× 40) fields in duplicate filters. Neutrophil chemotaxis was similarly evaluated but using a 90-min incubation period.

All agents tested were freshly prepared in Tyrode's buffer, pH 7.2–7.6. For inhibition studies with the H1-(mepyramine-) and H2-(burimamide-) antagonists, the drugs were either mixed throughout the assay with the test agents or preincubated with the leucocyte suspensions. In the latter instance, the cells were incubated for 30 min at 37 °C and the drugs removed by washing twice in Tyrode's buffer. The washed cells were then resuspended to a concentration of 2×10^6 ml^{-1} in Tyrode's buffer/0.5% ovalbumin. In deactivation studies, cells were similarly treated but with histamine in place of the drugs. Leucocytes from the same starting populations preincubated with Tyrode's buffer alone served as controls. Cell viability, as judged by trypan blue dye exclusion, was

unaffected by pretreatment with any of these agents.

Results

Chemotactic Activity of Histamine and its Metabolites

Histamine was selectively eosinophilotactic over a limited dose range, maximum activity being observed at $5\times10^{-6}\,M$; there was an apparent inhibition of response at higher doses (fig. 1), ImAA was also chemotactic for eosinophils but at relatively higher doses, peak activity being at $10^{-3}\,M$. *L*-Histidine, 1,4-MeHm, 1,4-MeImAA and N-AcHm were inactive at doses between 10^{-3} and $10^{-7}\,M$. None of the compounds tested possessed any activity for neutrophil-rich (> 94%) leucocyte suspensions.

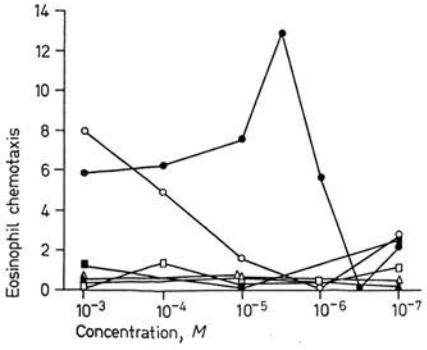

Fig. 1. Chemotaxis (per 5 high-power fields) of guinea pig eosinophils towards histamine (●; n = 8), ImAA (O; n = 4), *L*-histidine (■; n = 3), 1,4-MeHm (□; n = 3), 1.4-MeImAA (▲; n = 3) and N-AcHm (△; n = 3). Each point represents the pooled results of the numbers of experiments shown.

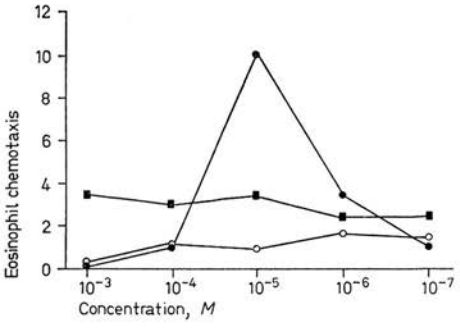

Fig. 2. Chemotaxis (per 5 high-power fields) of guinea pig eosinophils towards the ECF-A tetrapeptides AGSG (●), VGSG (O) and the analogue $VGAG_a$ (■). Each point represents the mean of 3 experiments.

Chemotactic Activity of AGSG, VGSG and $VGAG_a$

Chemotaxis was observed with AGSG at a concentration of $10^{-5}\,M$ (fig. 2). As with histamine the activity was selective, no response being observed with neutrophils, and it was apparently inhibited at high doses. Over a range of concentrations from 10^{-3} to $10^{-8}\,M$, VGSG and $VGAG_a$ were chemotactically inactive.

Effect of Cell Purity on Histamine-Induced Chemotaxis

The pattern and magnitude of the eosinophil response towards doses of histamine

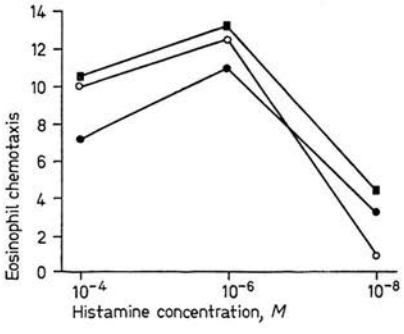

Fig. 3. Chemotactic response (per 5 high-power fields) to histamine of leucocyte suspensions containing 92% (●), 64% (O) and 30% (■) eosinophils. The cell counts and percent purity were pooled from 3 essentially similar experiments.

Table I. Chemotaxis of eosinophils, from initial populations of varying purity, towards a fixed (0.1 ml) dose of inulin-activated homologous serum; eosinophil chemotaxis is expressed as total cell counts per 5 high-power (×40) fields

Experiment	Eosinophils, %	Eosinophil chemotaxis
1	31	86
	67	106
	93	160
2	27	112
	69	191
	93	299
3	31	54
	55	68
	91	90

from 10^{-4} to 10^{-8} *M* were unaffected by the concentration of eosinophils in the leucocyte preparation (fig. 3). Thus the observed chemotaxis was similar whether the starting population contained 30, 64 or 92% eosinophils.

In contrast, the numbers of cells migrating towards an inulin-activated normal guinea pig serum were directly proportional to the eosinophil concentration (table I). This observation was not attributed to an increase in random migration since the presence of the same dose of histamine in both compartments of the Boyden chamber completely abrogated the eosinophil response.

Self-Deactivation of Histamine Chemotactic Activity

When cells were preincubated with histamine (5×10^{-5} *M*) prior to assay with various doses of the same agent as the chemoattractant, the number of migrating eosinophils was always markedly reduced (fig. 4). The dose-response pattern of the control preparations pretreated with Tyrode's buffer to histamine was similar but less pronounced than with untreated cells (see fig. 1).

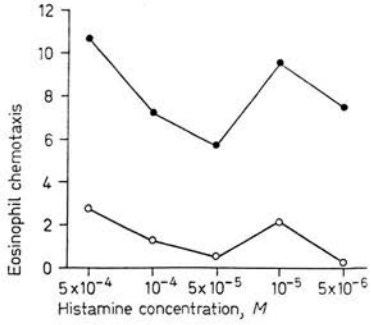

Fig. 4. Chemotactic response of eosinophils (per 5 high-power fields) to histamine following preincubation with Tyrode's buffer (●) or 5×10^{-5} *M* histamine (O). Each point represents the mean of 3 experiments.

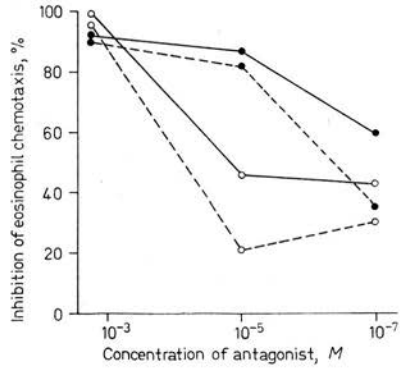

Fig. 5. Chemotactic response of eosinophils to histamine following preincubation with burimamide (●–●) and mepyramine (O–O), or with burimamide (●- - -●) and mepyramine (O- - -O) present in the stimulus compartment throughout.

Inhibition of Histamine-Mediated Chemotaxis

At high doses (10^{-3} *M*), both mepyramine and burimamide abrogated the eosinophil response to histamine (fig. 5). At lower concentrations (10^{-5} *M*), however, chemo-

taxis was selectively inhibited by burimamide. The effect of both drugs was similar irrespective of whether they were mixed with the chemoattractant throughout the assay or preincubated with the eosinophil suspensions and removed by washing before the introduction of the cells into Boyden chambers.

Discussion

Histamine was shown to be chemotactic for guinea pig peritoneal eosinophils over a narrow dose range with peak activity at 5×10^{-6} *M* and inhibition at higher concentrations (fig. 1). These results are comparable with those obtained using human peripheral blood eosinophils as target cells [2, 13]. Guinea pig eosinophils also migrated towards ImAA, showing a similarity in both the pattern of dose-response and magnitude to the response observed with human cells [13]. As in the latter study, no activity was demonstrable with several other histamine metabolites, including *L*-histidine, 1,4-MeHm, 1,4-MeImAA and N-AcHm.

In man, the eosinophil response to histamine was dependent on the absolute numbers of this cell in the starting population [2]. In the present study, there was no apparent correlation between the numbers of migrating cells and their starting concentration (fig. 3). This may be a species difference, but it is unlikely to be due to an increase in random mobility since the presence of identical doses of histamine in both compartments of the Boyden chamber abrogated eosinophil migration. In addition, it was shown that preincubation with histamine deactivated their migration towards the same agent (fig. 4), a property of chemotactic stimuli but not those which promote only random mobility [1]. Unlike histamine, eosinophilotactic response of the guinea pig cells towards an activated homologous serum was directly proportional to their initial concentration (table I).

Thus, eosinophil chemotactic activity joins the traditionally recognised pharmacological actions of histamine such as increasing vascular permeability, contraction of smooth muscle, bronchoconstriction, and the secretion of gastric HCl. The effects on both the vasculature and smooth muscle are blocked by classical (H1) antihistamines, whereas HCl secretion is abrogated in the presence of H2-receptor antagonists. The latter also blocks the self-inhibition of histamine release from anaphylactic mast cells [10]. It was of interest, therefore, to determine whether eosinophilotaxis was under the influence of H1 or H2 receptors. It was found that both mepyramine (H1) and burimamide (H2) were inhibitory at high concentrations (10^{-3} *M*) but that at lower doses (10^{-5} *M*) this effect was principally associated with burimamide (fig. 5). In man, however, neither pyrilamine (H1) or metiamide (H2) at a dose of 10^{-5} *M* had a direct blocking effect on chemotaxis, although the presence of metiamide did reduce the inhibition of cell migration observed with high doses of histamine [2].

The characterisation of human lung ECF-A as two related acidic tetrapeptides [4] has facilitated its synthesis in the laboratory. Synthetic 'ECF-A' (VGSG and AGSG) and an analogue, $VGAG_a$, were shown to possess selective chemotactic activity for human eosinophils [12]. In the present study, AGSG was shown to possess activity for guinea pig cells, but VGSG and $VGAG_a$ were without effect (fig. 2). The

chemotactic activity of histamine, ImAA and the ECF-A tetrapeptide, AGSG, was selective for eosinophils as judged by their total inability to attract neutrophils from pure (> 94%) suspensions.

Several conclusions may be drawn from the data presented. These may be summarised as follows: (a) in guinea pig, as in man, the accumulation of eosinophils at anaphylactic tissue sites involves the complex interaction of several components of the reaction, including histamine, ECF-A and possibly ImAA, in a manner not yet understood; (b) this interaction may be, in part, mediated through H2 receptors present on the target cells, although there is a difference between the human and guinea pig observations in this respect, and (c) guinea pig ECF-A may differ from its human counterpart, although it remains likely that both are families of closely related tetrapeptides.

Acknowledgements

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Human eosinophils, acidic tetrapeptides (ECF-A) and histamine

INTERACTIONS *IN VITRO* AND *IN VIVO*

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Summary. The ECF-A acidic tetrapeptides Val-Gly-Ser-Glu, Ala-Gly-Ser-Glu and the analogue Val-Gly-Asp-Glu were selectively chemotactic for human eosinophils over a narrow dose range although eosinophils from different individuals varied in their dose-response pattern. Histamine abrogated the chemotactic properties of the individual tetrapeptides. When Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu were combined in various concentrations the resultant chemotaxis was either negligible or no greater than that produced when each peptide was tested separately.

Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu both promoted eosinophil accumulation when applied to the abraded skin of man or i.d. to the marmoset. Biopsies of marmoset skin revealed that peptide-induced eosinophilia was not associated with mast-cell degranulation.

Histamine, which was chemotactic *in vitro*, did not lead to appreciable eosinophil accumulation *in vivo*, and combinations of histamine and the acidic tetrapeptides evoked little or no cutaneous eosinophil infiltration either in man or the marmoset.

These studies suggest that there is a complex interaction between histamine and the ECF-A tetrapeptides; however, the tetrapeptides alone can promote the recruitment and localization of eosinophils by a mechanism apparently independent of mast-cell degranulation.

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INTRODUCTION

It is now recognized that a number of chemical mediators associated with immediate-type (type I) hypersensitivity reactions can attract selectively human eosinophils *in vitro*. These include the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay, 1969; Kay & Austen, 1971), now identified as at least two acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (Goetzl & Austen, 1975), an analogue, Val-Gly-Asp-Glu (Kay, 1976), histamine (Clark, Gallin & Kaplan, 1975) and one of its major catabolites, imidazole acetic acid (Turnbull & Kay, 1976). In the present report we describe the effect of combining these peptides with histamine in terms of their capacity both to attract selectively the eosinophil in chemotaxis and to evoke eosinophil accumulation following administration to the skin of man and a non-human primate.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: (HCl)-Val-Gly-

Ser-Glu (mol. wt 427), (HCl)-Ala-Gly-Ser-Glu (mol. wt 497) and (HCl)-Val-Gly-Asp-Glu (mol. wt 455) (a gift from Dr R. Camble, ICI Ltd, Pharmaceuticals Division, Alderley Park, Macclesfield); histamine acid phosphate (BDH Chemicals Ltd., Poole); cellulose nitrate filters 8·0 μ (Sartorius-Membrane Filters, 34 Göttingen, W. Germany), Millipore filters 0·2 μ (Millipore Corp., Bedford, Massachusetts); Timothy grass pollen (TGP), lyophilized and defatted (a gift from Beecham Research Laboratories, Betchworth).

Eosinophil chemotaxis

Peripheral blood was obtained from individuals with an eosinophilia of between 10 and 25%. The preparation of eosinophil leucocytes and the chemotactic assay were performed as previously described (Turnbull & Kay, 1976).

Marmoset skin studies

Marmosets (*Callithrix jacchus*) of either sex, with an average weight of 350 g, were maintained on a balanced vegetable diet with added vitamins and salts. Six marmosets received i.d. injections of 0·05 ml of serum from an individual (R.B.) sensitive to TGP. Following a 48-h sensitization period the same sites were challenged i.d. with 1 μg of TGP contained in 0·05 ml of saline. At the same time other sites, all on the abdomen, received 0·05 ml of the following: histamine; Val-Gly-Ser-Glu (valyl-peptide); Ala-Gly-Ser-Glu (alanyl-peptide); valyl-peptide and histamine; alanyl-peptide and histamine; valyl-peptide, alanyl-peptide and histamine; TGP; or saline—all at a final concentration of 10^{-3} mol/l whether given alone or in combination; and TGP (1 mg/ml). Immediately after the injections, 0·5 ml of 1·0% Evans blue in saline was administered i.v. These procedures were carried out on six animals of which three were killed at 12 h and three at 24 h following injection of Evans blue. Immediately after the animals were killed, the full thickness of the skin sites were excised and fixed and stained for eosinophils and mast cells. This was performed on the same sections using a modification of the method of Lendrum (1944) in which Alcian blue and chromotrope 2R were used to identify mast cells and eosinophils respectively (Hogg, R.M. & Banks, D., manuscript in preparation). Cell counts were performed as described (Kay, 1970a). Neutrophil counts were expressed semi-quantitatively on a 'plus' basis.

Human skin studies

'Skin window' studies were performed on four atopic volunteers who had seasonal, allergic rhinitis and an immediate-type skin weal and flare reaction to TGP. The method used was slightly modified from that described by Felarca & Lowell (1968). Skin sites on the forearm were thoroughly cleaned with 70% alcohol, and minimally abraded with a high-speed drill. The penetration of skin was insufficient to cause bleeding. Skin sites were approximately 0·3 cm in diameter and 5 cm apart. Plastic 'hats' (10 mm diameter and 5 mm high) were packed with sterile cotton wool and sealed with a Millipore filter (0·2 μ) using a thermoplastic glue. The cotton wool acted as a reservoir for the test solutions. Between the abrasion and the plastic 'hat' a second 0·2 μ filter was introduced which was removed and replaced with a new filter at time intervals of 2, 4, 6, 8, 12, 16, 20 and 24 h. Fresh filters were dampened with the appropriate test solution and at each change the interior of the plastic 'hats' was moistened by injection of the same solution into the cotton-wool reservoir. The plastic 'hats' were securely attached to the skin with adhesive tape and crepe bandages. The solutions were as follows: Tyrode's diluent; histamine; valyl-peptide; alanyl-peptide; valyl-peptide and histamine; alanyl-peptide and histamine; valyl- and alanyl-peptide; valyl-peptide, alanyl-peptide and histamine—all at a concentration of 10^{-3} mol/l whether given alone or in combination; and TGP (1 mg/ml). The filters, removed at the time intervals described above, were fixed and stained as previously described (Kay, 1970b). Counts were expressed as the total of 10 random high-power fields (×90), per filter.

RESULTS

Eosinophil chemotaxis

The valyl- and alanyl-tetrapeptides, and the analogue Val-Gly-Asp-Glu, were tested for eosinophil chemotaxis over a wide dose range (Fig. 1). Eosinophils from six subjects gave two types of dose-response pattern with the valyl- and alanyl-peptides. Of these, four individuals gave peak activity to the valyl-peptide at 10^{-8} mol/l, with inhibition at higher doses, whereas two patients gave two peaks of activity, one at 10^{-4} mol/l and one at 10^{-7} mol/l. Eosinophils from individuals giving a double peak of activity with the valyl-peptide gave a similar pattern

of response to the alanyl-peptide; however the response at 10^{-4} mol/l was less marked. Similarly the four individuals whose eosinophils gave a single peak at 10^{-8} mol/l with the valyl-peptide gave a comparable response to Ala-Gly-Ser-Glu. Only those subjects who gave two peaks of activity with the valyl- and alanyl-peptides were tested against Val-Gly-Asp-Glu. The eosinophils from these individuals gave a single peak with this analogue which was maximal at 10^{-7} mol/l. The patterns of activity, in terms of the shape of the dose-response curve, remained constant for eosinophils from the same person tested against any of the individual peptides on three or more separate occasions.

Eosinophils from the four individuals who gave a single peak of activity to the valyl- or alanyl-peptides, were tested in chemotaxis against histamine, either alone or in combination with the peptides. With histamine alone these subjects gave a linear dose response with peak activity at the highest concentration. When the valyl- (Fig. 2) or alanyl- (Fig. 3) peptides were tested in combination with histamine at either 10^{-3}, 10^{-5} or 10^{-7} mol/l the resultant chemotaxis was negligible.

When the two peptides were mixed the resultant eosinophil chemotaxis was dependent on their relative concentrations (Fig. 4). Increasing concentrations of the alanyl-peptide were combined with the valyl-peptide at either 10^{-4}, 10^{-6} or 10^{-8} mol/l. Chemotaxis was only observed when the alanyl-peptide, at concentrations from 10^{-5} to 10^{-9} mol/l, was combined with the valyl-peptide at 10^{-8} mol/l.

Further experiments (not shown in the figures) using a variety of dose combinations of both the peptides and histamine again resulted in negative chemotaxis. A similar effect was observed when the analogue Val-Gly-Asp-Glu was combined with histamine.

In all studies neutrophil chemotaxis paralleled the eosinophil response although the counts for the former were considerably lower. Thus the pattern of the various dose responses was virtually identical for both cell types, there being neither neutrophil nor eosinophil chemotaxis using combinations of the peptides and histamine.

Marmoset skin studies

In preliminary experiments it was shown that following passive cutaneous anaphylactic (PCA) reactions in the marmoset a peak of eosinophil accumulation was seen at 12 h, whereas untreated skin examined at 4, 8, 12 and 24 h from animals given Evans blue dye i.v. contained neither eosinophils nor neutrophils. With increasing concentrations of serum from the TGP-sensitive individual (R.B.) there was a concomitant increase in both the 'blueing' reaction and the subsequent infiltration of eosinophils. The PCA reactions therefore served as a positive control enabling a comparison of the eosinophil promoting effects of histamine and the valyl- and alanyl-peptides. Following i.d. injection of these agents either alone or in combination, the resultant eosinophil and neutrophil accumulation was determined at 12 and 24 h (Fig. 5). At 12 h neutrophil infiltration was observed with the controls [antigen alone, antibody alone (not shown in Fig. 5) and saline] but considerably more was seen at the treatment sites and following the PCA reaction. At 24 h, few neutrophils were seen with saline, antigen, antibody or histamine alone but with the other treatments large numbers of neutrophils were still present. Few eosinophils were found at 12 or 24 h with antigen, antibody, saline or histamine alone; however, when these treatments were compared to the alanyl- and valyl-peptides the eosinophil accumulation at 24 h was significantly greater ($P < 0{\cdot}02$). At 12 h there was relatively little eosinophilia with the peptides. At 24 h, when the valyl-peptide was combined with histamine, the resultant eosinophil infiltration was significantly less than that obtained when the peptide was injected separately ($P < 0{\cdot}02$). When the alanyl-peptide was combined with histamine the infiltration although much less than the peptide alone was not significantly different. Similarly when all three agents were administered simultaneously inhibition of eosinophil accumulation was observed when this mixture was compared to the alanyl- ($P < 0{\cdot}02$) or valyl-peptide ($P < 0{\cdot}05$) alone. None of the differences was significant at 12 h. In the control PCA sites there were more eosinophils at 12 h than at 24 h, whereas the individual peptides gave a greater eosinophil response at 24 h.

In general there was little difference in the number of mast cells present in the test and control sites at 12 h. However at 24 h, there were fewer mast cells in the PCA site, compared to the control and treatment areas of skin. These studies indicate that the marmoset PCA reactions are associated with mast-cell depletion (presumably as a result of

degranulation) but that the acidic tetrapeptides administered either alone, or in combination with histamine, did not affect mast cells as assessed by light microscopy of paraffin sections. The 'blueing' reactions evoked by the peptides in the marmoset skin were usually diffuse and ill-defined and therefore it was not possible to quantify them with accuracy.

Human skin studies

The valyl- and alanyl-peptides and TGP all evoked eosinophil infiltration following application to the abraded skin of atopic individuals (Fig. 6), the TGP positive control giving the greatest response. The pattern of eosinophil infiltration to TGP was biphasic with peaks at 8 h and 20–24 h. A similar, but less marked, response was observed when either the valyl- or alanyl-peptides were administered separately. At 24 h the counts with these peptides were significantly greater than the Tyrode's control ($P < 0{\cdot}05$). However, even at the concentrations used (10^{-3} mol/l) the resultant eosinophilia was only approximately one-third that of the TGP control. When the two peptides were applied together there was an apparent inhibition of eosinophil accumulation when the values from this combination were compared to the agents administered separately ($P < 0{\cdot}02$). Applications of histamine led to minimal eosinophil infiltration but this was not significantly different to the Tyrode's control at any of the time intervals studied.

The response to Val-Gly-Ser-Glu or Ala-Gly-Ser-Glu administered in combination with histamine was significantly less ($P < 0{\cdot}05$ and $P < 0{\cdot}05$, respectively) than with the peptides alone. A mixture of either of the two peptides and histamine gave a smaller response than the combination of both peptides and histamine although this difference was not significant. Whatever the nature, or combination, of the agents giving a positive eosinophil response the patterns tended to be biphasic. The first peak was between 6 and 8 h and the second, which was always the more marked, between 20 and 24 h. Neither of the peptides, nor combinations of the peptides with histamine, gave an eosinophil response comparable in magnitude to the TGP control. With all treatments, excluding TGP, the predominant cell type was the neutrophil but the numbers were too numerous for accurate quantification.

DISCUSSION

A product of the anaphylactic reaction which selectively attracted eosinophils from a mixed leucocyte population (Kay, 1969) was later identified as the eosinophil chemotactic factor of anaphylaxis (ECF-A) and shown to be a unique mediator of type I hypersensitivity (Kay & Austen, 1971). It now appears that this activity is the property of a family of closely related acidic peptides, two of which were identified as Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu (Goetzl & Austen, 1975). A certain amount of aspartic acid was also present in highly purified ECF-A and it has subsequently been shown that the analogue Val-Gly-Asp-Glu possessed comparable eosinophilotactic activity (Kay, 1976). The observation that histamine (Clark, Gallin & Kaplan, 1975) and one of its major catabolites, imidazole acetic

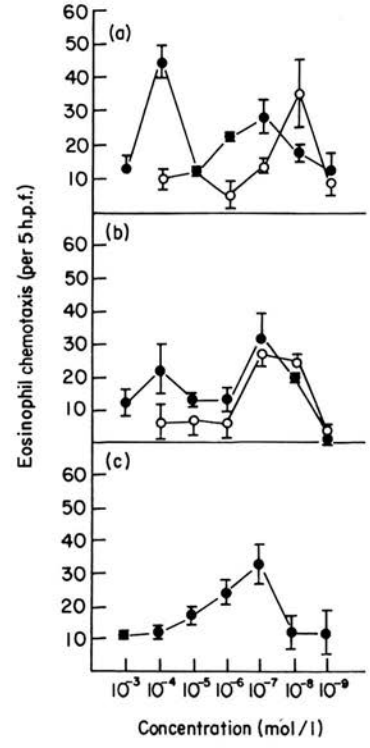

Figure 1. Eosinophil chemotaxis by Val-Gly-Ser-Glu (a), Ala-Gly-Ser-Glu (b) and the analogue Val-Gly-Asp-Glu (c). The two patterns of dose-response were from four individuals (●) with eosinophilia in association with non-Hodgkin's lymphoma, microfilariasis, carcinoma of colon and loa-loa, and two (○) in association with hypersensitivity to mefenamic acid and the 'hypereosinophilic syndrome'. Bars represent ± s.e.m.

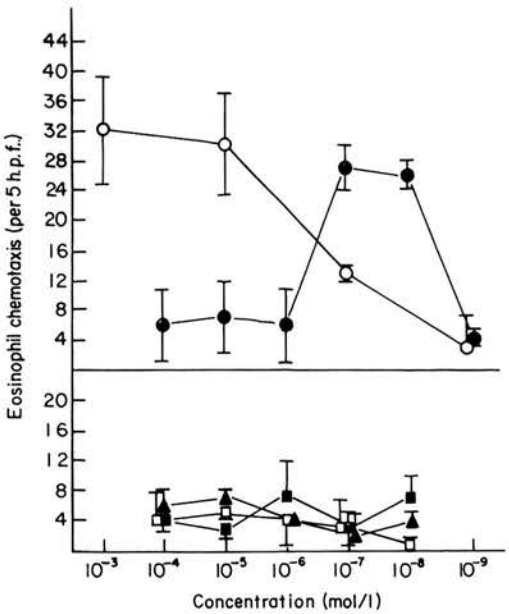

Figure 2. Eosinophil chemotaxis to Ala-Gly-Ser-Glu (●), or histamine (○), or the two agents in combination (■) Ala-Gly-Ser-Glu+histamine 10^{-3} mol/l; (□) Ala-Gly-Ser-Glu+Histamine 10^{-5} mol/l; (▲) Ala-Gly-Ser-Glu+histamine 10^{-7} mol/l.

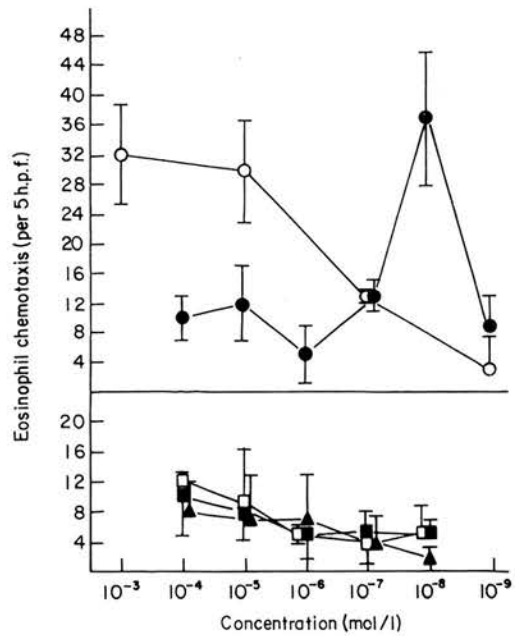

Figure 3. Eosinophil chemotaxis to Val-Gly-Ser-Glu (●), or histamine (○), or the two agents in combination. (■) Val-Gly-Ser-Glu+histamine 10^{-3} mol/l; (□) Val-Gly-Ser-Glu+histamine 10^{-5} mol/l; (▲) Val-Gly-Ser-Glu+histamine 10^{-7} mol/l.

acid (Turnbull & Kay, 1976) are also preferentially chemotactic for eosinophils points to the complexity of anaphylaxis-associated eosinophil chemotactic agents. In previous studies anaphylactic diffusates, in general, gave a linear dose response in chemotaxis, maximal activity being observed with the highest concentrations. In contrast, the peptides, or histamine, gave varying patterns of dose-response activities. Furthermore, different sources of target cells also gave differing chemotactic profiles to these agents. In general the peptides gave a peak of activity between 10^{-6} and 10^{-8} mol/l with apparent inhibition at higher doses (Fig. 1). Eosinophils from some individuals gave an additional peak with the valyl- and alanyl-peptides at 10^{-4} mol/l.

In this report various combinations of the peptides or histamine were tested in chemotaxis in order to ascertain whether the pattern of response was comparable to that of previously published studies employing anaphylactic diffusates. It was found that when histamine was combined with either the valyl- or alanyl-peptides the resultant chemotaxis was negligible (Figs 2 and 3). Thus not only did histamine and the peptides fail to act additively or synergistically but their combination abrogated the chemotactic response suggesting that there may be cross-deactivation between these agents. However we have recently shown that prior incubation of cells with histamine did not affect their response to the peptides and similarly incubation with the peptides did not abrogate the response to histamine (Turnbull & Kay, unpublished observations). In contrast prior incubation with histamine deactivated the cells for chemotaxis towards histamine or imidazole acetic acid (Turnbull & Kay, 1976) and prior incubation with the individual peptides deactivated to the same peptides (Goetzl & Austen, 1975). At the present time we are unable to explain these clearly complex interactions but suggest that *in vivo* there are possibly two processes which may require different relative concentrations and combinations of the various agents. Such events may be, firstly, directional migration of the cells and secondly, stabilization of the eosinophil at the site of allergic reactions. A possible role for histamine in localizing the eosinophil at the site of anaphylactic reactions has previously been suggested (Parish, 1974). It may be that there are also other, as yet unrecognized,

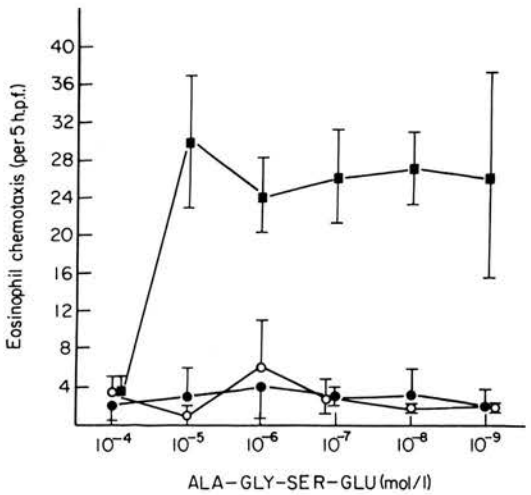

Figure 4. Chemotaxis of eosinophils to varying combinations of Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu. (●) Alanyl+ Val-Gly-Ser-Glu 10^{-4} mol/l; (○) analyl+ Val-Gly-Ser-Glu 10^{-6} mol/l; (■) alanyl+ Val-Gly-Ser-Glu 10^{-8} mol/l.

chemotactic agents in the anaphylactic diffusate which also contribute to directional migration and/or localization. In addition there may be, *in vivo*, differing pharmacokinetics in terms of histamine and ECF-A diffusion and/or inactivation, all of which may be critical for the observed eosinophil accumulation and localization. Many of these problems will not be solved until the relative

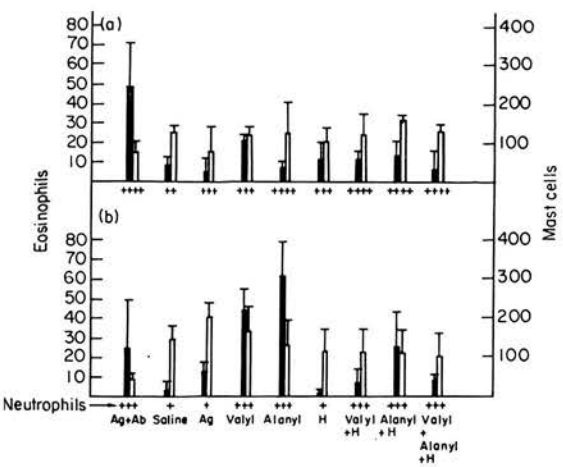

Figure 5. Eosinophil (solid columns) and neutrophil accumulation, and mast-cell counts (open columns) in the skin of marmosets at 12 h (a) and 24 h (b) following the i.d. injection of the acidic tetrapeptides, histamine or following passive cutaneous anaphylaxis (Ag+Ab).

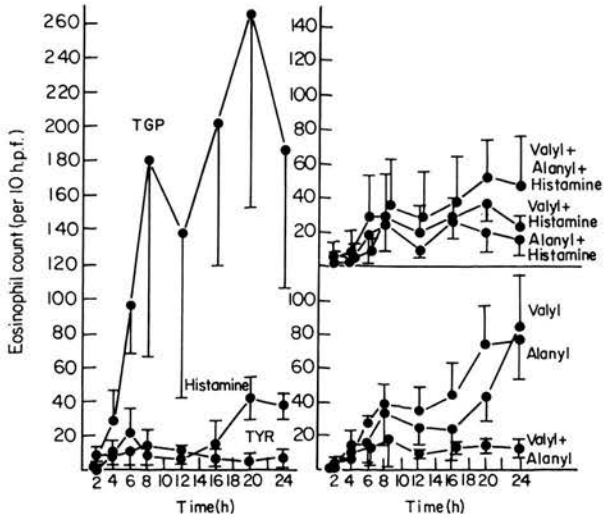

Figure 6. Eosinophil accumulation in human 'skin windows' following the application of the valyl-, the alanyl-, or histamine or these agents in various combinations. Timothy grass pollens (TGP) and Tyrode's (TYR) solution served as the positive and negative controls respectively.

amounts of the acidic peptides, histamine and possibly imidazole acetic acid present in anaphylactic diffusates are known. For this purpose the development of a quantitative assay for the peptides in biological fluids is required.

When the valyl- and alanyl-peptides were combined at low doses in the absence of histamine the resultant chemotaxis was similar to that of each peptide tested alone (Fig. 4). The apparent inhibition of chemotaxis by higher doses of the alanyl-peptide alone could be abrogated when this peptide was mixed with the valyl-peptide at 10^{-8} mol/l.

The studies *in vivo* using the skin of marmosets and humans had essential differences in their experimental design. Experiments in marmoset skin involved full thickness skin biopsies following a single injection of the agents either alone or in combination (Fig. 5). The human skin window studies, however, involved repeated applications of the agents to the skin site at 2- or 4-h intervals over the 24 h study period. These probably explain the major differences between the results obtained in humans and the non-human primate. In marmosets and man both the alanyl- or valyl-peptide could promote infiltration of eosinophils (Fig. 5). Combinations of the peptides with histamine gave lower eosinophil counts in both experimental situations. The exception was when histamine and

the valyl- and alanyl-peptides were combined together in human skin (Fig. 6). In this situation the resultant eosinophilia was comparable to the valyl- or alanyl-peptide alone and only slightly more than histamine alone. These, however, were the observations at 24 h. At 12 h, in human skin, histamine promoted very little eosinophil response whereas the peptides alone showed some activity. It was the impression both with the peptides and the positive control (Timothy grass pollen) that there was a biphasic response with a peak at 8 h, a slightly lower count at 12 h and then rising to a greater peak at 24 h. This dual response was not particularly marked but was a comparable finding to that of Hirashima & Hayashi (1976). These workers found a biphasic response in terms of eosinophil infiltration in active anaphylaxis in the guinea-pig. The first peak was associated with ECF-A-like material whereas the second was related to an eosinophilotactic protein of molecular size approximately 70,000, the nature of which was undetermined. The studies described in this report may be indicative of a comparable phenomenon in humans.

There have been a number of studies relating to capacity of histamine to promote a local eosinophilia in the skin of man. Both Eidinger, Wilkinson & Rose (1964) and Feinberg, Feinberg & Lee (1967) reported that histamine could induce a modest eosinophilia in atopic individuals but this response was far less than that produced by specific antigen. In contrast Felarca & Lowell (1968) found no such eosinophil promoting effect with histamine alone. In general, these studies, and those described in the present report suggest that in man histamine has slight eosinophil-attracting activities *in vivo* although we were unable to show that the response, by this agent, was significantly different from the Tyrode's control. In any event the response to histamine was far less than that of the individual tetrapeptides and these, in turn, evoked less eosinophil infiltration than the specific antigen.

Our demonstration of eosinophil recruitment in the skin of man by acidic tetrapeptides may possibly have clinical significance in terms of an individual's capacity to mobilize this cell type in various disease states associated with an eosinophilia.

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Eosinophil chemotaxis to an ECF-A tetrapeptide and histamine: the response in various disease states

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Summary

Using the micropore filter technique of Boyden, the eosinophil chemotactic responses to an ECF-A tetrapeptide (Val-Gly-Ser-Glu) and histamine were studied in thirty-two patients with a peripheral blood eosinophilia. The eosinophilia was associated with extrinsic asthma (twelve patients), neoplasia (seven), helminth disease (five) and a miscellaneous group (eight) which included three patients with pulmonary eosinophilia, one of whom also had asthma. Both the tetrapeptide and histamine gave two types of dose–response. With Val-Gly-Ser-Glu this was either a single peak at 10^{-8}, or two peaks at 10^{-4} and 10^{-7} mol/l, respectively. Histamine gave either a linear dose–response with maximal chemotaxis at the highest concentration or a peak response at 10^{-5} mol/l with inhibition at higher doses. The two types of response given by the peptide were not related to the disease state and were reproducible when tested on more than one occasion. However, with histamine, linear dose–responses were observed in ten out of twelve patients with asthma, four out of five with helminth disease and two of the three with pulmonary eosinophilia. This was also reproducible when tested on subsequent occasions. Therefore, in these diseases, which are known to be associated with exogenous antigens and raised IgE levels, eosinophils from 80% of the patients studied (sixteen out of twenty) gave a linear rather than a 'bell-shaped' response to histamine. In contrast, only four of twelve patients (33%) with eosinophilia in association with other diseases (which included seven with neoplasia) gave this type of response. If this observation can be confirmed with larger numbers of patients it may be useful diagnostically in eosinophilia of unknown origin.

Introduction

It has now been established that, using the appropriate micropore filter, both the ECF-A peptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) and histamine selectively attract eosinophils from a mixed leucocyte population (Kay & Austen, 1971; Goetzl & Austen, 1975; Clark, Gallin & Kaplan, 1975). In previous reports the authors have shown that cells obtained from six individuals with an eosinophilia gave two patterns of dose–response curve to histamine and two to the peptides (Turnbull & Kay, 1976;

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Turnbull, Evans & Kay, 1977). The purpose of the present study was to determine whether, in thirty-two individuals with an eosinophilia, the different patterns of chemotactic response could be related to the subjects' disease state.

Materials and methods

Materials

Materials were obtained as follows: (HCl)-Val-Gly-Ser-Glu, mol. wt. 427 (a gift from Dr R. Camble, I.C.I. Ltd, England); histamine acid phosphate (BDH Chemicals, Poole, England); ovalbumin five times crystallized (Koch-Light Laboratories, Colnbrook, Bucks., England); cellulose nitrate filters, 8·0 μm (Sartorius-Membrane Filters, 34 Göttingen, W. Germany).

Patients

Peripheral blood was obtained from thirty-two individuals with an eosinophilia of between 9 and 37%. Twelve of the patients had extrinsic bronchial asthma with positive prick test reactions to a variety of extracts of common inhalant allergens. None of these patients was taking corticosteroids or antihistamines. Seven patients had neoplasms. These were Hodgkin's disease (three), non-Hodgkin's lymphoma (one), bronchogenic carcinoma (two), acute lymphatic leukaemia (one) and carcinoma of the colon (one); none had received any chemotherapy. Five of the patients had helminth infestation. Three had filariasis, one had Loa-Loa and one had recently returned from central Africa and the nature of his helminth had not been precisely defined. Another group of eight patients had a miscellaneous group of disorders which included pulmonary eosinophilia (three), one of whom also had asthma, and rheumatoid arthritis (two) (Table 1).

Eosinophil chemotaxis

The preparation of eosinophil leucocytes for the chemotactic assay was performed as previously described (Turnbull & Kay, 1976). Leucocyte-rich plasma, obtained by dextran sedimentation of heparinized blood (Kay, 1970), was applied to a density gradient of 9% Ficoll solution and sodium diatrizoate (density 1·140) in the proportions of 2·4:1 (English & Anderson, 1974). Following centrifugation at 400 *g* for 40 min at 20°C the eosinophil-rich pellet was retained, washed in Tyrode's solution and resuspended in Tyrode's solution containing 0·5% ovalbumin. The cell counts were then adjusted to between 2 and 3×10^6 cells/ml, with a final eosinophil concentration of 20–30%. Histamine or the valyl-peptide were dissolved in Tyrode's solution and the pH adjusted to 7·24. All concentrations of each of these agents were assayed in duplicate. Following a 3 hr incubation, filters were removed, fixed and stained as previously described (Kay, 1970). The number of eosinophils which had migrated through each filter was then counted and the results expressed as the total number of cells in five random high power fields ($\times 40$) per filter. The mean from each pair of duplicate readings was used in the subsequent statistical analysis as previously described (Turnbull & Kay, 1976).

Results

The peripheral blood eosinophil count (mean $\pm$ 1 s.d.) for the groups of patients were:

Table 1. Eosinophil chemotactic response to histamine and valyl-peptide of three patients with pulmonary eosinophilia and of five patients with miscellaneous disorders

Subject	Diagnosis	Eosinophil counts/5 high power fields								
		Tyrode's solution	Histamine (mol/l)			Valyl-peptide (mol/l)				
			10^{-3}	10^{-5}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
E.A.	Pulmonary eosinophilia ?cause	1	18	11	9·5	22	22	19	26·5	11·5	11
J.H.	Pulmonary eosinophilia ?cause	1	10·5	11·5	9·5	39	27·5	14·5	46	30	21
A.D.	Pulmonary eosinophilia +asthma and allergic aspergillosis	6	36	20	19	45·5	33	23	22	41	15
	Mean	2·7	21·5	14·2	12·7	35·5	27·5	18·8	31·5	27·5	15·7
	±1 s.e.m.	1·7	7·7	3·0	3·2	7·0	3·2	2·5	7·3	8·6	2·9
M.C.	Drug reaction	1·5	4·5	19·5	9	7	8·5	8·5	14	19·5	6
F.F.	Hypereosinophilic syndrome	3·5	15·5	18	7	34	13·5	11	39	8·5	6
F.B.	Ulcerative colitis	3·5	14	27·5	11	14·5	27	13·5	16	28·5	18·5
C.P.	Rheumatoid arthritis	4·5	12·5	14	7·5	7·5	9	11	14·5	20	4·5
J.C.	Rheumatoid arthritis	4·5	12·5	17	6·5	12·5	5·5	9	21	13·5	5
	Mean	3·3	11·8	19·2	8·2	15·1	12·7	10·6	20·9	18·0	8·0
	±1 s.e.m.	0·5	1·9	2·3	0·8	5·0	3·8	0·9	4·7	3·4	2·6

asthma, 11·9±2·7%; neoplasia, 13·4±3·2%; helminth infestation, 13·4±3·2%; pulmonary eosinophilia and miscellaneous conditions (Table 1), 17·5±9·0%. The differences between these groups were not statistically significant (Wilcoxon test). Similarly there were no significant differences in the concentration of eosinophils, after partial separation, and purification between the different groups of subjects studied.

The results of the eosinophil chemotaxis experiments in the groups of patients with asthma (Fig. 1), helminth infestation (Fig. 2), neoplasia (Fig. 3), pulmonary eosinophilia and the miscellaneous group (Table 1) indicate that there were two types of dose–response with each chemoattractant. In the majority of patients with asthma, helminth disease and pulmonary eosinophilia the shape of the dose–response to histamine was linear while in others a 'bell-shaped' curve was obtained, maximum eosinophil chemotaxis being observed at a histamine concentration of 10^{-5} mol/l with apparent inhibition at higher concentrations (Table 2). Neither the numbers of eosinophils migrating or the shape of the dose–response curves were related to the blood eosinophil count (Fig. 4).

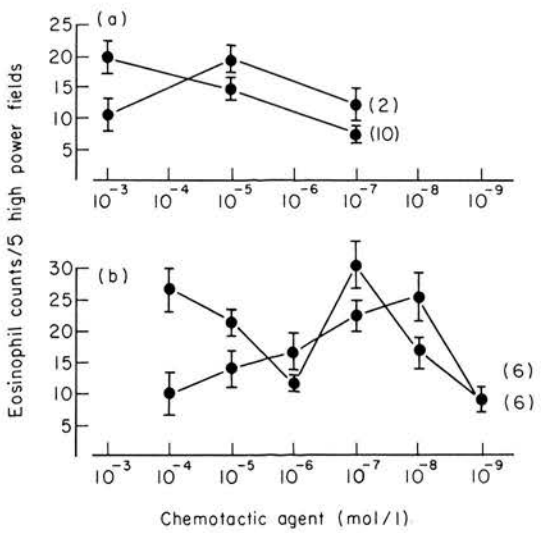

Fig. 1. Mean (±1 s.e.m.) eosinophil chemotactic response of twelve patients with extrinsic asthma. The numbers of patients giving each pattern of response are shown in parentheses. (a) Histamine; (b) Val-Gly-Ser-Glu.

When the asthma, helminth and pulmonary eosinophilia group were considered together, sixteen of the twenty patients (80%) gave a linear dose–response to histamine. Only 33% (four of the twelve) in the other groups, which included four patients with neoplasms, gave a straight line response.

Two patterns of eosinophil response were also seen when the valyl-peptide was used as the chemotactic agent. In some of the patients there was a single peak of eosinophil response at 10^{-8} mol/l whereas in others there were two peaks of activity, one at 10^{-4} mol/l the other at 10^{-7} mol/l. These two patterns of response were observed with similar frequency (Table 2) and could not be related to the disease state or the patients' peripheral blood eosinophil count (Fig. 4). Like histamine, the number

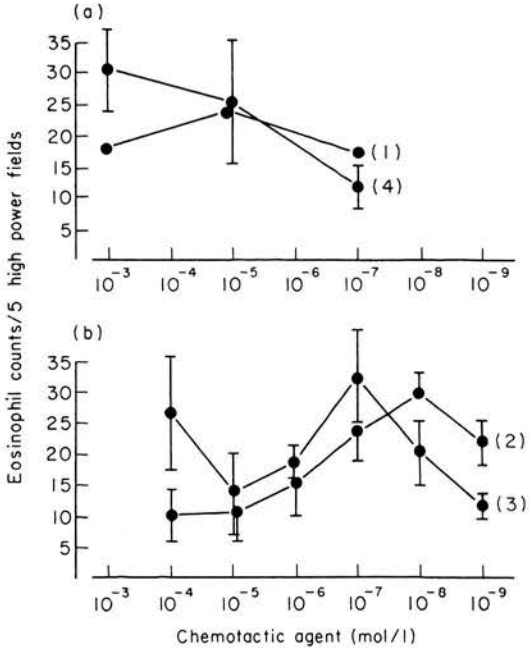

Fig. 2. Mean (±1 s.e.m.) eosinophil chemotactic response of five patients with helminth disease. The numbers of patients giving each pattern of response are shown in parentheses. (a) Histamine; (b) Val-Gly-Ser-Glu.

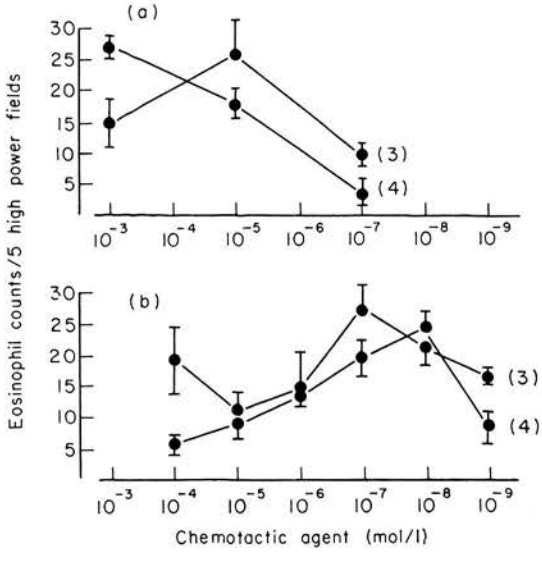

Fig. 3. Mean (±1 s.e.m.) eosinophil chemotactic response of seven patients with neoplastic disease. The numbers of patients giving each pattern of response are shown in parentheses. (a) Histamine; (b) Val-Gly-Ser-Glu.

Table 2. Patterns of the eosinophil chemotaxis dose-response to histamine or the ECF-A valyl-peptide in patients with diseases associated with exogenous antigens (extrinsic asthma, helminth disease and pulmonary eosinophilia) and others with eosinophilia (neoplasia and a miscellaneous group)

	No. of patients	Shape of dose–response curve to histamine	
		'Bell-shaped'	Straight line
Extrinsic asthma	12	2	10
Helminth disease	5	1	4
Pulmonary eosinophilia	3	1	2
Total	20	4	16
Neoplasia	7	3	4
Miscellaneous	5	5	0
Total	12	8	4
		Shape of dose–response curve to valyl-peptide	
		One peak of chemotactic activity	Two peaks of chemotactic activity
Extrinsic asthma	12	6	6
Helminth disease	5	2	3
Pulmonary eosinophilia	3	0	3
Total	20	8	12
Neoplasia	7	4	3
Miscellaneous	5	2	3
Total	12	6	6

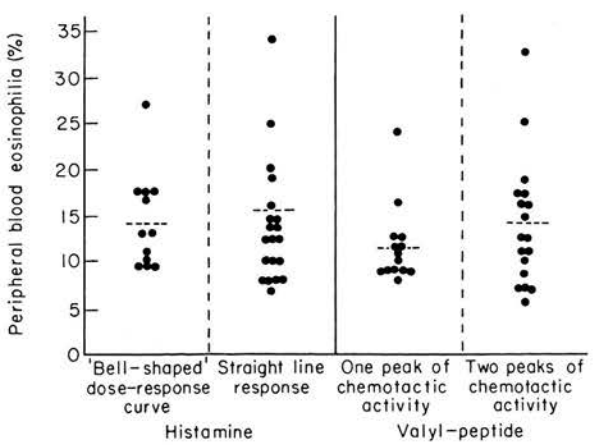

Fig. 4. Peripheral blood eosinophil counts of patients with different patterns of dose-response curve to histamine and the valyl-peptide.

of eosinophils migrating to this peptide were virtually identical irrespective of the disease state or the peripheral blood eosinophil count.

The individual results for eight of the thirty-two patients are shown in Table 1, but in the others mean values are given (Figs 1, 2 and 3). However, there was no apparent relationship between the pattern of response to histamine and the ECF-A peptide. For example, of the twenty patients in all who gave a straight line response to histamine, ten gave one peak and ten gave a two peak response to peptide.

The patterns of dose–response to both the peptide and histamine were identical when repeated on one or more occasions in eleven patients. These were helminth disease (three times with three patients, and twice with one patient), pulmonary eosinophilia (three times with two patients), asthma (twice with three patients), miscellaneous (twice with two patients).

Discussion

Under the conditions of these *in vitro* studies, using a modified Boyden chemotactic assay, we have observed two patterns of dose–response to histamine so confirming the observations in a previous report (Turnbull *et al.*, 1977). In that study, employing eosinophils from only six subjects, the linear dose–response with highest activity at the greatest concentration and the response giving a single peak of activity with inhibition at higher doses were equally divided among these numbers. In the present investigation, in which thirty-two patients were studied, the linear dose–response to histamine was far more frequent in those who had diseases associated with exogenous antigens (Figs 1 and 3, Tables 1 and 2). Although we did not measure IgE levels it is reasonable to assume that in this respect extrinsic asthma, helminth disease and pulmonary eosinophilia represent a homogenous group in terms of our present understanding of the mechanism of eosinophilia. That sixteen out of twenty (80%) gave a straight line response to histamine suggests that this observation may be useful diagnostically in the investigation of eosinophilia of unknown cause. Clearly larger numbers are required to substantiate this finding. However, the observation is supported by the fact that only four out of twelve (33%) of the remainder gave a straight line pattern.

Of the two recognized ECF-A tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (Goetzl & Austen, 1975), only one, the valyl-peptide, was employed in the present study since from previous work, and other unpublished observations, the pattern of response in a particular individual is the same for each peptide (Turnbull *et al.*, 1977). These two shapes of dose–response to the peptides, unlike histamine, did not appear to be related to the disease associated with the eosinophilia. Moreover, none of the responses appears to be related to the percentage of eosinophils in the cell population before migration (Fig. 4). The reasons for these different dose–response curves are unclear. However, the apparent inability of pharmacological or biological agents to exert their effects at high, but non-cytotoxic, concentrations is well documented. An example is the inhibition, by disodium cromoglycate, of histamine release from passively sensitized human lung fragments challenged with specific antigen (Orange & Austen, 1971). In this situation the pharmacological effect was only apparent at doses of 10 μg/ml or less, no effect being observed at higher doses. In the present study inhibition at high doses of the chemoattractant was unlikely to be a cytotoxic effect, since the migration of eosinophils into the micropore was unaffected by the higher doses of either histamine or ECF-A peptide.

In the accompanying paper differences between atopic and non-atopic individuals were described in terms of their 'skin window' responses to histamine and the valyl-peptide. Of considerable interest will be the relationship between the *in vivo* and *in vitro* response to these agents in the variety of eosinophilia-associated diseases and this is the subject of an ongoing study.

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Cutaneous eosinophil accumulation in atopic and non-atopic individuals: the effect of an ECF-A tetrapeptide and histamine

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Summary

The numbers of eosinophils recruited locally to abraded human skin were measured in eight atopic and eight non-atopic volunteers, at time intervals over 24 hr, following the application of an ECF-A tetrapeptide (Val-Gly-Ser-Glu) or histamine. In all subjects the higher doses of the peptide (10^{-4} and 10^{-6} mol/l) or histamine (10^{-3} and 10^{-5} mol/l) produced significantly greater counts than the Tyrode's diluent alone. The counts produced with the lowest dose of peptide (10^{-8} mol/l) or histamine (10^{-7} mol/l) were not significantly different from the control. The peptide or histamine evoked a greater local eosinophilia in the atopics than the non-atopics. This effect was probably independent of the peripheral blood eosinophil counts since at the time of study the numbers of circulating eosinophils between the two groups were not significantly different. In the atopics, histamine in doses of 10^{-3} and 10^{-5} mol/l were required to give the same eosinophil response as that obtained with 10^{-4} and 10^{-6} mol/l of the peptide, respectively.

It is suggested that the relative paucity of eosinophils recruited by locally applied ECF-A peptide or histamine, when compared to antigen-induced eosinophilia, is due either to an inability to mimic the events associated with the release of these mediators from mast-cells or the involvement of other pharmacological agents.

Introduction

Various human tissues can be sensitized passively by IgE for the antigen-induced release of a number of pharmacological mediators. These include histamine, a slow reacting substance of anaphylaxis and the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay & Austen, 1971). ECF-A selectively attracts eosinophils from a mixed leucocyte population and has been chemically characterized as two related acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (Goetzl & Austen, 1975). Recently it has been shown that, with certain types of micropore filter, histamine will also selectively attract human eosinophils (Clark, Gallin & Kaplan, 1975) as will one of its major catabolites, imidazole acetic acid (Turnbull & Kay, 1976).

We have also reported that the individual ECF-A tetrapeptides and, to a lesser

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extent, histamine evoked eosinophil accumulation *in vivo* when applied to the abraded skin of four atopic subjects (Turnbull, Evans & Kay, 1977). In the present investigation this study has been expanded to include greater numbers of subjects and to compare atopic with non-atopic individuals.

Materials and methods

Human volunteers

Eight healthy individuals who had no history of eczema, asthma, allergic rhinitis or any other allergic disorder and who had negative skin prick test reactions to extracts of five common inhalant allergens (house dust, the mite *Dermatophagoides pteronyssinus*, mixed grass pollens, *A. fumigatus* and cat fur—Bencard, Brentford, England) and eight subjects with perennial allergic rhinitis were studied. All of the subjects with rhinitis gave positive skin prick test reactions to two or more of the allergen extracts, one of which was the house dust mite *D. pteronyssinus*. The symptoms in all of these subjects were very mild and none had required any form of medication in the four weeks prior to the study.

Materials

Materials were obtained as follows: (HCl)-Val-Gly-Ser-Glu, mol. wt. 427 (a gift from Dr R. Camble, I.C.I. Ltd, England); histamine acid phosphate (B.D.H. Chemicals Ltd, England); millipore filters 0·2 μm (Millipore Corporation, U.S.A.); and lyophilized house dust mite extract (a gift from Beecham Research Laboratories, England).

'Skin window' studies

The 'skin window' technique used in this study has been described in detail elsewhere (Turnbull *et al.*, 1977). All of the studies were begun at noon and the subjects were not permitted any alcoholic beverages in the 24 hr prior to the study. Skin sites on the forearm were thoroughly cleaned with 70% ethanol, and minimally abraded with a high speed drill, insufficient to cause bleeding. The skin sites were approximately 0·3 cm in diameter and at least 5·0 cm apart. Plastic 'hats' (10 mm in diameter and 5 mm high) were packed with sterile cotton wool and sealed with a millipore filter (0·2 μm) using a thermoplastic glue. A second 0·2 μm filter was placed between the abrasion and the plastic 'hats' (Fig. 1). These filters were soaked with the appropriate test solution and were changed at intervals of 4, 8, 12, 20 and 24 hr after the initial abrasion. At the same time the appropriate test solution was injected into the plastic 'hats' which were securely attached to the skin with adhesive tape and crêpe bandages. The solutions

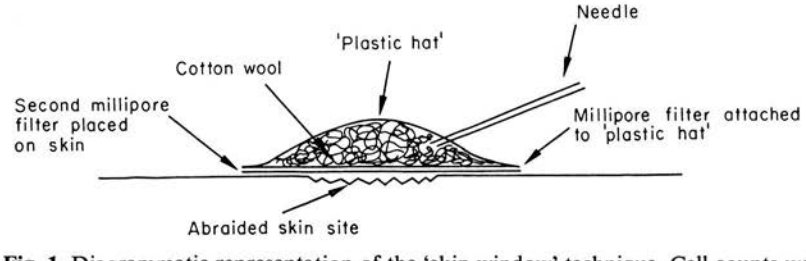

Fig. 1. Diagrammatic representation of the 'skin window' technique. Cell counts were performed on the second Millipore filter on the side in contact with the abraded skin.

tested in each subject were: Tyrode's diluent; Val-Gly-Ser-Glu (valyl-tetrapeptide) at concentrations of 10^{-4}, 10^{-6} and 10^{-8} mol/l; histamine at 10^{-3}, 10^{-5} and 10^{-7} mol/l; and 0·025 mg/ml solution of house dust mite extract. All of the agents were dissolved in Tyrode's diluent which had been prepared using sterile pyrogen-free distilled water. All solutions were then passed through a sterile bacterial filter (0·22 μm, Millipore Corp., U.S.A.) prior to use. The 'skin window' filters were removed at the time intervals described above and fixed and stained as previously described (Kay, 1970). The counts were expressed as the total number of eosinophils present in ten random high power fields ($\times 90$) per filter.

Logarithmic rather than linear values were used in the statistical analysis as this made the variance of the different groups comparable and enabled the Student's *t*-test to be used.

Results

The mean peripheral blood eosinophil count of the atopic subjects was higher than that of the non-atopic subjects but the difference between the groups was not statistically significant (Table 1). However the 'skin window' eosinophil response to house dust mite extract between these two groups was markedly different. In atopic subjects the response rose to a peak at 12 hr and then fell with a secondary rise being observed

Table 1. Peripheral blood eosinophil count and eosinophil response to house dust mite extract at 'skin window' sites in eight atopic and eight non-atopic subjects

Subject	Eosinophil count	'Skin window' Eosinophil count				
	(per μl)	4 hr	8 hr	12 hr	20 hr	24 hr
Atopic subjects						
D.B.	310	377	562	630	208	267
A.K.	550	180	132	139	22	47
S.M.	120	220	520	310	109	167
J.B.	280	71	153	140	53	88
T.B.	300	13	48	79	64	99
S.C.	240	16	38	70	56	115
D.R.	290	33	58	144	109	172
D.J.	170	67	85	102	52	155
Geometric mean	230	51·4	123·2	153·4	69·3	124·0
±1 s.e.m.	5	1·6	1·4	1·3	1·3	1·2
Non-atopic subjects						
D.P.	150	0	3	0	2	0
C.D.	250	13	6	2	2	2
R.B.	130	6	0	8	0	2
M.M.	340	2	4	0	0	3
J.S.	110	5	5	0	2	0
C.A.	100	2	0	3	4	0
D.H.	310	0	0	0	2	0
J.B.	110	0	3	0	0	0
Geometric mean	170	2·5	2·2	1·6	1·6	1·4
±1 s.e.m.	3	1·3	1·2	1·3	1·2	1·2

at 24 hr. The eosinophil response in the non-atopic subjects was negligible and not even different from that obtained with the Tyrode's diluent (Fig. 2). Although only eosinophils were counted these cells were far less numerous than neutrophils and monocytes. In the first 12–20 hr neutrophils predominated while towards the end of the 24 hr study period mononuclear cells became more frequent. In general these other cell types were far too numerous to count and this pattern of cellular accumulation was the same in response to allergen, peptide or histamine.

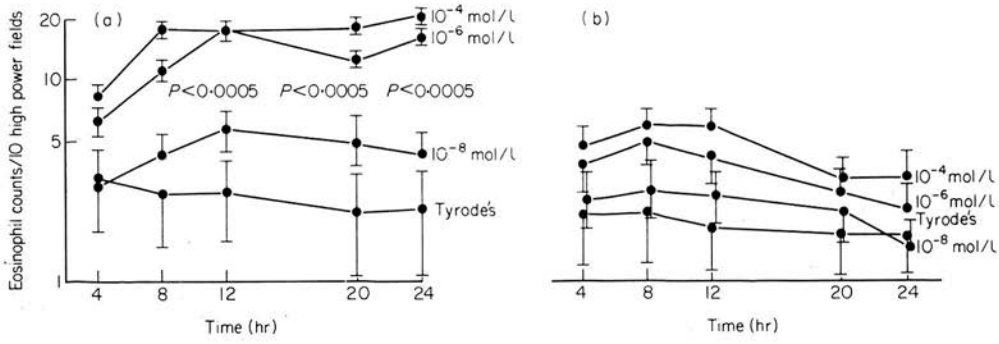

Fig. 2. Geometric mean (±1 s.e.m.) of the eosinophil response to valyl-tetrapeptide at 'skin window' sites of eight atopic (a) and eight non-atopic subjects (b).

The eosinophil response of the atopic and non-atopic subjects to the valyl-tetrapeptide is shown in Fig. 2. In the atopic subjects the mean eosinophil counts obtained with both the 10^{-4} and 10^{-6} mol/l solutions were significantly different ($P<0{\cdot}0005$) from the number obtained with the Tyrode's diluent at all of the time intervals studied. With the 10^{-8} mol/l solution only the counts obtained at the 12 and 20 hr intervals were significantly different ($P<0{\cdot}01$) from those obtained with the Tyrode's diluent. In the non-atopic subjects the mean number of counts obtained at the 8 and 12 hr intervals with the 10^{-4} mol/l and 10^{-6} mol/l solutions were significantly greater than the values obtained with the Tyrode's diluent ($P<0{\cdot}05$) but none of the mean values obtained with these two concentrations of valyl-tetrapeptide at the other times nor any of the mean values obtained with the 10^{-8} mol/l solution was significantly greater than that of the Tyrode's diluent.

The mean eosinophil counts obtained in the atopic subjects with 10^{-4} and 10^{-6} mol/l peptide solutions were significantly higher ($P<0{\cdot}005$) between 8 and 24 hr, than the mean counts in the non-atopic subjects at these times. The atopic group gave significantly higher counts at 12 hr and after with the 10^{-8} mol/l solutions. In both groups of subjects the counts rose to a maximum between 8 and 12 hr. With atopic subjects the eosinophil counts were maintained at this level for the 24 hr while in the non-atopics the eosinophil counts fell after 12 hr and by 20 hr the mean values obtained with all of the concentrations of valyl-tetrapeptide used were not significantly different from those obtained with the Tyrode's diluent.

The eosinophil responses of the atopic and non-atopic subjects to the histamine solutions are shown in Fig. 3 (and should be compared with the Tyrode's control shown in Fig. 2). The mean eosinophil counts obtained in the atopic subjects with the 10^{-7} mol/l solution were not significantly different from those obtained with the Tyrode's diluent at any of the time intervals studied while all of the values obtained

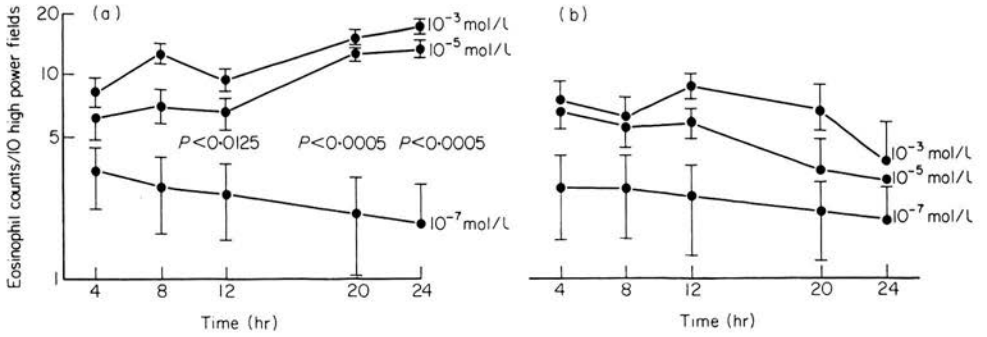

Fig. 3. Geometric mean (±1 s.e.m.) of the eosinophil response to histamine at 'skin window' sites of eight atopic (a) and eight non-atopic subjects (b). The Tyrode's diluent control is shown in Fig. 2.

with the 10^{-3} and 10^{-5} mol/l solutions were significantly higher from 8 hr and after. In the non-atopics the values between 4 and 20 hr, with the 10^{-3} mol/l solution, and those between 4 and 12 hr with the 10^{-5} mol/l solution were significantly greater ($P<0{\cdot}005$) than the control. The values with 10^{-7} mol/l in the non-atopics were not significantly greater than control.

In Table 2 the eosinophil responses (of the two groups) to the valyl-peptide and histamine are compared in terms of the maximal response regardless of the time at which this occurred. The maximal eosinophil response in the non-atopic subjects to the valyl-peptide was significantly less ($P<0{\cdot}005$) than that obtained in the atopic subjects. Moreover the maximal response in the non-atopic subjects occurred in the first 12 hr of the study while, in the atopic subjects, the eosinophil counts tended to increase with time. A similar pattern of results was obtained with histamine.

Although histamine and the valyl-peptide were not compared at identical molar concentrations in the two groups, doses of histamine at 10^{-3} and 10^{-5} mol/l were required to evoke the same eosinophil response as that obtained with 10^{-4} and 10^{-6} mol/l of the peptide respectively. This suggested that the eosinophil mobilizing capacity of the peptide was about ten times greater than that of histamine.

Table 2. Maximal eosinophil response to valyl-tetrapeptide and histamine at 'skin window' sites of eight atopic and eight non-atopic subjects

	Val-Gly-Ser-Glu		Histamine	
	Atopic subjects	Non-atopic subjects	Atopic subjects	Non-atopic subjects
Mean maximal number of eosinophils/10 high power fields	39·0	13·1	25·6	16·5
(±1 s.e.m.)	1·8	3·2	1·3	1·4
Median molar concentration of chemotactic agent giving peak response	10^{-4}	10^{-4}	10^{-3}	10^{-3}
Median time of peak response (hr)	22	6	22	10

Discussion

The main purpose of this study was to compare the eosinophil responses of atopic and non-atopic individuals. The results suggest that the valyl-tetrapeptide (Fig. 2) or histamine (Fig. 3) evoked a greater eosinophil response in atopic compared to non-atopic subjects. Thus the atopic individuals appear to have a greater eosinophil mobilizing capacity than 'normal' subjects (Table 2). The reasons remain unclear at present but are not explicable solely on differences in the peripheral blood eosinophil count as these were not significantly different between the two groups (Table 1).

In a previous study, with only four atopic subjects, it was reported that histamine had only slight eosinophil-attracting activity *in vivo* (Turnbull *et al.*, 1977). However, in this report, in which sixteen subjects were investigated, histamine clearly had a significant eosinophil-attracting effect even in non-atopics. Significant eosinophil accumulation in human skin, especially in that of atopics, was described in previous studies (Eiginger, Wilkinson & Rose, 1964; Cole & Roberts, 1966; Feinberg, Feinberg & Lee, 1967) while other workers have failed to demonstrate that this amine is capable of evoking eosinophil accumulation in human skin (Fowler & Lowell, 1966; Felarca & Lowell, 1968). However, in contrast to these other investigators, we have studied sixteen individuals and made measurements at 4, 8, 12, 20 and 24 hr following the application of three different concentrations of histamine. Therefore it is not possible to compare directly our results with these previous reports.

In view of the marked disparity between the number of eosinophils which accumulated in the abraded skin sites treated with allergen, and those treated with tetrapeptide or histamine, it is evident that it is not possible to explain fully the eosinophil accumulation which occurs at the site of immediate allergic reactions. A requirement for both histamine and peptide in promoting eosinophil accumulation seems unlikely as mixtures of these agents had a negative rather than additive effect both *in vivo* and *in vitro*. It was for this reason also that one only of the ECF-A peptides was used since mixing the valyl- and alanyl-peptide also had a negative effect under these same conditions (Turnbull *et al.*, 1977).

It is reasonable to suppose that eosinophilotactic anaphylaxis-associated mediators play a role in eosinophil accumulation *in vivo*. However, in the present study eosinophil counts obtained in atopics by the mite allergen were far greater than those in which exogenous histamine had been applied to the skin (Table 1). There was no evidence, from the appearance of the various skin sites, that there was more histamine release by antigen than the amount applied externally. The inability to mimic the antigen-induced eosinophil response by chemical mediators alone may be explained as follows. Subtle mixtures of histamine and peptides may be required although this is unlikely for the reasons explained above. The time course of release, the rate of diffusion and/or inactivation may be critical factors, or alternatively other mediators are involved which have yet to be defined.

Acknowledgments

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The effect of anti-eosinophil serum on skin histamine replenishment following passive cutaneous anaphylaxis in the guinea-pig

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Summary. Depletion of eosinophils by an anti-eosinophil serum resulted in the inhibition of histamine reaccumulation into guinea-pig skin sites following IgG1-mediated passive cutaneous anaphylaxis. This suggests that eosinophils may have a regulatory role in the repair mechanism which follows immediate-type hypersensitivity reactions.

INTRODUCTION

Following IgG1-mediated passive cutaneous anaphylaxis (PCA) in the guinea-pig, eosinophils accumulated in large numbers 8–12 h following antigen challenge (Kay, 1970a). We have suggested that eosinophils may play a role in repair following local anaphylaxis (Jones & Kay, 1975). In the present report evidence is provided to support this concept by showing that eosinophil depletion by a monospecific anti-eosinophil serum accelerates the replenishment of skin histamine levels following local anaphylaxis.

MATERIALS AND METHODS

Animals

Albino Dunkin–Hartley guinea-pigs of either sex weighing 300–400 g were used throughout this study

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Preparation of purified guinea-pig IgG1

Guinea-pig antiserum to ovalbumin was prepared and fractionated as previously described (Kay, 1970a, b). The functional purity of the IgG1-containing fractions was tested by PCA, passive haemolysis and passive haemagglutination (Bloch, Kourilsky, Ovary & Benacerraf, 1963) and by immunoelectrophoresis and gel diffusion using rabbit anti-7S IgG and specific anti-guinea-pig IgG1 (Kay, 1970a).

Preparation of rabbit anti-guinea-pig eosinophil serum (AES)

Eosinophils were recovered by peritoneal lavage of animals which had received weekly intraperitoneal injections of horse serum for a 4–6-week period (Kay, 1970b). Eosinophils were purified by density centrifugation on sodium diatrizoate (d = 1·140 at 20°) (Gleich & Loegering, 1973). Cell populations containing more than 95 per cent eosinophils were used for raising AES.

AES was prepared in the rabbit by three weekly injections of purified eosinophils in Freund's complete adjuvant. Each preparation, containing approximately 10^8 cells in a total volume of 2 ml of Tyrode's solution was administered to two subcutaneous areas of the neck. Animals were bled by cardiac puncture 1 week after the final injection, the serum inactivated by heating at 56° for 60 min and

stored in 1-ml aliquots at −85°. Normal rabbit serum (NRS) was similarly inactivated as a control for assessing the specificity of AES.

Measurement of histamine depletion following PCA

The shaved dorsa of guinea-pigs were divided into six equal areas of three pairs. One site of each pair was injected intradermally with 0·1 ml of IgG1, and a contralateral control site with 0·1 ml of Tyrode's solution. Following an 18-h sensitization period the animals were challenged intravenously with 0·75 ml of 2 per cent Evan's blue in Tyrode's solution containing 1 mg/ml of ovalbumin. Blueing reactions were measured and marked with indelible ink after 30–45 min. The dilution of IgG1 was adjusted to give blueing areas of approximately 15 mm in diameter (1:100 for the preparation used). At varying time intervals following challenge animals were killed, the IgG1-treated and equivalent control sites removed, dissected free of subcutaneous adipose tissue, weighed, chopped into small fragments, washed in Tyrode's solution, resuspended in 3 ml of the same buffer and placed in a boiling water bath for 15 min. The supernatants were then removed and assayed for histamine using the isolated, perfused guinea-pig ileum (Brocklehurst, 1960).

Changes in tissue histamine content (H) were defined as a percentage depletion (per cent D). The latter was computed as the mean value of

$$\frac{\text{H control} - \text{H test}}{\text{H control}} \times 100$$

for the three pairs where H was expressed as μg/g wet weight of skin. At least three animals were used for each measurement.

RESULTS

Cell specificity of AES

AES was shown to agglutinate suspensions of purified guinea-pig eosinophils to a dilution of 1 in 80 and to lyse these cells following the addition of fresh guinea-pig serum as a source of complement. At a dilution of 1 in 5 AES did not agglutinate, or 'complement-lyse', guinea-pig neutrophils, macrophages, lymphocytes or red cells.

Following PCA reactions, in which antigen and Evan's blue was administered with Tyrode's solutions, NRS or AES, the number of eosinophils migrating to the reaction sites at 18 h is shown in Table 1. With AES the number of eosinophils was markedly less than in animals pretreated with NRS. The numbers of cells in NRS or Tyrode's pretreated animals were virtually identical. With all treatments the numbers of infiltrating neutrophils and mononuclear cells were too numerous to count.

Table 1. Depletion of eosinophils from the sites of IgG1-mediated PCA reactions by anti-eosinophil serum

Animal no.	Treatment (ml)	Eosinophils/5 h.p.f.
1	AES (0·25)	9
2	AES (0·25)	7
3	AES (0.5)	1
4	AES (0·5)	2
5	AES (0·5)	12
6	NRS (0·25)	50
7	NRS (0·5)	24
8	NRS (0·5)	64
9	Tyrode's	44
10	Tyrode's	16
11	Tyrode's	70

Effect of AES on skin histamine levels following PCA

The depletion and replenishment of tissue histamine levels following PCA reactions is shown in Table 2. Irrespective of the treatment, the histamine content of the control sites (1–14) showed no significant differences. This indicated that AES had no effect on normal skin histamine content. There were also no significant differences in histamine content between contralateral sites in untreated animals (1); the same sites in animals challenged with antigen (2) and control and test sites pretreated with antibody in unchallenged animals (3). In those sets of animals in which a PCA reaction was evoked (4–14), there was considerable depletion of histamine at 1 h (4, 8 and 12). Those animals having Tyrode's (4–7) or 0·25 ml of NRS (8–11), injected intravenously with antigen, had comparable patterns of histamine replenishment and levels were not restored to control values until 48 h. In contrast, those animals receiving 0·25 ml of AES with antigen had recovered their histamine skin content by 9 h (13) and this was maintained at 24 h (14). Thus there was no significant difference between the Tyrode's and NRS 9- and 24-h points (5 and 9, 6 and 10 respectively) whereas there was a highly significant difference

Table 2. The effect of anti-eosinophil serum on skin histamine replenishment following passive cutaneous anaphylaxis

No.	Treatment	Prior preparation		Time (h) after antigen challenge	No. of animals	Histamine content* (± s.e.mean) of control sites	Percentage depletion (± s.e.mean) of test sites
		IgG1	Ag				
1	Tyrode's	None	None		6	3·7 (±0·4)	+6·0 (±4·4)
2	Tyrode's	−	+		6	3·1 (±0·7)	+4·0 (±4·7)
3	Tyrode's	+	−		6	3·9 (±0·3)	+0·4 (±5·3)
4	Tyrode's	+	+	1	6	3·3 (±0·6)	−56·2 (±3·9)
5	Tyrode's	+	+	9	4	3·6 (±0·4)	−34·0 (±1·0)
6	Tyrode's	+	+	24	6	3·9 (±0·5)	−33·0 (±4·3)
7	Tyrode's	+	+	48	6	3·3 (±0·2)	−10·5 (±3·4)
8	NRS	+	+	1	3	3·0 (±0·6)	−49·0 (±2·6)
9	NRS	+	+	9	4	3·0 (±0·6)	−34·0 (±14·4)
10	NRS	+	+	24	4	3·6 (±0·4)	−23·0 (±4·7)
11	NRS	+	+	48	4	2·9 (±0·5)	−1·7 (±2·2)
12	AES	+	+	1	3	3·4 (±1·4)	−47·3 (±2·0)
13	AES	+	+	9	3	3·3 (±0·2)	+6·0 (±5·9)
14	AES	+	+	24	11	3·1 (±0·4)	+6·4 (±14·4)

* μg/g wet weight of skin.

when the Tyrode's or NRS 9-h points (5 or 9) were compared with the AES 9-h point (13) ($P < 0{\cdot}001$). Similarly, the Tyrode's or NRS 24-h points (6 or 10) were significantly different when compared to AES at 24 h (14) ($P < 0{\cdot}05$).

DISCUSSION

In this report we suggest that the eosinophil may prevent replenishment of tissue histamine levels following cutaneous anaphylaxis (Table 2). The basis of this claim depends largely on the specificity of the anti-eosinophil serum. The AES used in these experiments was not absorbed with other cell types as this resulted in considerable loss in AES activity. However, cells used for immunoabsorption invariably contained eosinophils which could not be removed entirely even by separation procedures such as density centrifugation on sodium diatrizoate. Nevertheless, we feel that the AES used in this study was highly specific as shown by selective agglutination and lysis of eosinophils *in vitro* and selective depletion of this cell type *in vivo* following passive cutaneous anaphylaxis (Table 1). It was very unlikely that AES had a direct effect on mast cells in terms of their histamine content since the levels of this amine in the control sites of AES-treated animals were virtually identical to those in other control sites (Table 2). We were unable to ascertain conclusively the effect of local AES treatments since normal rabbit serum from a variety of sources evoked local dye extravasation (Kay, unpublished observation).

A rabbit anti-guinea-pig eosinophil serum has also been described by Gleich, Loegering & Olsen (1975). These workers and ourselves have found that AES activity, as analysed by *in vitro* cytotoxicity tests, was removed by adsorption with neutrophils. However, whereas the intraperitoneal injection of AES resulted in a complete absence of eosinophils from the peripheral blood and peritoneal cavity, there was only transient, or no, reduction in circulating neutrophils. However the effect of antisera directed against the neutrophil, monocyte or lymphocyte on mediator replenishment following anaphylaxis is yet to be ascertained but these studies are in progress.

The work described here, together with other reports on the role of the eosinophil in immediate-type hypersensitivity, suggest that this cell has a number of functions in allergic tissue reactions. Thus eosinophil-derived prostaglandins may inhibit further histamine release (Hubscher, 1975a, b), and eosinophil histaminase (Zeiger & Colten, 1974) and arylsulphatase (Wasserman, Goetzl & Austen, 1975) inactivate histamine and slow reacting substance of anaphylaxis respectively. Therefore the

eosinophil may have a regulatory role at all stages of the allergic response, namely mediator release, mediator inactivation and, as suggested here, mediator replenishment. The regulatory role of the eosinophil in the events following anaphylaxis may provide a homeostatic mechanism whereby the antigen-induced release of histamine and possibly other mediators is not perpetuated in situations where there is continuous antigenic stimulation.

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MEMBRANE RECEPTORS FOR IgG AND COMPLEMENT (C4, C3b AND C3d) ON HUMAN EOSINOPHILS AND NEUTROPHILS AND THEIR RELATION TO EOSINOPHILIA

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Receptors have been shown, on human eosinophils and neutrophils, for rabbit IgG, human IgG, and human C4, C3b and C3d. Both types of granulocyte formed rosettes with EA_G^{rab}. This was directly related to the amount of sensitizing antibody. No rosettes were formed with E alone or EA_M^{rab} Receptors for human IgG were also demonstrated by using heat-aggregated IgG and fluorescent-labeled anti-human IgG. In addition, aggregated human IgG inhibited both eosinophil and neutrophil rosettes with EA_G^{rab}.

Red cell intermediates were prepared with EA_M^{rab} (EA) and functionally pure human complement components. Eosinophil and neutrophil rosette formation was demonstrable with EAC14 and EAC1423b, prepared with limited amounts of C4 (EAC3b), and was related directly to the input of either C4 or C3. No granulocyte rosettes were formed with EAC1 or EAC142. By decay experiments it was shown that C2, in addition to forming the classical pathway C3 convertase with C1 and C4, also inhibited eosinophil and neutrophil EAC14 rosettes. EAC3d intermediates (prepared by treating EAC3b cells with the C3b inactivator and so rendering them immune adherence negative, unsusceptible to lysis with C5-C9 and more agglutinable with anti-C3d) formed rosettes with eosinophils and neutrophils in comparable numbers to EAC3b cells. In general, the percentage of neutrophils bearing receptors for rabbit IgG, human IgG, or human C4, C3b, and C3d was approximately two and one-half times greater than eosinophils.

There was a significantly reduced percentage of eosinophils forming rosettes with EAC14 or EAC3b when patients with eosinophilia of various etiology were compared to controls; however, no differences were found with EA_G^{rab}. When neutrophils from patients with eosinophilia were compared to controls in terms of percentage of EA_G^{rab}, EAC14, or EAC3b rosettes, there were no significant differences. These results, taken together with previously published findings on the monocyte, suggest that all circulating phagocytic cells have similar membrane receptors for promoting adherence of opsonized particles and that with eosinophils the numbers of complement receptors may be altered in disease.

Membrane surface receptors for immunoglobulin and complement components have been demonstrated on a number of hemopoietic cells. Most of the studies relate to cells of the lymphoid series and mononuclear phagocytes; however, the presence of surface receptors for IgG and complement on human or animal granulocytes has also been described. For example, the presence of receptors for human IgG (1), C3b (2) and C4 (3, 4) on human neutrophils has been described. Similarly, the presence of IgG (5) and C3 receptors on human eosinophils (5, 6) has been reported. It was also claimed that eosinophils have lymphocyte-like properties in that they bind sheep red blood cells (E)[3] alone (i.e., unsensitized) (6).

The need to define the presence of immunoglobulin and complement receptors on human eosinophils more precisely has been prompted largely by the work of Butterworth *et al.* (7) who showed that human eosinophils from normal individuals were the principal effector cells in antibody-dependent killing of schistosomula *in vitro*.

In the present study we report on the presence of IgG, C4, C3b, and C3d receptors on human eosinophils and neutrophils and their apparent alteration in individuals with blood eosinophilia.

MATERIALS AND METHODS

Buffers. Dextrose-gelatin-Veronal buffer ($DGVB^{++}$), pH 7.4, was prepared by mixing equal volumes of isotonic Veronal-buffered saline (containing 0.0015 M Ca^{++}, 0.0005 M Mg^{++}, and 0.1% gelatin Veronal buffer (GVB^{++})) with 5% dextrose in water containing the same concentration of Ca^{++} and Mg^{++}, as previously described (8). $GVB^{=}$ was prepared as for GVB^{++} but without the addition of divalent cations.

A stock solution of 0.086 M EDTA, pH 7.4, was prepared and diluted in $GVB^{=}$, to prepare 0.04 M EDTA $GVB^{=}$, or 0.01 M EDTA $GVB^{=}$.

Sheep red blood cells. Sheep blood was drawn aseptically into sterile Alsever's solution and kept at 4°C. Immediately before use the E were washed twice in cold saline, once in 0.01 M EDTA $GVB^{=}$, and adjusted to the required concentration in the same buffer.

Rabbit anti-sheep red blood cells (A). Multiple injections of E stroma were administered to rabbits as described by Rapp and Borsos (9). The IgG and IgM fractions were obtained by gel filtration on Sephadex G-200 (10).

Sensitization of E. Either rabbit IgG or IgM was added to E. Sensitization was achieved by selecting an antibody concentration which caused optimal sensitization but no agglutina-

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[3] Abbreviations used in this paper: E, sheep red blood cells; DGVB, dextrose-gelatin-Veronal buffer; GVB, gelatin Veronal buffer; A, anti-sheep red blood cells.

tion (9). The mixture was incubated at 37°C for 30 min in a shaking water bath, and 0°C for 30 min with frequent shaking. The cells were then washed once in 0.01 M EDTA GVB$^{=}$ and twice in DGVB^{++}.

Human IgG and complement components. Human IgG was prepared by diethylaminoethyl (DEAE)-cellulose chromatography and aggregated by the method of Dickler and Kunkel (11). The control, bovine serum albumin (BSA) (Miles Laboratories Inc., Kankakee, Ill.) was aggregated as described (12). All aggregated proteins were adjusted to a concentration of 1.2 mg/ml.

Human C1 was prepared by euglobulin precipitation according to the method described by Vroon *et al.* (13). Human C4 was prepared by sequential precipitation and ion-exchange chromatography on DEAE-Sephadex and carboxymethyl (CM)-Sephadex as previously described (13). Human C2, C5, C6, C7, C8, and C9 were purchased from Cordis Laboratories (Miami, Fla.) and purified human C3 was a generous gift from Dr. D. K. Peters. Purified C3b inactivator was kindly supplied by Dr. P. J. Lachmann, but in some experiments Cordis C3b inactivator was used. All complement components were functionally pure and free of other hemolytically active components as assayed by effective molecular titrations (9). Monospecific anti-C3d was purchased from the Netherlands Red Cross Blood Transfusion Service, Amsterdam.

Preparation of complement-coated intermediates. EA_M^{rab} was prepared as for EA_G^{rab}. For $EA_M^{rab}C1^{hu}$, EA was mixed with 400 effective molecules of human C1 per red cell. The mixture was incubated for 90 min at 0°C and then washed once in DGVB^{++}. $EA_M^{rab}C1^{hu}4^{hu}$ cells described in Table I, Table II, and Figure 4 were prepared by the addition of 4000 effective molecules of human C4 per EAC1 cell. The mixture was incubated for 30 min at 37°C. The cells were then washed four times in DGVB^{++}.

$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ cells used in the C2 decay experiments were prepared by adding 50 effective molecules of human C2 per EAC14 cell and the mixture was incubated at 30°C for 10 min without washing.

EAC1423b (EAC3b) cells were prepared as follows: 400 effective molecules of human C4 were added to EAC1 and incubated and washed as described. To this EAC14 intermediate (which had insufficient C4 to form rosettes with either neutrophils or eosinophils) were added 50 effective molecules of human C2 and the mixture was incubated at 30°C for 10 min without washing. In the experiments described in Table I, Table II, Figure 3, and Figure 4, 2500 effective molecules of purified human C3 were added to these EAC142 cells. The mixture was incubated at 37°C for 30 min and the cells were washed twice in DGVB^{++}.

EAC1423d intermediates were prepared by treating 0.5 ml of EAC3b cells containing 1×10^8 cells/ml with 0.5 ml of the C3b inactivator. The mixture was incubated for 1 hr at 37°C. The cells were then washed twice with cold DGVB^{++}. The quantity of C3b inactivator added was calculated by determining the amount required to cause 95% inhibition of immune adherence by using EAC3b cells and human group O, Rh-positive erythrocytes (14).

Eosinophils and neutrophils. Blood from patients with eosinophilia (Table III and Fig. 4) and from healthy controls was drawn into plastic tubes containing 10 units of preservative-free heparin (Evans Medical, Liverpool, England) per ml. The red cells were sedimented with 70% dextran (Lomodex 70, Fisons Pharmaceuticals, Loughborough, England); one part dextran to four parts blood v/v, for 60 min at room temperature. The leukocyte-rich plasma was then layered over sodium metrizoate (Nyegaard & Co., As, Oslo, Norway), specific gravity 1.148 at 4°C, in 15-ml plastic conical centrifuge tubes and centrifuged at 4°C for 40 min at 400 × G.

The pellet, containing red blood cells and granulocytes, was resuspended in 0.82% ammonium chloride solution as described by Boyle to lyse the red cells (15).

The cells were then washed twice in medium 199 (Flow Laboratories, Irvine, Scotland, U.K.), pH 7.4, and the cell count was finally adjusted to 2×10^6/ml in the same medium. These suspensions contained both eosinophils and neutrophils but were virtually free of mononuclear cells.

EA and EAC rosette formation. One-tenth milliliter of E, EA, or EAC containing 1×10^8 cells/ml in DGVB^{++} was added to equal volumes of granulocyte suspension in medium 199. The mixture was centrifuged at 100 × G for 10 min at 4°C. These mixtures, with the undisturbed pellet, were then incubated at 0°C (for EA rosettes) or 37°C (for EAC rosettes) for 30 min. The pellet was gently resuspended and smears were prepared on clean glass slides in duplicate. These were dried quickly in air, fixed in 95% methanol, and stained with May Grunwald/Giemsa. Only granulocytes with three or more adherent E were termed rosettes. In each slide 200 granulocytes were counted and the number of rosetting cells was expressed as a percentage of the total number of eosinophils or neutrophils counted.

Immunofluorescence. Granulocyte concentrations were adjusted to 5×10^6/ml in medium 199. Before use the cells were washed once in "FA buffer" (Difco Laboratories, Detroit, Mich.) and 0.5 ml of the granulocyte suspension was mixed with an equal volume of heat-aggregated human IgG, native IgG, or the control-aggregated bovine serum albumin. The mixture was incubated at 0°C for 30 min. The cells were then washed three times in FA buffer at 4°C. The fluorescent-labeled rabbit anti-human IgG (Miles Laboratories, Slough, England) was added to the cell suspension and the mixture incubated for a further 30 min at 0°C. The cells were washed three times in cold FA buffer and then resuspended in 2 drops of the "FA mounting medium" (Difco Laboratories). One drop of the cell suspension was mounted in a clean glass slide under a coverslip. The slides were prepared in duplicate, viewed under ultraviolet light, and the number of fluorescing cells counted. Cells with two or more peripheral fluorescing sites (i.e., nongranular fluorescence) were deemed positive. In each slide 200 cells were counted and the granulocytes showing positive fluorescence were expressed as a percentage of the total number of granulocytes counted.

RESULTS

E sensitized with rabbit IgG (EA_G^{rab}) formed rosettes with human neutrophils and eosinophils in a dose-response fashion by using increasing amounts of antibody (Fig. 1). The percentage of rosetting neutrophils was approximately two and one-half times the number of eosinophil rosettes. Heat-aggregated human IgG inhibited rosette formation of neutrophils and eosinophils with EA_G^{rab}. No rosette formation was observed with E or with EA_M^{rab} prepared with a comparable agglutinating titer to rabbit IgG.

When these experiments were performed at higher temperatures, (18° and 37°C), the number of rosetting cells was considerably less and was associated with phagocytosis of sensitized red cells by both neutrophils and eosinophils.

Surface receptors for human IgG were demonstrable by treating the granulocyte suspensions with heat-aggregated human IgG and counting the number of fluorescing cells after the addition of fluorescein-labeled monospecific rabbit anti-

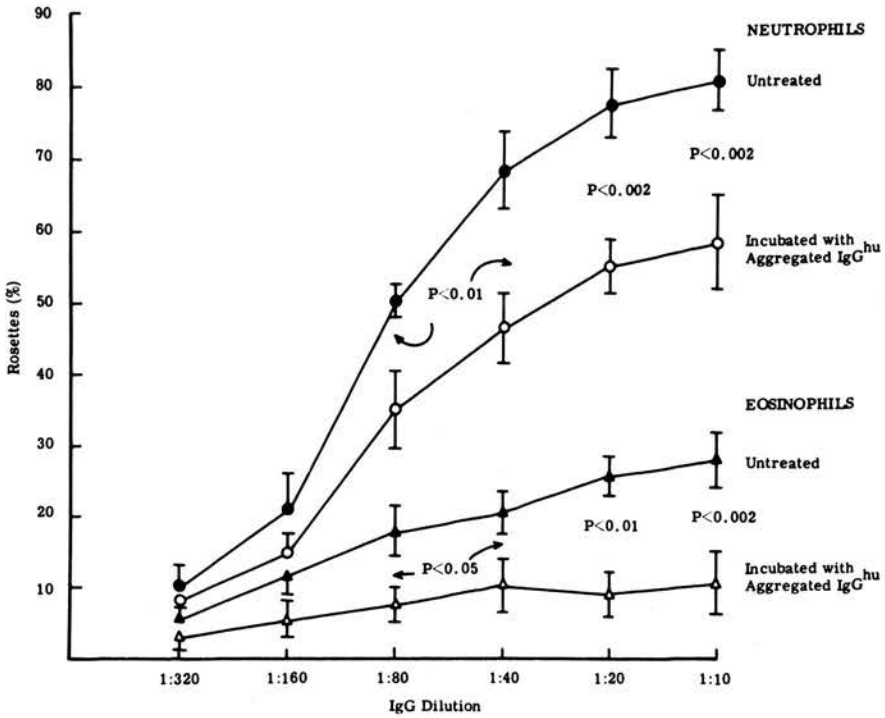

Figure 1. The effects of increasing concentrations of rabbit IgG on human neutrophil and eosinophil rosette formation by EA_G^{rab} and their inhibition by aggregated human IgG. The points represent the mean (± 1 S.D.) of five experiments. The degree of significnce of inhibition with aggregated IgG^{hu} is expressed as p value as calculated by the Student *t*-test.

human IgG (Fig. 2). The number of fluorescing cells with heat-aggregated IgG was significantly greater ($p < 0.001$) than with native IgG or with the aggregated bovine serum albumin control. As with the EA_G^{rab} rosettes (Fig. 1), the percentage of fluorescing neutrophils was approximately two and one-half times that of eosinophils.

The rosette-forming capacity of E coated with various human complement components with human neutrophils and eosinophils is shown in Table I. E, EA_M^{rab}, $EA_M^{rab}C1^{hu}$, and $EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ were virtually unreactive at 0°C and 37°C. the apparent ability of $EA_M^{rab}C142$ to form rosettes at 37°C was attributable to C2 decay (Table II). In contrast, EAC14 (prepared with 4000 effective molecules per cell), EAC1423b, and EAC1423d (prepared with limited amounts of C4) formed rosettes with neutrophils and eosinophils.

Rosette formation by EAC14 and EAC1423 was related to the input of C3 or C4 as shown in Figure 3. Increasing amounts of these complement components gave rise to increased numbers of eosinophil rosettes which paralleled the percentage of lysis as determined by the addition of the appropriate late components required to complete the hemolytic sequence. Similar results were found with the neutrophils although the number of rosetting cells was two to three times greater. The ability of eosinophils and neutrophils to recognize activated cell-bound human C4 or C3 was demonstrable in a dose-dependent fashion.

The capacity of C2 to inhibit the binding of EAC14 to eosinophils and neutrophils is shown in Table II. Binding of EAC14 to these granulocytes was restored by allowing C2 decay from EAC142 cells after incubation of this intermediate at 37°C for 30 min. The C2 preparation was free of the C3b inactivator. Therefore the addition of C2 to EAC14 promotes both the formation of the classical pathway C3 convertase and the inhibition of binding of C4 to these granulocytes.

Evidence for binding of red cell intermediates coated with C3d was achieved by treating the EAC1423b with purified C3b inactivator for 1 hr at 37°C. These C3d-coated cells, in contrast to untreated C3b cells, were no longer immune adherence positive and could not be lysed by the addition of C5-C9. However, they agglutinated with monospecific anti-C3d, as

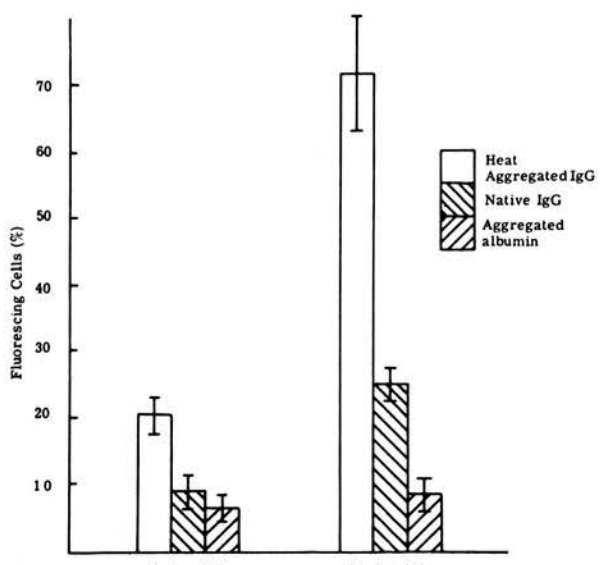

Figure 2. The percentage of fluorescing eosinophils and neutrophils after treatment with heat-aggregated IgG, or controls, and fluorescent anti-human IgG. The bars represent the mean (± 1 S.D.) of seven experiments.

did C3b cells, but to a greater extent. Both EAC3b and EAC3d formed rosettes with neutrophils and eosinophils as shown in Table III but only the numbers of EAC3d eosinophil rosettes were significantly less than that for EAC3b ($p < 0.05$).

The numbers of eosinophils and neutrophils from normal individuals, or those with eosinophilia, giving rosettes with EA_G^{rab}, EAC14, or EAC1423 are shown in Figure 4. With EA_G^{rab} there was no difference in the numbers of rosetting eosinophils between the two groups. However, the number of eosinophils rosetting with EAC14 or EAC1423 was significantly lower ($p < 0.05$) in the eosinophilia group. When the neutrophils from individuals with eosinophilia were compared with those having eosinophil counts of less than 4%, there was no significant difference in the percentage of rosetting cells with EA_G^{rab}, EAC14, or EAC1423. The percentage of blood

TABLE I

Eosinophil and neutrophil rosettes with E coated with rabbit antibody and various human complement intermediates[a]

Indicator Cells	Eosinophil Rosettes		Neutrophil Rosettes	
	0°C	37°C	0°C	37°C
	%	%	%	%
E	2.8 ± 1.0	5.1 ± 1.2	3.5 ± 2.0	6.2 ± 1.8
EA_G^{rab}	25.3 ± 3.6	16.3 ± 2.8	76.8 ± 7.6	58.3 ± 8.0
EA_M^{rab}	2.4 ± 1.2	3.3 ± 1.3	5.6 ± 2.2	4.5 ± 1.4
$EA_M^{rab}C1^{hu}$	4.0 ± 1.5	6.0 ± 1.6	3.5 ± 1.0	4.6 ± 1.3
$EA_M^{rab}C1^{hu}4^{hu}$[b]	10.0 ± 2.7	20.5 ± 1.8	54.8 ± 4.9	63.0 ± 7.5
$EA_M^{rab}C1^{hu}4^{hu}2^{huc}$	5.2 ± 1.6	(15.5 ± 3.6)	7.0 ± 1.8	(42.5 ± 6.9)
$EA^{rab}C1^{hu}4^{hu}2^{hu}3b^{huc}$	ND[d]	33.9 ± 2.4	ND	62.6 ± 2.6
$EA_M^{rab}C1^{hu}4^{hu}2^{hu}3d^{huc}$	ND	23.9 ± 1.6	ND	55.8 ± 4.4

[a] The figures represent the mean values of 15 experiments (± 1 S.D.) with the exception of the EAC3d experiments which were performed eight times. Rosette formation by EAC142 at 37°C was thought to represent EAC14 as a result of C2 decay and is therefore recorded in parenthesis.

[b] Prepared with 4000 effective molecules of C4 per cell.

[c] Prepared with 400 effective molecules of C4 per cell.

[d] ND, not done.

TABLE II

The effect of C2 decay on the binding of EAC142 cells (prepared with 4000 molecules of C4 and 50 molecules of C2 per cell) to human eosinophils and neutrophils[a]

Patient	Eosinophil Rosettes		Neutrophil Rosettes	
	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ (decay)	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$	$EA_M^{rab}C1^{hu}4^{hu}2^{hu}$ (decay)
	%	%	%	%
C. D.	6.0	26.0	9.0	46.0
J. A.	8.0	27.0	8.5	53.0
M. B.	5.0	26.0	6.5	61.0
A. M.	4.0	22.0	6.0	55.0
J. N.	5.0	26.5	9.0	51.0

[a] EAC14 cells were incubated with C2 as described. Before decay, EAC142 cells were hemolytically active as shown by total lysis after the addition of C3-C9. The C3b inactivator (as assayed by the method of Lachmann and Müller-Eberhard (1968)) was undetectable in the C2 preparation. All the rosette experiments were performed at 0°C.

eosinophilia and the percentage of rosetting neutrophils and eosinophils using EA_G^{rab}, EAC14, or EAC1423 in 15 patients were unrelated to the various disease states although the majority of patients had an eosinophilia in association with an exogenous antigen, i.e., bronchial asthma, pulmonary eosinophilia, or helminth disease. In addition, there was no association between the percentage of eosinophilia and the percentage of rosetting cells irrespective of the indicator erythrocytes.

The number of eosinophils and neutrophils from patients with eosinophilia-forming rosettes with EAC3d when compared to normal individuals were similar (Table III), but the numbers were smaller than with EAC14 or EAC3b.

DISCUSSION

The evidence for receptors for human IgG on human eosinophils and neutrophils is provided by both the capacity of heat-aggregated human IgG to inhibit EA_G^{rab} rosettes and its ability to adsorb directly on to these cells, as shown by immunofluorescence (Fig. 2). This binding is probably through the Fc portion of the IgG, but this is yet to be ascertained with certainty. Also, the subclass of the IgG which binds to granulocytes, and the possibility that these cells have receptors for other immunoglobulins also remains to be determined. However, the demonstration of IgG receptors on human eosinophils is consistent with the findings of Butterworth *et al.* (7) who have shown that the eosinophil is the principal effector cell which mediates antibody-dependent killing of schistosom-

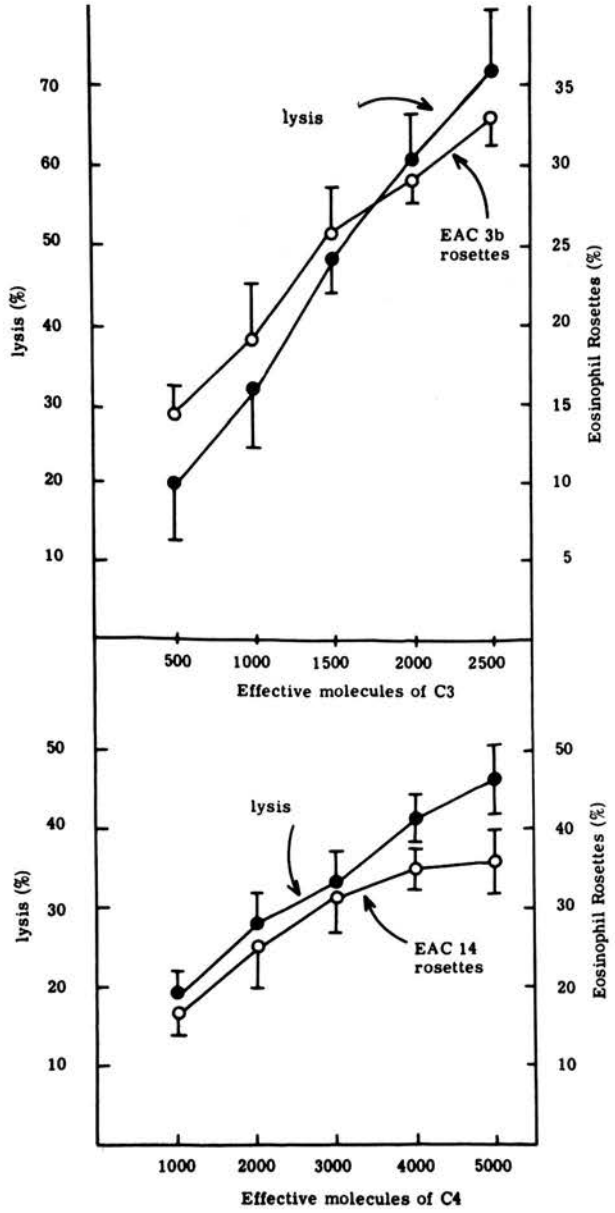

Figure 3. The effect of increasing concentrations of C3 or C4, with their appropriate red cell intermediate, on eosinophil rosette formation and lysis with terminal complement components. The EAC3b intermediate was prepared by using 400 effective molecules of C4 per cell. The points represent the mean (± 1 S.D.) of five experiments.

ula. The antibody, in this system, was identified as IgG (16) and the adherence of eosinophils to IgG-sensitized schistosomula may be dependent on the presence of freely available immunoglobulin receptors.

EA_G^{rab} rosettes were consistently shown both in healthy controls and patients with eosinophilia (Figs. 1 and 4). This contrasts with the report of Tai and Spry (5) who were unable to demonstrate appreciable numbers of EA_G^{rab} rosettes with human eosinophils from healthy donors. This apparent discrepancy could be due to differences in the preparation of EA_G^{rab}, and the time of incubation for rosette formation. Tai and Spry (5) reported on six normal individuals by using a 4-min incubation time, whereas in the present report 15 normal individuals were studied by using a total incubation time of 40 min (Fig. 4).

Unlike Sher and Glover (6) we were unable to demonstrate eosinophil rosettes with E alone (Table I). Furthermore when E were treated with 2-aminoethylisothiouronium bromide hydrobromide (AET), which is believed to enhance their T cell binding property (17), this did not evoke eosinophil rosetting (A.R.E. Anwar, unpublished observation).

Cell-bound C4 and C3 form rosettes with eosinophils and neutrophils (Table I and Fig. 3). Since increasing amounts of these components were associated with increased rosette formation and lysis, this suggests that the number of adherent cells was directly proportional to the input of C4 and C3. The relation between C4 and C3 receptors has yet to be ascertained, although appropriate inhibitory studies are in progress. C4 receptors on human neutrophils have been previously described (3, 4). Eosinophils have also been shown to have C4 receptors although details of the methodology and how the findings related to eosinophilia were not given (18). It should be emphasized that the amount of C4 (400 effective molecules) used to prepare the EAC3 intermediates gave the same number of rosettes as E alone. Therefore the rosettes observed with EAC3b and EAC3d in all the experiments cited are not due to C4 and thought to be entirely C3 dependent.

Rosette formation by neutrophils and eosinophils with EAC14 could be inhibited by functionally pure human C2 which was free of C3b inactivator (Table II). Although it was previously reported that C2 did not inhibit immune adherence by EAC14 cells (19), it should be noted that in our present study rosette inhibition was performed at 0°C to minimize C2

TABLE III

Eosinophil and neutrophil rosettes with EAC3b and EAC3d[a]

Patient	Diagnosis	Eosinophil Rosettes		Neutrophil Rosettes	
		EAC3b	EAC3d	EAC3b	EAC3d
		%	%	%	%
J. C.	Unknown	33	25	60	63
E. B.	Pulmonary eosinophilia	33	23	62	62
M. H.	Lymphoma	37	24	59	56
D. F.	Atopic eczema	32	22	65	51
S. L.	Bronchial asthma	36	25	64	54
J. S.	Hypersensitivity to sulfonamides	33	24	68	53
A. M.	Bronchial asthma	35	20	63	54
A. W.	Atopic eczema	34	22	64	53
D. B.	Normal	43	26	62	58
P. D.	Normal	41	27	64	54
J. N.	Normal	38	24	65	52
G. R.	Normal	37	25	67	50

[a] These intermediates were prepared with 400 effective molecules of C4 per cell.

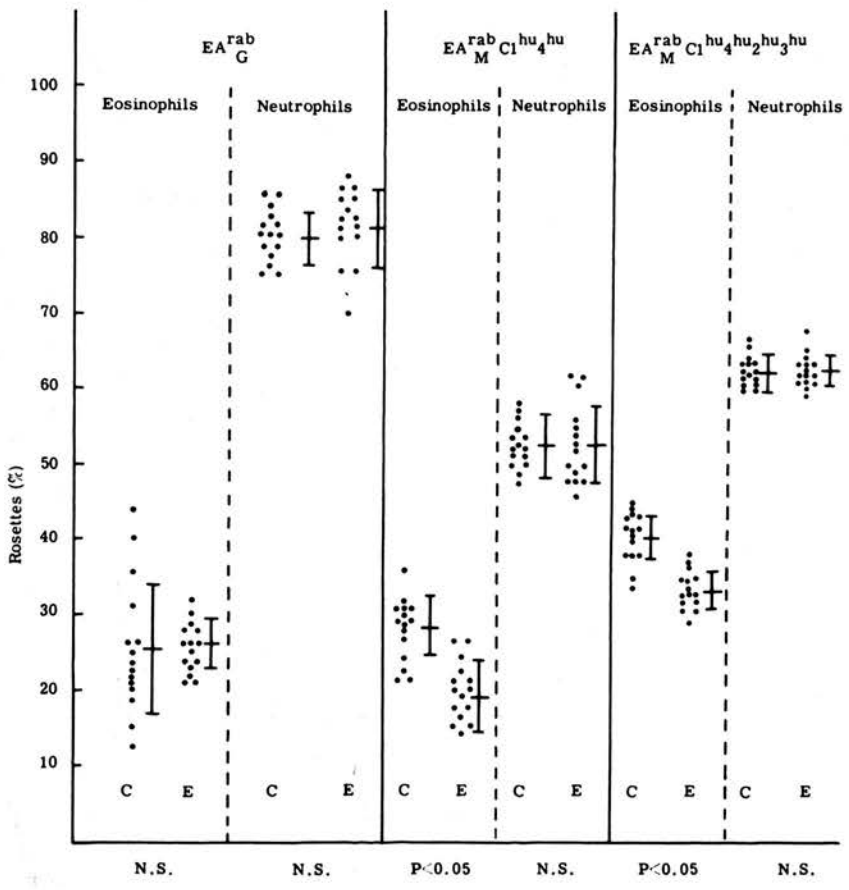

Figure 4. The percentage eosinophil or neutrophil rosettes with EA, EAC14, or EAC3b as compared between healthy normal controls (C) and patients with eosinophilia (E). The EAC14 cells contained 4000 effective molecules of C4 and the EAC3b were prepared with 400 effective molecules of C4 per cell. The bars represent the mean (± 1 S.D.). The p values were calculated by the Student *t*-test. (N.S. = not significant.)

decay. Binding capacity could be restored by allowing C2 decay. This suggests that C4 hemolytic- and granulocyte-binding sites are likely to be closely related.

The apparent inability of C3d cells to bind to human neutrophils had been previously reported (2, 20). However, in the present study, EAC3d cells prepared by treating EAC3b cells with C3b inactivator gave comparable rosette formation, with both neutrophils and eosinophils, to the untreated EAC3b cells (Table III). Nevertheless, unlike EAC3b, EAC3d cells were immune adherence negative, resistant to lysis by C5-C9 and gave stronger agglutination with anti-C3d than did the EAC3b cells. Differences in technology probably explain these discrepancies, particularly in the report by Ross *et al.* (20) where indicator and target cells were incubated for only 5 min. We have found that this incubation time is insufficient for rosette formation.

The relationship between granulocyte markers and eosinophilia was also studied. We found a decrease in rosetting eosinophils with EAC14 and EAC3b in patients with eosinophilia which was possibly due to smaller numbers of complement receptors on immature eosinophils (Fig. 4). Rabellino and Metcalf (21) were unable to demonstrate C3 receptors on mouse eosinophil colony cells grown *in vitro*, whereas in the same study IgG receptors were present. This is in agreement with the present study in which there was no difference between rosetting eosinophils with EA_G^{rab} in patients with eosinophilia compared to controls. Neutrophils from patients with eosinophilia had similar numbers of complement and IgG rosettes when compared to controls, providing further evidence that the stimulus for eosinophil production may be independent from the stimulus for neutrophil production.

We have considered the possibility that the granulocyte separation procedure may have yielded only a subpopulation of neutrophils and eosinophils. However, the recovery of granulocytes was always more than 80%. Moreover, in early experiments with unseparated leukocyte suspensions, similar ratios of eosinophil to neutrophil rosettes were observed.

There was no apparent association between the percentage of eosinophil and neutrophil rosettes and the degree of eosinophila with either EA_G^{rab} EAC14, or EAC3b. There was also no association between the degree of rosette formation and the disease states; however, the majority of individuals studied had either atopic disease or hypersensitivity reactions (Table III).

Our results are consistent with previous findings on surface markers on human monocytes (22, 23) in that, like monocytes, the eosinophil and neutrophil also have receptors for IgG, C3b, and C3d. Therefore, there would appear to be similarities in terms of recognition of opsonized particles by all circulating phagocytic cells. Phagocytosis by eosinophils is generally considered to be slower than the neutrophils in terms of the rate of ingestion (reviewed by Kay) (24). The relative paucity of receptors on human eosinophils as compared to the neutrophils may explain its relatively poor phagocytic response. Therefore, it is unlikely that the eosinophil plays a major role as a phagocytic cell. However, the presently recognized functions of eosinophils are that it acts as a cytotoxic-killer cell in helminth disease (7) and has a homeostatic or "dampening" effect in immediate hypersensitivity (25). These processes presumably involve a recognition mechanism enabling contact between eosinophils and target cells which in turn may depend on immunoglobulin and/or complement receptors.

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The ECF-A tetrapeptides and histamine selectively enhance human eosinophil complement receptors

VARIOUS products of the anaphylactic reaction such as the ECF-A tetrapeptides[1] (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu)[2] and histamine[3,4] preferentially attract the human eosinophil in chemotaxis *in vitro*. We have designed experiments to show whether these chemical mediators have a direct effect on the eosinophil cell membrane, as assessed by their capacity to alter receptors for either IgG or complement (C). We have established here that these two ECF-A peptides and histamine both enhance markedly the expression of eosinophil receptors for C3 but not for IgG. Neutrophil and monocyte receptors seemed to be unaltered and a number of other chemical mediators of hypersensitivity had no effect on these membrane markers when tested at comparable concentrations. Our demonstration of eosinophil membrane receptor enhancement by chemotactic factors may have more general biological significance in terms of how surface recognition mechanisms by phagocytic cells are regulated.

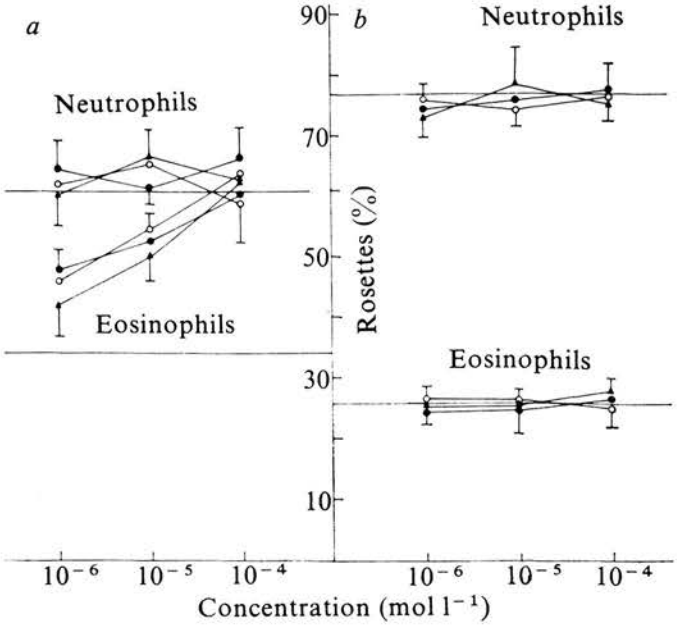

Fig. 1 The effect of increasing concentrations of the valyl-(●) and alanyl-(○) ECF-A peptides and histamine (▲) on *a*, EAC3b and *b*, EAG rosettes by human eosinophils and neutrophils. The lines represent the percentage rosettes of untreated eosinophils and neutrophils. Each point represents the mean of five experiments ± 1 s.d. Dextrose–gelatin–veronal buffer ($DGVB^{2+}$ *p*H 7.4) was used for washing sheep RBC during sensitisation and coating with various complement components. This was prepared by mixing equal volumes of isotonic veronal-buffered saline (containing 0.0015 M Ca^{2+}, 0.0005 M Mg^{2+} and 0.1% gelatin–veronal buffer (GVB^{2+}) with 5% dextrose in water containing the same concentration of Ca^{2+} and Mg^{2+} (ref. 10). The IgG and IgM fractions of rabbit antisera to sheep red cells were prepared by Sephadex G-200 gel filtration. Sheep cells were sensitised with either the IgG fraction for preparing EA_G^{rab} (EAG) or IgM (EA_M^{rab}) for EAC1423b rosettes. Functionally pure human complement components were added sequentially to EA_M^{rab} to prepare C3b-coated cells as described[11]. The amounts were as follows: 400 effective molecules of C1, 400 of C4, 50 of C2 and 2,500 of C3. This amount of C4 was insufficient to give EAC14 rosettes with neutrophils, eosinophils or monocytes[11]. The valyl- ((HCl)-Val-Gly-Ser-Glu, molecular weight 427) and alanylpeptides ((HCl)-Ala-Gly-Ser-Glu, molecular weight 497) were a gift from Dr R. Camble (ICI Limited, Pharmaceuticals Division, Alderley Park, Macclesfield); histamine acid phosphate (BDH Chemicals Limited, Poole).

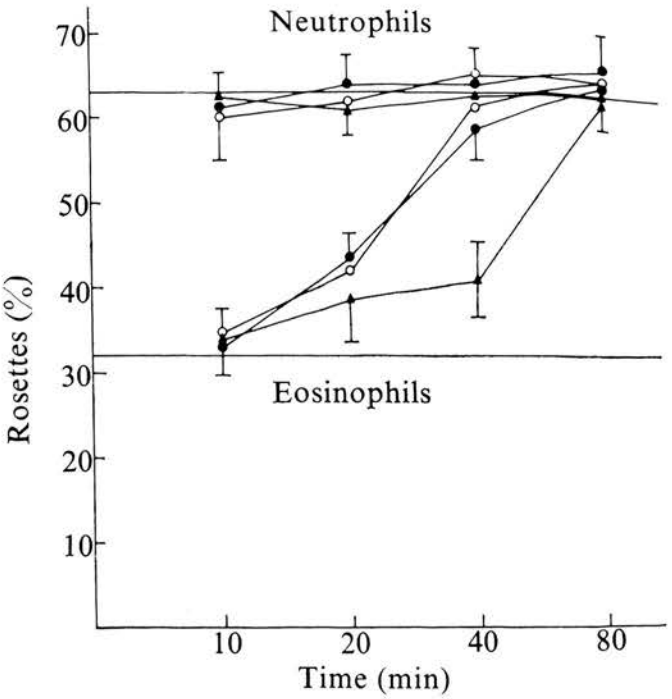

Fig. 2 The effect of time on enhancement of EAC3b rosettes by eosinophils and neutrophils following incubation with the valyl-(●) and alanyl-(○) ECF-A peptides and histamine (▲). Each mediator was tested at a concentration of 10^{-4} mol l^{-1}. The lines represent the percentage rosettes of untreated eosinophils and neutrophils. Each point represents the mean of five experiments ± 1 s.d.

Receptors were measured by the 'rosette technique' using sheep red blood cells (RBC) sensitised with either IgG or C3b (Fig. 1). Human eosinophils, neutrophils[5] and monocytes[6] were prepared from venous blood from patients with eosinophilia of various aetiology and adjusted to a concentration of 4×10^6 ml^{-1} in medium 199 (*p*H 7.4). Equal volumes of leukocyte suspensions and various concentrations of the pharmacological agents under study, or control medium alone, were mixed and incubated in a shaking water-bath at 37 °C for varying intervals. The cells were then washed twice in medium 199 and the numbers adjusted to 2×10^6 ml^{-1} in the same medium. An aliquot (0.1 ml) of IgG-(EAG) or complement-coated (EAC3b) red cells containing 1×10^8 ml^{-1} was then added to 0.1 ml of the cell suspension. The mixtures were centrifuged at 100*g* for 10 min at 4 °C and the pellets incubated at 0 °C (for EAG rosettes) or 37 °C (for EAC3b rosettes) for 30 min as described. The pellets were gently resuspended and smears were prepared in duplicate. The slides were dried in air, fixed in methanol and stained: Cells with three or more adherent erythrocytes were counted as rosettes. Two hundred cells were counted on each slide and the number of rosettes expressed as a percentage of the total number counted.

With no treatment the percentages of neutrophil, IgG or complement receptors were usually more than twice that of the eosinophil (Fig. 1). With increasing concentrations of the valyl- or alanyl-peptide, or histamine there was a dose-dependent enhancement of the numbers of eosinophils, but not of neutrophils, which formed rosettes with EAC3b. There was no increase in the numbers of eosinophils or neutrophils forming rosettes with EAG. When other pharmacological mediators, which included bradykinin and the prostaglandins PGE_1, E_2 and $F_{2\alpha}$, were incubated at comparable concentrations (10^{-4}–10^{-6} mol l^{-1}) to that of the ECF-A peptides or histamine, there was no appreciable increase in

Table 1 Percentage increase in the numbers of rosetting eosinophils, neutrophils and monocytes with EAC3b cells following incubation with increasing concentrations of various chemical mediators of hypersensitivity

	10^{-6} (mol l^{-1})			10^{-5} (mol l^{-1})			10^{-4} (mol l^{-1})		
	Eosinophils	Neutrophils	Monocytes	Eosinophils	Neutrophils	Monocytes	Eosinophils	Neutrophils	Monocytes
Valyl-peptide	43.3	5.6	(6.8)	55.6	0.5	(0.3)	77.8	8.1	(4.5)
Alanyl-peptide	37.0	1.0	5.3	60.6	8.1	(1.8)	87.8	(3.1)	(7.0)
Histamine	25.1	(1.1)	(3.3)	48.8	7.8	(3.3)	83.4	1.3	3.0
Bradykinin	0.5	7.8	—	0.5	(4.9)	—	3.0	2.3	—
PGE_1	5.6	4.2	—	(2.0)	(8.5)	—	14.7	1.3	—
PGE_2	(0.9)	(3.9)	—	6.5	2.9	—	6.8	(7.7)	—
$PGF_{2\alpha}$	5.6	(3.1)	—	13.3	1.3	—	(3.3)	2.1	—
5-HT	3.0	(5.2)	—	17.8	(3.4)	—	25.0	6.7	—

The figures represent the mean of five experiments, with the exception of bradykinin (three experiments). Decreases in rosette formation are shown in parentheses. Prostaglandins E_1, E_2 and $F_{2\alpha}$ were supplied by Dr John Pike, Upjohn Company, Kalamazoo, USA; bradykinin triacetate and 5-hydroxytryptamine were obtained from Sigma, Kingston-upon-Thames, UK.

the numbers of eosinophil- or neutrophil-EAC3b rosettes (Table 1). An increase of 25% was observed with 5-hydroxytryptamine, but only at the highest concentration (10^{-4} mol l^{-1}). The enhancement with the ECF-A peptides or histamine, with this dose was almost three times this value, thus achieving virtually the same percentage of rosetting eosinophils as neutrophils (Figs 1 and 2). The monocyte was also tested with the valyl- and alanyl-peptide and histamine but, unlike the eosinophil, there was no apparent alteration in the numbers of rosetting cells (Table 1). The enhancement of EAC3b eosinophil rosettes by the ECF-A peptides or histamine also increased with incubation time (Fig. 2). With the peptides, however, most of the increase in rosette formation was apparent at 40 min. In contrast, most of the histamine-induced rosette enhancement took place between 40 and 80 min.

These experiments suggest that the ECF-A peptides and histamine not only attract eosinophils selectively in chemotaxis but also have a direct effect on the eosinophil membrane as shown by the apparent increase in the numbers of complement receptors. It is not known whether this enhancement is caused by the generation of new receptors or whether receptors, previously 'hidden', are revealed as a result of membrane changes. The inability of these pharmacological agents to increase neutrophil and monocyte complement receptors emphasises further the unique, intimate relationship between the human eosinophil and these anaphylaxis-associated agents. We also explored the possibility that neutrophil and monocyte complement receptor enhancement may be revealed when the amount of C3 on the indicator cell was limited. In experiments in which the quantity of C3 used was adjusted to give approximately half the number of rosetting cells (that is, 1,000 effective molecules) however, there was no apparent increase in rosette formation following incubation with these agents. Similar negative results were found with neutrophils and monocytes when the amounts of IgG were decreased to give lower numbers of EAG rosettes. The reason why complement, and not IgG, receptors were altered by the ECF-A peptides and histamine is unknown. It was previously shown that IgG and C3b receptors act together to aid macrophage phagocytosis[7]. A similar mechanism may exist for eosinophils; optimal receptor ratio being determined by the presence of pharmacological mediators such as ECF-A and histamine.

The role of the eosinophil in immediate-type hypersensitivity is thought to be that of a homoeostatic cell affecting mediator release, mediator inactivation and mediator replenishment by mast cells[8]. Their function in helminth disease may be that of a cytotoxic cell requiring the participation of IgG, in an analogous fashion to lymphocyte–antibody-dependent cell-mediated cytotoxicity[9]. Although complement does not seem to be required in this system, it is nevertheless possible that adherence by way of the C3 receptor may amplify the parasiticidal properties of eosinophils. Our observations may therefore, be of relevance in helminth disease where the IgE-mediated release of chemical mediators is well recognised. In any event, our finding of a new biological activity for both the ECF-A peptides and histamine may provide further insight into both the essential biochemical differences between human eosinophils and other blood leukocytes, and the way in which chemical mediators modulate recognition mechanisms by cell membranes.

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SPECIAL ARTICLE

The Eosinophil in Infectious Diseases

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Although the role of the eosinophil leukocyte remains unknown, experimental situations have recently been described in which the cell has been shown to have characteristics distinct from those of other leukocytes. Thus the eosinophil migrates preferentially towards certain chemical stimuli and is dependent on various essential prerequisites for its maturation in the bone marrow and appearance in the peripheral blood.

Generally speaking, there are two situations in which eosinophils are associated with infection. In many parasitic diseases there is an increase in the number of eosinophils both in the tissues and in the circulation, whereas in a number of acute pyogenic processes the cell disappears from the peripheral blood. The eosinophilia which accompanies certain types of hypersensitivity reactions to drugs used to treat infectious diseases is a more indirect association, but one of considerable clinical importance.

In the present article morphologic and histochemical features of the eosinophil are described, and mechanisms by which the number of eosinophils fluctuates in the situations cited are discussed. In addition, the capacity of the eosinophil to ingest and kill microorganisms will be compared with that of the neutrophil. Finally, some speculations as to a possible role of the cell in the inflammatory response will be presented.

Morphology and Histochemistry

The eosinophil is characterized by the presence of large intracytoplasmic granules that have an affinity for acid or analine dyes [1]. The number of granules ranges from 25–50 in the horse to 200–400 in the rat [2]. The size and shape of human eosinophil granules are intermediate between those of the two species cited. The granules are biconvex disks bounded by a double-layer membrane and consist of a central crystalline core surrounded by a less electron-dense matrix [3, 4].

Miller et al. concluded that the crystalline "internum" that appears as parallel lines with regular spacing is a cubic lattice, the sides of the cube measuring approximately 30 Å in rodents and approximately 40 Å in man [5]. The core probably forms the basic material of Charcot-Leyden crystals, which are observed when wet eosinophil preparations undergo degeneration [6]. It was noted by Ghadially and Parry that in some electron-microscopic preparations the eosinophil granules have an electron-dense matrix that surrounds a less dense core, and that this reversal of electron density can be seen in cells from the same bone marrow preparation in a number of species examined [7]. This observation may indicate a functional heterogeneity of the eosinophil population.

The double-layer membrane surrounding the outer cortex of the granule contains lipid [8]. The cortex contains an abundance of the enzyme peroxidase [9], which differs from neutrophil myeloperoxidase (see below). The central crystalline core is rich in arginine, as shown by the Sakaguchi reaction. Recent studies by Gleich et al. have shown that this material has an isoelectric point of 10 and a molecular weight of 6,000–12,000 daltons [10]. Histochemical techniques revealed that the granules are Feulgen and pyronin negative [11] and contain only a small amount of material that stains with the periodic acid-Schiff reagent [12].

A number of enzymes are released following disruption of eosinophil granules [13]. They include cathepsin, ribonuclease, arylsulfatase, and β-glucuronidase. Peroxidase remains bound to the insoluble residue, from which it can be extracted by treatment with weak acid. Acid phosphatase has been located in the matrix, but not in the crystalline core. Since eosinophil granules are col-

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lections of enzymes contained within limited membranes, they have been considered as lysosomelike structures.

Peroxidase catalyzes the oxidation of a wide range of substrates. Many histochemical techniques depend on the capacity of peroxidase to cleave oxygen from H_2O_2, thereby oxidizing a suitable leuko-dye to a colored product. Kelsall suggested that eosinophils produce, store, and transport peroxidase to catalyze oxidation reactions in a number of tissues [14]. Rytömaa and Teir showed that tissues rich in eosinophils, such as lung, spleen, uterus, and the gastrointestinal tract, had a high peroxidase content [15]. Peroxidase has been shown to be discharged into phagosomes after phagocytosis by eosinophils [16]. The biological significance of eosinophil peroxidase is unknown. Unlike myeloperoxidase from the neutrophil, it is apparently unable to react in the bactericidal system involving H_2O_2 and a halide [17].

In most species the nucleus of the eosinophil is similar in appearance to that of the neutrophil [18], and the cell has mitochondria, golgi apparatus [19], ribosomes, and an endoplasmic reticulum [2]. Many of these intracellular organelles are more numerous and better developed than those of the neutrophil [18].

Compared with neutrophils, eosinophils contain a large amount of copper as well as other trace metals such as zinc, magnesium, manganese, and cobalt [20].

No lysozyme has been identified in eosinophils [11]. However, like neutrophils, human eosinophils also release leukocyte pyrogen [21].

The Eosinophil and Parasitic Infection

An increase in the number of eosinophils both in the tissues and in the circulation is a feature of parasitic disease in both man and experimental animals. In North America and continental Europe, the parasites that usually cause clinical eosinophilia are the nematodes, of which *Ascaris lumbricodes, Toxocara canis,* and *Trichuris trichura* are common examples; to a lesser extent, the cestodes such as *Taenia solium* and *Taenia saginata* are associated with eosinophilia. The parasitic causes of eosinophilia most commonly encountered in Europeans returning from Central Africa are filariasis and helminthiasis [22]. *Loa-Loa, Onchocerca volvulus, Dipetalonema perstans,* and lymphatic filariasis due to *Wuchereria bancrofti* account for the majority of cases of filarial eosinophila.

Helminthic infections [5], especially bilharzia, are also common forms of eosinophilia acquired in the tropics. The significance of the association of eosinophils with parasitic infection is unknown, although the mechanism of eosinophilia, in terms of both bone marrow turnover and acccumulation at tissue reaction sites, is becoming clearer from work with experimental animals.

Most studies on eosinophil turnover in normal and infected animals have been performed in the rat. These investigations have shown that, before birth, eosinophil production can be observed in both the thymus and the lymph nodes [23]. In adults the majority of the cells are produced in the bone marrow [24]. The precursor of the eosinophil in the bone marrow has not been identified, but it is known that the cell proceeds through several divisions before maturing and entering the bloodstream [25–30]. A stimulus for increased eosinophil production, such as the iv injection of larvae from *Trichinella spiralis*, appears to act at all stages of eosinophil maturation, with a resultant shortening of the phases of the cell cycle before final maturation. [29].

In the normal rat, labeled eosinophils appeared in the circulating blood 36–40 hr after a single pulse of tritiated thymidine. After a stimulus to increased production of eosinophils, this interval was reduced to about 18 hr. The same stimulus did not affect the time required for neutrophil emergence. There is evidence to suggest that after emergence from the bone marrow, the eosinophil matures further in the spleen. Peripheral blood eosinophils were found to be removed exponentially with a half-life of 6.7 hr, the same as the half-life of neutrophils in normal rats [30].

Recent studies have indicated that the lymphocyte may play a central role in the mechanism whereby injections of parasitic larvae lead to increased turnover of eosinophils in bone marrow of the rat [31, 32]. The eosinophil response could be depressed by chronic thoracic duct drainage, X irradiation, and thymectomy [33]. Furthermore, the lymphocytes from animals with an eosinophilia, when transferred to an irradiated recipient, led to an increase in the numbers of circulating eosinophils. Transfer of serum had no effect. An increase in the number of circulating eosinophils

could also be produced by placing the lymphocytes in diffusion chambers and implanting them ip. These experiments suggested that a soluble factor or factors, released from the lymphocytes of parasitized rats, can initiate increased turnover of eosinophils in bone marrow.

It has been found that a reproducible method of evoking an eosinophilia in the rat with *T. spiralis* is to inject iv living or dead muscle-stage larvae [31]. The larvae become arrested in the pulmonary vasculature. When the larvae are homogenized and thus pass through the pulmonary circulation, a subsequent blood eosinophilia is not observed. The larvae apparently have to be arrested in the lung in order to initiate the mechanisms leading to selective bone marrow turnover of eosinophil leukocytes. Even Sephadex beads trapped in the lung after iv injection give rise to a modest eosinophilia [34]. It has been suggested that in this experimental model, Sephadex is reacting with natural antibody directed against polysaccharide antigens, thereby producing an eosinophilia in a similar fashion to that observed with parasitic larvae.

The local cellular reaction to parasites depends on a number of factors such as the type of organism, the route of administration, and the species involved. Thus, the local accumulation of cells around muscle-stage *Trichinella* larvae takes place relatively quickly after iv administration [35]. The 12–24-hr lesion is essentially granulomatous, the parasite being encased by large mononuclear cells. Eosinophils are seen in large numbers but at a distance from the parasite, as are lymphocytes and a few neutrophils.

The majority of eosinophils reside within the tissues. It has been estimated that for every circulating cell there are 300–500 cells within the tissues [36]. The fate of eosinophils depends largely on the stimulus to their production. In acute allergic reactions the cell undergoes extensive degranulation at the site of mast cell disruption [37]. Normally, eosinophils find their way to mucosal surfaces where they shed their contents into the lumen [24].

A feature of parasitic infection in both man and experimental animals is the production of circulating IgE antibody. Thus, elevations in circulating IgE have been demonstrated in Ethiopian school children with ascariasis [38], and in patients with visceral larva migrans [39]. In *Nippostrongylus brasiliensis* infection in the rat, IgE production has an apparent protective effect, as shown by massive expulsion of worms between the 10th and 18th days after administration of the worm (the so-called "self-cure" phenomenon) [40–42]. This effect can be transferred by tissue-sensitizing antibody, which adsorbs onto mast cells of the gastrointestinal tract, thus leading to histamine release by the worm antigen. The release of this amine apparently leads to expulsion of the worms. The production of IgE and the associated eosinophilia suggests that there is an allergic element in the host reaction to many parasitic diseases. For example, rats injected with *Trichinella* larvae developed systemic anaphylaxis when challenged with trichinella extract, and the survivors subsequently developed a blood eosinophilia [43]. These observations suggest that a product of the anaphylactic reaction, possibly the eosinophil chemotactic factor of anaphylaxis (ECF-A), participates in parasite-mediated eosinophilia [44–46].

In addition to IgE, antibodies capable of activating the complement system are produced in a number of parasitic infections; advantage is taken of this fact in serodiagnosis. A fragment cleaved from the fifth component of complement (C5a) has been shown to attract the eosinophil selectively under defined experimental conditions [47]. When ECF-A and C5a were combined, marked synergism was observed in their ability to attract eosinophils. Possibly this mechanism is operative in the local accumulation of eosinophils in certain parasitic states [46].

It is not possible to give a unifying hypothesis to explain the continued eosinophil response found in association with parasitic disease. Present evidence suggests that the lymphocyte plays a role in production of eosinophils by the bone marrow and that continued local infiltration of eosinophils may be accentuated by allergic responses to the parasite involving both IgE and complement. A possible role for the eosinophil in parasitic and other states is discussed below.

Eosinophils and Acute Infective Processes

It has long been recognized that a peripheral blood eosinopenia accompanies acute infection. It was shown by Spink that the eosinophilia evoked by trichinosis in the guinea pig could be obliterated

by a superimposed staphylococcal, trypanozomal, or tuberculous infection [48]. Until recently the eosinopenia of infection was regarded as a response to stress and thus a reflection of increased production of adrenal glucocorticosteroids.

Bass has shown recently that the eosinopenia of various types of acute inflammation are mediated by a mechanism independent of adrenal glucocorticosteroids [49]. Using mice infected with *T. spiralis*, he showed that the resultant eosinophilia could be depressed by the superimposition of a pneumococcal abscess, staphylococcal infection, *Escherichia coli* pyelonephritis, *Coxsackie* B4 pancreatitis, or acute sc inflammation due to turpentine. The eosinophils that disappeared from the peripheral blood appeared to accumulate at the site of inflammation. Splenectomy caused an even greater fall in peripheral eosinophil counts. When the acute inflammatory procedures were terminated at an early stage, eosinophil production was restored to normal.

The theory that the eosinopenia of an acute inflammation was the result of the adrenal response was tested directly by assay of cortisone in serum. Increased levels of serum cortisone were found after bacterial, viral, and rickettsial infections. However, these were smaller than the increase in serum cortisone observed after the response to trichinosis during the early infective period. It was thus concluded that the eosinopenia of acute inflammation could not be ascribed to stimulation of the adrenal glands.

Bass then showed that material present in the exudate from the pneumococcal abscess produced a decrease in the number of circulating eosinophils 4–24 hr after injection. The material responsible for this effect was referred to as an "eosinopenic factor" (EF), although it is not certain whether this is the property of one or several molecules. The eosinopenic effect could not be transferred by heat-killed pneumococci, pneumococcal-culture filtrate, or normal serum. Adrenalectomized mice responded to EF in a way similar to that of normal animals. EF had no effect on the number of circulating neutrophils but caused a reduction of about 30% in the number of circulating lymphocytes. The significance of this shift in the eosinophil population is unknown. Further studies on EF may help to explain this effect.

Other Associations of the Eosinophil with Infective Agents

Although a blood eosinopenia is a feature of the acute stage of many infections, the number of white blood cells returns to normal when the inflammatory process resolves [50]. Some infections, notably scarlatina [51], pneumococcal pneumonia, and tuberculosis [52], are occasionally associated with a blood eosinophilia during the convalescent phase of the illness. These situations are probably a reflection of some form of hypersensitivity reaction to bacterial products.

Adverse reactions to drugs used in the treatment of infectious diseases are often associated with a blood eosinophilia. This is invariably a manifestation of a hypersensitivity state. The mechanisms by which this occurs are poorly understood, but such reactions are probably mediated by a number of factors including IgE, complement, and possibly a delayed-type hypersensitivity component [53].

Some of the types of "pulmonary eosinophilia," which include simple pulmonary (Loffler's syndrome), asthmatic, and tropical eosinophilia, are associated with what is presumed to be an alveolar hypersensitivity to parasites [54, 55]. *A. lumbricoides, Ancylostomum braziliense, T. trichura, T. saginata*, and *Fasciola hepaticum* have all been implicated in simple pulmonary eosinophilia. Evidence of hypersensitivity to *Aspergillus fumigatus* can be demonstrated in about 50% of cases of asthmatic pulmonary eosinophilia. Convincing evidence exists that tropical pulmonary eosinophilia is associated with a filaria that is unable to mature further in the human host.

The Phagocytic and Bactericidal Properties of the Eosinophil

The morphology of eosinophil and neutrophil phagocytosis is similar. The granules coalesce with the phagosomes following membrane fusion [16].

A number of recent studies have compared the capacity of eosinophils and neutrophils to ingest and kill various microorganisms. The events leading to phagocytosis and destruction of particles are often considered in terms of chemotaxis, adherence, engulfment, and digestion. Agents capable of selectively attracting the eosinophil include ECF-A [44], C5a [47], and a lymphocyte-medi-

ated chemotactic factor [56]. The eosinophil has a receptor for immune adherence [57], but not for the Fc portion of IgG [58].

Studies comparing the capacity of eosinophils and neutrophils to phagocytose microorganisms and inert particles have all concluded that the eosinophil is less efficient than the neutrophil [21, 59, 60]. Similarly, the bactericidal activity of eosinophils is less than that of the neutrophil in terms of intracellular killing of *E. coli*, *Staphylococcus aureus, Staphylococcus albus,* and *Listeria monocytogenes* [17, 21, 60].

In contrast, the metabolic activity of the resting, unstimulated eosinophil is greater than that of the neutrophil [21]. Oxidation of 1-^{14}C glucose, 6-^{14}C glucose, and ^{14}C formate, which reflect activity of the hexosemonophosphate shunt, Kreb's cycle, and formation of H_2O_2, respectively, are greater in the resting eosinophil than in the resting neutrophil. Similarly, following stimulation of eosinophils, either by contact with a particle or by engulfment, a greater increase in the hexosemonophosphate shunt and production of intracellular H_2O_2 can be demonstrated [21, 59, 61]. The oxidative response of the eosinophil is thought to be due to increased nicotinamide-adenine dinucleatide phosphate-H oxidase of granular origin rather than soluble nicotinamide-adenine dinucleotide phosphate-H oxidase, which is apparently operative in the neutrophil [60].

Despite the availability of H_2O_2 and peroxidase, the eosinophil is apparently unable to utilize efficiently the peroxidase-H_2O_2 bactericidal system that is thought to play an important role in the killing of microorganisms by the neutrophil [62]. This relatively poor bactericidal response by the eosinophil may be related to the nature of its peroxidase, which differs in several respects from myeloperoxidase of the neutrophil. Thus it was found that the optical spectra of the reduced form of eosinophil peroxidase differed from that of the reduced form of neutrophil myeloperoxidase [63]. The two peroxidases are also antigenically distinct [64], and unlike neutrophil myeloperoxidase, eosinophil peroxidase is not inhibited by cyanide [65].

It should be noted that many of the studies on the capacity of eosinophils and neutrophils to ingest and kill microorganisms have used populations of cells that have ranged between 50% and 90% in purity. These are essentially mixed cell populations, and a possible influence of one cell type on another, in terms of phagocytosis and killing of microorganisms, cannot be excluded.

Concluding Comments

The eosinophil has distinct morphologic and histochemical features, but these have yet to be equated with a unique function. The material which comprises the central core of the eosinophil granule is a major histochemical feature of the cell, and therefore it will be of interest to see whether the isolated protein can modulate inflammatory processes associated with eosinophilia. Why the eosinophil is unable to take up and ingest common pathogens efficiently is unknown, but this deficiency may indicate that engulfment is a property the cell has maintained during evolution and is therefore incidental to its main functions(s) [66].

The association of eosinophils with parasitic infections has been discussed, and it was suggested that this association is due in part to an allergic response to parasitic antigens, which are able regularly to produce IgE-type antibodies in man and a number of experimental animals [41, 67]. When IgE becomes adsorbed to cell membranes of the mast cell-basophil series and reacts with specific antigen, chemical mediators are released, with subsequent accumulation of eosinophils.

It has been suggested that a role of the eosinophil is to inactivate pharmacologic agents [68]. However, we have been unable to show that eosinophils inactivate histamine. Purified suspensions of eosinophils from humans, guinea pigs, and horses, when incubated with histamine, did not affect the capacity of the amine to contract the isolated perfused guinea pig ileum [69]. We have shown, furthermore, that histamine is inactivated very rapidly after local release in the skin, which is several hours before the infiltration of eosinophils [70].

We regard the eosinophil more as a repair cell which restores the status quo of tissues that have undergone an allergic response. Thus, in studies in the rat, eosinophil leukocytes were seen to accumulate around degranulated mast cells and to ingest their granules [71]. These observations have been confirmed by Mann, who further showed that after the accumulation of eosinophils around mast cells and phagocytosis of the gran-

ules there was phagosome formation followed by intracellular breakdown of the phagosome membrane [72]. These observations are consistent with the view that the eosinophil acts as a repair cell in immediate-type hypersensitivity reactions. The repair mechanism may be restricted to phagocytosis of mast cell contents, although the eosinophil may also participate in the restoration of mast cell contents, thus enabling the cell to react again in immediate-type hypersensitivity.

A previous study has shown that eosinophils can adhere directly to certain parasites in vitro [73]. Possibly eosinophils have the capacity to destroy parasites or inactivate their products. However, the association of eosinophils and parasitic diseases is usually long-term, suggesting that whereas the eosinophil may contain the spread of parasites, it probably does not have a profound effect of the viability of the organisms. The recent description of an antiserum specific for the eosinophil cell membrane may help to answer these questions [74].

Another possibility is that the eosinophil influences the synthesis and assembly of the IgE molecule. It would, however, be of interest to have details of correlations of fluctuations in eosinophil levels with the amount of circulating IgE and to determine the effect of antiserum to eosinophils or exogenous administration of eosinophils on the IgE response.

In conclusion, it is unlikely that the eosinophil plays a major role in the phagocytosis and destruction of common microorganisms or that it inactivates chemical mediators such as histamine. Some investigations are mentioned that have suggested possible roles of the cell; these include the capacity of the eosinophil to repair those cells participating in immediate-type hypersensitivity, the direct influence of this leukocyte on parasites and their products, the ability of the eosinophil to modulate the production of IgE-type antibodies, and the biochemical properties of the granular material.

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Chemotaxis of Eosinophil Leucocytes in Relation to Immediate-Type Hypersensitivity and the Complement System[1]

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Introduction

An increase in the number of eosinophils in the tissues and the circulation is a feature of various clinical conditions. These include diseases associated with high levels of IgE, such as extrinsic (or allergic) asthma, hay fever and parasitic infections [1–4], and disorders in which there is direct or circumstantial evidence of the presence of circulating, or fixed antigen antibody complexes capable of activating the complement system. The latter group of diseases includes polyarteritis nodosa with lung involvement, pulmonary aspergillosis and rheumatoid arthritis [5–8].

In experimental animals, an eosinophilia can be evoked following injection of specific antigen into a sensitised animal. Thus, an eosinophilia can occur following general or local anaphylaxis [9–11] or following the implantation of anaphylactic tissue into normal animals [12, 13]. Injections of antigen-antibody complexes into the peritoneal cavity of normal animals also results in a local eosinophilia [14].

In order to study the relationship between immediate-type (anaphylactic) hypersensitivity, the complement system and the accumulation of eosinophils, initial experiments were performed in guinea-pig skin [15]. It was shown that IgG_1 and IgG_2, as preformed complexes, prepared skin for a subsequent local eosinophilia 12 h after injection. However, if antibody was

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first placed in the skin and after a variant latent period the animal was challenged by antigen and Evans blue dye intravenously (as in a usual passive cutaneous anaphylactic reaction) IgG_1, but not IgG_2, elicited a local eosinophil response 8–12 h after the initial blueing reaction. Intradermal histamine given at different skin sites in the same animals in doses which gave a comparable blueing reaction to the IgG_1-mediated response did not lead to an accumulation of eosinophils. Eosinophils also accumulated around the sites of injections of compound 48/80, an agent which depletes mast cell granules. Low mast cell counts also accompanied the infiltration of eosinophils observed with IgG_1-mediated passive cutaneous anaphylaxis (PCA) reactions. It was suggested at this time that the accumulation of eosinophils following PCA reactions was by a complement-independent mechanism subsequent to the release from mast cells of an agent other than histamine. The participation of the complement system was suggested in antigen-antibody complex mediated eosinophilia.

When animals were decomplemented with cobra venom factor this had no effect on eosinophil accumulation into the site of IgG_1-mediated PCA reactions in the guinea-pig [16]. Furthermore, eosinophil accumulation also followed PCA reactions in animals partially or totally deficient in the fourth component of complement. Surprisingly, intradermal injections of preformed antigen-antibody complexes prepared from guinea-pig IgG_1 or IgG_2 were also unaffected by decomplementation with purified cobra venom factor. The lesions produced with either immunoglobulin complex were similar in appearance in decomplemented and normal animals and were followed by comparable tissue accumulation of eosinophils. Since IgG_2 is tunable to sensitise skin for PCA reactions [17] or lung fragments for histamine [18] and slow-reacting substance of anaphylaxis (SRS-A) release [19], but can fix complement by the classical pathway, it was expected that complement may play a part in the cellular infiltration following intradermal injections of IgG_2 complexes. These apparent discrepancies could be explained on a weight basis since IgG_1 is far more efficient in preparing tissue for an eosinophilia when administered in PCA reactions than when injected as preformed complexes. It was previously demonstrated that soluble complexes prepared from guinea-pig IgG_1 or IgG_2 both have the capacity to liberate histamine from perfused guinea-pig lung [20]. This suggests that IgG_2 may have similar properties to IgG_1, in terms of its effect on mast cells, when presented to tissue as a complex.

These observations *in vivo* prompted studies on the identification of chemotactic agents for eosinophils released during the anaphylactic reaction

or following complement activation. A modification of the Millipore technique of Boyden was used for these experiments [21]. Eosinophils were obtained from the peritoneal cavity of the guinea-pig following multiple injections of horse serum. In the human studies peripheral blood leucocytes from patients with eosinophilia were used as a source of target cells.

An Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A)

As an experimental model of anaphylaxis, sensitised guinea-pig lung was chosen since this has been employed by numerous workers as a model of immediate-type hypersensitivity and the organ can be perfused free of blood thereby providing a serum-free system. Similarly, human lung fragments taken at pneumonectomy can be thoroughly washed before and after sensitisation with serum from an allergic individual. When sensitised lung was challenged with specific antigen there appeared in the diffusate, along with histamine and SRS-A, an agent which selectively attracted eosinophils. This eosinophil chemotactic factor of anaphylaxis has been termed ECF-A [22].

Formation Mechanism

The release of ECF-A was accompanied by the release of histamine and SRS-A, but differed in its time course of release. In the experiment depicted in figure 1 guinea-pig lung was perfused free of blood by cannulating the pulmonary artery. Specific antigen was injected *via* the same route. Approximately 10 ml volumes of perfusate were collected every 15 min for 1 h. Virtually all the histamine and SRS-A was released during the first 15 min, whereas ECF-A continued to be released for the duration of the experiment. A similar time-course of release was observed with human lung fragments passively sensitised with serum from a ragweed-sensitive individual and challenged with ragweed antigen E [23]. Histamine, SRS-A and ECF-A were similar in terms of the time-course of passive sensitisation and the amount of antigen required for optimal release. ECF-A release was dependent on the presence of divalent cations and was strikingly enhanced by the presence of succinate or maleate; features characteristic of the release of histamine and SRS-A [24].

In the guinea-pig, decomplementation by the administration of purified cobra venom factor had no effect on the antigen-induced release of ECF-A

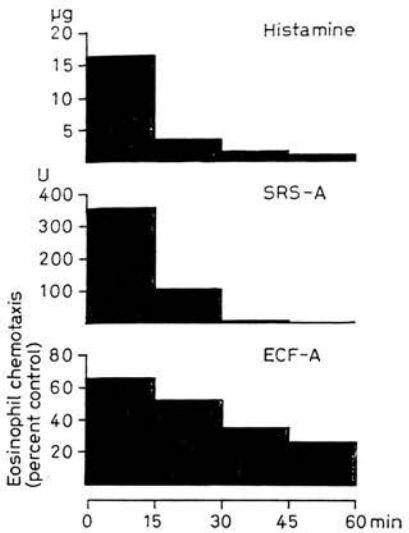

Fig. 1. Time-course of release of chemical mediators of anaphylaxis following antigen challenge of perfused, actively sensitised guinea-pig lung. The measurements represent the mean values from 16 animals. The experimental technique and the expression of results are described in ref. No. 22. SRS-A = slow-reacting substance of anaphylaxis, ECF-A = eosinophil chemotactic factor of anaphylaxis.

from actively or passively sensitised lung fragments. Since the lungs were then perfused free of blood and the fragments thoroughly washed this strongly suggests that ECF-A release is complement-independent [22].

There is evidence that increase in the level of intracellular 3′–5′ cyclic adenosine monophosphate (cyclic AMP) inhibits the release of ECF-A since the antigen-induced release of ECF-A was inhibited from passively sensitised human lung fragments by either isoprenaline, in doses ranging from 10^{-5} to 10^{-8} M, or with dibutyryl cyclic AMP in a concentration of 5×10^{-4} M [25].

Antibody Requirement

ECF-A from the guinea-pig can be released from normal lung passively sensitised with IgG_1. The same immunoglobulin has been shown to sensitise

guinea-pig lung slices for the antigen-induced release of histamine and SRS-A. The fractions of IgG_1 were prepared by ion exchange chromatography and lost no activity after heating at 56°C for 4 h, a property characteristic of IgG_1 but not of an IgE-like immunoglobulin. Comparable amounts of IgG_2-containing fractions failed to sensitise lung fragments for the antigen-induced release of histamine, SRS-A or ECF-A. The capacity of guinea-pig IgE to sensitise tissue for ECF-A release is yet to be ascertained due to difficulties in raising antibodies of this class and in obtaining guinea-pig IgE free from IgG_1.

In the human, the release of ECF-A was shown to be mediated by IgE [23]. This was demonstrated by absorption of ragweed-sensitive serum with a rabbit antibody specific for IgE. Absorbed antibody could no longer passively sensitise lung for the release of ECF-A, histamine or SRS-A. ECF-A could also be released from normal lung by the reversed-type reaction employing a specific anti-IgE prepared in the rabbit.

The Identification of ECF-A as a Distinct Pharmacological Agent

Various chemical mediators of anaphylaxis were tested directly for their ability to evoke the migration of eosinophils [22]. It was found that histamine, bradykinin, serotonin and prostaglandins (PGE) PGE_1, PGE_2 and $PGF_{2\alpha}$ over a wide dose range, were not chemotactic for eosinophils *per se;* and, furthermore, when incubated with sensitised lung in the absence of antigen these agents did not secondarily affect the release of ECF-A. Although SRS-A is yet to be chemically characterised there were several reasons for considering ECF-A to be distinct from this agent. SRS-A survived boiling in alkaline solution for 20 min, whereas ECF-A activity was abolished by this procedure and, in addition, SRS-A and ECF-A in the guinea-pig could be separated by gel-filtration. Human ECF-A appears to be more closely associated with human SRS-A, but they could be separated functionally since the latter was partially destroyed by lyophilisation and boiling in acid solution, whereas ECF-A was inactivated by boiling for 10 min in alkaline solution. Further evidence that ECF-A is distinct from SRS-A and histamine was apparent from measurements of mediators in the perfusates described for figure 1. In 4 of the 16 lungs no SRS-A or histamine was detected in the 15- to 30-min perfusate, although this contained large quantities of ECF-A. Histamine and SRS-A were released during the first 15 min only.

Physico-Chemical Properties of ECF-A

Guinea-pig and human ECF-A were relatively resistant to heat, only about 25% of the activity being lost following boiling for 10 min or heating at 56°C for 1 h. Both human and guinea-pig ECF-A activity was totally recovered following lyophilisation and multiple freezing and thawing. About 30% of their activity was destroyed by extraction in 80% ethanol, evaporation to dryness and re-suspension of the residue to the original volume. Some preliminary evidence suggested that ECF-A may be a small peptide [26]. It is fully recovered from a column of Sephadex G-25 and had an estimated molecular weight of between 500 and 1,000. The peak of ECF-A activity contained materials which had free amino groups (ninhydrin reaction) and peptide bonds (starch-iodide reaction). Recent experiments have shown, however, that guinea-pig ECF-A activity is *not* destroyed by relatively large doses of pronase, chymotrypsin and trypsin [27]. In these experiments advantage was taken of the relative heat stability of ECF-A. Partially purified guinea-pig ECF-A, prepared by G-25 Sephadex chromatography, and a control Tyrode's buffer with antigen subjected to the same procedure, were incubated with enzymes for 1 h at 30°C and then heated at 56°C for 30 min. Both chymotrypsin and trypsin completely lost activity by this heat treatment although pronase was unaffected. However, the heated enzymes were inactive in chemotaxis when tested alone, and when mixed with ECF-A did not affect its activity. The proteolytic activity of the enzymes was monitored by following the release of trichloroacetic acid insoluble material from casein using the same buffer and identical incubation conditions to those used for the samples.

Selectivity of ECF-A for Eosinophil Leucocytes

ECF-A selectively attracts eosinophils from a mixed leucocyte population. Guinea-pig neutrophils from a pure population (>96% purity) will migrate towards ECF-A, although when eosinophils are introduced into the suspension they are selectively attracted when they comprise 10% or more of the mixed cell population [28]. Human ECF-A also selectively attracts eosinophils but neutrophils and basophils will migrate when eosinophils are present in very small numbers. Thus, a broad specificity for ECF-A, as of other chemotactic factors can be shown when conditions for migration are optimal and when other cells which may be preferentially attracted are absent.

Table I. Tissue eosinophil counts at the sites of intrapulmonary injections of guinea-pig anaphylactic lung diffusates

Animal No.	Anaphylactic diffusate (right lung)	Control (left lung)
1	240	50
2	187	95
3	80	35
4	277	28
5	39	24
6	9	11
7	61	41
8	82	32
9	73	23
Mean	116	38

Volumes of 0.2 ml were injected into each lung, and tissue eosinophil counts were performed 12 h later using the technique previously described [39]. The anaphylactic diffusate contained 1.0 μg/ml of histamine, 60 U/ml of slow-reacting substance of anaphylaxis (SRS-A) and 50 μg/ml of ovalbumin. An equivalent amount of histamine and antigen was injected into the control lung.

Other Features of ECF-A

The source of lung ECF-A is not known; however, a comparable material has been identified from highly purified sensitised human peripheral blood basophils challenged with antigen [29]. There is evidence that ECF-A exists in a pre-formed state in highly sensitised human basophils [30] or guinea-pig lung [31], i.e. cells or tissue which will release relatively large amount of mediators following antigen challenge. In these situations slight mechanical manipulation, in the absence of antigen challenge, results in the elaboration of an eosinophil-attracting substance.

When a guinea-pig anaphylactic diffusate was injected into one lung of an animal and the appropriate control administered into the other lung, a 3-fold increase in local eosinophil accumulation could be demonstrated (table I) [32]. A similar local eosinophilia could be seen in the guinea-pig peritoneal cavity following injection of partially purified ECF-A (table II). A summary of the features of ECF-A is depicted in table III.

Table II. Eosinophilia following intraperitoneal injections of partially purified ECF-A

Animal No.	Treatment	Eosinophil count per millilitre of fluid recovered, $\times 10^3$
1		500
2	partially purified ECF-A	245
3	in Tyrode's solution	293
Mean		346
4		85
5	Tyrode's solution	140
6		120
Mean		115

ECF-A = eosinophil chemotactic factor of anaphylaxis.
Volumes of 5 ml were injected and eosinophil counts were performed 12 h later. Partially purified ECF-A, giving a chemotactic count of 45 cells/ml [22], was prepared by Sephadex G-25 chromatography and was free of histamine and SRS-A.

Table III. Some properties of human and guinea-pig ECF-A

Released by antigen challenge of actively or passively sensitised lung.
Passive sensitisation mediated by IgE in human lung and IgG_1 in the guinea-pig.
Release of ECF-A accompanied by histamine and SRS-A.
Release of ECF-A from perfused whole guinea-pig lung slower than histamine and SRS-A.
Optimal conditions for ECF-A release, in terms of antigen and antibody dose, similar to histamine and SRS-A.
Requirement for divalent cations.
Enhanced by succinate and maleate.
Complement not required.
Probably modulated by levels of intracellular cyclic AMP.
Distinct from histamine, bradykinin, serotonin, SRS-A, prostaglandins (PGE) PGE_1, PGE_2, $PGF_{2\alpha}$ and C5a.
Labile in alkaline solution.
Resistant to heat, lyophilisation and multiple freezing and thawing.
Molecular weight 500–1,000.

Complement-Derived Eosinophil Chemotactic Factors

Factors Generated from Serum by Antigen-Antibody Complexes

Although the *in vivo* significance of complement-dependent chemotactic factors is unknown, an eosinophil specific chemo-attractant can be generated from serum [21]. Preformed antigen-antibody complexes prepared either from guinea-pig IgG_2 or IgG_1 were equally capable of generating, from normal serum, heat-stable activities that were chemotactic for guinea-pig eosinophils and neutrophils. The generation of this activity was apparently dependent on the presence of an intact complement system. When serum, activated by complexes prepared from either of these sub-classes, was passed over a column of Sephadex G-100, two peaks of chemotactic activity could be demonstrated. The peak of activity for guinea-pig neutrophils had a molecular weight of approximately 75,000. The eosinophil chemotactic activity eluted with molecules having a molecular weight of between 15,000 and 20,000. The nature of the neutrophil chemotactic activity is unknown but a comparable activity has been generated from rat serum and is thought to be a cleavage product of the 5th component of complement [33]. The smaller fragment which was predominately chemotactic for eosinophils had a similar molecular size and sedimentation constant to that of C5a. It was subsequently confirmed that C5a prepared from highly purified C5 did, in fact, preferentially attract eosinophils from a mixed leucocyte population [28]. Although C5a, like ECF-A, is chemotactic for neutrophils, when eosinophils comprised approximately 10% or more of a mixed population they were preferentially attracted by this complement fragment.

Interaction of ECF-A with C5a

Since ECF-A and C5a are distinct both in their molecular weight and formation mechanism, it was of interest to determine the effect of combining the two agents in eosinophilotaxis [28]. When these agents were mixed together in the test compartment of the chemotactic chamber the resultant counts were three times or more greater than would have been expected by summation of counts when the agents were assayed alone. This suggested that ECF-A and C5a act synergistically in their ability to attract eosinophils. It is possible that eosinophils have more than one receptor for chemotaxis and that, if different types of receptors are stimulated at a low threshold,

this produces an increased chemotactic response. These observations on synergism may be of significance in parasitic infestations, many of which are associated with a pronounced eosinophilia. Homocytotropic antibody and complement-fixing antibody can occur together in a variety of parasitic diseases, situations in which ECF-A and C5a might act together.

Other Complement-Derived Eosinophil Chemotactic Factors

A fragment from the third component of complement (C3a) [34] and the trimolecular complex of $\overline{C567}$ [35] have also been shown to attract eosinophils. When purified $\overline{C56}$ and $\overline{C7}$ were prepared as in the 'reactive-lysis' procedure their combination resulted in attraction of neutrophils, eosinophils [36] and basophils [37]. There was no evidence that $\overline{C567}$ was preferentially chemotactic for any of these cell types.

Cell-specific attraction for eosinophils or neutrophils has recently been demonstrated by activating serum from the hog, rat or guinea-pig with antigen-antibody complexes, yeast or dextran [38]. Following progressive purification of the activated serum, two peptides have been isolated which have been termed 'classical anaphylatoxin' and 'cocytotaxin'. By themselves these peptides have little chemotactic activity but on re-combination and depending on their molar ratio, cell-specific chemotactic activity for eosinophils or neutrophils can be demonstrated. These interesting observations suggest that low molecular weight anaphylatoxins are distinct from chemotactic agents although closely related in molecular size, and that the ratio in which they are combined determines the expression of various biological activities. Although the peptides are probably products of complement activation this has yet to be demonstrated conclusively.

Conclusion

Two chemotactic factors have been described which selectively attract eosinophil leucocytes. They differ both in molecular size and in formation mechanism. ECF-A is a product of the anaphylactic reaction and is distinct from previously described pharmacological mediators. The complement-derived factor, C5a, can show preferential attraction of eosinophils under certain experimental conditions. Marked synergism was observed between

ECF-A and C5a in their ability to attract eosinophil leucocytes. The elaboration of these agents in various hypersensitivity states may in part account for the infiltration of eosinophils.

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THE ROLE OF EOSINOPHILS

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Discussing the nature of eosinophil structure, G. Gleich reported that the major basic protein recovered from a pool of isolated cytoplasmic granules in the guinea-pig is a more potent sulphydryl agent than mercaptoethanol. In several biologic systems the protein was found to be devoid of enzymatic activity, inflammatory or anti-inflammatory effects.

Regarding the kinetics of eosinophil development, G. Hudson raised many unresolved problems particularly the importance of cell death as a regulator of eosinopoesis.

D.G. Colley offered preliminary evidence that lymphocyte culture fluid containing eosinophil stimulating promotor (ESP) also contains *in vitro* colony stimulating activity in bone marrow cultures.

R.S. Speirs reported, in the mouse, that the secondary eosinophil response can be transferred by T-cells primed with tetanus toxoid. In neonatally thymectomized mouse models, D.A. Cohn's observation supports this finding. In contrast, Colley in a non-transfer system in T-cell depleted *Schistosoma mansoni* infected mice stated that these animals were still capable of mounting an intermittent peripheral blood eosinophilia. Rosette formation of eosinophils around macrophages induced by the injection of *Trichnella* larvae was reported by R.S. Walls. Other investigators (Speirs, Cohn) agree with this observation; Speirs suggesting that the development of this phenomenon leads to the killing of macrophage target cells by eosinophils participating in the inflammatory response. The biologic significance of this observation is not known.

A number of experimental animal models of eosinophilia were discussed. D.L. Boros found a fall in circulating eosinophils in mice following the administration of a monospecific anti-eosinophil serum (AES); the effect on other cell types *in vivo* was not reported. This same AES also prevented the appearance of eosinophils within

granulomatous cells responses engendered around schistosome ova. D.G. Jones and A.B. Kay also reported on the inhibition of eosinophil accumulations by AES in vivo following PCA reactions in the guinea-pig.

In another experiment model D. Zucker-Franklin reported on an association between eosinophilia in rats induced by intravenous injections of protein antigen-coated latex particles and in vitro stimulation of lymphocytes by corresponding antigen on the particles. Ultrastuctural study of the lesions surrounding the particles showed a characteristic delayed type reaction with blast cell transformation of lymphocytes. In this regard W.E. Parish showed that "naked" particles can also induce a reaction with some of these histological characteristics; this phenomenon was attributed to surface absorption and possible aggregation of autologous serum proteins.

Parish stated that eosinophil chemotactic factor of anaphylaxis (ECF-A) while promoting a local eosinophilia does not provide the stimulus for engendering systemic eosinophilia reflected in the peripheral blood. By contrast, a distinct mediator having an approximate molecular weight of 30,000 which was also derived from anaphylactic lung diffusates elicited a blood eosinophilia but this agent was not chemotactic for eosinophils in vitro. M. Samter and Kay's observation on the infiltration of eosinophils into lung and peritoneal cavity following the administration of ECF-A is relevant to a consideration of an in vivo physiologic role for this mediator.

Attempts have been made to characterise ECF-A by the susceptibility of this mediator to treatment by a variety of enzymes. M.K. Bach, Jones and Kay reported on guinea-pig studies in which no demonstrable loss of activity resulted from treatment with trypsin, chymotrypsin, pronase, alkaline phosphatase and sialidase. In contrast loss of activity in a dose-dependent fashion was shown with tyrosinase, leucine aminopeptidase and aryl sulfatase; thus suggesting that ECF-A contains a phenolic hydroxyl group, a sulfate ester and a peptide linkage with a free α- amino group. The enzymes employed had specificity only for their appropriate substrates. Thus, although carboxypeptidase A and B inactivated ECF-A the arylsulfatase present in these preparations was sufficient to account for the loss of chemotactic activity. E. Goetzl, S. Wasserman and K.F. Austen reported that human ECF-A is inactivated by aminopeptidases, carboxypeptidase A, subtilisin, pronase and possibly the arylsulphatase

derived from eosinophils but not trypsin or chymotrypsin; thus confirming its suspected peptide nature.

Goetzl and his co-workers also reported that pre-incubation of human eosinophils with corticosteroids inhibit random migration and chemotaxis but this effect was not seen with the cells of guinea-pig origin. This observation was confirmed by Jones and Kay with respect to non-inhibition of migration of hydrocortisone in the guinea-pig system. These same workers were also unable to demonstrate inhibition by disodium chromoglycate but did find that an anti-SRS-A compound, FLP 55712, inhibited chemotaxis at a considerably lower dosage than that required for the selective inhibition of SRS-A.

Studying eosinophil infiltrations into skin sites subsequently biopsied, B. Zweiman noted that one week of moderage dosages of short acting corticosteroids in either daily or alternate day regimes is followed by significantly less eosinophil accumllations than that resulting from intradermal injections of compound 48/80 or of antigen in specifically sensitized individuals. The whealing response however was not significantly affected.

Comments by S.G. Cohen were directed to difficulties in his experience, in reconciling putative eosinophil functions visualised by the induction of animal models of eosinohilia and in vitro experiments with known pathophysologic alterations in eosinophil-related syndromes and disorders. Specifically mentioned in the discussion were eosinophil properties of phagocytosis of antigen-antibody complexes, relationships to basophil and mononuclear leukocytes and lymphocytes, and the association with tissue sensitising antibody.

A number of multi-systemic diseases of unknown aetiology associated with hypereosinophilia were reported (Zucker-Franklin) D.M. Geunther).

Thus an eosinophilia occurs in association with a number of clinical states and is evoked by injection of antigen using a variety of animal models. It is clear that under certain circumstances the cell is associated with the antigen induction phase. The role of the cell is associated with the antigen ineuction phase. The role of the cell in the modulation of the humoral and/or cellular response is unknown. This aspect of eosinophil study is possibly the most confusing due partly to the variety of experimental models used. A number of

issues were raised in regard to the participation of the cell in the inflammatory response. Does the eosinophil have a primary function in inactivating chemical mediators or is it a repair cell? The kinetics of eosinophil infiltration following local anaphylaxis would suggest the latter. Therefore it was not felt possible at this time to interpret the role of eosinophil granuolcytes in either physiologic states or human disorders on the basis of our present knowledge of eosinophil function.

A.B. Kay and S.G. Cohen

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were further purified by density centrifugation on sodium metrizoate (Hypaque) gradients[12, 13].

Eosinophil chemotactic factor of anaphylaxis (ECF-A)

ECF-A like histamine is a preformed mediator and preferentially attracts eosinophils from a mixed leucocyte population (Fig. 2). The agent was first identified in anaphylactic diffusates derived from actively or passively sensitised guinea-pig lung challenged with specific antigen[14]. The factor was shown to be distinct from previously recognised chemical mediators of anaphylaxis. A comparable agent was subsequently recognised in human lung tissue[15]. The antibody mediating the release of ECF-A was shown to be IgE in man and IgG_1 in the guinea-pig. In the guinea-pig it has recently been shown that other tissues including skin, uterus, heart and intestine in addition to the lung can be passively sensitised with IgG_1 for the subsequent antigen induced release of a factor with similar biological and chemical properties to ECF-A[16, 17]. In contrast this mediator was not released in detectable amounts from sensitised kidney, liver, brain and skeletal muscle. *In vivo* the injection of guinea-pig anaphylactic diffusate into normal lung or partially purified ECF-A into the guinea-pig peritoneal cavity provoked the local accumulation of eosinophils[1]. The release of ECF-A from human lung apparently required an intact glycolytic pathway, a DFP inhibitable esterase and interaction with the cyclic AMP pathway[18, 19].

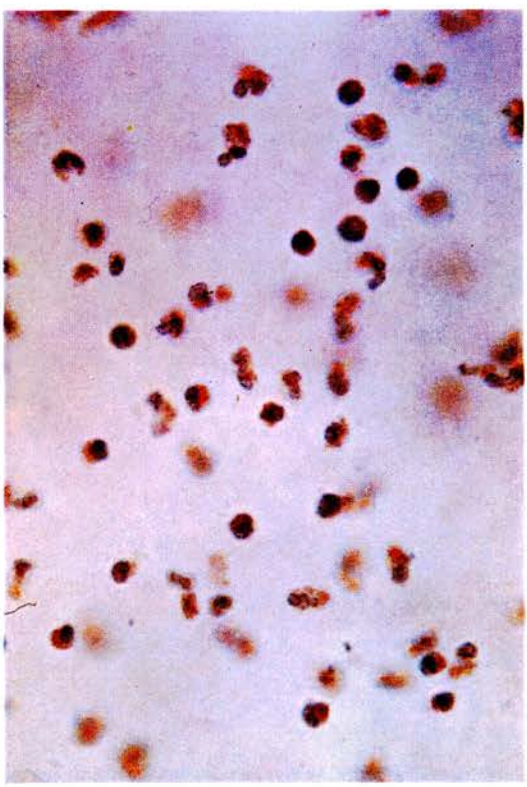

Fig. 2: Millipore filter showing the chemotaxis of guinea-pig eosinophils towards ECF-A. (Haematoxylin and Chromotrope 2 R x 40 magnification).

Biologically ECF-A shows considerable cellular specificity. Thus guinea-pig eosinophils were preferentially attracted when they comprised 10 % or more of mixed leucocyte populations[20]. However ECF-A would attract neutrophils from pure (> 96 %) populations. Similarly human ECF-A selectively attracted human eosinophils, but neutrophils and basophils would migrate when eosinophils were present in low numbers[21]. Recent work in this laboratory has shown that ECF-A has a functional similarity between species[22]. Thus, human ECF-A selectively attracted guinea-pig eosinophils and ECF-A of human origin was chemotactic for guinea-pig cells. In addition rat mast cell ECF-A preferentially attracted guinea-pig eosinophils.

The chemical structure of ECF-A is unknown but it is likely to be, in part, a peptide-like molecule. Human and guinea-pig ECF-A were fully recovered as a single peak of activity from Sephadex G-25 and had an estimated molecular size of between 500 and 1,000 daltons[14, 15]. ECF-A was relatively heat stable, retaining 75 % of its activity following boiling for 10 minutes or heating at 56° C for an hour. Chemotactic activity was fully recovered following lyophilisation and multiple freeze-thawing, and about 70 % of the original activity remained following extraction in 80 % ethanol.

An inhibitor of eosinophil migration

Compound FPL 55712 has been shown to antagonise the contractile effect of SRS-A on the isolated guinea-pig ileum[36]. In a recent study we have shown that the same agent potently inhibits (IC_{50} 2×10^{-7} g/ml^{-1}; 3.8×10^{-7} M) the migration of eosinophils towards partially purified guinea-pig ECF-A[37]. The agent did not affect the IgG_1-mediated release of ECF-A or histamine from guinea-pig lung *in vitro*. High doses of FPL 55712 had a selective cytotoxic effect on the eosinophil as shown by Trypan blue exclusion. However lower doses which still markedly inhibited eosinophil chemotaxis did not exhibit cytotoxicity[38].

Studies on the role of the eosinophil

A number of reports have suggested that the eosinophil inactivates chemical mediators of anaphylaxis[39, 40]. However we were unable to confirm previous studies on the capacity of the eosinophil to inactivate histamine using cells from the horse, guinea-pig and human[1]. Arylsulphatases of various origin including the mollusc[41], human eosinophils[40], guinea-pig and human lung tissue[42] have been shown to inactivate SRS-A. This group of enzymes has a wide biological distribution[43]. Thus although the eosinophil contains appreciably more arylsulphatase than other granulocytes[44] it is unlikely that the content of this enzyme in eosinophils points to a unique function. Furthermore maximal eosinophil infiltration *in vivo* did not occur until several hours after mediator release[8].

This laboratory has developed a model for studying the repair mechanism following PCA reactions in the guinea-pig by measuring the restoration of histamine in depleted skin sites[45]. Preliminary evidence suggests that eosinophil infiltration correlates with the restoration of histamine following the local anaphylactic reaction suggesting that the cell may play a part in the repair mechanism by which the functional integrity of the mast cell is resorted. Attempts to modulate the "repair time" in terms of histamine depletion following PCA reactions are being undertaken employing a monospecific anti-eosinophil serum and injections of viable eosinophils.

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Annotation

FUNCTIONS OF THE EOSINOPHIL LEUCOCYTE

An increase in the number of eosinophils both in the tissues and the circulation is a feature of many clinical conditions. They include diseases associated with high serum levels of IgE, such as extrinsic (or allergic) bronchial asthma, allergic rhinitis and certain parasitic infections, and disorders in which there is evidence of circulating antigen–antibody complexes capable of activating the complement system. The latter group of diseases include polyarteritis nodosa with lung involvement, pulmonary aspergillosis and rheumatoid arthritis. Until recently, little was known about the function of these cells but now several laboratories have suggested possible functions for the eosinophil both in parasitic disease and immediate-type hypersensitivity.

The main obstacle in attributing a unique role to the eosinophil had been the difficulty in demonstrating a biological activity for the cell distinct from those of other leucocytes. For example, eosinophils and neutrophils are both motile, respond in chemotaxis to a variety of agents, engulf particles, undergo degranulation and kill micro-organisms (reviewed by Kay, 1974a; Clark & Kaplan, 1975). Certain agents such as eosinophil chemotactic factor of anaphylaxis (ECF-A) preferentially attract the eosinophil from a mixed leucocyte population but there is no marked difference between neutrophils and eosinophils in terms of the type of particles these cells ingest, their rates of engulfment, the mode of degranulation or the types of micro-organism they kill. If anything the eosinophil is more sluggish than the neutrophil in its rate of ingestion (Cline *et al*, 1968) and bacterial killing but paradoxically has a higher metabolic activity in terms of H_2O_2 production, oxidative response and iodination (Baehner & Johnston, 1971; Mickenberg *et al*, 1972).

It was hoped that the special structural features of the eosinophil might offer an insight into its specific function(s). The granules, with their affinity for acid or analine dyes, are the central feature of the cell. Contained in the granular core is a major basic protein, rich in arginine, having a molecular size of about 11 000 daltons (Gleich *et al*, 1973). This material has been isolated in a highly purified form but in a variety of test systems no biological activity or anti-inflammatory properties could be shown (Gleich *et al*, 1974). The granule is also characterized by its high content of a peroxidase which is present mainly in the cortex. This enzyme is clearly different both chemically and antigenically from the myeloperoxidase of the neutrophil and does not appear to participate in the peroxidase–H_2O_2–halide bactericidal system (Bujak & Root, 1974).

The clear association between eosinophils and parasites has encouraged many investigators to examine both the mechanisms of parasite-induced eosinophilia and the possibility that the eosinophil is an effector cell in parasite destruction. Following administration of homogenized larvae from trichinella, bone marrow turnover of eosinophils was shown to be dependent on viable (probably T-dependent) lymphocytes (Basten *et al*, 1970; Basten & Beeson, 1970). When lymphocytes from animals with an eosinophilia were transferred to

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irradiated recipients this evoked an increase in the number of circulating eosinophils. Further studies indicated that a soluble mediator from lymphocytes sensitized to trichinella antigen evoked selective eosinophil production by the bone marrow. Raised serum levels of IgE and complement-fixing antibodies are a feature of many parasitic infections associated with an eosinophilia. The mobilization of eosinophils around the parasite in tissues may be a result of eosinophil chemotactic factor of anaphylaxis and complement-dependent chemotactic factors acting synergistically since *in vitro* there is marked synergism between ECF-A and a fragment cleaved from the fifth component of complement (Kay *et al*, 1973).

A role for the eosinophil in the destruction of parasites has been suggested (Kay, 1974a) and recently there have been a number of studies to support this concept. It is known that normal leucocytes (normal in the sense of being unsensitized) have the capacity to lyse antibody-coated target cells (reviewed by Perlmann *et al*, 1972). In most systems the effector cell has been shown to be a subpopulation of lymphocytes and this reaction is usually termed lymphocyte-dependent antibody mediated cytotoxicity (LDAC) or K (killer)-cell cytolysis. In some systems the neutrophil (Dean *et al*, 1974) or macrophage (Holm & Hammarström, 1973) have been identified as effector cells but all require antibody on target cells with an intact Fc region. Using an *in vitro* assay normal human peripheral blood leucocytes were shown to release ^{51}chromium from labelled immature schistosomes (schistosomula) (Butterworth *et al*, 1974). When the leucocytes were separated into eosinophil, neutrophil or mononuclear cell rich preparations K-cell activity was associated with the eosinophil (Butterworth *et al*, 1975). It is of particular interest that, in contrast to eosinophils from normal subjects, eosinophils from patients with high numbers of circulating eosinophils are apparently ineffective in this system, suggesting that in eosinophilia the cells may be altered possibly by interference with cell surface receptors by circulating antigen–antibody complexes.

Further evidence for the eosinophil as an effector cell in antibody dependent damage to schistosomula has been demonstrated in an *in vivo* system (Mahmoud *et al*, 1975). In *Schistosoma mansoni* infection in mice partial immunity can be transferred by immune serum which contains specific antibody directed against the schistosomula. The protective effect of serum was abrogated by prior treatment of the mice with anti-eosinophil serum but not with antisera directed against the lymphocyte, monocyte or neutrophil. Therefore the partial immunity transferred by serum would also appear to require the participation of the eosinophil. Although these findings on the relationship between the eosinophil and parasitic infection will need to be substantiated (particularly in other parasitic states where an eosinophil response is more marked) they strongly support previous suggestions that eosinophils have parasiticidal properties. There would also appear to be a role for complement in the destruction of sensitized schistosomula (Dean *et al*, 1974) and therefore it is probable that both humoral and cellular effector systems play a part in parasite destruction (Fig 1).

The functions of the eosinophil in immediate-type (allergic or anaphylactic) hypersensitivity reactions are probably diverse and therefore point to the versatility of the cell in various inflammatory states. It may have a regulatory role at all stages of the allergic response, namely mediator release, mediator inactivation and mediator replenishment. This is depicted diagramatically in Fig 2. Tissue containing mast cells can be sensitized by IgE (or equivalent tissue sensitizing antibody) for the antigen-induced release of chemical mediators of anaphylaxis. These pharmacological agents include histamine, slow reacting substance of

anaphylaxis (SRS-A) and an eosinophil chemotactic factor of anaphylaxis (ECF-A). ECF-A selectively attracts eosinophils from a mixed leucocyte population *in vitro* (Kay, 1969; Kay *et al*, 1971) and following administration *in vivo* also leads to eosinophil recruitment at the site of injection (Kay, 1974b). Histamine itself may also contribute to the infiltration and localization of eosinophils (Clark *et al*, 1975). Following mediator release an eosinophil derived inhibitor of histamine release (EDI)—probably a prostaglandin—may then inhibit further histamine release (Hubscher, 1975a, b). Eosinophil histaminase (Zeiger & Colten, 1974) and arylsulphatase (Wasserman *et al*, 1975) are then available for histamine and SRS-A inactivation; SRS-A having a free sulphate group. It is difficult to ascertain the relative importance of

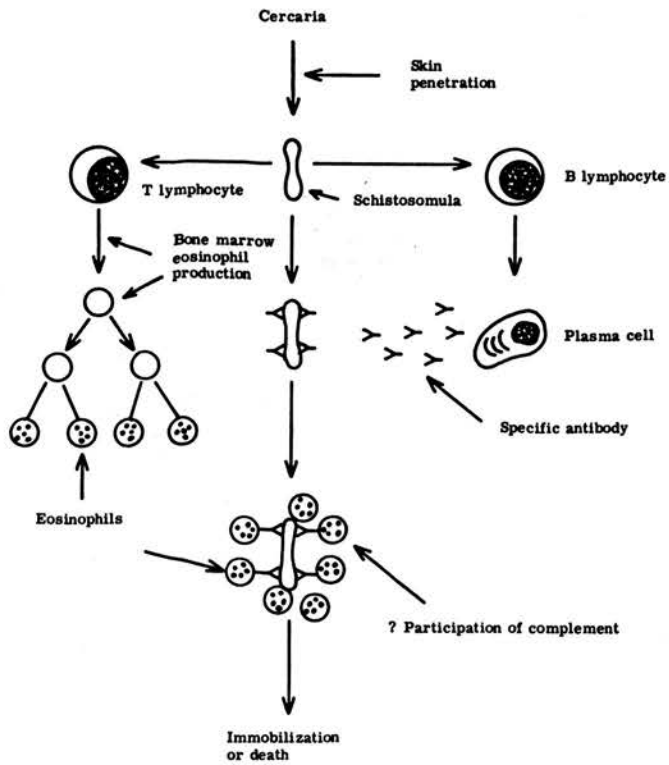

FIG 1. Diagrammatic representation of the possible participation of the eosinophil in the destruction of schistosomula.

tissue stores of these enzymes in mediator inactivation. Many tissues including the lung, skin, ileum, spleen and liver are rich in both arylsulphatase II B (Kay *et al*, 1976) and histaminase (Buffoni, 1966). Furthermore in kinetic studies in which the infiltration of eosinophils has been observed following a local anaphylactic response, appreciable eosinophil infiltration does not occur until several hours after antigen challenge at a time when mediator release has almost certainly terminated and the repair process initiated (Kay, 1970). In addition it should be emphasized that although the eosinophil contains about eight times more arylsulphatase II B than the neutrophil (Tanaka *et al*, 1962), the neutrophil is initially the predominant cell following an anaphylactic tissue response (Kay, 1970). However, the role of eosinophil

arylsulphatase in SRS-A inactivation in the subacute or chronic state where eosinophilia is more pronounced is yet to be ascertained. Eosinophil derived histaminase can be markedly induced by parasitic infestations but there was no difference in the cell content of this enzyme between normal and allergic individuals (R. S. Zeiger and H. R. Colten, personal communication). This raises the possibility that eosinophil arylsulphatase may also be an inducable enzyme.

Following mediator release and inactivation a repair process is initiated which leads to mediator replenishment. A role for the eosinophil is also suggested in repair in which it may have a 'negative' or 'dampening' effect as with mediator release and inactivation. Depletion of eosinophils by anti-eosinophil serum (AES) resulted in more rapid histamine accumulation following cutaneous anaphylaxis when compared to controls (Jones & Kay, 1976). In these experiments AES or control rabbit serum (CRS) was injected intravenously along

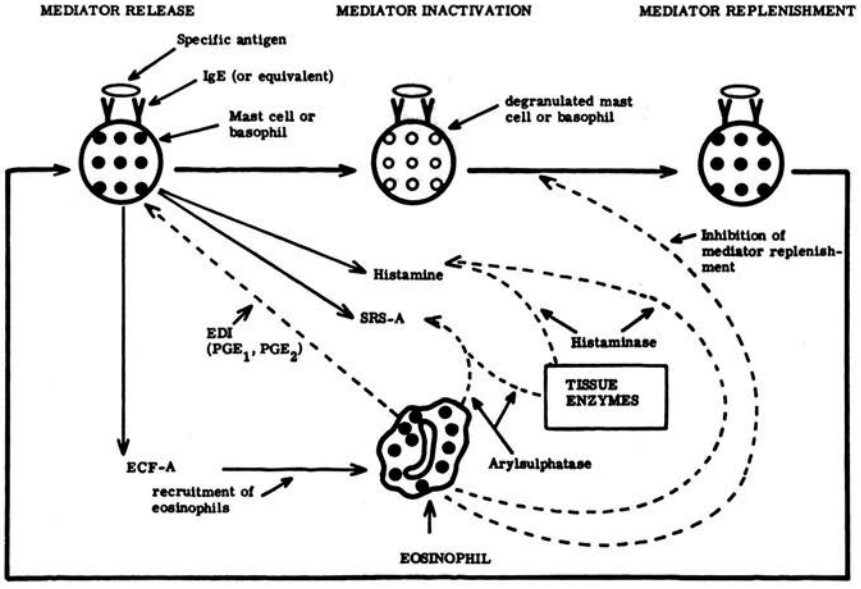

FIG 2. Diagrammatic representation of the functions of the eosinophil during immediate-type (anaphylactic) hypersensitivity. The 'inhibitory' effects of the eosinophil are represented by the interrupted lines.

with specific antigen into guinea-pigs previously sensitized with homocytotropic antibody. These were then excised, histamine extracted, and the levels compared with those in unsensitized contralateral skin sites. At 1 h, with control rabbit serum, there was approximately 55% depletion and histamine stores were not fully replenished until 48 h. With anti-eosinophil serum there was a comparable histamine depletion at 1 h but levels were fully restored by 9 h. Therefore eosinophils may also have a regulatory role in repair following anaphylaxis and so provide a homeostatic mechanism whereby the antigen-induced release of mediators, presumably derived from mast cells, is not perpetuated in situations where there is continuous antigenic stimulation.

In conclusion, whereas the functions of the eosinophil in parasitic disease and immediate-type hypersensitivity are becoming clearer, these observations do not explain its association

with other disease states such as malignancies, connective tissue disorders and the hypereosinophilic syndrome. Possibly in these conditions, other biochemical features of the cell are operative which are unconnected with mechanisms pertinent to parasitic and allergic states. Notwithstanding, the cell can no longer be regarded as an innocent bystander in inflammatory responses, but rather an active participant, and probably a very effective one.

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Eosinophil Leucocytes: Recruitment, Localization and Function in Immediate-Type Hypersensitivity[1]

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Over the years, a number of laboratories have addressed themselves to the following series of closely related questions regarding the eosinophil and immediate-type hypersensitivity. What is the mechanism by which eosinophils accumulate at the site of allergic tissue reactions? What is the relationship of eosinophils to recognised chemical mediators of anaphylaxis? What role does the eosinophil play in immediate-type reactions?

In a variety of models of experimental anaphylaxis *in vivo*, the eosinophil appears at the site of allergic tissue reactions following antigen challenge (*Samter et al.*, 1953; *Parish and Coombs*, 1968). When antibody was injected into the skin of the guinea-pig and after a variable latent period the animal challenged with specific antigen and Evan's blue dye intravenously (as in a usual passive cutaneous anaphylactic [PCA] reaction), IgG_1 but not IgG_2 elicited a local eosinophil response which was maximal 8–12 h following the initial blueing reaction (*Kay*, 1970). Therefore, IgG_1 which prepares guinea-pig skin *in vivo* for PCA (*Ovary et al.*, 1963) and *in vitro* for the antigen-induced release of chemical mediators of anaphylaxis (*Jones and Kay*, 1974) could also evoke a local eosinophil response. In the same species, intradermal injections of histamine, in doses which gave a comparable blueing reaction to the IgG_1-mediated response, did not lead to the accumulation of eosinophils (*Kay*, 1970). Eosinophils did accumulate, however, at the site of injections of compound 48/80, an agent which depletes mast cell granules. It was suggested therefore that the accumulation of eosinophils following PCA reactions in the guinea-pig was subsequent to the release from mast cells of an agent other than histamine.

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Identification and Characterization of Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A)

The *in vivo* observations prompted a search for chemotactic agents for eosinophils possibly released during the anaphylactic reaction. For this purpose, a modification of the micropore technique of *Boyden* (1962) was used. It was shown that an agent, released by specific antigen from sensitized guinea-pig lung, selectively attracted eosinophil leucocytes from a mixed cell population (*Kay*, 1969). This factor was later shown to be distinct from other chemical mediators such as histamine, slow reacting substance (SRS-A), bradykinin, 5-hydroxytryptamine and the prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ and termed an 'eosinophil chemotactic factor of anaphylaxis' (ECF-A) (*Kay et al.*, 1971). Material released from human lung sensitized with IgE, and challenged with specific antigen was comparable to guinea-pig ECF-A in terms of its capacity to attract selectively this cell from a mixed population, its molecular size and its identification as a previously unrecognized mediator of anaphylaxis (*Kay and Austen*, 1971). Human ECF-A could also be released from lung by antibody to IgE as in the 'reverse-type' reaction.

ECF-A, like histamine, resides in tissues in a preformed state from which it can readily be extracted by multiple freezing and thawing or homogenization (*Wasserman et al.*, 1974). Using isolated cell preparations from the rat, ECF-A was shown to be localized in mast cells probably in association with the granules. When cell free homogenates from human lung were applied to a column of Sephadex G-25, several peaks of eosinophilotactic activity were found including one in the molecular size range 500–1,000, corresponding to 'anaphylactic ECF-A'. Following desalting and further purification of this peak by QAE-Sephadex chromatography and high voltage paper electrophoresis, several acidic peptide-like components could be eluted which possessed ECF-A activity (*Kay*, 1976a). Using similar procedures *Goetzl and Austen* (1975) have purified human ECF-A and synthesized two acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu. These workers and ourselves also recognized the presence of aspartic acid in the purified peptide preparations. We have found that the analogue, Val-Gly-Asp-Glu, also had chemotactic activity for human eosinophils which was comparable to the other tetrapeptides (*Kay*, 1976a). The evidence therefore seems to point to ECF-A being a family of closely related peptides, but there may be species difference in their chemical structure.

The ECF-A acidic tetrapeptides Val-Gly-Ser-Glu, Ala-Gly-Ser-Glu and the analogue Val-Gly-Asp-Glu were selectively chemotactic for human eosinophils over a narrow dose range, although eosinophils from different individuals varied in their dose response pattern (*Turnbull et al.*, 1977). For instance, eosinophils from some patients gave two peaks of activity, one at 10^{-4} *M* and one at 10^{-7} *M*.

When the valyl- and alanyl-peptides were combined at low doses in the absence of histamine, resultant chemotaxis was similar to that of each peptide tested alone. The apparent inhibition of chemotaxis by higher doses of the alanyl-peptide alone could be abrogated when this peptide was mixed with the valyl-peptide at low concentrations (10^{-8} *M*).

Guinea-pig ECF-A may have chemical dissimilarities with its human counterpart. Val-Gly-Ser-Glu and the analogue Val-Gly-Asp-Glu had no appreciable activity for guinea-pig eosinophils over a wide dose range, although some activity was seen with Ala-Gly-Ser-Glu (*Jones and Kay,* 1976). In previous studies on the effect of enzyme digestions on the activity of ECF-A from the guinea-pig, it was shown that partially purified material lost activity following incubation with tyrosinase, arylsulphatase and leucine aminopeptidase (*Bach et al.,* 1975). This suggested that guinea-pig ECF-A contained a phenolic hydroxyl group, a sulphate ester and a peptide linkage with a free α-amino group. It could be that in the guinea-pig, the full expression of eosinophil chemotactic activity requires traces of SRS-A and this possibility is being explored.

Histamine and Imidazole Acetic Acid as Eosinophil Chemotactic Factors

The role of histamine in the recruitment of eosinophils to the site of allergic tissue reactions has been the subject of some controversy (*Parish,* 1974). Whereas subcutaneous injections of histamine into the horse resulted in eosinophil accumulation, these observations have not been confirmed in the guinea-pig (*Kay,* 1970) or man (*Felarca and Lowell,* 1968). Similarly as described above, *in vitro* experiments failed to show chemo-attractant properties for histamine when eosinophils from the guinea-pig and man were used as target cells. However, *Clark et al.* (1975) were able to show human eosinophilotaxis by histamine with peak activity of concentrations of approximately 5×10^{-5} *M*, there being inhibition with higher doses. It now appears that these discrepancies on the chemotactic property of histamine can be explained largely on the type of micropore filter used in the assays. Thus, with the more traditional 'Millipore' filter chemotaxis by histamine cannot be demonstrated, whereas these membranes were suitable for the identification and characterization of ECF-A (*Kay and Austen,* 1971). In contrast, cellulose nitrate filters were satisfactory for the assay of both ECF-A peptides and histamine (*Kay,* 1976a). Our laboratory has confirmed and extended these observations to show that in addition to histamine, one of its major catabolites, imidazole acetic acid, is also selectively chemotactic for the human eosinophil (*Turnbull and Kay,* 1976). *L*-histidine and other histamine catabolites including 1,4-methylhistamine, 1-methyl-4-imidazole acetic acid and *N*-acetylhistamine were inactive in eosinophilotaxis over a large dose range. The

dose response for histamine was dependent on the chemotaxis incubation time and the source of eosinophils, although the latter was not clearly associated with particular disease states. When histamine and imidazole acetic acid were combined, the chemotactic response was similar to that obtained when one agent was assayed alone, no additive or synergistic effects being observed. There was also cross-deactivation between histamine and imidazole acetic acid. These experiments seem to suggest that histamine and imidazole acetic acid may activate the same chemotactic recognition mechanism for eosinophils and, therefore, it was of considerable interest to determine how histamine and the acidic tetrapeptides reacted both *in vivo* and *in vitro* following combination at various doses.

Histamine and imidazole acetic acid were also chemotactic for the guinea-pig eosinophil, the dose-response patterns being similar to those obtained with human cells (*Jones and Kay,* 1977). Similarly, histidine and the major histamine catabolites were inactive in guinea-pig eosinophilotaxis. Chemotaxis of guinea-pig eosinophils to histamine could be inhibited by both H_1- and H_2-receptor antagonists at high concentrations (10^{-3} *M*), but the effect was more pronounced with the H_2-receptor antagonist, burimamide at lower doses (10^{-5} *M*). A similar but more complex dependence on H_2-receptors by human eosinophils in terms of their migration to histamine has been reported by *Clark et al.* (1975).

Eosinophil Chemotaxis by Combinations of the ECF-A Peptides and Histamine

In previous studies, anaphylactic diffusates, in general, gave a linear dose response in eosinophil chemotaxis, maximal activity being observed with the highest concentrations (*Kay and Austen,* 1971). We therefore tested various combinations of the ECF-A peptides and histamine in order to ascertain whether the pattern of response was comparable to that of anaphylactic diffusates. It was found that when histamine was combined with either the valyl- or alanyl-peptides, the resultant chemotaxis was negligible (*Turnbull et al.,* 1977). Thus, not only did histamine and the peptides fail to act additively or synergistically, but their combination abrogated the chemotactic response, suggesting that there may be cross-deactivaton between these agents. However, prior incubation of cells with histamine did not affect their response to the peptides and similarly incubation with the peptides did not abrogate the response to histamine. In contrast, as stated above, prior incubation with histamine deactivated the cells for chemotaxis towards histamine or imidazole acetic acid and similarly prior incubation with the individual peptides deactivated to the same peptides. At the present time, we are unable to explain these clearly complex interactions, but

suggest that *in vivo* there are possibly two processes which may require different relative concentrations and combinations of the various agents. Such events may be, firstly, directional migration of cells and secondly, stabilization of the eosinophil at the site of allergic reactions. A possible role for histamine in localizing the eosinophil at the site of anaphylactic reactions has previously been suggested (*Parish,* 1974). It may be that there are also other, as yet unrecognized, chemotactic agents in the anaphylactic diffusate which also contribute to directional migration and/or localization. In addition, there may be, *in vivo,* differing pharmokinetics in terms of histamine and ECF-A diffusion and/or inactivation, all of which may be critical for the observed eosinophil accumulation and localization. Many of these problems will not be solved until the relative amounts of the acidic peptides, histamine and possibly imidazole acetic acid present in the anaphylactic diffusates are known. For this purpose, the development of a quantitative assay for the peptides in biological fluids is required.

Eosinophil Accumulation following the Administration of the ECF-A Peptides and Histamine to Human and Non-Human Primate Skin

The acidic tetrapeptides both promoted eosinophil accumulation when introduced into the skin of man and a non-human primate (marmoset) (*Turnbull et al.,* 1977). Experiments in marmoset skin involved full thickness skin biopsies following a single intradermal injection of the agent either alone, or in combination. Human studies were performed in timothy grass pollen (TGP) sensitive individuals using the 'skin window' technique. In these experiments, there were repeated applications of the agents to the skin sites at 2- or 4-hour intervals over a 24-hour study period. In marmosets and man, both the alanyl- and valyl-peptide evoked infiltration of eosinophils. Combinations of the peptides with histamine gave lower eosinophil counts in both experimental situations. The exception was when histamine and the valyl- and alanyl-peptides were combined together in human skin. In this situation, the resultant eosinophilia was comparable to the valyl- or alanyl-peptide alone and only slightly more than histamine alone. These were the observations at 24 h; however, at 12 h, in human skin, histamine promoted little eosinophil response whereas the peptides alone showed some activity. It was the impression both with the peptides and the positive control (TGP) that there was a biphasic response with a peak at 8 h, a slightly lower count at 12 h and then rising to a greater peak at 24 h. A more striking dual response has recently been reported by *Hirashima and Hayashi* (1976) in terms of eosinophil infiltration in active anaphylaxis in the guinea-pig. In this study, the initial peak was associated with ECF-A-like material, whereas the second was related to an eosinophilotactic protein of molecular size approxi-

mately 70,000, the nature of which was undetermined. Our studies may be indicative of a comparable phenomenon in humans.

In general, histamine did not evoke eosinophil accumulation in the skin of marmosets or humans, although with the latter, using repeated applications, some cells were seen at 20–24 h (*Turnbull et al.*, 1977). These observations are in agreement with other studies on eosinophil accumulation by histamine in man (*Felarca and Lowell*, 1968) and the guinea-pig (*Kay*, 1970). In contrast, the individual peptides had considerable activity both *in vivo* and *in vitro*. It was unlikely that the peptides exerted their effect by releasing other mediators, as in the marmoset mast cell disruption by these agents was not apparent (*Turnbull et al.*, 1977).

Eosinophil Function in Immediate-Type Hypersensitivity

The role of the eosinophil in immediate-type (allergic or anaphylactic) hypersensitivity reactions is probably diverse and therefore points to the versatility of the cell in various inflammatory states (reviewed by *Kay*, 1976b). It may have a regulatory role at all stages of the allergic response, namely mediator release, mediator inactivation and mediator replenishment. Thus, following mediator release, an eosinophil-derived inhibitor of histamine release (EDI) – probably a prostaglandin – may inhibit the further liberation of histamine (*Hubscher*, 1975a, b). Eosinophil histaminase (*Zeiger and Colten*, 1974) and arylsulphatase (*Wasserman et al.*, 1975) are then available for histamine and SRS-A inactivation – SRS-A having a free sulphate group. It is difficult to ascertain the relative importance of tissue stores of these enzymes in mediator inactivation. Many tissues, including the lung, skin, ileum, spleen and liver are rich in both arylsulphatase IIB (*Kay et al.*, 1976) and histaminase (*Buffoni*, 1966). Furthermore, in kinetic studies in which the infiltration of eosinophils has been observed following a local anaphylactic response, appreciable eosinophil infiltration does not occur until several hours after antigen challenge at a time when mediator release has almost certainly terminated and the repair process initiated (*Kay*, 1970). In addition, it should be emphasized that although the eosinophil contains about eight times more arylsulphatase IIB than the neutrophil (*Tanaka et al.*, 1962), the neutrophil is initially the predominant cell following an anaphylactic tissue response (*Kay*, 1970). However, the role of eosinophil arylsulphatase in SRS-A inactivation in the subacute or chronic state where eosinophilia is more pronounced is yet to be ascertained. Eosinophil-derived histaminase can be markedly induced by parasitic infestations, but there was no difference in the cell content of this enzyme between normal and allergic individuals (*Zeiger and Colten*, 1976). It is therefore possible that eosinophil arylsulphatase is also an inducible enzyme.

Following mediator release and inactivation, a repair process is initiated which leads to mediator replenishment. A role for the eosinophil is also suggested in repair in which it may have a 'negative' or 'dampening' effect, as with mediator release and inactivation. Depletion of eosinophils by anti-eosinophil serum (AES) resulted in more rapid histamine accumulation following cutaneous anaphylaxis when compared to controls (*Jones and Kay,* 1976). In these experiments, AES or control rabbit serum (CRS) was injected i.v. along with specific antigen into guinea-pigs previously sensitized with homocytotropic antibody. These were then excised, histamine extracted, and the levels compared with those in unsensitized contralateral skin sites. At 1 h, with CRS, there was approximately 55% depletion and histamine stores were not fully replenished until 48 h. With AES, there was a comparable histamine depletion at 1 h, but levels were fully restored by 9 h. Therefore, eosinophils may provide a homeostatic mechanism whereby the antigen-induced release of mediators, presumably derived from mast cells, is not perpetuated in situations where there is continuous antigenic stimulation.

Concluding Comments

It seems probable that recruitment and localization of eosinophils to the site of allergic reactions is dependent on the interaction between the acidic tetrapeptides (ECF-A), histamine and possibly other anaphylaxis-associated mediators.

The functions of the eosinophil in immediate-type hypersensitivity (and helminth disease) are becoming clearer, although this does not explain the association of this cell with other disease states such as malignancies, connective tissue disorders and the hypereosinophilic syndrome. Possibly in these conditions, other biochemical features of the cell are operative which are unconnected with mechanisms pertinent to parasitic and allergic states. Notwithstanding, the cell can no longer be regarded as an innocent bystander in inflammatory responses, but rather an active participant, and probably a very effective one.

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THE EOSINOPHIL LEUCOCYTE; FORMATION, FUNCTION AND FATE

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An increase in the number of eosinophils both in the tissues and the blood is a feature of many clinical conditions. They include the allergic diseases such as extrinsic bronchial asthma, atopic dermatitis and allergic rhinitis; hypersensitivity to numerous drugs; pulmonary eosinophilia; certain neoplasms including Hodgkin's disease; a number of connective tissue disorders and the hypereosinophilic syndrome. In terms of world population the commonest association with eosinophilia are almost certainly helminth diseases, the more prevalent being intestinal infestation with nematodes (e.g. Ascaris lumbricoides, Toxocara canis and Trichuris trichura), filariasis (e.g. Loa-Loa, Onchocerca volvulus, Dipetalonema perstans and Wuchereria bancrofti), schistosomiasis, and infestation with trematodes and, less commonly, the cestodes (1).

Recently, several functional properties of the eosinophil have been described which are quite distinct from that of the neutrophil and other leucocytes and may help to explain the role of this cell both in helminth disease and allergic tissue reactions. The main emphasis in this chapter will be on the formation, function and fate of eosinophils in relation to helminth disease.

Formation of the eosinophil

It was shown in the rat that, before birth, eosinophil production occurs in both the thymus and the lymph nodes (2); whereas in adults the majority of cells are produced in the bone marrow (3). The precursor of the eosinophil in the bone marrow has not been identified with certainty, but it is known that the cell proceeds through several divisions before maturing and entering the blood-stream (4). During early stages of development protein synthesis is apparently very active as shown by a highly developed endoplasmic reticulum and the presence of up to four nucleoli. With maturation and the appearance of granules, morphological signs of protein synthesis disappear (5).

Structural features and enzyme content

The hallmark of the mature eosinophil is its content of large intracytoplasmic granules which have an affinity for acid or aniline dyes. Contained in the granular core is a major basic protein, rich in arginine, having a molecular size of about 11,000 daltons (6). This material has been isolated in a highly purified form but in a variety of test systems no biological activity or anti-inflammatory properties could be shown (7). The granule is also characterised by its high content of a peroxidase which is present mainly in the cortex. This enzyme is different both chemically and antigenically from the myeloperoxidase of the neutrophil and does not appear to participate in the peroxidase-H_2O_2-halide bactericidal system (8). "Late" eosinophils contain a second type of smaller granule which has no crystalline core and stains intensely for acid phosphatase and arylsulphatase (9). There is evidence that these granules are derived from the Golgi apparatus.

A number of eosinophil-derived lysosomal enzymes are thought to be located primarily in the large granules, although this is yet to be shown conclusively. Compared to the neutrophil, the human eosinophil contains eight times as much arylsulphatase (10), two and a half times as much β-glucuronidase and twice the amount of β-glycerophosphatase (11). Phospholipase D, previously recognised only in plants has also been shown to be present in human eosinophils (12).

Function of the eosinophil

(i) General properties

The main obstacle in attributing a unique role to the eosinophil has been the difficulty in demonstrating biological activity for the cell distinct from those of other leucocytes. For example, eosinophils and neutrophils are both motile, respond in chemotaxis to a variety of agents, engulf particles, undergo degranulation and kill micro-organisms. Certain agents such as the eosinophil chemotactic factor of anaphylaxis (ECF-A) (13), histamine (14, 15) and imidazole acetic acid (15) preferentially attract the human eosinophil from human peripheral blood leucocytes but there is no marked difference between neutrophils and eosinophils in terms of the nature of the particle these cells ingest (whether these are micro-organisms or inert substances), their rate of engulfment, the mode of degranulation or the variety of micro-organisms they kill. If anything, the eosinophil is less efficient than the neutrophil in terms of its rate of ingestion (16) and bacterial killing although, perhaps paradoxically, it has a higher metabolic activity as determined by H_2O_2 production, oxidative response and iodination (17, 18).

Human eosinophils contain receptors for rabbit and human IgG and the human complement components C4, C3b and C3d (19). There are about two and a half times more detectable receptors for immunoglobulin and complement on neutrophils when compared to the eosinophil. In eosinophilia of various aetiology the number of eosinophil C4 and C3b receptors was reduced when compared to the eosinophil from the blood of normal individuals.

Both human (20, 21) and mouse (22) eosinophils bear cell surface specific antigens which has enabled the production of monospecific anti-eosinophil serum. Monospecific antisera against guinea pig eosinophils have been more difficult to prepare (23, 24). However, all these various anti-eosinophil sera have proved useful reagents for studying the function of the cell in helminth disease and immediate-type hypersensitivity.

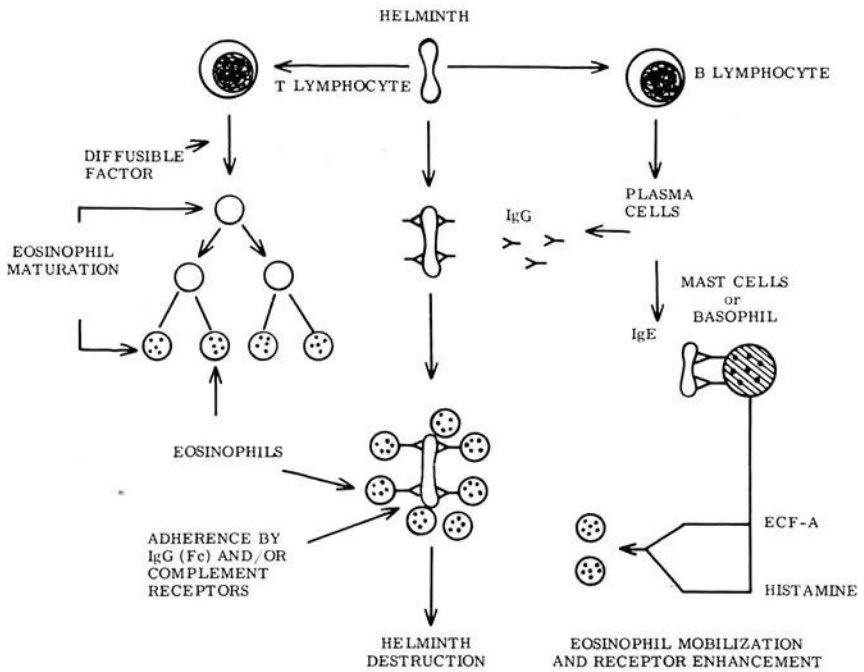

Fig. 1. Diagrammatic representation of the possible mechanisms involved in helminth destruction by eosinophils.

(ii) Helminths

In figure 1 a scheme is presented which attempts to explain, diagrammatically, some of the events which may follow the introduction of a helminth into the mammalian host. Data are taken from various sources, some from work with in vitro models and others with in vivo systems. Investigators concerned with the events leading to eosinopoiesis by the bone marrow have often used the administration of trichinella to the rat, whereas studies on eosinophil-killing have, in general, focussed on the larval stages of Schistosoma mansoni.

Following administration of homogenised larvae from trichinella, bone marrow turnover of eosinophils was shown to be dependent on viable (probably T dependent) lymphocytes (25, 26). When lymphocytes from animals with an eosinophilia were transferred to irradiated recipients this evoked an increase in the number of circulating eosinophils. Further studies indicated that a soluble mediator (diffusible factor) from lymphocytes sensitised to trichinella antigen evoked selective eosinophil production by the bone marrow. Exactly how helminths induce a T-cell dependent eosinophilia is unknown although it is probably determined by unique structural configurations of the parasite antigen.

Helminths also evoke an antibody response, particularly IgE but also IgG and probably other immunoglobulin classes. The way in which eosinophils are recruited to the site of tissue parasites is probably complex but the results of in vitro experiments suggest a number of possible mechanisms. For instance, parasite antigens may release, from IgE-sensitised mast cells, various chemical mediators of anaphylaxis which increase selectively human eosinophil locomotion. These include the ECF-A tetrapeptides (now characterised as Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu) (27), histamine, and one of its major catabolites, imidazole acetic acid (15). The ECF-A tetrapeptides and, to a much lesser extent, histamine elicit a local eosinophilia when applied to the abraded skin of man (28, 29) suggesting that they have both in vivo as well as in vitro activity. Various components of the complement system are also known to be chemotactic for the eosinophil and are possibly generated in vivo as a result of IgG interaction with the appropriate helminth antigen. The components include human C5a (30) and the trimolecular complex C567 (31). In general these agents have no particular preferential eosinophil attracting properties in vitro, with the exception of the guinea pig in which C5a evokes selective eosinophil migration (32). Furthermore, synergism was reported in in vitro chemotaxis between guinea pig C5a and ECF-A suggesting that in some circumstances these agents may act together (33). Other chemotactic factors for the eosinophil include a product which is released by the interaction of a T-lymphocyte product and immune complexes (34).

Another lymphocyte mediator of considerable interest enhances the migration of eosinophils out of agarose droplets *in vitro*. This has been referred to as the eosinophil stimulation promoter (ESP) and was first observed in supernatants of mouse lymphocytes stimulated with phytohaemagglutinin or specific antigen (35). It is derived from T-cells and is dependent on protein synthesis (36). Secretion of ESP could be demonstrated by isolated schistosome egg granulomas cultured *in vitro* (37). It has yet to be determined what biological property this agent confers on the eosinophil leucocyte and what its relationship is to the diffusible factor (25, 26).

Recent work suggests that eosinophils act as cytotoxic "killer" cells for helminths sensitised by IgG. Using an *in vitro* assay normal human peripheral blood leucocytes were shown to release ^{51}Cr from labelled immature schistosomes (schistosomula) (38). When the leucocytes were separated into eosinophil, neutrophil or mononuclear cell rich preparations cytotoxic activity was only associated with the eosinophil (39). Eosinophils from patients with schistosomiasis and other helminths were, however, ineffective in this system and there is evidence to suggest that eosinophil receptors may be blocked by circulating antigen-antibody complexes in certain parasitic diseases.

Although this system requires human IgG and the eosinophil as the effector cell, a similar system has been described using rat cells and serum in which killing is mediated by the macrophage sensitised with IgE (40). However, in general, (see below), *in vivo* experiments in mice support *in vitro* findings in humans in that the eosinophil appears to be the principal effector cell in schistosomula killing. Antibody-dependent killing of schistosomula by eosinophils does not apparently require the participation of other cells, such as neutrophils and mononuclear cells (41), and the release of ^{51}Cr correlates well with damage to parasites as judged by phase contrast and electron microscopy (42). The association between eosinophils and schistosomula is one of tight adherence accompanied by eosinophil degranulation and the appearance of electron dense material on the surface of the parasite.

The biochemical mechanisms by which human eosinophils kill schistosomula in the presence of antibody has been studied using metabolic inhibitors and other agents which alter cell function (43). There would appear to be no requirement for oxidative phosphorylation, protein synthesis, intact microtubules, DNA and mRNA synthesis or prostaglandin synthesis; parasite killing was not inhibited with prednisolone. In contrast, agents which act on microfilaments such as cytochalasin B rendered eosinophils ineffective in this system and inhibitors of glycolysis decreased markedly eosinophil parasiticidal activity. There was also a dependence for agents which

modulate the levels of intracellular cyclic AMP. In addition inhibition of certain cell membrane-associated esterases also rendered eosinophils ineffective in this system. It would appear, therefore, that the killing of antibody coated schistosomula by eosinophils is a complex phenomenon requiring the integrity of certain metabolic pathways, tight adherence of eosinophils to target cells and presumably an intact Fc receptor.

Schistosome eggs are also destroyed by eosinophils in an antigen specific system (44). Only eosinophils from schistosome infected mice were active whereas those from normal mice or those infected with T. spiralis were ineffective. These experiments suggest that eosinophils from infected animals become "armed" by a cytophilic antibody directed against the appropriate antigen.

Eosinophils have also been shown to be the effector cells in the killing of the epimastigotes from Trypanosoma cruzii (45). Using a technique which depends on the release of RNA from T. cruzii, eosinophils, in the presence of specific antibody, mediated killing whereas lymphoid cells had insignificant activity.

Although these various in vitro systems do not have the requirement for complement the possibility that complement enhances this reaction has not been excluded. In other systems, where cells of the lymphoid series are antibody-dependent killer cells, the sensitised target cell (usually erythrocytes) can be rendered more susceptible to lysis if they are coated with the early complement components up to C3 (46).

Recent experiments suggest that it may be possible to explain the association between helminths, eosinophils, the IgE response and complement. Receptors for C3b (but not IgG) were enhanced by incubating eosinophils with either the ECF-A tetrapeptides, or histamine, and this enhancement was demonstrable in a time- and dose-dependent fashion (47). Other mediators of anaphylaxis such as bradykinin, the prostaglandins PGE_1, E_2 and $F_{2\alpha}$ as well as serotonin were ineffective in this system. Neutrophils and monocyte receptors were unaffected by any of these treatments. Whether the enhanced eosinophils are more effective in the various other systems described is currently under investigation but as is shown in figure 1 it is suggested that the increase in receptors may represent an amplification mechanism whereby eosinophils are rendered more efficient in killing parasites.

There are a number of in vivo studies which support the contention that there is a requirement for eosinophils and specific antibody in the killing of certain helminths especially schistosomula. In Schistosoma mansoni infection in mice partial immunity can be transferred by immune serum which contains specific antibody directed against the

schistosomula (48). The protective effect of serum was abrogated by prior treatment of mice with anti-eosinophil serum but not with antisera directed against the lymphocyte, monocyte or neutrophil. Therefore, the partial immunity transferred by serum would also appear to require the participation of the eosinophil. Not only was immunity to reinfection abolished but treatment with anti-eosinophil serum also reduced the size and numbers of eosinophils in granulomas formed after intravenous injection of schistosome eggs.

The participation of eosinophils in damage to other helminths has been suggested and of particular interest is the apparent requirement for this cell in containing the number of muscle stage larvae in T. spiralis infection in mice (49).

In view of these findings it will not be surprising if a number of helminths, associated with eosinophilia, are shown to be destroyed by this cell under the appropriate experimental conditions. One of the many questions which arises is at what stage in the life cycle, or development of a parasite, is destruction by eosinophils most effective and how does this relate to eosinophil-independent mechanisms which are also thought to have significance in immunity to parasites.

(iii) Allergic disease

The functions of the eosinophil in allergic tissue reactions are also becoming clearer from recent experimental work. Many consider that the cell has a negative or "dampening" effect in type I or immediate-type hypersensitivity reactions (50). Thus an eosinophil derived product (EDI), possibly prostaglandin in nature, apparently inhibits further histamine release from sensitised basophils challenged with antigen (51, 52). The eosinophil also contains a number of enzymes which inactivate various chemical mediators associated with hypersensitivity. These include an arylsulphatase (53) which destroys the biological activity of slow reacting substance of anaphylaxis and histaminase (54). The eosinophil may also have a preferential phagocytic capacity for mast cell granules although it has yet to be shown with certainty that this cell is any more effective than other phagocytes in this respect (55). Eosinophils may also play a part in the regeneration of mast cells following type I reactions (23). Depletion of guinea pig eosinophils by anti-eosinophil serum greatly accelerated the restoration of local skin histamine levels following an IgG_1 mediated passive cutaneous anaphylactic reaction. Possibly this is a way in which the organism regulates the perpetuation of immediate-type reactions in situations where there is continuous antigenic stimulation. These possible functions of the cell in allergic reactions are depicted diagrammatically in figure 2.

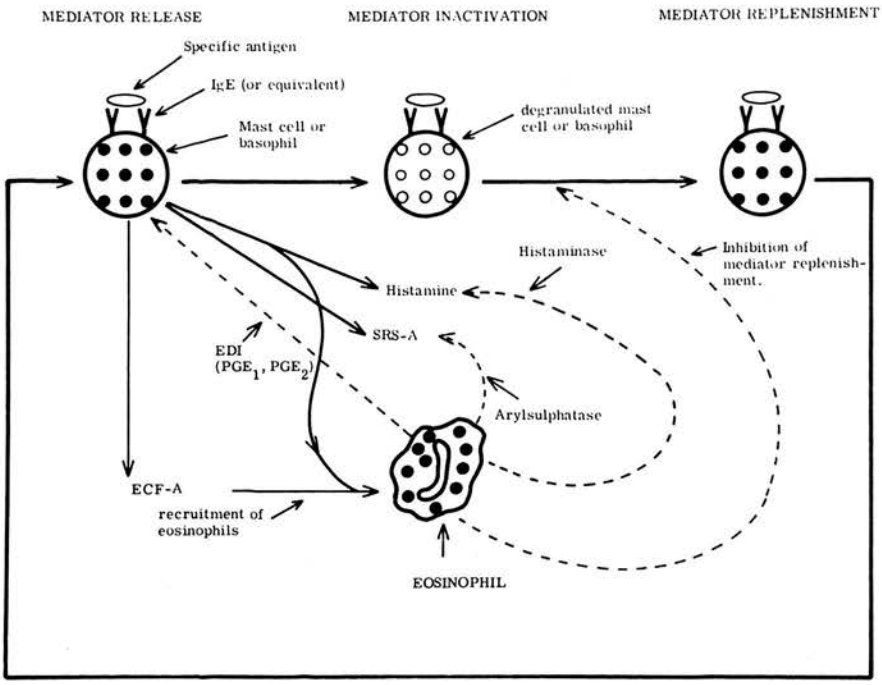

Fig. 2. Diagrammatic representation of the functions of the eosinophil during immediate-type (anaphylactic) hypersensitivity. The "inhibitory" effects of the eosinophil are represented by the interrupted lines.

Conclusion

The function of the eosinophil is becoming clearer both in helminth disease and allergic tissue reactions. Currently, the role of the eosinophil as antibody-dependent killer cell is receiving much attention and there are many experiments to suggest that in immediate-type hypersensitivity eosinophils have a "homeostatic" role. However, much is to be learnt as to the exact biochemical pathways by which these cells exert their various effects.

Fate of the eosinophil

The fate of human eosinophils from a patient with the hypereosinophilic syndrome was studied by reinjection of the cells labelled with ^{51}chromium (56). Following infusion, radioactivity attributed to the eosinophil declined rapidly within the first 3 hours but was followed by an increase after 6-24 hours. Thereafter the radiolabelled cells were removed from the circulation in an exponential fashion, with a half-life of approximately 45 hours. One explanation of these findings was the existence of a recirculating pool of, or marginal compartment for, eosinophils although surface counting data did not indicate specific tissue localisation of these cells. These findings are yet to be substantiated in eosinophilia in association with the more common diseases.

Eosinophils comprise between 1 and 5% of the total circulating leucocytes in normal healthy individuals. However, for every circulating eosinophil there are approximately 200 mature cells in the bone marrow reserve and 500 in loose submucosal connective tissue. There is, therefore, a dynamic turnover of eosinophils, with production and maintenance of a reserve pool in the marrow, and transport via the blood to certain tissue sites. The fate of these cells is not fully appreciated either in health or disease. However, "damaged" eosinophils may be engulfed by macrophages or may pass through the intestinal and respiratory mucosae and be excreted. This would be similar to neutrophil elimination where the bulk of cells are probably excreted or removed by tissue macrophages.

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Résumé :

Eosinophiles et réponses immunitaires.

Une augmentation du nombre des éosinophiles est caractéristique d'un grand nombre de parasitoses. L'augmentation du "turn over" des éosinophiles dans la moelle des os à la suite d'une agression parasitaire dépend vraisemblablement de la libération de facteurs solubles par une sous-population de lymphocytes T. Plusieurs laboratoires ont suggéré que les eosinophiles agiraient en tant que cellule cytotoxique sous l'action d'anticorps (IgG). Le rôle des IgE est mal défini quoique l'action de produits de la réaction anaphylactiques : histamine et tetrapeptides ECF-A (Val-Gly-Ser-Glu et Ala-Gly-Ser-Glu) se traduise par une augmentation des récepteurs de membrane des éosinophiles. Ceci peut représenter une étape d'amplification par laquelle les éosinophiles accomplissent leur effet cytotoxique. Les mécanismes d'activité des éosinophiles en tant que cellules effectrices dans la destruction des parasites sont discutés au niveau des réponses immunitaires humorales et cellulaires.

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IMMEDIATE HYPERSENSITIVITY

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CHAPTER 21

THE MAST CELL DERIVED PHARMACOLOGIC MEDIATORS OF ANAPHYLAXIS: EOSINOPHIL CHEMOTACTIC FACTOR OF ANAPHYLAXIS

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The abbreviations used are:
PCA; passive cutaneous anaphylaxis
ECF-A; eosinophil chemotactic factor of anaphylaxis
DFP; diisopropylphosphofluoridate
EDI; eosinophil-derived inhibitor

I. INTRODUCTION

It has been appreciated for many years that there is a clear association between eosinophil leukocytes and immediate-type hypersensitivity reactions. In a variety of models of experimental anaphylaxis *in vivo* the eosinophil appears at the site of allergic tissue reactions following antigen challenge (1,2). When antibody was placed in the skin of the guinea-pig and after variable latent period the animal challenged by specific antigen and Evan's blue dye intravenously (as in a usual passive cutaneous anaphylactic reaction), IgG_1 but not IgG_2 elicited a local eosinophil response which was maximal 8-12 hr following the initial bluing reaction (3). Therefore IgG_1, which prepares guinea-pig skin *in vivo* for passive cutaneous anaphylaxis (PCA) (4) and *in vitro* for the antigen-induced release of chemical mediators of anaphylaxis (5), could also evoke a local eosinophil response. In the same species intradermal injections of histamine in doses which gave a comparable bluing reaction to the IgG_1-mediated response did not lead to the accumulation of eosinophils (3). Eosinophils did accumulate, however, at the sites of injection of Compound 48/80, an agent which depletes mast cell granules. It was suggested therefore that the accumulation of eosinophils following PCA reactions in the guinea-pig was subsequent to the release from mast cells of an agent other than histamine.

These *in vivo* observations prompted studies on the identification of chemotactic agents for eosinophils released during the anaphylactic reaction and for this purpose a modification of the micropore technique of Boyden was used (6,7). The Boyden chambers consist essentially of two compartments divided by a micropore filter. A suspension of eosinophil-rich leukocytes is placed in the upper compartment and the chemotactic agent is introduced into the lower part of the chamber. Following an incubation period the filter is removed, fixed and stained and chemotaxis is measured by counting the number of cells which have traversed the entire thickness of the micropore or by measuring the distance the "leading front" of cells has migrated from the origin (8).

Other refinements to the technique include a method in which leukocytes are labelled with ^{51}chromium and chemotaxis is expressed in terms of radioactivity of a membrane placed between the filter and the chemo-attractant (9). Suspensions of eosinophil-rich leukocytes from the guinea-pig are obtained by twice weekly intraperitoneal injections of horse serum for 3-6 weeks. Human eosinophils were taken from the peripheral blood from patients with eosinophilia in association with a variety of conditions. Eosinophils can be further purified following centrifugation on sodium metrizoate cushions (10).

II. EOSINOPHIL CHEMOTACTIC FACTOR OF ANAPHYLAXIS

A. Identification

An agent released by specific antigen from sensitized guinea-pig lung which selectively attracted eosinophil leukocytes from a mixed cell population was first described in 1969 (11). This factor was later shown to be distinct from other chemical mediators such as histamine, slow-reacting substance (SRS-A), bradykinin, 5-hydroxytryptamine and the prostaglandins PGE_1, PGE_2 and $PGF_{2\alpha}$ and was termed an "eosinophil chemotactic factor of anaphylaxis" (ECF-A) (12). ECF-A (or ECF-A-like material), was then shown to be present in a number of tissues including human lung (13) and nasal polyps (14), basophil-rich human leukocytes (15), isolated rat mast cells (16), guinea-pig skin (5), uterus, heart and intestine (17).

ECF-A was shown to be distinct from a fragment cleaved from the fifth component of complement (C5a; 12), which also, under certain conditions, selectively attracts the eosinophil *in vitro* (18). Furthermore, prior decomplementation *in vivo* by purified cobra venom factor did not affect the antigen-induced release of ECF-A (12).

It was shown that guinea-pig ECF-A had a molecular size of approximately 500 (12) and probably had a peptide-like structure (19).

Material released from human lung sensitized with IgE and challenged with specific antigen was comparable to guinea-pig ECF-A in

terms of its capacity to attract selectively this cell from a mixed population, its molecular size and its identification as a previously unrecognized mediator of anaphylaxis (13). Human ECF-A could also be released from lung by antibody to IgE as in the "reversed type" reaction.

B. Biochemistry of ECF-A Release

Generally speaking the biochemical variables for the release of ECF-A are similar to those for histamine and SRS-A release. These include a requirement for calcium ions (12), an intact glycolytic pathway, and prior activation of a DFP-inhibitable serine esterase (20). Similarly the release of ECF-A, like histamine and SRS-A, can be modulated by alterations in the intracellular levels of the cyclic nucleotides, adenosine monophosphate (cyclic-AMP) and guanosine monophosphate (cyclic-GMP) (20). As in other systems, levels of these nucleotides have opposing effects; increased levels of cyclic AMP leading to inhibition of ECF-A release and enhancement of release by raised concentration of cyclic GMP.

C. ECF-A Release in Relation to Histamine and SRS-A

Studies on the time course of mediator release from tissue fragments are difficult to interpret since ECF-A, like histamine and SRS-A, is highly charged (see below). However when sensitized whole guinea-pig lung was perfused with specific antigen through the pulmonary artery and mediators measured in the perfusate there was a more protracted release of ECF-A, compared to histamine and SRS-A (1). This is not surprising if ECF-A does indeed play a role in the recruitment of eosinophils to the site of allergic tissue reactions *in vivo*.

D. Selectivity of Action of ECF-A for the Eosinophil

The remarkable property of ECF-A is its capacity to attract selectively eosinophils from a mixed leukocyte population. In general this is more striking with a homologous guniea-pig system than when studying human ECF-A with human eosinophils as target cells. Thus, although guinea-pig ECF-A will attract the neutrophil from pure (>96%) preparations it selectively attracted the eosinophils when the comprised 10% or more of mixed cell suspension (18). Selective attraction of human eosinophils by homologous human ECF-A was less striking.

Guinea-pig ECF-A was also shown to act synergistically with C5a (18). The capacity of ECF-A and the complement-derived fragment to act together in chemotaxis may be of significance in parasitic infections associated with a pronounced eosinophilia in which homocytotropic and complement-fixing antibodies occur together.

Further evidence for the selective effect of ECF-A on the eosinophil was provided by studies on diminished eosinophil chemotactic responsiveness (deactivation). Thus, eosinophils pretreated, and then washed free of ECF-A showed a marked inhibition of chemotaxis when subsequently tested against ECF-A in micropore chambers (22).

These observations point to a special recognition process between ECF-A and the eosinophil, the nature of which is yet to be ascertained.

E. Chemical Characterization of ECF-A

It is now appreciated that ECF-A, like histamine, resides in tissues in a preformed state from which it can be readily extracted by multiple freezing and thawing or homogenization (16). Using isolated cell preparations from the rat, ECF-A was shown to be localized in mast cells probably in association with the granules. When cell-free homogenates from human lung were applied to a column of Sephadex

G-25 several peaks of eosinophilotactic activity were found including one in the molecular size range 500-1000 corresponding to "anaphylactic ECF-A". Following desalting and further purification of this peak by QAE-Sephadex chromatography and high voltage paper electrophoresis, several acidic peptide-like components could be eluted which possessed ECF-A activity (L.W. Turnbull, D.G. Jones and A.B. Kay - unpublished). Using similar procedures Goetzl and Austen have purified human ECF-A and synthesized two acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (23) possessing ECF-A-like activity. Both groups have also recognized the presence of aspartic acid in the purified peptide preparations. We have found that Val-Gly-Asp-Glu also has chemotactic activity for human eosinophils comparable to the other tetrapeptides (24). The evidence therefore seems to point to ECF-A being a family of closely related peptides. Furthermore there may be species differences in ECF-A. Thus, the three tetrapeptides mentioned above had little or no activity for guinea-pig eosinophils (D.G. Jones and A.B. Kay - unpublished) and there is evidence that amongst guinea-pig ECF-A(s) there may be phenolic hydroxyl groups, sulfate esters and groups susceptible to the effects of leucine aminopeptidase (25).

It is now recognized that other pharmacological mediators associated with anaphylaxis have selective chemotactic activity for human eosinophils. These include histamine (26) and one of its major metabolites, imidazole acetic acid (24). Histidine and other histamine catabolites including N-acetylhistamine, 1,4-methylhistamine and 1,4-methyl imidazole acetic acid had no eosinophil or neutrophil chemotactic activity over the dose range 10^{-3} to 10^{-7}M.

Because of their quite different dose-response patterns it is difficult to compare the relative eosinophil chemotactic activities of the tetrapeptides with that of histamine and imidazole acetic acid (Fig. 1). With Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu there were two peaks of eosinophil chemotactic activity, one at 10^{-3}M and one at 10^{-6}M. Between these doses there was apparent inhibition of chemotaxis. These two peptides also evoked some neutrophil chemotaxis

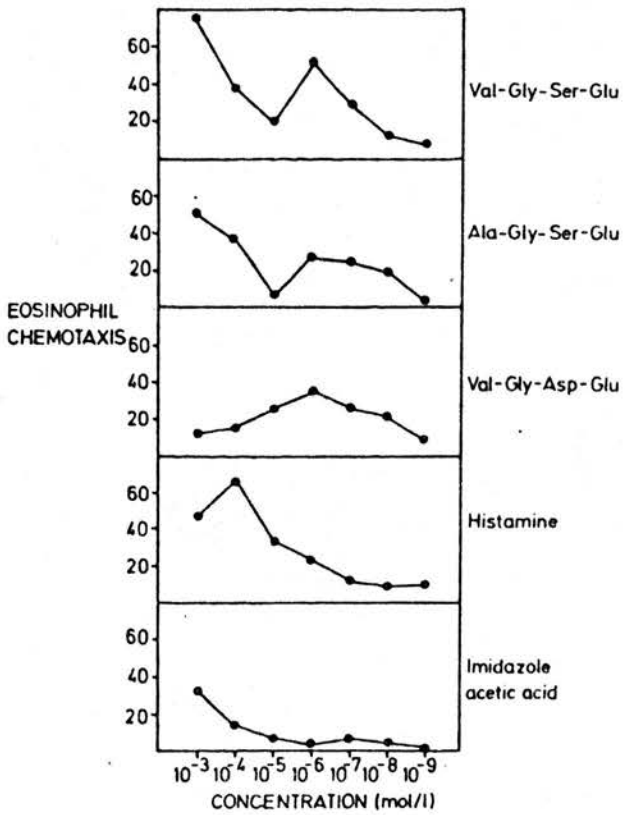

Fig. 1. Chemotaxis for human eosinophils by acidic tetrapeptides, histamine and imidazole acetic acid. (The synthetic peptides were kindly supplied by Dr. R. Camble, I.C.I. Pharmaceuticals, Alderley Park, England.)

but only at 10^{-3}M. The pattern of eosinophil chemotaxis with Val-Gly-Asp-Glu was similar except that there was little activity for either the eosinophil or the neutrophil at high doses (10^{-3}M). Maximal activity with histamine was at 10^{-4}M with some apparent inhibition at 10^{-3}M. With imidazole acetic acid there was a linear dose-response at concentrations higher than 10^{-5}M. In the study depicted, the results represent the pooled values from three different preparations of target cells, i.e. each agent, at seven concentrations, was set

up in chemotaxis against three different preparations of eosinophils. We have observed, in both these and other experiments, that the peptides and imidazole acetic acid give similar patterns (or shape) of dose-response irrespective of the source of target cells. With histamine, however, the concentration giving maximal chemotaxis can vary between sources of eosinophils although for a particular patient the pattern remains constant. Thus, the concentration of histamine giving maximal activity may be 10^{-7}, 10^{-4} or 10^{-6}M, with inhibition at higher doses. The reasons for these variations in eosinophil response to histamine are unclear but may be related to the concentrations of leukocyte histaminase. The complex dose response patterns for both the tetrapeptides and histamine are difficult to explain but may be related to optimal *in vivo* concentrations required for cell accumulation. Furthermore it is likely that these agents act in combination in the events leading to local eosinophilia in allergic tissue reactions.

F. Tumor-associated ECF-A-like Material

Eosinophilia is found in association with a number of neoplastic conditions including various carcinomas and Hodgkin's disease. An eosinophilotactic agent extracted from a large-cell anaplastic carcinoma of the lung from a patient with eosinophilia has been described (27). The active material was shown to be peptide-like and had many of the characteristics of ECF-A.

Selective eosinophilotactic activity was also demonstrable from cultured Hodgkin's lymph node supernatants (28). Control supernatants prepared from lymphocytic lymphoma and reactive hypoplasia nodes were chemotactic for neutrophils but had little eosinophil chemotactic activity. In most instances the degree of eosinophil infiltration observed histologically in the Hodgkin's lymph node correlated with elaboration of eosinophil chemotactic activity from the cultured cells. Following gel filtration of the Hodgkin's lymph node supernatants, several peaks of eosinophil chemotactic activity were observed, one of which corresponded in molecular size to ECF-A.

Whether tumor-derived ECF-A is chemically identical to the tetrapeptides is yet to be determined.

III. CONCLUSION

Despite the identification of ECF-A and the appreciation that histamine and imidazole acetic acid are also selectively chemotactic for eosinophils, the precise contributions of these agents to the recruitment and accumulation of the cell *in vivo* are not known. Following administration of partially purified ECF-A to the lung and peritoneal cavity of the guinea-pig, there was a subsequent eosinophil infiltration (21). However these studies will have to be corroborated using the synthetic peptides and other eosinophilotactic agents, either alone, or in combination. Furthermore, the events leading to eosinophil infiltration in the anaphylactic situation are clearly complex and chemotaxis may only contribute to a limited extent in cellular localization. "Stickiness" of the vasculature, passage through the endothelium and entrapment of eosinophils at the reaction site are other prerequisites for cell recruitment and may be dependent on agents other than chemotactic factors. In the subacute and chronic situation there is, in addition to cell recruitment, a signal for turnover of eosinophils by the bone marrow.

Despite the uncertainty of the exact *in vivo* role for ECF-A the agent(s) have the potential for providing considerable information on the biology and biochemistry of eosinophils due to their unique selective action on this cell.

The central issue is the role of the eosinophil. In anaphylaxis it has been proposed that eosinophils play a part both in mediator release, mediator inactivation and mediator replenishment. At all stages this is thought to be a "negative" or "dampening" effect. Thus an eosinophil-derived inhibitor (EDI) - probably a prostaglandin - can inhibit the anaphylactic release of histamine (29,30); eosinophil histaminase and arylsulfatase inactivate histamine (31) and SRS-A respectively (32); histamine replenishment by tissue which has

undergone an immediate hypersensitivity response, is more rapid in the absence of eosinophils (33). It would seem therefore that eosinophils are more than colorful onlookers in the anaphylactic response but rather active participants (see also Zucker-Franklin, Chapter 15 this volume).

IV. RECENT DEVELOPMENTS

Since this chapter was written there have been a number of further studies on the biology of ECF-A and its association with disease. The variation in the *in vitro* dose-response pattern by eosinophils to ECF-A and histamine from different individuals, referred to above, has been investigated further. The eosinophil chemotactic response to Val-Gly-Ser-Glu and histamine was studied in 32 patients with a peripheral blood eosinophilia (34). Their eosinophilia was associated with extrinsic asthma (12 patients), neoplasia (7), helminths (5) and a miscellaneous group (8) which included three patients with pulmonary eosinophilia, one of whom had asthma. Both the tetrapeptide and histamine gave two types of dose-response with Val-Gly-Ser-Glu. This was either a single peak at 10^{-6}M, or two peaks at 10^{-4} and 10^{-7}M, respectively. Histamine gave either a linear dose-response with maximal chemotaxis at the highest concentration or a peak response at 10^{-5}M with inhibition at higher doses. The two types of response given by the peptide were not related to the disease state and were reproducible when tested on more than one occasion. However, with histamine, linear dose-responses were observed in ten out of twelve patients with asthma, four out of five with helminth disease and two of the three with pulmonary eosinophilia. This was also reproducible when tested on subsequent occasions. Therefore in these diseases, which are known to be associated with exogenous antigens and raised IgE levels, eosinophils from 80% of the patients (16 out of 20) gave a linear rather than a "bell-shaped" response to histamine. In contrast only four of twelve (33%) with eosinophilia in association with other diseases (which included seven with neoplasia) gave this

type of response. If this observation can be confirmed with a larger number of patients it may be useful diagnostically in eosinophilia of unknown origin. Although the differences with histamine in terms of their association with disease were more apparent than with Val-Gly-Ser-Glu, the association of the dose-response curve with disease states may be apparent with the peptide response when greater numbers of patients are studied.

We have also found that when Val-Gly-Ser-Glu or Ala-Gly-Ser-Glu were combined in various concentrations the resultant chemotaxis was either negligible or no greater than that produced when each peptide was tested separately. (35). Thus not only did histamine and the peptides fail to act additively or synergistically but their combination abrogated the chemotactic response suggesting that there may be cross-deactivation between these agents. However prior incubation of cells with histamine did not affect their response to the peptides and similarly incubation with the peptides did not abrogate the response to histamine. In contrast, prior incubation with histamine deactivated the cells for chemotaxis towards histamine or imidazole acetic acid, (24) and prior incubation with the individual peptides deactivated to the same peptide. (23) At the present time we are unable to explain these clearly complex interactions but suggest that *in vivo* there are possibly two processes which may require different relative concentrations and combinations of the various agents. Such events may be, firstly, directional migration of the cells and secondly, stabilization of the eosinophil at the site of allergic reactions. It may be that there are also, as yet unrecognized, chemotactic agents in the anaphylactic diffusate which also contribute to directional migration and/or localization. In addition there may be, *in vivo*, differing pharmacokinetics in terms of histamine and EFC-A diffusion and/or inactivation, all of which may be critical for the observed eosinophil accumulation and localization. Many of these problems will not be solved until the relative amounts of the acidic peptides, histamine and possibly imidazole acetic acid present in the anaphylactic diffusates are known. For this purpose, the development

of a quantitative assay for the peptides in biological fluids is required.

We were also able to show that Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu both promoted eosinophil accumulation when applied to the abraded skin of man or intradermally to the marmoset. (35) Biopsies of marmoset skin revealed that peptide-induced eosinophilia was not a result of mast cell degranulation. These experiments have been extended and the number of eosinophils recruited locally to abraded human skin were measured in eight atopic and eight non-atopic volunteers, at time intervals over 24 hr, following the application of Val-Gly-Ser-Glu or histamine. (36) In all subjects the higher doses of the peptide (10^{-4} and 10^{-6}M) or histamine (10^{-3} and 10^{-5}M) produced significantly greater counts than the Tyrode's diluent alone. The counts produced with the lower dose of peptide (10^{-8}M) or histamine (10^{-7}M) were not significantly different from the control. The peptide or histamine evoked a greater local eosinophilia in the atopics than the non-atopics. This effect was probably independent of the peripheral blood eosinophil count since at the time of study the numbers of circulating eosinophils between the two groups were not significantly different. In the atopics, histamine in doses of 10^{-3} and 10^{-5}M were required to give the same eosinophil response as that obtained with 10^{-4} and 10^{-6}M of the peptide, respectively. It is suggested that the relative paucity of eosinophils recruited by locally applied ECF-A peptide or histamine, when compared to antigen-induced eosinophilia, is due either to an inability to mimic the events associated with the release of these mediators from mast cells or the involvement of other as yet undetermined pharmacological agents. For instance, we have also found that histamine, when combined with the acidic tetrapeptides, evoked little or no cutaneous eosinophil infiltration either in man or in the marmoset. Therefore, these recent studies suggest that there is a complex interaction between histamine and the ECF-A tetrapeptides; however, the tetrapeptides alone can promote the recruitment and localization of eosinophils by a mechanism apparently independent of mast cell degranulation. The dose-response pattern

of the peptides and histamine *in vitro* has considerable individual variation, which in the case of histamine may be related to the disease state.

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SECTION B - MEDIATORS OF HYPERSENSITIVITY

Clin. exp. Immunol. (1974) **16,** 213–222.

PASSIVE SENSITIZATION OF GUINEA-PIG SKIN *IN VITRO* FOR THE ANTIGEN-INDUCED RELEASE OF ANAPHYLACTIC MEDIATORS

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SUMMARY

Guinea-pig skin fragments were passively sensitized for the antigen-induced release of histamine, SRS-A and ECF-A. Skin histamine was chemically identified by fluorimetry; SRS-A gave a characteristic dose-dependent contraction of the guinea-pig ileum; and ECF-A selectively attracted eosinophils. The antibody mediating the release of these agents was shown to be IgG_1. Following antigen challenge different time courses of release were demonstrable for histamine, SRS-A and ECF-A. Skin SRS-A was resistant to treatment with the enzyme pronase and skin ECF-A had an estimated molecular size of between 500 and 1000. They were therefore comparable to similar agents released from the lung. Thus SRS-A and ECF-A join histamine as chemical mediators in cutaneous anaphylaxis.

INTRODUCTION

It has long been recognized that the skin is a target organ for immediate-type hypersensitivity reactions in man and experimental animals. The antigen-induced release of a histamine-like substance from sensitized skin *in vitro* has been described in a number of species (Mongar & Schild, 1952; Greaves, Fairley & Yamamoto, 1971; Yeoh, Tay & Greaves, 1972; Greaves, Yamamoto & Fairley, 1972). However, anti-histamines only partially suppress cutaneous anaphylaxis (Fisher & Cooke, 1957) suggesting the possible participation of other chemical mediators.

In this report it is shown that agents other than histamine are released when passively sensitized skin fragments are challenged with specific antigen. These include a slow reacting substance of anaphylaxis (SRS-A) and an eosinophil chemotactic factor of anaphylaxis (ECF-A). Both are shown to have properties comparable with agents previously described in the lung (Brocklehurst, 1962; Kay, Stechschulte & Austen, 1971). In addition, the optimal conditions and the antibody mediating the release of these mediators from skin are described.

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MATERIALS AND METHODS

Animals

Dunkin–Hartley strain guinea-pigs, of either sex, were used throughout the study.

Reagents

Histamine acid phosphate, Blue Dextran, vitamin B_{12}, glycogen, ovalbumin five times crystallized and horse serum were obtained as previously described (Kay, Shin & Austen, 1973). Other materials were obtained as follows. Amberlite IRC-50, *n*-butanol and orthophthalaldehyde (BDH Chemicals Ltd, Poole, England); casein (Merck, Darmstadt, Germany); mepyramine maleate (May & Baker Ltd, Dagenham, England); inulin (Schwarz–Mann, Orangeburg, New York, U.S.A.); Ring-[2-^{14}C]histamine dihydrochloride (Radiochemical Centre, Amersham, England), and pronase P (Serva, Heidelburg, Germany).

Preparation of guinea-pig IgG1 and IgG2 immunoglobulins

Antiserum to ovalbumin was prepared in guinea-pigs following an injection schedule previously described (Kay, 1970a), and fractionated by diethylaminoethyl (DEAE)-cellulose chromatography (Kay, 1970b). All fractions were diluted in Tyrode's solution before use. The functional purity of the IgG1- and IgG2-containing fractions was tested by passive cutaneous anaphylaxis and passive haemolysis (Bloch *et al.*, 1963); immunoelectrophoresis and gel diffusion using rabbit anti-7S IgG and specific anti-guinea-pig IgG1 (Kay, 1970a); and passive haemagglutination (Bloch *et al.*, 1963).

Preparation of skin for the in vitro *release of mediators*

Guinea-pigs were killed by stunning and exsanguination. Shaved abdominal and thoracic skin was separated from subcutaneous tissue and cut into fragments of 10–20 mm^2 in area. Portions of skin (0·5–2·0 g) washed twice in Tyrode's solution and sensitized in 3·6 ml of antibody per gram wet weight of skin, were challenged with ovalbumin in a total volume of 3 ml. The characteristics of the antibody solution are described in the results. The diffusate was then removed and the residual histamine extracted by suspending the fragments in an equal volume of Tyrode's solution and placing in a boiling water-bath for 15 min.

Assay of anaphylactic mediators

Histamine was measured fluorometrically or by bioassay using the guinea-pig ileum suspended in oxygenated atropinized Tyrode's solution (0·5 μmole atropine/litre) in a 2-ml capacity organ bath maintained at 37°C by a continuous flow water-jacket (Brocklehurst, 1960). Fluorimetric assay was performed by a modification (Jones, 1973) of the method of Oates, Marsh & Sjoerdsma (1962) in which [^{14}C]histamine was used to estimate recovery. Allowance was made for the contribution of non-histamine fluorescence by the addition of the fluorometric reagents in reverse order (reverse–blank).

SRS-A was assayed by the method of Brocklehurst (1960).

Chemotaxis was measured using a previously described modification of the Millipore technique of Boyden (Kay, 1970b), with the exception that a 4-hour incubation period was used. Guinea-pig eosinophils were obtained by peritoneal lavage from animals which had received multiple injections of horse serum. Neutrophils were harvested from the peritoneal cavity of animals injected with glycogen 3–6 hr previously. Eosinophil and neutrophil

migration were both estimated using an 8·0 μ pore size and total cell counts adjusted to $0{\cdot}5\text{–}2{\cdot}0 \times 10^6$/ml.

Inulin-treated guinea-pig serum possessing both eosinophil and neutrophil chemotactic activity was used as a positive control in all the experiments described. A pool of this material was prepared by incubating fresh guinea-pig serum with inulin (20 mg/ml serum) for 30 min at 37°C. The inulin was then removed by centrifugation and the serum subsequently heated at 56°C for 30 min. Samples were stored at −85°C until used. ECF-A activity was expressed as the mean cell count of five high power fields from duplicate analyses (Kay, 1970b). Counts were performed either in the body of the filter or on the surface to which the cells had migrated.

Activity of pronase

Pronase activity was tested using casein as a substrate. The enzyme was dissolved in Tyrode's solution to a concentration of 100 PUK units/ml. (1 PUK unit = the amount of enzyme required to give an optical density of 1·0 at 660 mμ, 40°C and pH 7·4 as determined by the casein–Folin method.)

Gel filtration

A cell-free anaphylactic skin diffusate (20 ml) was evaporated to dryness under vacuum, at 60°C, using a rotary evaporator. The residue was reconstituted in 2 ml of distilled water, centrifuged free of particulate matter and the supernatant applied to a column of Sephadex G-25 ($90 \times 2{\cdot}0$ cm). Alternate 2 ml fractions were tested for eosinophil chemotaxis using 1 ml volumes. The column was calibrated as previously described using Blue Dextran, vitamin B_{12} and histamine as molecular markers (Kay *et al.*, 1973).

RESULTS

Histamine

Content in normal guinea-pig skin. The mean abdominal skin histamine content from twenty-eight normal animals weighing between 200 and 600 g was $1{\cdot}85 \pm 0{\cdot}89$ (μg/g wet

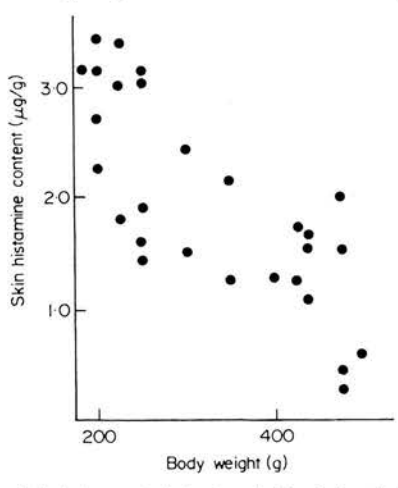

FIG. 1. The inverse relationship between total extractable abdominal skin histamine and body weight. Correlation coefficient, −0·75. Number of samples, twenty-eight. $P < 0{\cdot}001$.

weight ± S.E.M.). An inverse relationship was found between total body weight and the skin histamine content (Fig. 1). The amount of extractable histamine in the skin of younger animals (300 g or less) was significantly higher (2·43 ± 0·73) and therefore smaller animals were used in further studies.

In all experiments abdominal skin was chosen since in five animals the histamine content of the dorsal region was approximately 20% lower than that of the abdomen. In addition, abdominal skin was more easily dissected free of subcutaneous tissue.

Chemical identification in anaphylactic diffusates. When passively sensitized guinea-pig skin was challenged with specific antigen, there appeared in the diffusate an agent which gave a characteristic histamine-like contraction of the isolated guinea-pig ileum. The identity of this material was confirmed by its fluorimetric characteristics when complexed with orthophthalaldehyde following extraction using Amberlite IRC-50 and *n*-butanol. The fluorimetric spectra obtained with synthetic histamine and the skin diffusate are illustrated in Fig. 2. The spectra for synthetic histamine, a skin diffusate and a mixture of the two were

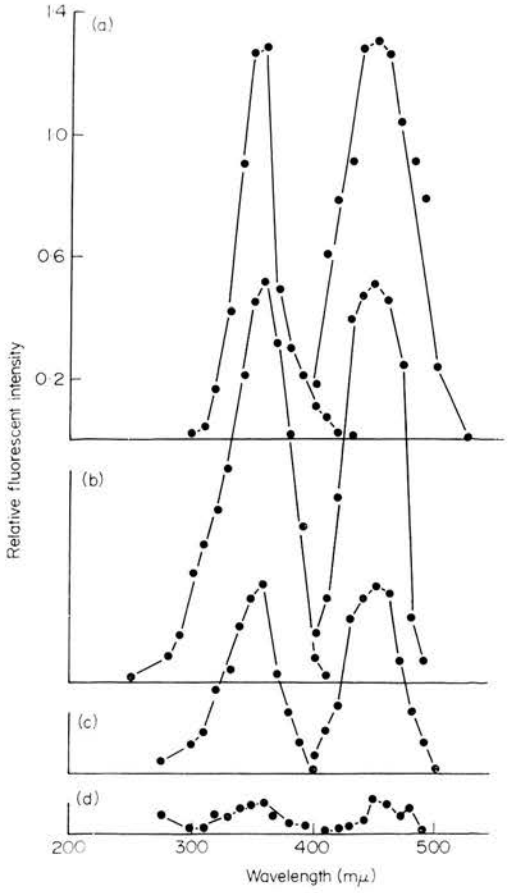

FIG. 2. Fluorometric spectra of synthetic histamine and skin diffusates. (a) Synthetic histamine (0·2 μg/ml). (b) Graphs (a) + (c). (c) Skin diffusate (0·06 μg/ml histamine by bioassay). (d) Skin diffusate reverse-blank (see Materials and Methods section).

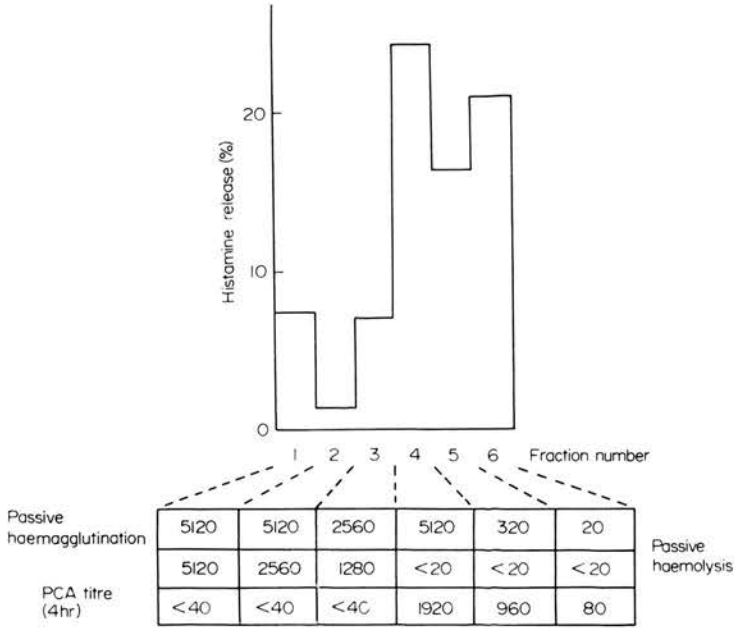

FIG. 3. The capacity of fractions of guinea-pig IgG1 and IgG2 passively to sensitize skin fragments for the antigen-induced release of histamine. All antibody fractions were diluted 1 in 20.

identical, all having optimal excitation and fluorescent wavelengths of 360 and 450 mμ respectively.

Since measurement of histamine by fluorimetry or bioassay gave similar values ($\pm 5\%$) the bioassay was used for convenience in further experiments.

Optimal conditions for antigen-induced release

Antibody mediating release. Fractions of IgG1 and IgG2 obtained from guinea-pig antiserum to ovalbumin by DEAE–cellulose chromatography were tested for their ability to prepare guinea-pig skin fragments for the antigen-induced release of histamine (Fig. 3). Only skin sensitized with IgG1-containing fractions (possessing high PCA and low passive haemolysis titres) evoked an appreciable release of histamine. An IgG1-containing fraction

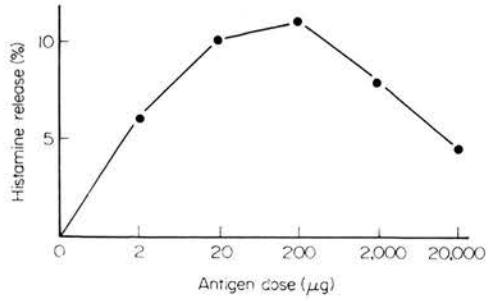

FIG. 4. The effect of antigen concentration on the release of histamine from guinea-pig skin sensitized with IgG_1 (fraction 4 diluted 1 in 20).

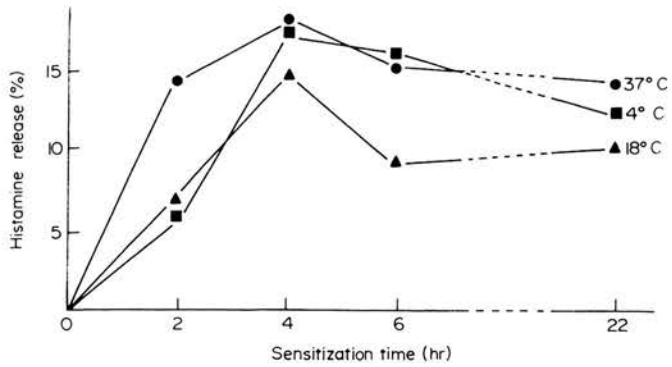

FIG. 5. Time and temperature course of passive sensitization of skin fragments by an IgG1-containing fraction (fraction 4 diluted 1 in 20) for the antigen-induced release of histamine.

(fraction 4) was used in further experiments at a concentration of 1 in 20, since this gave optimal release when this fraction was tested in doubling dilutions from 1 in 5 to 1 in 160.

Antigen dose. The effect of increasing doses of specific antigen on the release of histamine from skin fragments passively sensitized with guinea-pig anti-ovalbumin (fraction 4) is shown in Fig. 4. Maximal liberation of histamine was observed when 0·5 g of skin was challenged with 200 μg of ovalbumin in a total volume of 3 ml. These conditions were used in all further studies on histamine release. An inhibitory effect was observed with the higher doses of antigen.

Temperature and time of sensitization. Skin fragments were sensitized at varying time intervals at either 4°C, 18°C or 37°C and challenged with antigen for 15 min at 37°C. Optimal histamine release was obtained using a 4-hr incubation period at a temperature of 37°C (Fig. 5).

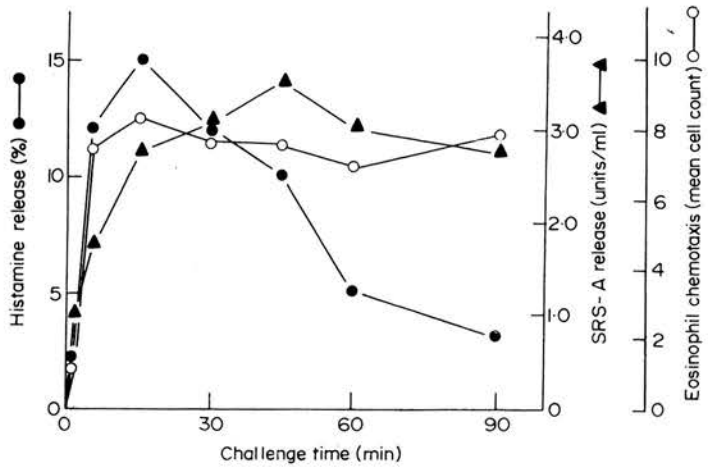

FIG. 6. Time course of release of histamine, SRS-A and ECF-A following antigen challenge of guinea-pig skin fragments passively sensitized with an IgG1-containing fraction (fraction 4 diluted 1 in 20).

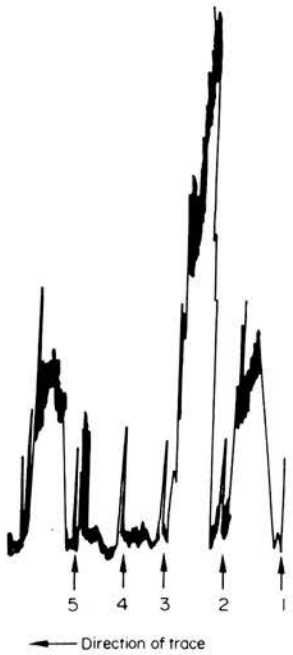

FIG. 7. Kymograph tracing showing the effect of a skin diffusate on the mepyramized guinea-pig ileum: 1 and 5 = diffusate from IgG1-sensitized skin challenged with antigen, diluted 1 in 2; 2 = As above, undiluted; 3 = Tyrode's solution alone; 4 = Undiluted diffusate from IgG1-sensitized skin incubated without antigen.

Time course of release. Skin, sensitized at 37°C for 4 hr, was challenged with ovalbumin for periods of up to 90 min. Maximal release of histamine occurred following a 15-minute incubation at 37°C (Fig. 6). Unlike SRS-A and ECF-A (see below) the percentage release and the absolute release of histamine steadily declined following continued incubation with antigen.

Quantitative release. In fourteen experiments using the optimal conditions defined above, the mean $\pm$S.E.M. percentage release of histamine was $14{\cdot}3 \pm 4{\cdot}8$ (range 6–24·5) or, in terms of μg/g wet weight skin, $0{\cdot}20 \pm 0{\cdot}06$ (range 0·10–0·33). In all experiments under the defined conditions there was no release of histamine from untreated skin, or from skin incubated with antigen or antibody alone.

TABLE 1. The capacity of fractions of guinea-pig IgG1 and IgG2 (Fig. 3) to passively sensitize skin fragments for the antigen-induced release of SRS-A and ECF-A. Fractions were diluted 1 in 20

	Histamine (μg/g)	SRS-A activity (units/g)	Eosinophil chemotactic activity (mean cell count)
IgG1 (fraction 4)	12	10	10
IgG2 (fraction 1)	0	<5	0

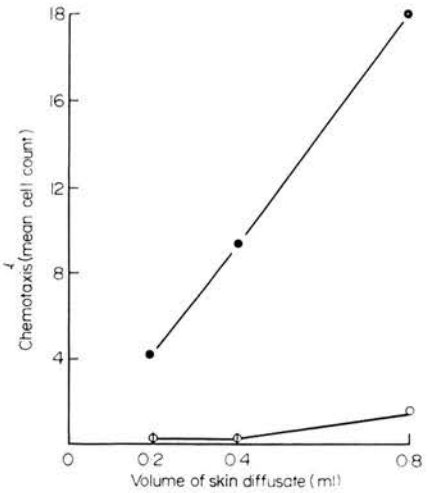

FIG. 8. Chemotactic dose–response following increasing concentrations of skin diffusate. Cell counts were adjusted so that both the (●) eosinophil and (○) neutrophil suspensions contained $1{\cdot}6 \times 10^6$ cells per ml. The mean cell counts of eosinophils and neutrophils migrating towards 0·1 ml of inulin-treated guinea-pig serum were both > 50.

Slow reacting substance

Passively sensitized skin challenged with specific antigen under the conditions described above for histamine release contained an agent which produced a contraction of the guinea-pig ileum, in the presence of mepyramine maleate, which was similar in character to contractions produced by SRS-A from the lung. SRS-A from the skin was demonstrable in a dose–response fashion (Fig. 7). No SRS-A activity was detected in samples incubated with antigen or antibody alone. In order to obtain skin SRS-A in appreciable amounts it was necessary to halve the reaction volume. Thus 2 g of sensitized skin was challenged in a total volume of 6 ml.

As with histamine the antibody mediating the release of skin SRS-A was shown to be IgG1. In the experiment depicted (Table 1) fractions containing IgG2 were unable passively to sensitize skin for the antigen-induced release of SRS-A (or ECF-A—see below). The experiment was repeated twice for all of the mediators and gave similar results.

In contrast to histamine maximal release of skin SRS-A was detected at 45 min and was virtually maintained for at least 90 min (Fig. 6).

In six experiments the mean ± S.E.M. release of SRS-A, expressed in units/g wet weight skin, was 14 ± 10 (range 3–32).

Skin SRS-A was unaffected by treatment with the enzyme pronase. In three experiments diffusates were incubated at 37°C for 30 min with pronase at a concentration of 10 PUK units per unit of SRS-A. The diffusates contained 15, 12 and 20 units per ml of SRS-A. No loss of activity was observed following treatment with the enzyme.

Eosinophil chemotactic factor

In addition to histamine and SRS-A, diffusates from passively sensitized guinea-pig skin challenged with antigen contained a factor specifically chemotactic for guinea-pig eosinophil leucocytes (Fig. 8). Chemotactic activity was demonstrable in a dose–response fashion, no

chemotaxis being observed when neutrophils were used as target cells. In all experiments skin ECF-A was prepared under the same conditions as those described for SRS-A. Only fractions containing IgG_1 prepared skin for the antigen-induced release of ECF-A (Table 1).

The time course of release of ECF-A is shown in Fig. 6. Maximal release was observed after 15 min and was maintained for the duration of the experiment.

Following passage of a concentrated skin diffusate through a column of Sephadex G-25, chemotactic activity eluted between a vitamin B_{12} marker (mol. wt 1357) and histamine (mol. wt 310). The estimated molecular size of ECF-A from the skin was therefore 500–1000.

DISCUSSION

In previous reports (Mongar & Schild, 1952; Yeoh *et al.*, 1972) the release of a histamine-like substance from the skin of actively sensitized guinea-pigs, challenged with specific antigen, was described. Active sensitization in the guinea-pig induces several types of cutaneous hypersensitivity reactions. These include delayed-type reactions and cutaneous basophil hypersensitivity (Dvorak *et al.*, 1970). In order to study immediate-type hypersensitivity we have eliminated these variables by passively sensitizing skin fragments *in vitro* with purified antibody. In addition, we have found that passive sensitization was not associated with the spontaneous release of histamine previously reported when actively sensitized guinea-pigs were used (Yeoh *et al.*, 1972).

Extractable histamine was inversely related to total body weight (Fig. 1). This may reflect age-dependent changes in the synthesis and storage of this amine in the skin.

In a previous report (Yeoh *et al.*, 1972) the histamine-like substance released from guinea-pig skin was identified by its capacity to contract the guinea-pig ileum. In order to show that this effect was not due to other ileum-contracting agents, we chemically defined the presence of histamine by fluorimetry (Fig. 2).

In studies using guinea-pig lung, IgG1 was shown to mediate the antigen-induced release of histamine (Baker, Bloch & Austen, 1964), SRS-A (Stechschulte, Austen & Bloch, 1967) and ECF-A (Kay *et al.*, 1971). Using skin from the guinea-pig we have similarly shown that IgG1 mediates the antigen-induced release of these agents from passively sensitized fragments.

High doses of antigen inhibited the release of histamine from passively sensitized skin fragments (Fig. 4). A similar effect was reported in the guinea-pig lung system (Kay *et al.*, 1971). It has been suggested that inhibition by high doses of antigen may be a result of an inability to effect the necessary conformational changes on cell-bound immunoglobulins which lead to mediator release (Osler, 1971).

Skin SRS-A was shown to be resistant to proteolytic activity and in this respect is similar to SRS-A from the lung (Brocklehurst, 1962). This treatment also distinguishes SRS-A from bradykinin, an agent which also contracts the guinea-pig ileum (Rocha e Silva, Beraldo & Rosenfeld, 1949).

Skin ECF-A also has properties in common with ECF-A from the lung (Fig. 8). These include its estimated molecular size and its capacity to attract guinea-pig eosinophils selectively (Kay *et al.*, 1971). The presence of ECF-A may partly explain the histology of cutaneous anaphylaxis in terms of infiltration of eosinophils (Kay, 1970a).

Thus histamine, SRS-A and ECF-A have been identified from guinea-pig skin passively sensitized *in vitro* and challenged with antigen.

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Tissue inactivation of slow reacting substance of anaphylaxis

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Summary. The release of slow reacting substance of anaphylaxis (SRS-A) from sensitized guinea-pig lung challenged with antigen was followed by a fall in the activity of this mediator in the diffusate when the incubations were continued for 3 h. The inactivating principle was also present in normal lung in addition to other normal guinea-pig tissues such as the liver, kidney, spleen, ileum and skin. No activity was found in muscle. Evidence is provided that some of this SRS-A-inactivating activity was due to tissue arylsulphatases. These were measured by hydrolysis of *p*-nitrophenyl sulphate (*p*-NPS) and *p*-nitrocatechol sulphate (*p*-NCS), which reflect activities of arylsulphatase II A and II B respectively. Hydrolytic activity for p-NCS was present in all tissues with SRS-A-inactivating properties, whereas only lung, liver and skin tissue hydrolysed *p*-NCS and *p*-NPS. Following passage of a cell-free lung homogenate through a column of Sephadex G-200 the *p*-NCS hydrolysing and SRS-A-inactivating activities eluted together, with molecules having a molecular size of approximately 150,000 Daltons, *p*-NPS hydrolysing activity being destroyed during the preparation of the homogenate. The release of arylsulphatase from sensitized tissue was not dependent on the presence of specific antigen. These experiments suggest that inactivation of SRS-A is related to tissue arylsulphatase and that these enzymes may play a role in the expression of the effective levels of this mediator within the tissues.

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INTRODUCTION

Slow reacting substance of anaphylaxis (SRS-A) is a low molecular weight acidic lipid which gives a characteristic contraction of guinea-pig ileal, and human bronchial, smooth muscle (Brocklehurst, 1960, 1962). Unlike preformed anaphylactic mediators such as histamine and eosinophil chemotactic factor (ECF-A), the formation of SRS-A requires the interaction of specific antigen with sensitized tissue.

SRS-A is known to contain a free sulphate group as shown by its inactivation by arylsulphatases of various origins including the mollusc (Orange, Murphy and Austen, 1974), human eosinophils (Wasserman, Goetzl and Austen, 1975), guinea-pig and human lung (Jones and Kay, 1975; Wasserman and Austen, 1975). During experiments on the time courses of mediator release from sensitized tissue we observed that there was a fall in the amount of SRS-A when the incubations were continued up to 3 h. It is the purpose of the present report to show that this inactivation of SRS-A by lung and other tissues is probably related to their content of arylsulphatase and that the enzyme is released non-specifically from

tissues and not as a result of an anaphylactic (type I) reaction.

MATERIALS AND METHODS

Animals

Dunkin–Hartley strain guinea-pigs, of either sex, were used throughout the study.

Reagents

Materials were obtained as follows. Ovalbumin five times crystallized (Koch-Light Laboratories, Colnbrook), Freund's complete adjuvant (Wellcome Reagents Ltd, Beckenham, Kent), Amberlite XAD-2 (BDH Chemicals Ltd, Poole), *p*-nitrocatechol sulphate, 4-nitrocatechol, *p*-nitrophenyl sulphate, 4-nitrophenol and quinol (Sigma London Chemical Company Ltd, Kingston-upon-Thames, Surrey).

Preparation of SRS-A

Animals were actively sensitized with ovalbumin in Freund's complete adjuvant and an anaphylactic diffusate prepared by challenging sliced perfused lung, with 670 μg of antigen per gram of tissue as previously described (Kay, Stechschulte and Austen, 1971).

SRS-A inhibition assay

Inactivation of SRS-A was performed by incubating increasing quantities of various tissue fragments, washed free of visible blood, with 2 ml of purified SRS-A in Tyrode's solution at 37° for 2 h. The remaining activity was compared with the same volume of SRS-A alone incubated under the same conditions and subsequently extracted from the tissues in 80 per cent ethanol prior to assay. For inactivation of SRS-A by cell-free lung homogenates, 1 ml of purified SRS-A was incubated with 1 ml of homogenate under the same conditions. The loss in SRS-A activity was compared with 1 ml of SRS-A and 1 ml of homogenate incubated separately and then mixed prior to ethanol extraction and assay.

Homogenates were prepared from wet lung fragments on ice, using an M.S.E. tissue blender. Resulting supernatants were centrifuged at 15,000 *g* for 2 h to remove particulate matter.

Assay of arylsulphatases

Arylsulphatases were measured by their capacity to hydrolyse either *p*-nitrocatechol sulphate (*p*-NCS) or *p*-nitrophenyl sulphate (*p*-NPS) which reflect activity of the type II B and II A enzymes respectively (Roy, 1960; Rammler, Grado and Fowler, 1964). Volumes of 0·4 ml of diffusate or 0·4 ml of 0·5 M sodium acetate containing 400 μg tissue fragments were incubated with 0·4 ml of the appropriate substrate at a concentration of $6{\cdot}25 \times 10^{-3}$ M in 0·5 M sodium acetate, pH 5·6. Following 60 min incubation at 37° the reaction was stopped by the addition of 2 ml of an 'alkaline-quinol' solution (20 ml of 5 per cent $Na_2SO_3 . 7H_2O$ in 0·5 N NaOH mixed with 1 ml of 4 per cent quinol in 0·1 N HCl immediately before use). The amount of 4-nitrocatechol (4-NC) or 4-nitrophenol (4-NP) liberated was measured at 515 nm or 410 nm respectively on a Gilford 300-N microsampling spectrophotometer. A standard curve of increasing concentrations of 4-NC or 4-NP, in a mixture of 0·8 ml of 0·5 M sodium acetate and 2 ml of 'alkaline-quinol' solution, was used as a reference. One unit of arylsulphatase activity is defined as the amount which liberates 1 μmole of 4-NC or 4-NP per hour from the appropriate substrate under the conditions described above.

Gel filtration chromatography

A volume of 2 ml of lung homogenate was applied to a column of Sephadex G-200 ($95 \times 3{\cdot}5$ cm) equilibrated in 0·5 M sodium acetate buffer, pH 5.6. Fractions of 2 ml were collected and tested for arylsulphatase and SRS-A-inhibiting activity. The column was calibrated by using guinea-pig serum as a source of molecular markers. The serum gave three distinct peaks which corresponded to V_0 and the elution volumes of IgG (molecular weight 150,000) and albumin (molecular weight 60,000).

RESULTS

SRS-A levels following antigen challenge

When actively sensitized lung fragments were incubated with specific antigen at time intervals up to 3 h, maximal SRS-A activity was found in the diffusates sampled between 30 and 60 min (Figs 1 and 4). When the incubation was continued up to 3 h the activity was depleted by 40 per cent. If the diffusate was removed at 30 min, centrifuged to remove particulate matter, and the incubation continued at 37° for up to 3 h there was only a 20 per

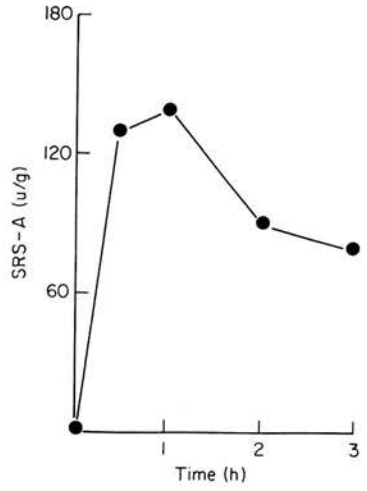

Figure 1. The release and inactivation of SRS-A following antigen challenge of sensitized lung.

cent loss in the amount of remaining SRS-A, suggesting that the majority of the SRS-A-inactivating principle was associated with the lung fragments. Both these experiments were performed three times and gave essentially similar results.

The capacity of normal lung fragments to inactivate SRS-A in a dose-dependent fashion was determined as shown in Fig. 2. In these four experiments

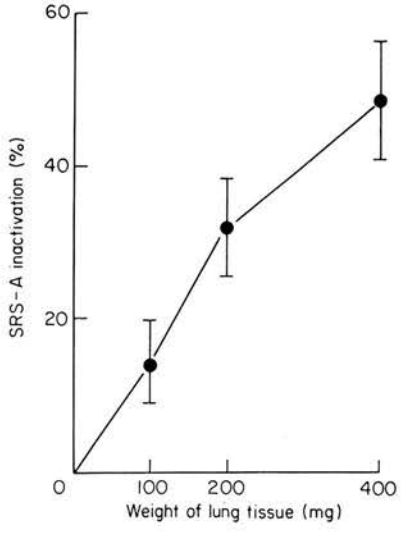

Figure 2. Inactivation of SRS-A by increasing quantities of normal lung fragments. The points represent the mean of four experiments ± two standard errors.

the remaining SRS-A was extracted in 80 per cent ethanol to exclude the non-specific adherence of SRS-A to the increasing quantities of tissue.

Since arylsulphatases are known to inactivate SRS-A, several tissues were tested for the presence of arylsulphatases and SRS-A-inactivating properties (Table 1). The lung, liver, kidney, spleen, ileum

Table 1. The arylsulphatase and SRS-A-inactivating activity present in various tissue and organs

	Percentage SRS-A inactivation	Arylsulphatase (u/g)	
		p-NCS	*p*-NPS
Lung	55	0·61	0·70
Liver	58	0·34	0·43
Kidney	20	0·84	0·10
Spleen	37	0·81	0·00
Ileum	25	0·44	0·09
Skin	10	0·52	0·21
Muscle	4	0·04	0·03

These values represent the average results from three experiments.

and skin had both SRS-A-inactivating activity and sulphatase as assessed by hydrolysis of *p*-NCS which represents the activity of arylsulphatase type II B. In contrast p-NPS which is used as a substrate for type II A sulphatase was hydrolysed by lung, liver and skin tissue only. Muscle contained little or no SRS-A-inactivating or sulphatase activities. There was no clear correlation between the amounts of sulphatases in tissues and their SRS-A-inactivating activities.

A cell-free homogenate of normal lung with SRS-A-inactivating and sulphatase activity was applied to a column of Sephadex G-200 (Fig. 3). During the preparation of this cell-free lung homogenate the *p*-NPS hydrolysing capacity was destroyed but arylsulphatase as assessed by *p*-NCS hydrolysis was unaffected. The latter eluted from the column in the same position as the SRS-A-inactivating principle and had a molecular size of approximately 150,000 Daltons. This was repeated twice with essentially the same results.

Experiments were undertaken to determine whether arylsulphatase was released following antigen challenge of sensitized lung. Fig. 4 shows that the release of this enzyme was not affected by

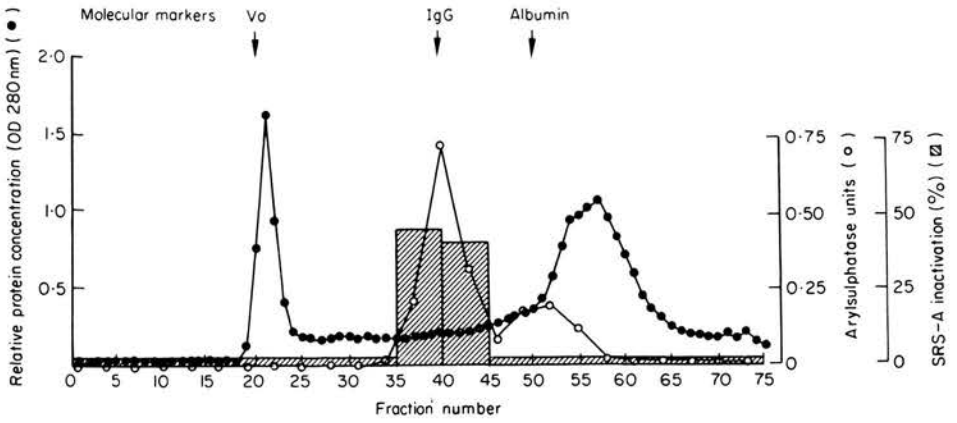

Figure 3. Sephadex G-200 chromatography of a cell-free lung homogenate showing the elution profiles of arylsulphate II B and SRS-A-inactivating activities.

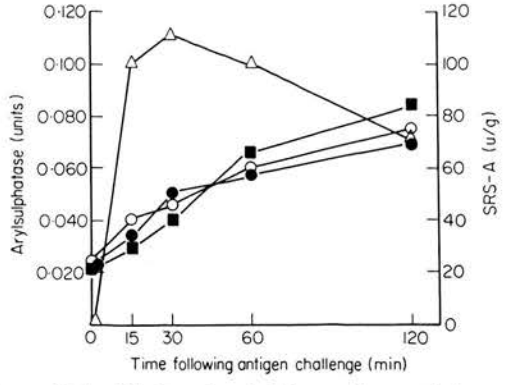

Figure 4. The diffusion of arylsulphatase from actively sensitized lung challenged with antigen (●), actively sensitized lungs alone (○) and normal lung (■). The amount of SRS-A appearing in the diffusate of the sensitized, challenged lung is also shown (△).

the presence of specific antigen and that normal lung also releases arylsulphatase following incubation in Tyrode's balanced salt solution.

DISCUSSION

It is clear that a number of factors influence the release of SRS-A into the diffusate when sensitized tissue fragments are challenged with specific antigen. SRS-A is an acidic lipid which combines non-specifically with tissue and plasma proteins from which it can be recovered by extraction in 80 per cent ethanol (Brocklehurst, 1962). Furthermore it has been shown that a proportion of SRS-A formed during a type I (anaphylactic) reaction remains within the cell from which it can be extracted by organic solvents (Lewis, Wasserman, Goetzl and Austen, 1974). There would now appear to be a further influence on the amount of SRS-A activity in the diffusate due to the presence of tissue derived arylsulphatase. Thus our observation that the amount of SRS-A appearing in the diffusate diminished when the incubation time was prolonged to 3 hours, probably reflects the subsequent action of sulphatases which have diffused from the tissue (Figs 1 and 4). A recent report by Wasserman and Austen (1975) has shown that arylsulphatases II A and II B with estimated molecular sizes of 100,000 and 65,000 respectively can be extracted from human lung tissue. In the present report we show that these sulphatases are also present in guinea-pig lung (Table 1) but arylsulphatase II B had an estimated molecular size of 150,000 Daltons (Fig. 3). In the present study the apparent lability of arylsulphatase II A prevented determination of its molecular size by gel filtration.

Lung tissue contained both sulphatase II A and II B activity and could inactivate SRS-A in a dose-dependent fashion (Fig. 2, Table 1). Liver and skin also contained both these activities but kidney, spleen and ileum had only sulphatase II B activity. Under the experimental conditions herein muscle had no sulphatase activity and did not affect SRS-A.

In general tissues containing arylsulphatases had the capacity to inactivate SRS-A but there was no clear correlation between enzymatic content and the ability to destroy SRS-A.

The enzyme cannot be regarded as a mediator of anaphylaxis since the rate of diffusion from sensitized tissue was not affected by the presence of

specific antigen (Fig. 4). Therefore although sulphatases may have a regulatory role in the expression of the pharmacological effects of SRS-A the release of the enzyme(s) are presumably independent of the biochemical pathways which lead to the elaboration of pharmacological mediators of anaphylaxis.

Previous studies have shown that the infiltration of the eosinophil into sensitized tissue is maximal 8–12 h following challenge with specific antigen (Kay, 1970). Since arylsulphatases are widely distributed throughout the tissues it is unlikely that the high content of this enzyme in the eosinophil points to a unique function of this cell (Wasserman *et al.*, 1975).

Whether tissue or circulating levels of arylsulphatase have importance in clinical conditions mediated by SRS-A, such as extrinsic (allergic) asthma, is yet to be determined.

It should be emphasized that in these experiments on tissue inactivation by SRS-A, the mediator was extracted in 80 per cent ethanol prior to assay and therefore it is unlikely that the observed loss of activity was due to non-specific binding by this acidic lipid to the tissues.

ACKNOWLEDGMENTS

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Slow reacting substance as a preformed mediator from human lung

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Summary. Homogenates from human lung contained a preformed slow reacting substance (pSRS). The pattern of contraction on the guinea-pig ileum by pSRS was indistinguishable from that of SRS-A. The activity of pSRS could not be attributed to the presence of K^+, Na^+, Ca^{2+} and Mg^{2+} ions, or any prostaglandin including $PGF_{2\alpha}$ or its 15-oxo derivative. As with SRS-A, pSRS could be adsorbed onto Amberlite XAD-2 and silicic acid. Both were eluted from the former with 80 per cent ethanol and from the latter with a mixture of ethanol, ammonia and water. Both pSRS and SRS-A were resistant to the action of NaOH whereas their activities were destroyed by boiling in HCl. Arylsulphatase II B destroyed the activities of both pSRS and SRS-A. An antagonist of SRS-A, FPL 55712, inhibited the action of pSRS at comparable concentrations to that of SRS-A. These experiments suggest that pSRS and SRS-A are identical. Thus SRS joins histamine and ECF-A as a preformed mediator. Although SRS was present in a preformed state the amount of material extractable was more than doubled by the anaphylactic reaction. The extraction of slow reacting substance from human lung without apparent requirement for antigen or antibody points to a possible role of this mediator in inflammatory reactions evoked by mechanisms independent of IgE and other tissue-sensitizing antibodies.

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INTRODUCTION

The term 'slow reacting substance' was first used to describe material released from guinea-pig or cat lung perfused by cobra venom (Feldberg & Kellaway, 1938). Subsequently an agent with similar pharmacological properties was identified *in vitro* from perfusates of sensitized guinea-pig lung challenged with specific antigen (Brocklehurst, 1960). This was designated 'slow reacting substance of anaphylaxis' (SRS-A) to distinguish it from other slow reacting substances released by non-immunological mechanisms.

A number of workers have since reported that SRS-A, unlike histamine and eosinophil chemotactic factor (ECF-A) which are recognized as preformed mediators of anaphylaxis, is present in tissue only in a precursor state from which the active form must be generated either immunologically (Brocklehurst, 1962; Austen & Orange, 1975) or by a calcium ionophore (Bach & Brashler, 1974).

Although SRS-A must await full chemical characterization, it can now be obtained in a highly purified form by a combination of non-ionic chromatography and differential solubility in a variety of organic solvents (Orange, Murphy, Karnovsky & Austen, 1973). Therefore SRS-A can be distinguished from other slow reacting substances which evoke a similar pattern of contraction of the guinea-pig ileum.

We have found that fresh human lung obtained

at surgery contains a preformed SRS (pSRS) which can be extracted non-immunologically. Since SRS-A is considered to be a primary mediator in allergic (extrinsic) bronchial asthma (Brocklehurst, 1956), due largely to its capacity to produce sustained contraction of bronchial smooth muscle, it seemed important to determine the relationship of pSRS to SRS released by anaphylaxis. In the present report we show that pSRS and SRS-A are identical, so raising the possibility that SRS may participate in those forms of bronchial asthma in which there is no evidence for the participation of IgE and other tissue-sensitizing antibodies.

MATERIALS AND METHODS

Materials were obtained as follows: Amberlite XAD-2 (BDH Chemicals Ltd, Poole), silicic acid (SIL-R, Sigma Chemical Company, St. Louis), arylsulphatase II B and N,N-bistrimethylsilyltrifluoroacetamide BSTFA, Sigma T.6381- (Sigma Chemical Company, St. Louis), FPL 55712-sodium 7-((3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxyl))-4-oxo-8-propyl-4H-chromene-2-carboxylate—(a gift from Fisons Research Laboratories, Loughborough), prostaglandins (PG) $F_{2\alpha}$, 15-oxo $F_{2\alpha}$ (a gift from Professor E. Horton, Department of Pharmacology, University of Edinburgh), Timothy grass pollen (a gift from Beecham Research Laboratories, Betchworth).

Preparation of SRS-A and pSRS

Macroscopically normal human lung obtained at surgery, usually in association with bronchiogenic carcinoma, was dissected free of pleura, bronchi and large blood vessels, sliced into fragments of approximately 50 mg and washed thoroughly in Tyrode's buffer. For SRS-A, each gram wet weight of sliced lung fragment was incubated with 5·4 ml of a 1 in 8 dilution of serum (R.B.) from an individual sensitive to Timothy grass pollen. Following an 18 h incubation period at room temperature the fragments were washed twice in Tyrode's solution and challenged in 9 ml of the same buffer, containing 0·2 μg/ml of Timothy grass pollen antigen. Following incubation for 15 min at 37° the diffusate was removed and stored at −85° until use.

pSRS was prepared from thoroughly washed, unsensitized, unchallenged lung by gentle homogenization on ice using an MSE tissue blender, freezing and thawing twice and heating at 56° for 1 h in a water bath. The preparation was centrifuged at 15,000 *g* for 30 min to remove particulate matter and stored at −85° until use.

For experiments on the generation of SRS, 350 mg of lung fragments were sensitized with 1·8 ml of varying dilutions of serum (R.B.) and challenged with 3 ml of the antigen/Tyrode's solution as described above. After the removal of the diffusate, residual SRS was extracted by resuspending the fragments in a further 3 ml of Tyrode's solution and boiling for 15 min in a water bath.

Measurement of SRS

SRS was measured by the method of Brocklehurst (1960) using the isolated guinea-pig ileum in the presence of atropine and mepyramine maleate as previously described. One unit of SRS-A or pSRS was defined as the concentration required to give a contraction of the ileum of equal amplitude to 5 ng of histamine in that assay.

Progressive purification of SRS-A and pSRS

SRS-A and pSRS were purified in parallel by extraction in 80 per cent ethanol, hydrolysis in 0·1 N NaOH for 60 min at 37° and chromatography on Amberlite XAD-2 (Orange *et al.*, 1973). These partially purified preparations were used in subsequent inactivation and inhibition studies. Where necessary further purification was achieved following the application of the samples to activated silicic acid and sequential elution with hexane, dichloromethane, acetone, n-propanol and a mixture of ethanol, concentrated ammonia and water (6:3:1 v/v) as described (Orange *et al.*, 1973). Eluates were evaporated to dryness and resuspended in Tyrode's buffer for bioassay or distilled H_2O for prostaglandin analysis.

Inactivation by arylsulphatase

Arylsulphatase II B was partially purified by chromatography on Sephadex G-200 in 0·5 M acetate buffer, pH 5·5, as described (Kay, Roberts & Jones, 1976). One-millilitre volumes of partially purified SRS-A and pSRS of equivalent ileal contracting activity (approximately 55 u/ml) were adjusted to pH 5·5 with 0·1 N HCl and incubated for 1 h with 1 ml of increasing concentrations of arylsulphatase. Following incubation the samples were readjusted to pH 7·4–7·7 with 0·1 N NaOH and assayed immediately for SRS activity.

Inhibition by FPL 55712

Dilutions of FPL 55712, freshly prepared in Tyrode's solution, were applied to the perfused guinea-pig ileum for 2 min. The organ bath was then refilled with partially purified SRS-A, or pSRS, containing the same concentration of drug and contractile activity measured (Brocklehurst, 1960). Before the addition of subsequent samples the ileum was returned to its original sensitivity by repeated washing with mepyramised Tyrode's.

Detection of prostaglandins

Prostaglandins were measured using an LKB 9000 Gas Chromatograph Mass Spectrometer as described (Green, 1969) with $PGF_{2\alpha}$ and 15-oxo $F_{2\alpha}$ as internal standards. All samples contained in 1 to 2 ml volumes were initially brought to pH 3, using N HCl and then extracted three times with 20 ml of ether. A three-step derivatization procedure was then carried out.

Methyl ester formation. 0·3–0·4 ml of diazomethane (nine parts diethyl ether, one part methanol) were incubated with the test samples for 5 min at room temperature and then removed using a nitrogen jet and vacuum desiccation.

O-alkyloxime formation. O-alkylhydroxylamine hydrochloride (1 mg in 0·2 ml) in dry pyridine was added to the desiccates. The samples were then heated at 60° for 90 min. The pyridine was then removed with a nitrogen jet and the samples vacuum desiccated.

Trimethylsilyl ether formation. Finally, 25 μl of BSTFA were added to the samples, which were then either heated for 15 min at 60° or left overnight at room temperature. The BSTFA was then removed with a nitrogen jet and the residues redissolved in n-heptane, before analysis.

Measurement of Na^+, K^+, Ca^{2+} and Mg^{2+} levels

Na^+ and K^+ levels were determined using a standard flame photometry technique, using lithium as the internal standard.

Ca^{2+} levels were measured by a modified Kessler and Wolfman technique (1964) using cresophthalein and 8-hydroxyquinoline. Blue colouration was measured using a spectrophotometer, the readings being made at 580 nm.

Mg^{2+} levels were determined by atomic absorption.

RESULTS

Comparison of SRS-A and pSRS

Contraction of the guinea-pig ileum

The pattern of contraction of the guinea-pig ileum of SRS-A and pSRS is shown in Fig. 1. Both agents gave identical types of contraction which were demonstrable in a dose-dependent fashion. These effects could not be attributed to the presence of potassium since the concentration of this ion in both the SRS-A and pSRS preparations was invariably less than that present in Tyrode's solution (3·0 mmol/litre). Furthermore the pattern of contraction observed with high concentrations of K^+ (20 mmol/

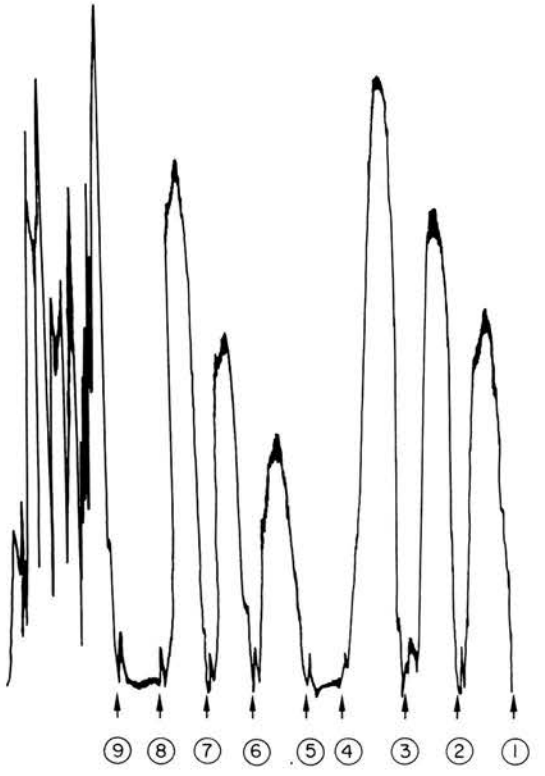

Figure 1. Contraction of the guinea-pig ileum for SRS-A, pSRS and K^+. (1, 2 and 3) SRS-A diluted 1 in 35, 20 and 10, respectively. (4) Tyrode's buffer (5, 6 and 7) pSRS diluted 1 in 20, 10 and 5, respectively. (8 and 9) K^+ at 3·0 and 20·0 mmol/l in. Tyrode's, respectively. Direction of trace from right to left.

Table 1. Progressive purification of SRS-A and pSRS

Procedure	SRS-A		pSRS	
	Units/g	Percentage recovery	Units/g	Percentage recovery
Starting material	380	—	240	—
Ethanol extraction	106	28	75	31·5
0·1 N NaOH (37° × 30 min)	76	20	56	23
Amberlite XAD-2 chromatography	67	18	48	20
Silicic acid chromatography—				
Hexane	6·0	1·5	5·5	2·3
Dichloromethane	4·5	1·2	4·0	1·7
Acetone	4·5	1·2	3·5	1·5
n-Propanol	3·0	0·8	3·0	1·3
Ethanol/conc. NH_3/H_2O 6:3:1 v/v	20·5	5·4	18·0	7·5

litre) was clearly different from that produced by pSRS or SRS-A. The levels of other ions including Na^+, Ca^{2+} and Mg^{2+} were also lower in the test preparations than in Tyrode's solution.

Progressive purification

SRS-A and pSRS were progressively purified as shown in Table 1. Comparable amounts were recovered following extraction in 80 per cent ethanol, alkaline hydrolysis and adsorption and elution from Amberlite XAD-2. Following further purification by adsorption onto activated silicic acid, appreciable amounts of SRS-A or pSRS were recovered in the ethanol:ammonia:water eluate but considerably less was present in the hexane, dichloromethane, acetone and *n*-propanol fractions.

Effect of acid hydrolysis

Preparations of SRS-A and pSRS which had been extracted in 80 per cent ethanol, hydrolysed with 0·1 N NaOH and subjected to adsorption and elution on Amberlite XAD-2 as in Table 1, were rapidly inactivated by boiling in 0·05 N HCl. Thus 75 and 60 per cent of activity, respectively, was lost within 5 min.

Effect of arylsulphatase

The effect of increasing concentrations of arylsulphatase II B on the ileal-contracting properties of SRS-A and pSRS is shown in Fig. 2. The inactivation profiles for both preparations were virtually identical. The time course inactivation by arylsulphatase is shown in Fig. 3. A similar pattern of inactivation of both preparations was observed, both SRS-A and pSRS being totally destroyed after 30 min.

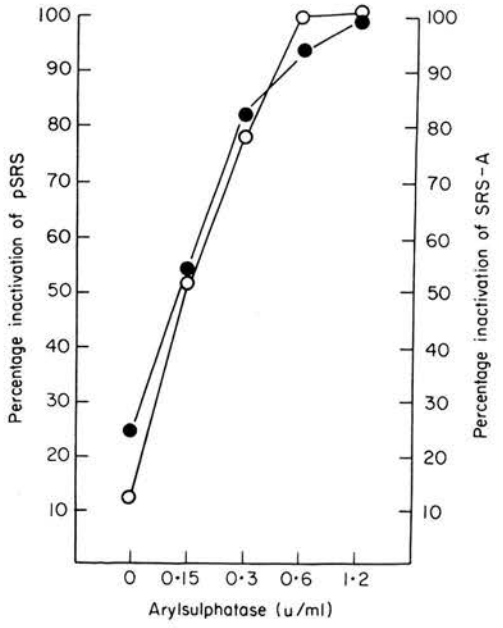

Figure 2. Inactivation of SRS-A (●) and pSRS (○) by increasing concentrations of arylsulphatase.

Effect of FPL 55712

When SRS-A and pSRS were applied to the ileum in the presence of increasing concentrations of FPL

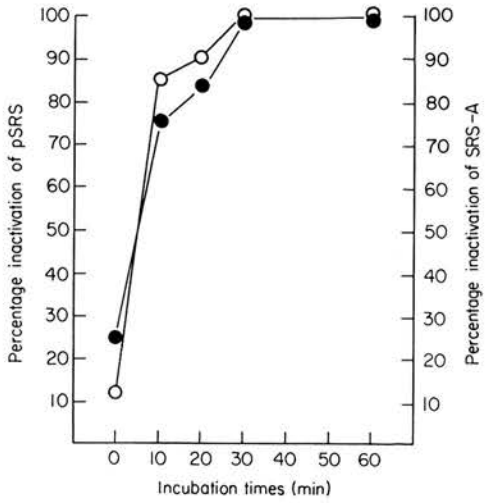

Figure 3. Time course of inactivation of SRS-A (●) and pSRS (○) by arylsulphatase. One-millilitre volumes of SRS were incubated with 1 ml of arylsulphatase containing 1·2 units/ml.

55712 following a 2 min preincubation with the same agent, there was a similar dose-dependent inhibition of both activities, with an IC_{50} of approximately 10^{-7} g/l ($1{\cdot}9 \times 10^{-7}$ M).

pSRS, SRS-A and prostaglandins

SRS-A and pSRS activities were not due to the presence of the prostaglandins $PGF_{2\alpha}$ and 15-oxo $F_{2\alpha}$ as shown in Table 2. One sample of pSRS before extraction by solvents on silicic acid contained 100 ng/ml of $PGF_{2\alpha}$. However in a separate experiment eluates obtained by the sequential elution from silicic acid by various organic solvents possessing SRS activity contained no detectable prostaglandin as assessed by mass spectrometry, the smallest amounts detectable being 50 ng of $PGF_{2\alpha}$ and 15-oxo $F_{2\alpha}$ and 100 ng/ml of PGE and E_2 per millilitre of sample. The amounts of pure $PGF_{2\alpha}$ and 15-oxo $F_{2\alpha}$ required to give contraction of the ileum of equal amplitude to that of SRS was far greater than the actual amounts present.

Generation of SRS

Although SRS was present in a preformed state, the total amount of SRS recoverable from lung following *in vitro* sensitization and antigen challenge was more than double that extractable from an untreated sample of the same lung (Fig. 5). The amount of antigen-induced SRS release decreased with increasing dilutions of tissue-sensitizing antibody. However the amount of the residual SRS, in both test and control samples, was relatively constant.

DISCUSSION

We have been able to show that material extracted from human lung non-specifically by homogeniza-

Table 2. The prostaglandin content of pSRS and SRS-A

		Amount of prostaglandin required to give a contraction of equal amplitude (ng/ml)		Prostaglandin content of sample (ng/ml)	
Number	SRS (u/ml)	$PGF_{2\alpha}$	15-oxo $F_{2\alpha}$	$PGF_{2\alpha}$	15-oxo $F_{2\alpha}$
1	SRS-A (28)	560	700	< 50	< 50
2	pSRS (15)	300	375	100	< 50
3	pSRS (15)	300	375	< 50	< 50
4	pSRS (10)	200	250	< 50	< 50
5	pSRS (8)	160	200	< 50	< 50
6	pSRS (6)	120	150	< 50	< 50
7	pSRS (19)	380	475	< 50	< 50

All samples were extracted in 80 per cent ethanol, hydrolysed with 0·1 N NaOH and adsorbed and eluted from Amberlite XAD-2. In addition samples 3–7 were adsorbed onto activated silicic acid. Sample 3 was eluted with hexane, 4 with dichloromethane, 5 with acetone, 6 with *n*-propanol and 7 with a mixture of ethanol, concentrated ammonia and water (v/v 6:3:1).

tion and freezing and thawing (pSRS), had biological and chemical properties identical to SRS-A. It has been suggested that potassium ions may account for the gut-contracting property of material extracted from 'unshocked' tissue (Brocklehurst, 1962). However, in the present investigations potassium ion concentration in pSRS was less than that of the Tyrode's buffer and the effect on the ileum of high concentrations of potassium was an irregular pattern of brisk, spontaneous-like contractions, which did not dose-respond and usually led to irreversible damage of the gut (Fig. 1). Similarly the levels of Na^+, Ca^{2+} and Mg^{2+} in pSRS were always less than those present in Tyrode's solution.

When SRS-A and pSRS were purified progressively by a combination of non-ionic chromatography and differential solubility in a variety of organic solvents the recovery at each step was comparable for the two preparations (Table 1). Thus both agents could be extracted in 80 per cent ethanol, survived alkaline hydrolysis and adsorbed onto Amberlite XAD-2 from which they could be eluted with 80 per cent ethanol. At the stage both preparations adsorbed onto activated silicic acid from which they could not be eluted in appreciable amounts by hexane, dichloromethane, acetone or *n*-propanol but could be recovered in larger quantities by an ethanol: ammonia: water mixture.

A number of agents which are known to destroy the activity of SRS-A, or inhibit its action on target tissue, had a comparable effect on pSRS. Thus SRS-A and pSRS were destroyed by acid hydrolysis and the enzyme arylsulphatase (Figs 2 and 3). It is known that SRS-A contains a free sulphate group which is essential for its biological activity (Orange *et al.*, 1974) and which can be cleaved by arylsulphatases derived from a variety of sources such as normal lung, skin, liver and spleen (Kay *et al.*, 1976) and preparations of purified eosinophils (Wasserman, Goetzl & Austen, 1975).

An antagonist of SRS-A, FPL 55712 (Augstein, Farmer, Lee, Sheard & Tattersall, 1973), which also inhibits the migration of guinea-pig eosinophils towards ECF-A (Jones & Kay, 1974), was found to inhibit pSRS activity at comparable concentrations to those affecting SRS-A (Fig. 4).

Prostaglandins $PGF_{2\alpha}$ and 15-oxo $F_{2\alpha}$ are known to contract the guinea-pig ileum and to be present in human lung in a preformed state (Horton & Main, 1963). There was the possibility that activity of pSRS was due to the presence of prostaglandins $PGF_{2\alpha}$ and

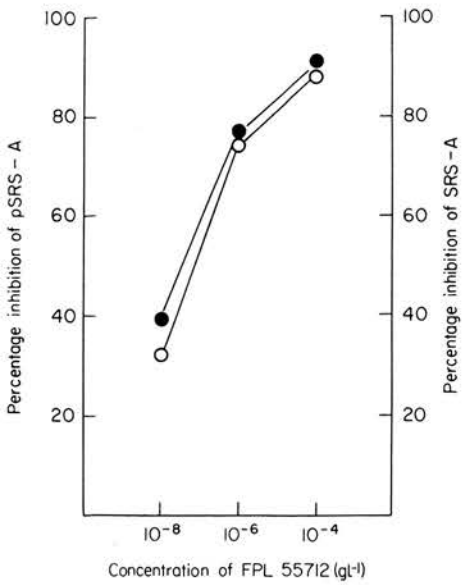

Figure 4. Inhibition of SRS-A (●) and pSRS (○) by increasing concentrations of FPL 55712.

15-oxo $F_{2\alpha}$ which were retained throughout the extraction and purification steps. Although destruction of pSRS activity by arylsulphatase and its inhibition by FPL 55712 made it unlikely that prostaglandins were accounting for the observed effects, it was necessary to measure the amounts present. This was achieved by applying samples of preparations of pSRS and SRS-A to the mass spectrometer after the appropriate extraction procedures (Green, 1969). There was no detectable prostaglandin material in any of the preparations which had passed through the 'silicic acid step' and had been extracted by various solvents (Table 2). Although one 'pre-silicic acid' sample contained 100 ng/ml of $PGF_{2\alpha}$, none of the preparations contained sufficient prostaglandin material to account for their ileal-contracting activity.

These experiments indicate that pSRS and SRS-A were identical. However, it was necessary to determine if all the SRS was present in a preformed state or whether some was generated by the anaphylactic mechanism. It was shown that more than twice the amount of SRS could be generated from lungs sensitized by IgE and challenged with specific antigen than could be extracted from fragments from the same lung by non-immunological means (Fig. 5). Thus a large amount of SRS-A was formed

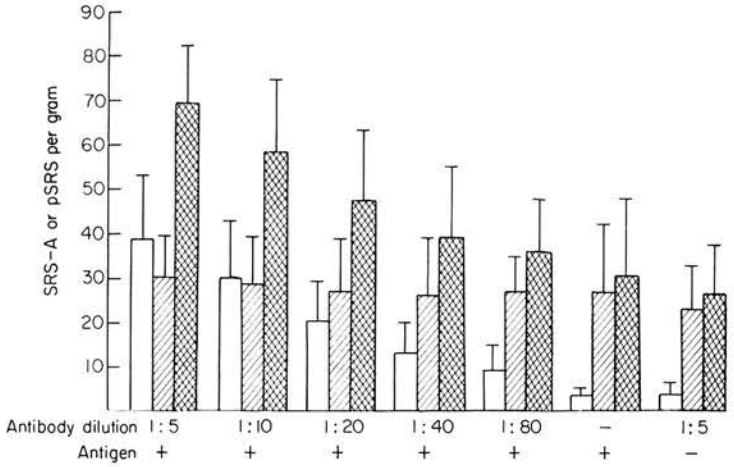

Figure 5. The antigen-induced, and residual SRS, released from human lung fragments sensitized with dilutions of serum from a Timothy grass pollen-sensitive individual and challenged with specific antigen. The results are the pooled values from four experiments. For each experiment each point was performed in triplicate. The bars represent one S.E.M. Open columns, antigen-induced SRS; hatched columns, residual SRS; cross-hatched columns, total SRS.

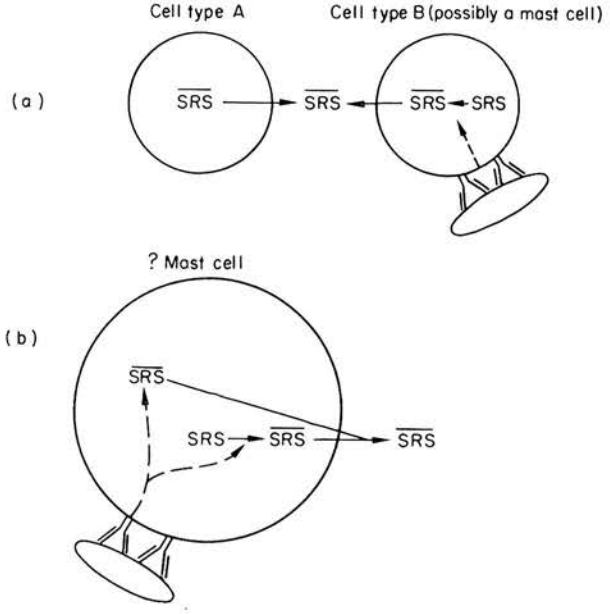

Figure 6. A diagramatic representation of the possible origins of $\overline{\text{SRS}}$. In (a) two cell types, A and B, are proposed and in (b) one cell type having both SRS and $\overline{\text{SRS}}$ is postulated.

as a direct result of the anaphylactic reaction. Two suggestions as to the origin of SRS are depicted diagrammatically in Fig. 6. Since pSRS and SRS-A are apparently identical it is proposed that, in its precursor form, the agent should be referred to as SRS and that $\overline{SRS}$ be used to describe the active state. One possibility is that there are two distinct intracellular pools of SRS contained in two different cell types, one in the form of $\overline{SRS}$ and the other SRS which is converted to $\overline{SRS}$ following anaphylaxis. Alternatively the same cell may contain both SRS and $\overline{SRS}$, the anaphylactic reaction stimulating the conversion of SRS to more $\overline{SRS}$. The concept of two pools is based on the finding that the amount of extractable (or residual) $\overline{SRS}$ remained fairly constant whereas the amounts of $\overline{SRS}$ released immunologically decreased with dilutions of antibody (Fig. 5).

The possibility exists that activation of SRS to $\overline{SRS}$ occurred in the non-anaphylactic lung during the initial preparatory stages by an artificial combination of, as yet undetermined, enzyme and substrate. However this is unlikely since the extraction procedures were performed at 0° or 100°.

Finally, these observations may have considerable implications in the mechanisms of bronchial smooth muscle contraction in situations where IgE-mediated release of $\overline{SRS}$ is probably not operative, for example, chronic or intrinsic bronchial asthma. Since $\overline{SRS}$ can now no longer be regarded as exclusive to the anaphylactic reaction the possibility remains that it is released from lung stores by acute or chronic non-specific inflammatory reactions.

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ACTIVATION OF THE CLASSICAL AND ALTERNATE PATHWAYS OF COMPLEMENT BY *CORYNEBACTERIUM PARVUM*

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SUMMARY

The immunological adjuvant *Corynebacterium parvum* has been shown to activate the alternate pathway of complement in human and guinea-pig serum. Human serum in addition contains anti-*C. parvum* antibodies leading to activation of the classical complement pathway.

The possible role of a *C. parvum* derived polysaccharide in this activation is considered in relation to the biological effects of the micro-organism.

INTRODUCTION

A number of biological effects are associated with the administration of a killed vaccine of *Corynebacterium parvum* to experimental animals. These include adjuvant effect for both T cell- and B cell-dependent antigens (Howard, Scott & Christie, 1973) and the ability to induce 'anti-self' red cell activity in CBA mice (McCracken, McBride & Weir, 1971). There is also widespread proliferation of lymphoid cells, including macrophages, in response to *C. parvum* injection which results in marked hypertrophy of lymphoid tissues (Halpern *et al.*, 1964). Of particular interest is the ability of *C. parvum* to inhibit tumour growth in mice (Woodruff & Boak, 1966; Halpern *et al.*, 1966), and the effectiveness of this agent to influence dissemination of tumour in man is currently under investigation (*New Scientist*, 1973).

The mechanisms underlying these effects are still obscure as are the particular constituents of the micro-organism responsible. Our recent observation that a polysaccharide moiety extractable from a strain of *C. parvum* binds to tissue cells (McBride, Jones & Weir, 1974) suggests the need to consider the possible involvement of this material in the biological

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effects of the organism. Various polysaccharides such as inulin, zymosan and endotoxin are known to activate complement. In addition various polysaccharides have anti-tumour effects which are possibly a result of activation of complement components (Okuda *et al.*, 1972). In the present study we report a further biological activity of *C. parvum*, that is its capacity to activate directly the complement system in both human and guinea-pig serum.

MATERIALS AND METHODS

Assay of complement components

Individual components were assayed by the effective molecular titration method described by Rapp & Borsos (1970).

C3 proactivator was identified in immunoelectrophoresis using anti-C3 proactivator antibody (Hoechst Pharmaceuticals) (Götze & Muller-Eberhard, 1971).

Agglutination tests

Direct agglutination tests were performed in the presence of Difco latex 0·8 μm, 100 μl of latex suspension in 1 ml of suspension of *C. parvum* 3×10^{11} organisms/ml. The stock suspension is finally diluted 1/20 before use in the agglutination test (Woodruff, McBride & Dunbar, 1974).

C. parvum strain 10390 was obtained from the National Collection of type cultures (Colindale, England) and cultured as described previously (McBride *et al.*, 1974).

RESULTS

When increasing concentrations of a washed suspension of killed *C. parvum* 10390 were incubated with guinea-pig or human serum for 1 hr at 37°C and centrifuged, there was a dose-dependent decrease in the total haemolytic complement (CH_{50}) of the supernatant (Fig. 1). The levels of the individual complement (C) components C1, C4, C2 and C3 were then measured in pooled sera before and after treatment with a dose of 5×10^9 organisms per ml. Following treatment of human serum the level of C3 was greatly depleted as were the components of the 'classical pathway' C1, C4 and C2. In contrast, whereas guinea-pig C3 was also depleted after treatment there was apparent sparing of C1, C4 and C2 in this serum indicating activation via the 'alternate pathway' of complement (Table 1).

Evidence that human, but not guinea-pig serum, contained 'natural antibodies' to *C. parvum* was provided by direct agglutination tests. Agglutination of *C. parvum* was observed at a 1 in 32 dilution of the human serum pool whereas no agglutination was observed with the guinea-pig pool. Further evidence that antibodies to *C. parvum* were present in human serum and could account for 'classical pathway' activation was obtained by testing sera that had been repeatedly absorbed with the organisms in the presence of 0·04 M EDTA. The absorbed serum was dialysed against dextrose–gelatin veronal buffer containing Ca^{++} and Mg^{++} and divided into two. One half was treated with *C. parvum* at a concentration of 5×10^9 organisms per ml, while the other was left untreated. The C4 titres of the treated and the untreated control sera were similar, being 7750 and 6940 respectively. In contrast, the titres of the terminal components (C3–9) (Borsos & Rapp, 1967) were 41 and 194 respectively indicating activation of the 'alternate pathway' of complement. These experiments therefore suggest 'that *C. parvum* has the capacity, in human serum, of activating

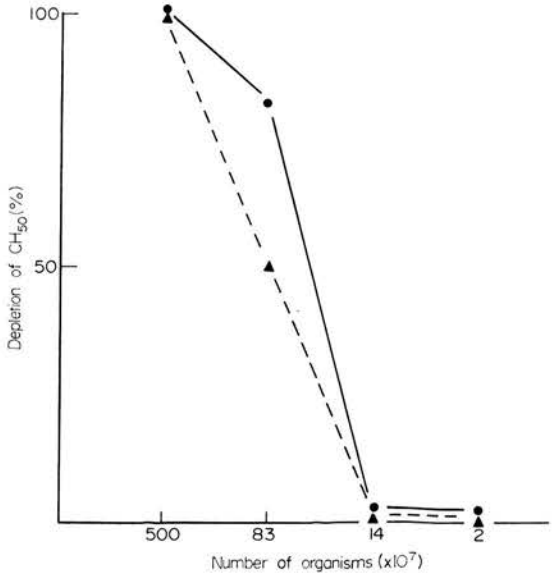

FIG. 1. Depletion of the CH_{50} of human (▲) and guinea-pig (●) serum by *C. parvum*. The CH_{50} was measured by the method of Mayer (1961). Each estimation was performed on a pool prepared from equal volumes of fifty human and twenty guinea-pig sera.

both pathways of complement'. These observations were further substantiated by incubating the human serum with the organisms in the presence of Mg^{++} EDTA, which permits 'alternate' (but not the Ca^{++}-dependent 'classical') pathway activation. The C3–9 titre of the serum that had been treated with *C. parvum* in the presence of Mg^{++} EDTA was <5 compared with the untreated control titre of 115, again indicating terminal component activation. Following immunoelectrophoresis using an antibody against the C3 proactivator (Hoechst Pharmaceuticals) the electrophoretic mobility of this protein had changed in the serum treated with *C. parvum* from the β to the γ region; an observation compatible with 'alternate pathway' activation (Götze & Muller-Eberhard, 1971). Thus the difference in the pathways of complement activation between the sera of man and guinea-pig is most likely due to the presence of 'natural antibodies' to *C. parvum* present in the human but not in the guinea-pig serum.

TABLE 1. The effect of *C. parvum* on the titre of the individual complement components C1, C4, C2 and C3 in human and guinea-pig serum

	Human serum		Guinea-pig serum	
	C. parvum treated	Control	*C. parvum* treated	Control
C1	11700	208000	22261	19321
C4	<5000	17021	9142	10240
C2	<50	1143	30567	35310
C3	305	2250	<50	1650

DISCUSSION

These results show that the immunological adjuvant *C. parvum* is capable of activating the alternate pathway of complement in both human and guinea-pig serum. The activation of the classical pathway in human serum is likely to be due to anti-*C. parvum* antibodies present in such sera.

It has been suggested that receptors for C3 on lymphoid cells may play a role in the antigen induction phase of the immune response (Pepys, 1972; Dukor *et al.*, 1974). A *C. parvum*-derived polysaccharide, bound to these cells (McBride *et al.*, 1974), and activating C3 may result in an amplification of the activity of these cells and also account for some of the biological effects of this agent. Whether or not the observed anti-tumour effect referred to above depends upon the presence of an intact complement system has yet to be determined. This study raises a number of issues relating to the biological activities of corynebacteria, some of which may be concerned in the initiation and amplification of cellular defence mechanisms. In addition this report points to a possible hazard in the use of *C. parvum* as a therapeutic agent, due to the formation of immune complexes with subsequent activation of the complement system.

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Chromatographic and Electrophoretic Properties of Synthetic Human Fibrinopeptides

From the University Department of Respiratory Diseases, City Hospital, Edinburgh, and the South-East Scotland Regional Blood Transfusion Service, Royal Infirmary of Edinburgh

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Summary

Some properties of synthetic human fibrinopeptides were studied by thin-layer chromatography, thin-layer electrophoresis and low voltage and high voltage paper electrophoresis. The Rf values and electrophoretic mobilities of the peptides in these systems were determined. In high voltage electrophoresis synthetic and natural (fibrinogen-derived) peptides migrated in an identical fashion.

When gel filtration was performed in 0.05 M pyridine or 0.1 N ammonia, synthetic fibrinopeptides A and B appeared to be aggregated. In contrast, when filtration was performed in 1.3 M formic acid, the peptides eluted in positions corresponding to their monomeric molecular weights.

In addition it was possible to quantitate synthetic fibrinopeptides by two colorimetric assays, the Sakaguchi reaction and the Folin-Ciocalteu method. Ultraviolet extinction coefficients for each peptide were also determined.

Introduction

In the final stages of blood coagulation, thrombin cleaves several small peptides from the N-terminus of the α- and β-chains of fibrinogen (Blombäck and Vestermark 1958). These peptides have been designated fibrinopeptides A, AP, AY and B and are recognised on the basis of their electrophoretic mobilities. These acidic peptides are associated with a number of biological activities which include the potentiation of bradykinin induced contraction of the oestrous rat uterus, prolonged rhythmic vaso-constriction and chemotaxis of leucocytes (Gladner et al. 1968; Colman et al. 1967; Kay et al. 1974).

Synthetic human fibrinopeptides have recently become available thereby providing preparations of high purity suitable for investigation of their physical and chemical properties. In the present study some chromatographic and electrophoretic properties of these synthetic peptides are reported. In one system (high voltage electrophoresis) their electrophoretic mobilities are compared with natural (fibrinogen-derived) peptides A and B.

Materials and Methods

Synthetic Fibrinopeptides

Synthetic fibrinopeptides A, B, and fibrinopeptide B (1–Glu), an analogue of fibrinopeptide B in which glutamic acid was substituted for pyrrolidone carboxylic acid at the

N-terminus, were obtained from Schwarz/Mann (Orangeburg, New York). Amino acid analyses of the fibrinopeptides in μ moles/mg were as follows: Fibrinopeptide A, Arg–0.72, Asp–1.50, Glu–1.50, Ser–0.62, Gly–3.64, Ala–1.79, Val–0.72, Leu–0.80, Phe–0.72; Fibrinopeptide B, Arg–0.53, Asp–1.50, Glu–1.67, Ser–0.50, Gly–1.03, Ala–0.53, Val–0.55, Phe–0.94; (1–Glu) Fibrinopeptide B, Arg–0.61, Asp–1.68, Glu–1.70, Ser–0.53, Gly–1.14, Ala–0.60, Val–0.59, Phe–1.11.

Human Fibrinogen-derived Fibrinopeptides

were prepared as previously described (Kay et al. 1974). Human fibrinogen, purified by the method of Blombäck and Blombäck (1956), was clotted with bovine thrombin (Parke-Davis, Detroit, Michigan). Following centrifugation of the clot, the resulting supernatant was precipitated with trichloroacetic acid. Fibrinopeptides A and B were then separated from the TCA-soluble fraction of the clot supernatant by high voltage electrophoresis in two dimensions (Kay et al. 1974).

Gel Filtration

Sephadex G–25 columns were prepared at 4° C with the following eluants: 0.05 M pyridine, pH 8.0; 0.1 M ammonia, pH 9.0 and 1.3 M formic acid, pH 2.0. Mixtures of blue dextran, vitamin B_{12} (M. W. 1357) and sodium chloride were first passed through the columns as markers and subsequently detected by spectrophotometric absorption and conductivity. Samples of 0.5 mg of each of the synthetic fibrinopeptides A and B were then chromatographed individually. For gel filtration of the (1–Glu) analogue of fibrinopeptide B, a G–75 column was prepared at room temperature with 0.05 M pyridine as the eluant. Following elution the peptides were detected in pyridine by the Folin-Ciocalteau method, in ammonia solution by absorption at 215 nm, and in formic acid by the Folin-Ciocalteau reaction of samples which had been evaporated to dryness and re-dissolved in PBS (phosphate buffered saline, pH 7.2, containing 6.80 g sodium chloride, 1.48 g disodium hydrogen orthophosphate and 0.43 g potassium dihydrogen orthophosphate per litre of distilled water).

Fibrinopeptide Detection and Quantitation

For quantitation of fibrinopeptides in solution two techniques were used: the Folin-Ciocalteau method (Legget-Bailey 1967) using human serum albumin for the standard curve and the Sakaguchi reaction (Shainoff and Page 1960) with arginine for the standard curve. Sakaguchi spray solutions were used to detect peptides following thin-layer chromatography and thin-layer or paper electrophoresis (Leggett-Bailey 1967).

Thin-layer and Paper Chromatography

Silica gel pre-coated aluminium sheets (Merck), 0.25 mm thick, and Whatman No. 1 filter paper were cut into 20 cm × 2 cm strips. Fifty μg of synthetic fibrinopeptides A and B, dissolved in 5 μl of 0.05 M pyridine, were applied at the origin. Arginine was also added as a standard. The strips were chromatographed at room temperature in an airtight tank saturated with one of the following solutions: n-propanol: water, 7:3 v/v (Blombäck et al. 1972): n-butanol:acetic acid:water, 2:2:1 or 2 – methoxyethanol:acetic acid: sec-butanol:water, 2:1:8:3. After five hours the sheets were dried at 85° C and sprayed with Sakaguchi reagents.

Thin-layer Electrophoresis

The peptides were applied to cellulose pre-coated TLC glass plates, 0.1 mm thick, 10 cm × 20 cm (Merck), as described above. Electrophoresis was performed at 31 V/cm for 30 minutes in either 4% v/v acetic acid and 1% v/v formic acid, pH 2.0 (Herzig et al. 1972) or pyridine: acetic acid: water, 1:30:89, pH 3.0.

Low Voltage Paper Electrophoresis

Samples were applied to Whatman No. 1 filter paper. A potential difference of 23 V/cm was maintained for 85 minutes using the pH 2.0 buffer as described for thin-layer electrophoresis.

High Voltage Paper Electrophoresis

Electrophoresis was performed in vertical tanks using the following buffers: pH 6.5, pyridine-acetic acid-water (25:1:225 by volume); pH 3.5, pyridine-acetic acid-water (1:10:8); pH 2.1 acetic acid-formic acid-water (35:10:355) (Ambler 1963).

For electrophoresis at pH 2.1, 50 to 100 μg of synthetic fibrinopeptides A and B or the natural (fibrinogen-derived) peptides, plus a mixture of ninhydrin-positive amino acid markers (Milstein and Milstein, 1968) were applied to Whatman No. 1 paper (57 cm × 16 cm). For electrophoresis at pH 3.5 and 6.5 Whatman No. 3 paper was used. In each tank a potential difference of approximately 60 V/cm was maintained for one hour, at which time the paper strips were dried at 45° C and sprayed with Sakaguchi reagents. The Sakaguchi-positive spots were marked with a lead pencil, and the paper was then immersed in 0.2% ninhydrin in acetone and dried at 60° C. Migration was measured from the centre of the taurine marker.

Results

Gel Filtration

When synthetic fibrinopeptides were chromatographed on G-25 Sephadex with three different solvents, different elution patterns were observed (Fig. 1). The K_d values of A and B respectively were 0 and 0 in 0.05 M pyridine, 0.09 and 0.18 in 0.1 M ammonia, and 0.32 and 0.54 in 1.3 M formic acid.

Thin-layer and Paper Chromatography

After chromatography on silica gel or paper strips for 5½–7 hours, the solvent front had moved 12–16 cm and Rf values were calculated as described in Table 1.

Table 1. The Rf values of synthetic fibrinopeptides A and B obtained by thin layer chromatography (TLC) and paper chromatography using various solvent systems.

System	Solvent	Rf Values A	Rf Values B	Arg
1. TLC	n-propanol:water, 7:3	0.20	0.16	—
2. TLC	n-butanol:acetic acid: water, 2:2:1	0.30	0.31	0.23
3. Paper chromatography	n-butanol:acetic acid: water, 2:2:1	0.86	0.90	0.67
4. TLC	2-methoxyethanol:acetic acid:sec-butanol:water, 2:1:8:3	0.14	0.14	0.08

Several techniques were investigated in attempting to improve the silica gel separation. When strips were first heated at 85° C for four hours before application of the sample, the determined Rf's of B and A in solvent 1 were 0.20 and 0.15. After a five-hour separation, silica gel strips were dried and re-run a second, third or fourth time, but this modification did not improve the separation.

Thin-layer Electrophoresis and Low Voltage Paper Electrophoresis

Electrophoretic migration of the peptides determined by these methods is shown in Table 2. Of the three systems the greatest separation of the peptide was achieved by paper electrophoresis at pH 2.0.

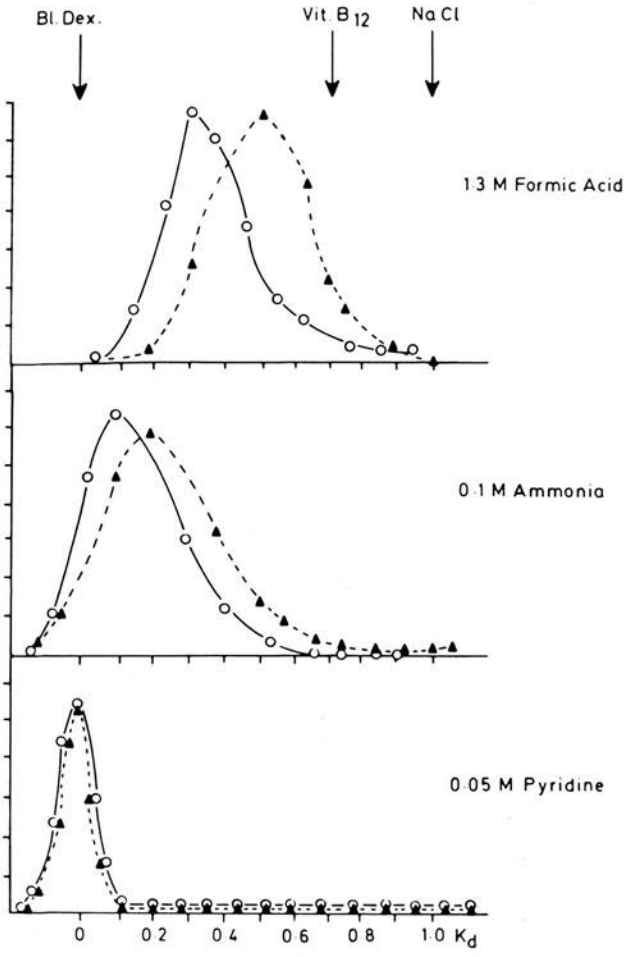

Fig. 1. Sephadex G–25 chromatography of synthetic fibrinopeptides A (O – O) and B (▲ – ▲) using three solvents. The K_d is shown on the abscissa and on the ordinate the optical density of the fractions measured either directly or after Folin-Ciocalteau assay. Each column was first calibrated with three markers. blue dextran (Bl. Dex.), vitamin B_{12} (Vit. B_{12}), and sodium chloride (NaCl).

High Voltage Paper Electrophoresis

As shown in Fig. 2 synthetic fibrinopeptides A and B migrated together at pH 6.5 but separated at pH 3.5 and pH 2.1. The separation at the lower pH was slightly greater. Natural (fibrinogen-derived) peptides migrated to identical positions as the synthetic peptides in each system.

Quantitation of Fibrinopeptides in Solution

Since accurate weighing of small amounts of the hygroscopic fibrinopeptides was difficult, three methods were used to determine the concentration of peptides in solution. Solutions of peptides A and B were first quantitated by the Sakaguchi

Table 2. The distance of migration of synthetic fibrinopeptides A and B by thin-layer and paper electrophoresis using different solvent systems.

System	Solvent	pH	Voltage	Time	Distance of migration	
					A	*B*
1. Paper electrophoresis	4% v/v acetic acid 1% formic acid	2.0	23 V/cm	85 min	9.0	5.4
2. Thin-layer electrophoresis	4% acetic acid 1% formic acid	2.0	31 V/cm	30 min	4.1	2.8
3. Thin-layer electrophoresis	pyridine:acetic acid:water, 1:30:89	3.0	31 V/cm	30 min	1.2	0

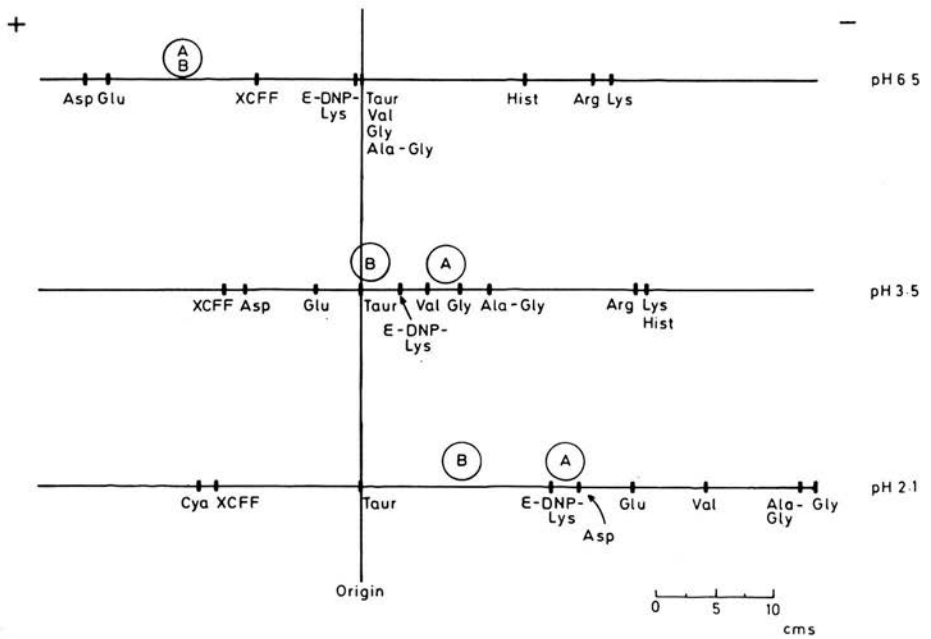

Fig. 2. High voltage electrophoresis of fibrinopeptides A and B at pH 6.5, 3.5 and 2.1. A, B, Cya, XCFF, Taur, ε-DNP-Lys, Asp, Glu, Val, Ala-Gly, Gly, Hist, Arg, Lys indicate respectively the positions reached by synthetic and natural fibrinopeptide A, synthetic and natural fibrinopeptide B, cysteic acid, xylene cyanol FF, taurine, epsilon-dinitrophenol lysine, aspartic acid, glutamic acid, valine, alanine-glycine, glycine, histidine, arginine, and lysine.

reaction using an arginine standard curve. Since one mole of each peptide contains one mole of arginine, equimolar solutions produced the same colour yield.

By the Folin method 1 μg of fibrinopeptide A or B, previously quantitated by the Sakaguchi reaction, gave the same optical density as 1.00 μg or 0.56 μg of human serum albumin respectively.

To determine the extinction coefficients, each peptide was dissolved in PBS and quantitated by the Sakaguchi reaction. The optical density of the quantitated solution

was then measured at 215 nm. From these measurements, $E^{1\%}_{215nm}$ values of 100 and 125 were calculated for peptides A and B respectively.

Discussion

In a previous study, Johnson and May (1969) reported that in water synthetic fibrinopeptide A eluted from Sephadex G–25 as the first major fraction. In the present work synthetic fibrinopeptides were filtered on Sephadex G–25 using three solvents each of which gave a different elution pattern. As shown in Fig. 1, fibrinopeptides A and B were excluded by G–25 Sephadex in 0.05 M pyridine. Since the gel is reported to exclude molecules of molecular weights greater than 5000 daltons, the peak at V_o may have resulted from aggregation of the peptides (molecular weights ca. 1500). In 0.1 N ammonia both peptides were retarded by the gel perhaps as dimeric aggregates. In 1.3 M formic acid, however, peptides A and B eluted with the same K_d values expected for the monomeric molecular weights of peptides which contain one (peptide A) and two (peptide B) aromatic residues. According to Ambler (personal communication) peptides may be retarded on Sephadex G–25 due to binding of aromatic groups by the gel. With pyridine, however, hydrophobic binding of aromatic groups to Sephadex G–25 may either be averted by saturation of the sites on the gel by the aromatic groups of the eluant, or possibly masked by aggregation of the peptides.

We have also shown that by gel filtration on Sephadex G–75 the [1–Glu] analogue of fibrinopeptide B eluted with the same K_d as a "marker" of molecular weight 6000 (Trasylol, Bayer, Germany) when 0.05 M pyridine was used as the solvent. This finding is consistent with tetramer aggregation under these conditions.

In the purification of fibrinopeptides this aggregating property can be exploited by desalting on G–25 in 0.05 M pyridine followed by subsequent G–25 filtration in 1.2 M formic acid.

Comparing a propanol solvent used by Blombäck et al. (1972) with several other thin-layer chromatography solvents, we found that the diluted propanol gave the best separation of A and B. When a butanol-acetic acid solvent was used for both silica gel and paper chromatography, a higher Rf value was found on paper chromatography, but the spots migrated less compactly showing some "tailing." In this latter system Johnson and May (1969) reported an Rf value of 0.75 for synthetic fibrinopeptide A. Differences in our results may be due to differences in temperature or chromatographic paper.

In the present study we obtained the best electrophoretic separation of fibrinopeptides A and B using a pH 2.0 solvent. This system proved more suitable for qualitative identification of fibrinopeptides A and B than thin-layer chromatography or thin-layer or paper electrophoresis. Natural (fibrinogen-derived) and synthetic fibrinopeptides A and B gave identical migration on high voltage electrophoresis. As described in a previous report, synthetic peptides can be used as markers in the purification of fibrinogen-derived peptides (Kay et al. 1974).

Three methods were standardized for the quantitation of fibrinopeptides in solution. When the sensitivities of these assays were compared, the Folin reaction produced the highest O.D. for peptide A whereas the absorbance of B was greatest when measured directly at 215 nm.

Résumé

On a étudié quelques propriétés des fibrinopeptides humains synthétiques par chromatographie et électrophorèse en couche mince, et par électrophorèse sur papier

à bas et haut voltage. On a déterminé les valeurs de Rf et la mobilité électrophorétique des peptides dans les différents systèmes. Au cours de l'électrophorèse à haut voltage les peptides synthétiques et naturels (dérivés du fibrinogène) migrent de façon identique.

Quand la filtration sur gel est faite dans la pyridine 0.005 M ou l'ammoniaque 0.1 N les fibrinopeptides synthétiques semblent être agrégés. Au contraire avec l'acide formique 1.3 M les peptides sont élués de façon correspondante à leur poids moléculaire de monomère.

De plus il est possible de déterminer quantitativement les fibrinopeptides synthétiques par deux méthodes colorimétriques, la réaction de Sakaguchi et la méthode de Folin-Ciocalteu. On a également déterminé les coefficients d'extinction dans l'ultraviolet pour les deux peptides.

Zusammenfassung

Es wurden einige Eigenschaften der synthetischen Menschenfibrinopeptide mit Hilfe der Dünnschichtchromatographie, der Dünnschichtelektrophorese, der Tief- und Hochspannungs-Papierelektrophorese studiert. Die Rf-Werte und die elektrophoretische Mobilität wurden in diesen Systemen bestimmt. In der Hochspannungselektrophorese bewegten sich die synthetischen und die natürlichen (aus Fibrinogen) Peptide in identischer Weise.

Wenn die Gelfiltration entweder 0.05 M Pyridin oder in 0.1 M Ammoniaklösung durchgeführt wurde, schienen die Fibrinopeptide A und B aggregiert zu sein. Hingegen wurden sie in 1.3 Ameisensäure als Peptidmonomer eluiert.

Zusätzlich war es möglich, die synthetischen Fibrinopeptide mit zwei kolorimetrischen Methoden, mit der Sakaguchi-Reaktion und mit der Folin-Ciocalteu-Methode, quantitativ zu messen. Der UV-Extinktionskoeffizient wurde für beide Peptide auch bestimmt.

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Passive Sensitization of Tissue Cells
III. A Primate Macrophage-Cytophilic Antibody

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Introduction

As a result of the original studies of BOYDEN and SORKIN [1960], our laboratory, and others, have devoted much effort to the study of macrophage-cytophilic antibody in guinea pigs and mice [see NELSON and BOYDEN ,1967; JONAS, GURNER, NELSON and COOMBS, 1965; and TIZARD, 1969]. Our main interest lies in the still unknown role of this antibody and the model it supplies for the phenomenon of passive sensitisation (allergisation) of tissue cells in general.

Another special interest for us, is the selectivity of uptake by the macrophage membrane of this type of antibody and the use of this reaction to characterise macrophage-like properties. A recent report by COULSON, GURNER and COOMBS [1967] has suggested that a percentage of guinea-pig lymphocytes undergoing 'transformation' may acquire such macrophage-like properties, as demonstrated by the ability of such cells to passively adsorb cytophilic antibody onto their cell membrane.

For various reasons, not the least being the already existing knowledge on the human immunoglobulins and the interest in having the fullest understanding of allergic reactions in man, we have attempted to produce this type of antibody in a primate – namely the baboon.

Materials and Methods

Baboons. All baboons (*Papio cynocephalus*) were aged between 6 and 12 months and weighed between 3 and 4 kg. They were maintained on a balanced vegetable diet with added vitamins and salts.

Preparation of cytophilic antisera. These were produced by injecting baboons with four-times washed red cells of the rabbit in Freund's complete adjuvant under the experimental schedules shown in table II and III. The animals were lightly sedated with 5 mg of Phenycyclidine (Parke Davis) before injection. The antisera were stored at –20° C.

Fractionation of antisera. The conditions for *Sephadex G 200* filtration and *DEAE cellulose chromatography* are described under section 4 of *Experimental Study.*

Procurement and storage of baboon and human macrophages. Baboon peritoneal macrophages: The peritoneal cavity of baboons which had had abdominal operations 7 days previously were washed out with 200 ml of heparinised Hanks solution. The operation involved the placing of various suturing material into the small bowel. A stimulus such as this was a necessary step in the procurement of macrophages in a sufficient quantity. The peritoneal cells were then centrifuged in siliconed glassware at 100 g for 5 min at 4° C and resuspended in Hanks solution. To an equal volume of the cell suspension was added, dropwise, an equal volume of 20% dimethyl sulphoxide at 4° C, the cell count having previously been adjusted to give a final concentration of 5×10^6 cells per ml. The cells were then cooled at 1° C/min using the Nagington cylinder [NAGINGTON and GREAVES, 1962] and stored at –180° C in liquid nitrogen. For use in the tests the cells were thawed quickly at 37° C and slowly diluted with Hanks solution to a final concentration of approximately 1,000 cells per mm^3.

Human peritoneal macrophages were obtained from human peritoneal washings obtained during the course of peritoneal dialysis in the treatment of renal failure. A balanced salt solution is introduced into the peritoneal cavity and removed after 30 min. The process is repeated hourly. Macrophages and neutrophils usually appear in the dialysate after the third day. The cells were centrifuged at 100 *g* for 5 min and resuspended in Hanks solution. The cells were stored in the same manner as described for baboon peritoneal cells.

Baboon alveolar macrophages were obtained by the method outlined by MYRVIK, LEAKE and FARISS [1961].

Baboon and human blood leucocyte monolayers were prepared in a similar manner to that described by JONAS *et al.* [1965].

Papain-treated rabbit red cells for cytophilic tests. Rabbit red cells were papain treated in essentially the same way as described by HAWES and COOMBS [1960].

Special antiglobulin reagents for experiment 5. Anti-γ (R4785), the two anti-α sera (R83 and R179) and the anti-μ (R199) were prepared as described by COOMBS, JONAS, LACHMANN and FEINSTEIN [1965].

Sheep 198 anti-IgA was raised against colostral IgA, purified by ion exchange chromatography and Sephadex G 200 filtration. It was made heavy chain specific by absorbing with human cord serum. This serum was kindly given to us by Dr. A. FEINSTEIN. The anti-human albumin serum was raised in a rabbit injected with crystallized human serum albumin (Mann Laboratories).

Procedure used for testing sera for antibodies cytophilic for macrophages and other cells. Both the monolayer technique the more sensitive 'suspension-centrifugation method' and the method of scoring the reaction were used as described by JONAS *et al.* [1965] in studies on guinea-pig macrophage-cytophilic antibody. Essentially, dilutions of antiserum were added to the macrophage suspension or monolayer. After incubation at room temperature the cells were washed. The rabbit red cells next added were previously treated with papain to render them less sticky to glass and to increase their agglutinability. In the monolayer test the red cell suspension was allowed to sediment on the serum-treated monolayer, while in the suspension-centrifugation method the red cells and serum-treated macrophage suspension were mixed and centrifuged together and the deposited cells subsequently mounted on a slide.

Experimental Study

1. Attempted Production in the Baboon of Macrophage-Cytophilic Antibody to Sheep Red Blood Cell Antigens

Attempts to stimulate and demonstrate cytophilic antibody directed against sheep red blood cells were unsuccessful since peritoneal and alveolar macrophages from three normal baboons without any treatment with serum, gathered sheep red cells in rosette formation around them. We therefore tested the ability of various species of red blood cells to form rosette reactions with baboon macrophages in the absence of cytophilic antibody and, as may be seen from table I, only sheep red cells acted in this way. This natural affinity reaction was greatly reduced by pre-treatment of the sheep red cells with papain but, even so, it was decided that this particular system was not a satisfactory model for further study. The titres of directly agglutinating antibody and sensitizing antibody (detectable by the antiglobulin test) in baboon sera against sheep red blood cells were only 5 and 10 respectively and since there were also rosettes around neutrophils it was considered unlikely that this was a naturally occurring cytophilic antibody.

It was subsequently found that sheep red cells had no natural affinity for human macrophages.

2. Stimulation of Macrophage Cytophilic Antibody in Baboons against Rabbit Red Cells

Rabbit red cells in a saline suspension have the disadvantage of being 'sticky' on glass and have a tendency to non-specific aggregation, but these difficulties are largely overcome by pretreatment of the washed cells with papain.

A total of 5 baboons were injected at different times with washed rabbit red cells emulsified in Freund's complete adjuvant according to the schedules given in tables II and III. The schedule followed that used successfully for the stimulation of macrophage-cytophilic antibody in the guinea pig [Jonas *et al.*, 1965]. The titre of cytophilic antibody produced is also given in these tables. It may be seen that very little cytophilic antibody was produced in these baboons without the intradermal booster injections of red cells given at multiple sites.

Table I. The reaction between red cells of various species and a monolayer of baboon macrophages. Only red cells of the sheep form rosettes around untreated macrophages

Red cells	Forming rosettes around untreated baboon macrophages
Baboon	–
Human	–
Sheep	++
Guinea pig	–
Rabbit	–
Ox	–
Fowl	–

Table II. Injection schedule and titre of macrophage-cytophilic antibody to rabbit red cells produced in baboon (Willis)

Day	Treatment	Titre cytophilic antibody 5	20	100	500	dil
0	bled and 1st FCA injection[1]	–	–			
9	bled	NT				
15	bled and booster i.d. injection[2]	NT				
22	bled	+++[3]	+++	++	+	–
30	bled	+++	++	+	–	–
68	bled and 2nd FCA injection[1]	++	+	w	–	–
68 + 9	bled	+++	++	+	+	–
68 + 28	bled	+++	+++	++	+	–
68 + 36	bled	+++	+++	++	w	–

[1] 2 ml 30% suspension rabbit red cells emulsified with equal volume of Freund's Complete Adjuvant and injected intramuscularly.

[2] 0.2 ml of a 30% suspension of washed rabbit red cells in saline were injected intradermally into 6 sites on the anterior abdominal wall.

[3] +++, ++, +, w, strength of rosette formation, – negative, N.T. = not tested.

3. Selectivity as Regards Cell Type onto Which This Cytophilic Antibody Is Passively Adsorbed

The preliminary tests with these antisera were done on monolayers of macrophages obtained from the peritoneal cavity of baboons seven days following laparotomy for other purposes. Fixation and staining with Leishman's stain of the rosettes showed the involved cell to be the

Table III. The effect of the 17th day intradermal booster injection on stimulation of macrophage-cytophilic antibody

Baboon	Day	Treatment	Titre of cytophilic antibody 5	20	100	500	dil
Humphrey	0	bled and 1st FCA injection[1]	–	–			
	10	bled	–	–			
	17	bled					
	25	bled	+[3]	w	–	–	–
Joe	0	bled and 1st FCA injection	–				
	10	bled	+	–			
	17	bled	++	–			
	25	bled	+	–			
Spike	0	bled and 1st FCA injection	–				
	10	bled	–	–			
	17	bled and booster i.d. injection[2]	+++	++	–	–	
	25	bled	+++	+++	+	+	–
Jook	0	bled and 1st FCA injection	–				
	10	bled	–	–			
	17	bled and booster i.d. injection[2]					
	25	bled	+++	++	+	–	–

[1] 2 ml 30% suspension rabbit red cells emulsified with equal volume of Freund's Complete Adjuvant and injected intramuscularly.

[2] 0.2 ml of a 30% suspension of washed rabbit red cells in saline were injected intradermally in to 6 sites on the anterior abdominal wall.

[3] +++, ++, +, w, strength of rosette formation, – negative.

macrophage. The cytophilic antibody was also adsorbed on to alveolar macrophages. Using monolayers of blood leucocytes prepared by the method described by JONAS *et al.* [1965] it was clear that although the antibody was passively adsorbed by blood monocytes it was not taken up by polymorphonuclear cells. Using the suspension-centrifugation technique no reaction was obtained on lymphocytes or eosinophils or polymorphonuclear leucocytes. These findings are similar to those found in the guinea pig [JONAS *et al.*, 1965] and in the mouse [TIZARD, 1969] using macrophage-cytophilic antibody produced in the respective species.

These baboon cytophilic antibodies had a similar pattern of reactivity on human cells. Both polymorphonuclear leucocytes and

Table IV. Distribution of cytophilic activity following Sephadex G 200 filtration of antiserum

		Sephadex G 200 I	II	III
Immuno-electrophoresis[1]	IgG	–	+++	(+)
	IgA	–	+	–
	IgM	+	–	–
Micro-Ouchterlony[2]	IgG	–	+++	++
	IgA	–	++	–
	IgM	++	–	–
Cytophilic antibody titre[3]		0	500	(5)

[1] Using polyvalent anti-human serum in the troughs and an anti-α serum.
[2] Using specific anti-human immunoglobulin sera (see Methods).
[3] Titre of unfractionated serum 500.

macrophages were obtained from peritoneal washing during dialysis; only the macrophages reacted. These macrophages were also found to be adequate in 'monolayer' or 'suspension-centrifugation' tests after storage at –180° C in 10% dimethyl sulphoxide.

4. Physical Characteristics of Baboon Macrophage-Cytophilic Antibody

a) Filtration through Sephadex G 200. 2.5 ml of a baboon serum with a cytophilic antibody titre of 500 was added to a Sephadex G 200 column (2.5 × 94 cm) which was equilibrated with 0.1 M Tris and 0.5 M NaCl pH 7.2. Two further peaks followed the exclusion peak. Cuts were made to separate the material under each peak.

After concentration to the original volume fractions 1, 2 and 3 were tested by immuno-electrophoresis and in micro-Ouchterlony plates. The location of IgG, IgA and IgM was as shown in table IV. The cytophilic antibody was located in the second peak (table IV) suggesting that cytophilic antibody resided in either IgG or IgA.

b) DEAE chromatography. 4.0 ml of the cytophilic antiserum was dialysed against 0.0175 M phosphate buffer, pH 7.2, and added to an equilibrated DEAE cellulose column (1.43 × 15 cm). Following the exclusion peak a step elution followed with 0.2 M phosphate buffer.

Table V. Distribution of cytophilic activity following DEAE chromatography of antiserum

		DEAE cellulose I	II
Immuno-electrophoresis[1]	IgG	+++ slow	++ fast
	IgA	–	+
	IgM	–	+
Micro-Ouchterlony[2]	IgG	+++	++
	IgA	–	++
	IgM	–	++
Cytophilic antibody titre[3]		500	100

[1] Using polyvalent anti-human serum in the troughs and an anti-α serum.
[2] Using specific anti-human immunoglobulin sera.
[3] Titre of unfractionated serum 500.

The eluates under the two peaks were pooled and concentrated to the original volume to give fractions I and II. Testing these fractions by immuno-electrophoresis and in micro-Ouchterlony tests (table V) showed the cytophilic activity to be fairly evenly divided between fractions I and II the titre being 500 and 100 respectively. These results taken in conjunction with those on the Sephadex fractions suggest that macrophage-cytophilic antibody is to be associated with the 7S IgG antibodies.

c) Treatment with 2-mercapto-ethanol. Treatment of the DEAE exclusion fraction with 0.1 M 2-mercapto-ethanol left the macrophage-cytophilic antibody unaffected.

The antibody was also heat-stable to heating at 56° C for ½ h.

d) Digestion of Fc piece by pepsin. The DEAE '7S' fraction of cytophilic baboon serum was digested with pepsin according to the method of NISONOFF, WISSLER and WOERNLEY [1959].

After treatment the $F(ab)^2$ antibody fraction agglutinated papain-treated rabbit red cells to a titre of 1,280 ($>$ 2,560 by the antiglobulin test on untreated rabbit red cells) as compared with 2,560 and $>$ 2,560 respectively using the undigested antibody. This indicated very little reduction in the agglutinating activity of the $F(ab)^2$ antibody. However the pepsin-treated antibody had no cytophilic activity in either the monolayer or in the more sensitive 'suspension-centrifugation technique' using dilutions of $^1/_{10}$ and $^1/_{100}$ at which the untreated anti-

Table VI. Attempt to inhibit uptake of baboon macrophage-cytophilic antibody by specific anti-human immunoglobulin sera

Monolayer incubated with class-specific anti-immunoglobulin sera			Subsequent rosette formation indicating uptake on cell membrane of cytophilic antibody
Hanks			+
Normal rabbit serum	1:40		+
R4785 anti-IgG, not abs.	1:40		(+)
R216 polyvalent	1:40		−
R4785 anti-γ	1:40		+
R179 anti-α^2	1:40		−
R179 anti-α^2	1:160		−
R83 anti-α^3	1:40	Cytophilic antiserum added diluted either $^1/_{50}$ or $^1/_{250}$[1]	−
R83 anti-α^3	1:160		−
Sheep 198 anti-α^4	1:40		+
R199 anti-μ	1:40		+
R × 516 anti-δ[5]	1:40		+
R anti-ε[6]	1:40		+
R anti-human albumin	1:40		+

[1] $^1/_{250}$ represented 2 doses of cytophilic antibody, $^1/_{50}$ = 10 doses.
[2 3 4] See 'Materials'.
[5] Anti-δ was a gift from Dr. D. ROWE.
[6] Anti-ε was a gift from Dr. S. G. O. JOHANSSON.

body produced a strong reaction. It thus seems that as with guinea pig macrophage-cytophilic antibody [BERKEN and BENACERRAF, 1966] and mouse cytophilic antibody (TIZARD, 1969] the cytophilia depends on an intact Fc piece.

5. Attempts to Identify the Immunoglobulin Class of Baboon Cytophilic Antibody by Specific Inhibition Studies

After formation of monolayers of baboon peritoneal cavity macrophages the cells were washed and specific antiglobulin sera added to the chambers as shown in table VI. Diluted cytophilic antiserum was then added and following incubation the cells washed and tested for uptake of cytophilic antibody by the usual procedure. Table VI shows

the pattern of reactivity which summarises the results of 5 experiments each with differing doses of the various reagents.

Should cytophilic antibody be one of the common immunoglobulins it is probable that the doses of antiglobulin used would not have been sufficient to neutralize the activity. However a neutralizing effect was produced consistently by one polyvalent serum and by two supposedly class-specific anti-heavy chain reagents, both anti-*a* sera. Should these inhibitory effects recorded reflect neutralization of the operative immunoglobulin it would seem that the antibody is not 'common' IgG not IgM, IgD or IgE.

The inhibitory results with the two anti-*a* reagents (R179 and R83) pointed to IgA but no inhibition was produced by the sheep 198 anti-*a* reagent. This latter serum was raised against ion exchange and sephadex purified colostral IgA while R179 was raised against whole colostrum and R83 which was raised against myeloma protein in urine. This could suggest that the inhibition was not due to anti-*a* heavy chain but to some other minor immunoglobulin.

A further piece of evidence that cytophilic antibody is not IgA came from an experiment in which normal human serum and human colostrum (as a source of IgA) was added to a macrophage monolayer to complete with cytophilic antibody for receptor sites on the macrophages [see Jonas *et al.*, 1965]. Colostrum inhibited very poorly while normal human serum gave inhibition. A human serum (Ro) known to be naturally free of IgA gave especially good inhibition.

No firm conclusions as to immunoglobulin class of primate macrophage-cytophilic antibody can be drawn from these experiments but they rather indicate that baboon macrophage-cytophilic antibody is not IgM IgD or IgE, but is a minor immunoglobulin residing within the 7S IgG fraction. It must be remembered however the antibodies were raised not in man, but in the baboon and the specific antiglobulin reagents were against human immunoglobulins.

Discussion

We have mentioned in the introduction that there are many reasons to encourage a search for macrophage-cytophilic antibody in man. There are however many difficulties, not the least being that Freund's complete adjuvant may be needed to produce it experimentally. This

made us use another primate, namely the baboon where this can be done. Fortunately, as with reaginic antibody where human antibody reacts in baboon skin, the baboon macrophage-cytophilic antibody reacts on human macrophages. This interchangeability of cells and reagents within the primate species has made the present investigation possible. Even so the system is not an easy one to work with. Neither human nor baboon macrophages are easy to obtain fresh or on demand, but fortunately we found that a source may be preserved frozen at –180° C in 10% dimethyl sulphoxide, but such cells have not quite the 'viability' of freshly procured cells. Another difficulty of course is that, despite this interchangeability, baboon immunoglobulins may differ considerably and in many details from their human counterparts.

We would now wish to see if man produced this type of cytophilic antibody under natural conditions but the system to test is not obvious. An antibody with such a distinctive property may well form a distinct sub-class or even class of immunoglobulin and its study could make a physico-chemical definition possible.

We have already made use of the antibody in an analytical sense to reveal changes in the membrane of human lymphocytes undergoing antigen-induced 'transformation'. A percentage of the cells acquire macrophage-like properties in that they stick to glass, engage in phagocytosis and passively take up baboon macrophage-cytophilic antibody [COOMBS, GURNER, CHALMERS and Kay, 1969].

The observed natural affinity of sheep red cells to baboon macrophages (but not to human macrophages as subsequently found) precluded using sheep red cells and their membrane antigens in these tests. Annoying as this was, this 'affinity' reaction is an interesting phenomenon. Other examples of this type of reaction have been discussed by COOMBS and FRANKS [1969].

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Summary

1. Injection of rabbit red cells into baboons has been successful in stimulating macrophage-cytophilic antibodies. These passively adsorb selectively on to both baboon and human cells of the macrophage/monocyte series.

2. The macrophage-cytophilic antibody resists heating at 56° C for ½ h, resists the action of 0.1 M 2-mercapto-ethanol, but loses its cytophilic activity on pepsin-treatment.

3. The immunoglobulin class of the antibody is not yet ascertained but, from preliminary filtration, chromatographic and biological inhibition studies, there is a suggestion that it may not belong to any of the existing classes although it is closely associated with '7S' IgG.

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11

Some Complications Associated with the Administration of Blood and Blood Products

A. B. KAY

Although it is difficult to assess the frequency of the complications of blood transfusions the ever increasing demand for blood and blood products has made associated adverse effects following administration of these agents one of the more common clinical problems. The transmission of hepatitis B virus remains the most serious complication, and this is dealt with elsewhere as are the effects of massive transfusion. This chapter deals largely with immunological aspects of blood transfusion with particular consideration of the mechanisms leading to the clinical manifestations of adverse clinical effects.

The understanding of the various blood group systems in man and the technology for grouping and matching compatible red cells for transfusion have made erythrocyte associated transfusion reactions a relatively rare event. During 1974 the Blood Transfusion Laboratories of the Royal Infirmary of Edinburgh supplied 57 462 units of whole blood or red cell concentrates to 16 815 individuals (Table 1). Signs and symptoms which were *probably* a

Table 1. *The incidence of transfusion reactions (Blood Transfusion Laboratories, Royal Infirmary of Edinburgh, 1974)*

	Number
Patients receiving whole blood or red cell concentrates	16 815
No. of units transfused	36 424
Transfusion reactions	114
Associated with red cell antibodies	1
Associated with leucocyte or plasma protein antibodies	27
No cause found	86

result of these infusions developed in 114 patients (0.68 per cent). Only one recipient had a reaction which was clearly attributable to red cell incompatibility. Apart from this patient, leucocyte or plasma protein antibodies were found in 27 persons who had allegedly reacted, but in the remaining 86 individuals laboratory tests were unhelpful in the investigation of the cause

Table 2. *Antibodies to immunoglobulin determinants and leucocyte antigens found in association with adverse transfusion reactions (Blood Transfusion Laboratories, Royal Infirmary of Edinburgh, 1974)*

Immunoglobulin antibodies	No.	Leucocyte antibodies (anti HL-A)	No.
Gm 1	2	HL-A 1	2
Gm 4 + 17	1	HL-A 2	3
Gm 4	1	HL-A 2 + 9	1
Gm 5	1	HL-A 7	1
Gm 25	1	HL-A 12	2
Inv 1	2	Polyspecific anti HL-A	4
Polyspecific anti-Gm	5	Polyspecific anti HL-A + Polyspecific anti-Gm	1

(Tables 1 and 2). A considerable degree of sophistication has been achieved by the red cell serologists in the grouping and cross-matching of erythrocytes for transfusion, but this technology is not yet available on a practical routine basis for the detection of antibodies or specifically sensitised cells present in donor or recipient blood which are directed against antigens on granulocytes, platelets or plasma proteins. It should be emphasised that the evidence for attributing reactions to the infusion of blood or blood products is often largely conjectural. Although it is reasonable to assume that features such as fever, rigors and urticaria are a direct result of transfusion if they appear during or after the administration of blood it is usually impossible to prove this beyond peradventure.

A number of factors determine the response of a particular individual to an antigenic stimulus. These include the amount of antigen administered and the activity of various regulatory systems which determine the degree of the 'immune response'. Thus the existence of specific immune response (IR) genes to specific antigens or groups of antigens has been described and shown to be linked to the major histocompatibility systems in several animal species (reviewed by McDevitt and Bodmer, 1974). Also of relevance in this context is the recognition of the suppressive effect of certain populations of T lymphocytes in terms of their effect on the production of specific antibody or specifically sensitised cells. There are therefore many complex interactions which finally determine the response of an individual to various antigens. These various regulatory mechanisms are dynamic and so the contributions of the biological systems to the final expression of the immune response will vary in any one individual.

THE RED CELL AND TRANSFUSION REACTIONS

As stated the major obstacles to the successful transfusion of blood and its products have been overcome by the pioneering work of the red cell serologists. Indeed the situation is such that the majority of red cell associated transfusion reactions are usually a result of technical or clerical errors. All blood transfusion centres, therefore, must have procedures which ensure that the correct donor blood is delivered to the appropriate recipients. Physicians and surgeons

have a special responsibility in ensuring that the samples of blood sent for grouping and cross-matching are fully and correctly labelled.

The precise mechanism by which the administration of incompatible red cells are destroyed in vivo and in turn evoke adverse reactions is not fully appreciated. The participation of various enzyme cascades such as complement (C) probably play a central role as does red cell destruction by mononuclear cells both in the circulation and in organs such as the liver and spleen.

The term complement (or alexin) was introduced about 70 years ago to describe an activity present in normal serum (normal in the sense of not being 'immunised') which led to the lysis of red cells or bacteria previously sensitised with specific antibody. It soon became apparent that this 'activity' was not the property of a single factor but of several components most of which are now available in highly purified forms. A certain amount is now known about their molecular structure. In many respects the complement system is similar to the blood coagulation pathway in which components become activated and depleted in turn. Like the coagulation pathway the complement system has a number of inactivators and inhibitors which act at various stages during the reaction sequence thereby maintaining homeostasis. The order in which components of the 'classical pathway' of complement act is different from the order in which they were discovered. A red cell sensitised with the appropriate antibody will activate C1 and the order of reaction thereafter is C4, C2, C3, C5, C6, C7 with lysis being brought about by the terminal components C8 and C9. When an antigen–antibody complex is incubated with a source of complement the reaction sequence will proceed in a similar fashion with activation and depletion of the components in turn (reviewed by Ruddy, Gigli and Austen, 1972).

As a result of complement activation a number of factors are released which have biological activity. These include chemotactic agents and peptides which release histamine (and other pharmacological agents) directly from mast cells (e.g., anaphylatoxins).

Most of our knowledge of the complement system in terms of the identification and chemical characterisation of the individual components, the nature of the biological agents released during the reaction sequence and the nature of the interactions of the components has been gained from a standard in vitro model using sheep red blood cells, a source of sensitising antibody (haemolysin usually prepared in the horse or rabbit) and complement from various species. As discussed below extrapolation of this model using animal reagents to gain insight into the human complement cascade has led to a number of erroneous conclusions and a certain amount of confusion.

As stated, the classical pathway* of complement (C) activation involves the

* The term 'classical pathway' is used to distinguish this reaction sequence from the 'alternate pathway'. The alternate pathway of complement activation activates C3 and the terminal components thereafter with apparent sparing of C1, C4 and C2. The components of the alternate pathway include properdin, factor B (also termed the C3 proactivator or glycine-rich β-glycoprotein) and other activating enzymes. Their reaction sequence is not fully defined and since there is no direct evidence at present that the alternate pathway participates in the transfusion reactions considered in this chapter it will not be discussed further. Readers are referred to a review by Müller-Eberhard (1974) for a full account of the patterns of complement activation.

sequential interaction of at least nine plasma proteins (Figure 1). An erythrocyte (E) when coated with the appropriate antibody (A) activates the first component (C1). In general antibodies capable of activating C1 are IgG_1, IgG_3 and IgM. IgG_2 is relatively inefficient in activating C1 whereas IgG_4, IgA, IgD and IgE do not 'fix' complement via the classical pathway. In the human

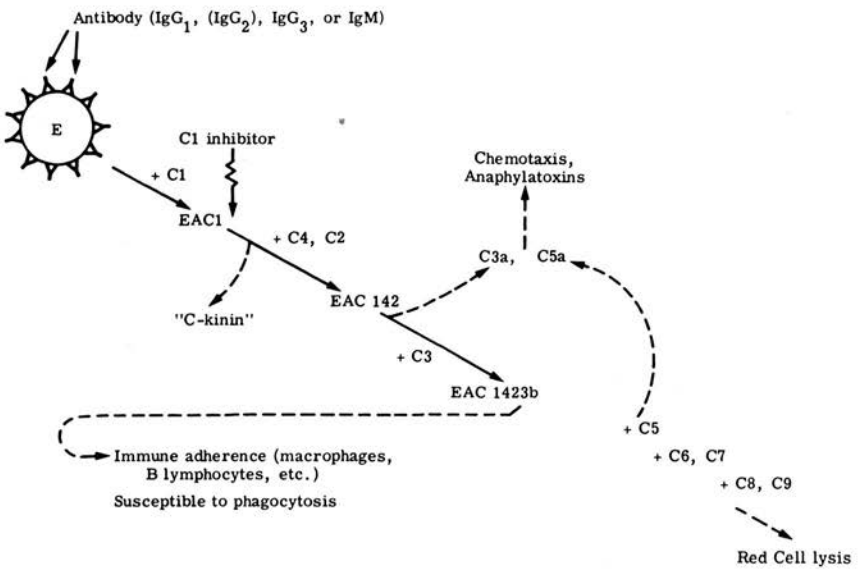


Figure 1. The classical pathway of complement activation. Associated biological properties are indicated by the interrupted lines.

situation there are exceptions to this general rule as discussed below. Following the action of C1 on its natural substrates C4 and C2 an EAC 142 enzymatic intermediate is formed which then activates C3. Activated C3 acts on C5 leading to consumption of the terminal components C6, C7, C8 and C9. The action of C8 and C9 on the red cell membrane leads to lysis and release of intracellular constituents. Following the interaction of C1, C4 and C2 a small 'kinin-like vasoactive peptide' is released which evokes tissue swelling. The agent is thought to be of importance in the pathogenesis of the hereditary form of angio-oedema in which there is an absence of the natural inhibitor of C1 (C1 inh). Similarly two peptides are released from C3 and C5 (C3a and C5a). These are both chemotactic for leucocytes and are anaphylatoxins. C3 in its activated form on the cell membrane is termed C3b and has the capacity of adhering to various cells including B lymphocytes and macrophages. C3b also renders cells highly susceptible to phagocytosis. Thus cells which escape extravascularly in the form EAC 1423b presumably adhere to macrophages of the reticuloendothelial (RE) system where they are engulfed and destroyed. C3b is extremely sensitive to the action of the C3b inactivator and can cleave this molecule into C3c (containing the A antigen) and C3d (containing the D

antigen*, also termed α_2d) (Figure 2). C3d remains on the cell and is inactive both in adherence and in its action on C5 and subsequent completion of the 'haemolytic sequence'. Macrophages apparently have receptors for the A and D antigens present on C3b but not for the D antigen alone. These mononuclear cells also have receptors for IgG_1 and IgG_3 but not for other IgG subclasses or IgM. The interaction of antibody-coated erythrocyte with cells of the macrophage/monocyte series apparently results in either phagocytosis or, where the

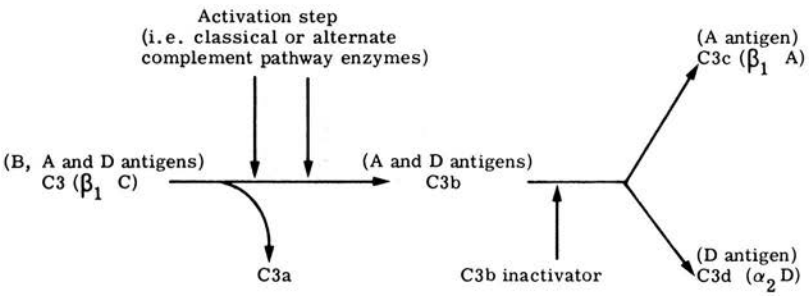


Figure 2. The antigens and electrophoretic mobilities of C3 and its breakdown products. During the conversion of C3 to C3b the B antigen is lost but this antigenic determinant is apparently not on the C3a fragment cleaved during the reaction.

contact is more transient, the fragmentation of red cells with the appearance of spherocytes and fragmentation of red cells. Spherocytes are particularly sensitive to osmotic lysis which takes place either extravascularly or in the circulation.

In general human red cell antibodies which are 'haemolytic' in vitro are associated with immediate intravascular destruction of red cells in vivo. These antibodies have the capacity to lyse human cells (containing the corresponding antigen) in the presence of a source of human complement and therefore presumably activate C1. Antibodies which do not 'fix complement' or are only slowly haemolytic in vitro are associated with extravascular haemolysis which occurs mainly in the spleen.

The rapid intravascular reactions associated with, for example, anti-A and anti-B red cell antibodies are probably largely complement mediated. The clinical features produced such as pain, fever and shock may be a result of the liberation of vasoactive peptides ('C kinin', C3a and C5a) in addition to the rapid release of haemoglobin. In these situations there is usually some degree of extravascular destruction and it is assumed that EAC 1423b cells can be removed by erythrophagocytosis before the C3b inactivator can act. A similar mode of action may be operative in the extravascular destruction of cells coated with anti-Le^a and Le^b in which the antibody is usually IgM and haemolysis in vitro is slow.

One molecule of IgM or two adjacent molecules of IgG_1 or IgG_3 are capable of activating the complement cascade. It has been stated that if the

* The terms A and D antigens are unfortunate for the red cell serologist since they are terms used both for antigens on C3 and antigens of the ABO and Rhesus (Rh) blood group systems.

antigenic sites on the red cell are too sparse, such as in the Rh system where the antibodies are IgG_1 or IgG_3, C1 binding cannot occur (Rosse, 1968). This is unlikely to be the whole explanation for 'non-complement fixation' by anti-Rh antibodies. A rare variant of the Rh system (Ripley antibody) is a potent 'haemolytic' antibody. In practice destruction of cells coated with anti-D, -E, -e, -C and -c takes place mainly in the spleen presumably by RE macrophages containing IgG receptors.

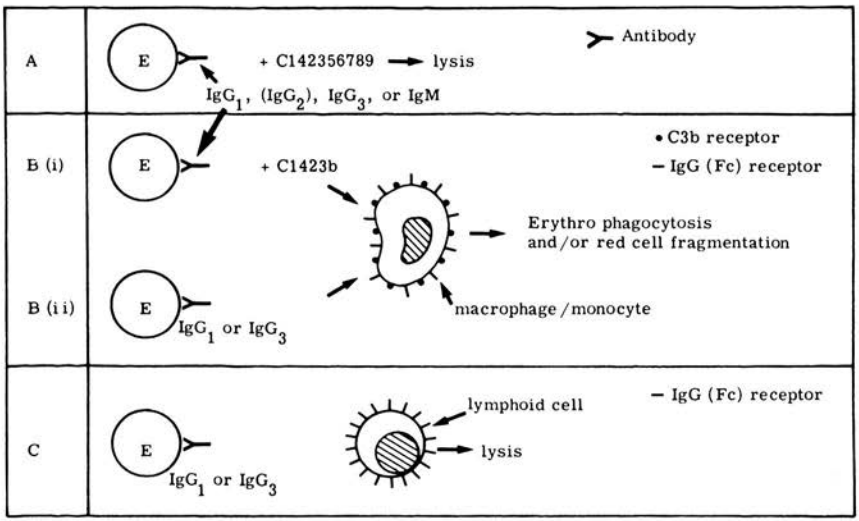
There are obviously a number of factors that determine the rate and site of destruction of antibody-coated red cells. These include the optimal temperature of antibody binding, the class or subclass, the amount of incompatible cells infused, the distribution of antigens and the relative amounts of circulating complement components and their cofactors.

Another form of complement independent red cell lysis which may be of importance in the destruction of sensitised human red cells is the phenomenon of lymphoid cell-mediated antibody-dependent cytotoxicity (LDAC). This is apparently distinct from thymus-dependent T lymphocyte-mediated cytotoxicity with its requirement for specifically sensitised effector cells. In this respect LDAC is 'nonspecific' and appears to be the property of an as yet unidentified subpopulation of lymphocytes which recognise and destroy target cells coated with IgG. Urbaniak, S. J. (personal communication) has recently shown that human peripheral blood lymphoid cells have the capacity to lyse CDe/CDe cells coated with anti-C plus anti-D. Thus LDAC (sometimes called K-cell cytotoxicity) may play a role in human red cell destruction in vivo. These various proposed mechanisms of red cell damage are diagramatically summarised in Figure 3.

THE LEUCOCYTE AND TRANSFUSION REACTIONS

Following pregnancy or the administration of blood products containing white cells many individuals will show evidence of sensitisation to allogeneic leucocytes. Because of the complexity of the leucocyte antigen systems and individual variations in the immune response (see above) the exact incidence of sensitisation both quantitatively and qualitatively is extremely difficult to assess. Chromosomes of leucocytes have an area referred to as the major histocompatibility complex which is made up of at least four separate regions (two defined serologically (SD) and two defined by mixed lymphocyte culture (MLR) techniques (LD)). The SD system has been termed traditionally the human leucocyte (HL-A) antigenic system and is an extremely polymorphic genetic system controlled by two closely linked segregant series of genes. The first (also called LA series) and second segregant series (also called Four) are inherited by autosomal mendelian inheritance. HL-A antigens (or antibodies) are usually detected by a simple cytotoxic technique which depends on the capacity of separated human lymphocytes to undergo lysis by the appropriate antibody in the presence of a source of rabbit complement. Following a transfusion or pregnancy, individuals may produce HL-A antibodies (lymphocytotoxins and leucoagglutinins) not present on their own leucocytes (or other cells such as platelets which also contain HL-A antigens). The original

technique for detecting HL-A antibodies was by leucoagglutination. Some authorities find leucoagglutination, as a method for detecting anti-leucocyte antibodies following transfusion, to be more sensitive than the lymphocytotoxic test (McCullough et al, 1974).



A - Complement mediated with completion of the haemolytic pathway
B (i) - C3b coated cells and macrophages
B (ii)- IgG coated cells and macrophages
C - Lymphoid cell dependent, antibody mediated cytotoxicity (K-cell cytolysis)

Figure 3. Some immune mechanisms of red cell (E) destruction.

It is now apparent that these leucocyte antigenic systems are even more complex as it has come to be appreciated that some individuals can be immunised to antigens specific for the neutrophil. Neutropenia in association with isoimmune neonatal sensitisation or following transfusions has been reported and the mode of inheritance and nomenclature of various neutrophil antigens is described (Lalezari and Radel, 1974).

Whereas a mixed lymphocyte reaction can be used for determining the genetics of the LD loci and for determining compatibility, or otherwise, between donor and recipient prior to grafting, the technique cannot be used retrospectively for identifying lymphocytes specifically sensitised against leucocyte antigens. One approach to this problem is by the use of a cytotoxic assay in which recipient lymphocytes are incubated for between four and 24 hours with ^{51}Cr labelled allogeneic lymphocytes, lysis being assessed by measuring chromium release.

Generally speaking transfusion reactions associated with granulocyte incompatibilities are mild and can often be minimised or prevented either by the use of corticosteroids and antihistamines or by giving washed or frozen and thawed red cells. Whereas the presence of leucocyte antibodies in recipients requiring red cells is usually of little clinical importance in the majority

of patients it has serious implications in those individuals awaiting an organ transplant, bone marrow transplant or granulocyte therapy. In order to minimise sensitisation by leucocyte antigens it is common practice to give thawed and frozen red cells to patients on chronic dialysis awaiting a kidney graft. In these individuals the administration of frozen and thawed red cells poor in leucocytes greatly minimises the risk of developing lymphocytotoxic antibodies (Miller et al, 1975). However, white cell-poor blood usually contains an appreciable amount of leucocyte antigenic material (Engelfreit et al, 1975). When frozen and thawed red cells were injected into rabbits, they produced both anti-lymphocyte and anti-granulocyte antibodies. However, in the clinical situation there is apparently considerable variability in the capacity of individual patients to form lymphocytotoxic antibodies against various preparations of leucocyte-poor blood (Perkins et al, 1974). Methods of rendering 'erythrocyte-rich' blood as free as possible from viable leucocytes or their fragments by filtration procedures is discussed elsewhere (Chapter 3).

It has recently been stated that patients on chronic dialysis receiving frozen and thawed red cells have a *higher* rate of subsequent graft rejection than those receiving whole blood (Opelz and Terasaki, 1974). This was attributable to a state of immunological tolerance to white cell antigens in those individuals receiving whole blood. It has also been suggested that HL-A antigens present in partially purified albumin solution may also produce tolerance (Pattison, Hindman and Maynard, 1974). These observations will require long-term ratification and substantiation before a unified policy is introduced in terms of the most beneficial blood products for patients awaiting organ transplantation. It does however raise a number of questions in regard to the efficacy and economics of the use of special blood products in these patients. It must be emphasised nevertheless that the use of frozen and thawed red cells greatly reduces the risk of transmitting hepatitis B infection and therefore most centres having these facilities will probably continue to use frozen and thawed cells in patients receiving long-term immunosuppressive therapy.

In renal allograft recipients there is a marked variation in the production of leucocyte cytotoxic antibodies. Presumably the response of the individual patient is governed by the same general immunological principles as outlined above.

A less common transfusion complication found in association with circulating leucoagglutinins is a syndrome characterised by pulmonary infiltrates and the abrupt onset of fever, tachycardia, cough and dyspnoea (Ward, Lipscomb and Cawley, 1968). This reaction is distinguished from the 'post-perfusion lung' syndrome where hypoxia is the prominent feature and which is recognised in patients undergoing cardiac bypass surgery and thought to be a result of the occlusion of small pulmonary vessels by microaggregates (Nahas et al, 1965a, b).

Particular care must be taken with the use of blood in immunosuppressed individuals because of the potential hazard of a graft vs host reaction. Even after 22 days of storage at 4°C viable dividing mononuclear cells have been detected in whole blood (Petrakis and Politis, 1962). Blood which has been washed and the red cells frozen and thawed still contains a number of lymphocytes capable of responding in mitosis in the presence of phytohaemagglutinin

(Crowley, Skrabut and Valeri, 1974). Transfusions of fresh blood to the human fetus and newborn infant also carry the risk of evoking a graft vs host reaction (Parkman et al, 1974). These problems can be minimised or alleviated by prior irradiation of the blood. It has been reported that the administration of whole blood to non-immunosuppressed adults leads to an increase in the capacity of lymphocytes to undergo spontaneous DNA synthesis (Schechter, Soehnlen and McFarland, 1972). This was assessed by incorporation of ^{3}H-thymidine by lymphocytes and was maximal one week after transfusion. This increased lymphocyte reactivity was not found with leucocyte-poor blood suggesting that in routine whole blood transfusions there is a degree of sensitisation to donor white cell antigens in the majority of patients.

The administration of granulocytes as therapy for the infected neutropenic individual is considered elsewhere. Adverse reactions can usually be minimised by giving cells from HL-A compatible donors, the majority of complications of granulocyte transfusions being associated with the presence of leucoagglutinins or leucocytotoxins in the recipient's serum. Since many of the patients requiring granulocyte transfusions are undergoing immunosuppressive therapy, graft vs host reaction is a particular hazard. Again this can be minimised by irradiating cells prior to transfusion. Granulocytes for infusion are usually prepared by continuous flow centrifugation using a cell separator or by the less expensive and simpler method of filtration leucaphoresis in which granulocytes are trapped on nylon filters from which they are subsequently eluted. Although filtration leucaphoresis gives high yields of granulocytes and has the advantage of low cost, the survival of cells in vivo is shorter and cells prepared in this fashion are more commonly associated with febrile reactions.

The use of red cells, granulocytes or platelets in relation to bone marrow transplantation requires special consideration. Generally speaking transfusions should be avoided if possible in patients in whom this treatment is indicated. Cells given for supportive therapy following transplantation before the graft is established should also be HL-A and MLC compatible. Ideally the bone marrow donor should also supply the platelets and granulocytes for supportive therapy after transplantation and in these situations it is essential to have access to a continuous flow cell separator. Failure of the bone marrow grafting is the rule in the previously multitransfused individual. Since bone marrow transplantation is becoming an established line of treatment in a number of conditions including selected cases of aplastic anaemia and certain leukaemias every effort should be made to avoid prior sensitisation of potential recipients to leucocyte antigens.

COMPLICATIONS OF PLATELET THERAPY

This has been discussed in detail in Chapter 5 and will only be summarised here.

Since platelets contain the A and B blood group antigens they should always, when possible, be given to ABO compatible individuals. Platelet rich preparations also contain considerable amounts of contaminating red cells

and therefore should also be matched for the Rhesus D antigen. In addition platelets carry HL-A antigens but in most cases it is impracticable to HL-A type donor and recipient in the context of routine platelet therapy. There are conditions, however, when this is highly desirable as discussed above in the context of bone marrow transplantation. Individuals who require long-term platelet therapy often become refractory to treatment due to the development of leucocyte/platelet antibodies. HL-A compatible platelets will often survive normally in these patients (McCredie et al, 1974). Some patients with aplastic anaemia and thrombocytopenia seem at particular risk of developing antibodies. In all these situations an HL-A compatible individual, preferably a sibling or another relative, is the most suitable donor (Herzig, Poplack and Yankee, 1974).

A rare complication of platelet therapy is 'post-transfusion purpura' (Shulman et al, 1964). In this condition an episode of thrombocytopenia occurs approximately seven days after the administration of blood and occurs almost exclusively in women who lack the PL^{A1} platelet antigen. The exact mechanism of post-transfusion purpura is not understood but anti-PL^{A1} antibodies are often detected. It has been suggested that complexes of this antigen and specific antibody become absorbed onto recipient platelets leading to destruction possibly by complement or phagocytosis. The condition has been successfully treated by repeated plasmapheresis, the antibody presumably being 'washed-out' (Abramson, Eisenberg and Aster, 1974).

PLASMA PROTEINS AND TRANSFUSION REACTIONS

The recognition of genetic polymorphism amongst a number of plasma proteins has led to an appreciation of their possible role in various adverse transfusion reactions. Antibodies against allotypic* determinants on immunoglobulins are often detectable in multitransfused individuals but usually have to be present in high titre to be associated with adverse effects following transfusion. Anti-Gm antibodies, directed solely against the Fc fragment of IgG, are more commonly found after multiple transfusions than anti-Inv antibodies, which recognise determinants on light chains (Table 2). Such reactions are usually mild in contrast to reactions against purified gamma globulin which occasionally occur in individuals receiving long-term gamma globulin therapy. These patients, who are usually those with acquired or sex-linked agammaglobulinaemia, develop anaphylactic-type reactions within minutes following intramuscular gamma globulin. Although some of these individuals have circulating anti-Gm and/or Inv antibodies their serum may also contain antibodies against denatured gamma globulin (Ellis and Henney, 1969). It would appear that the preparation of gamma globulin for clinical use results in a degree of denaturation which reveals antigenic sites not present on native IgG. Individuals with persistent anaphylactoid reactions to gamma globulin will often require infusions of fresh frozen plasma as a source of IgG.

* Allotypic specificities are those antigenic specificities on various plasma proteins which are distinct between different groups of individuals within the same species.

This has the attendant risk of transmitting hepatitis B virus not present with fractionated gamma globulin and we have recently described a case in which such a person acquired a dual infection as demonstrated by the presence of both the ay and ad antigens on circulating virus particles (Lawton et al, 1975).

The anaphylactic reactions which follow union of the globulin antigen with recipient antibody are possibly complement-mediated. Since the complement cascade can also be activated by soluble antigen–antibody complexes in the fluid phase (see above), vasoactive peptides such as anaphylatoxins may be liberated. This concept is compatible with the work of Barandun et al (1975) who were able to show that plasmin-treated gamma globulin was well tolerated by immunodeficient patients highly sensitive to native IgG. Plasmin treatment presumably removed the C1 binding site on the Fc region of IgG thus preventing complement activation.

The evidence that complement plays a major role in these types of reactions and in the reactions involving anti-IgA (described below) is still circumstantial and there is little data to support the concept. It is therefore worth considering the possible participation of the kinin-forming system in immune-complex induced anaphylactoid reactions since activation of the kinin system has been demonstrated in vivo in monkeys following the infusion of anti-red cell antibodies (Lopas et al, 1973). Bradykinin and related peptides are cleaved from circulating kininogen by the action of the enzyme kallikrein (kininogenase) (Figure 4). Prekallikrein is converted to kallikrein by the action of fragments

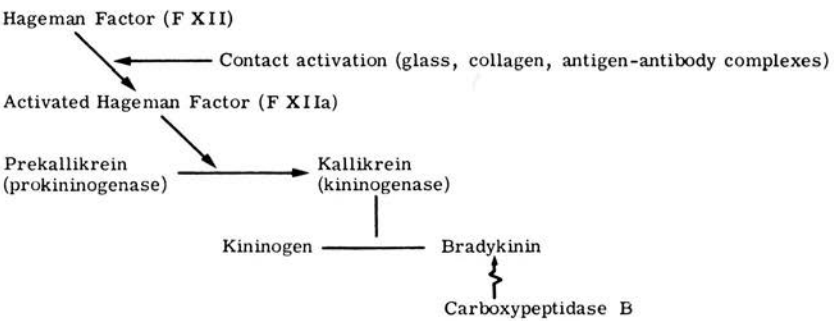


Figure 4. The kinin-forming system in human plasma.

derived from activated Hageman factor (factor XII). Factor XII is activated following contact with a variety of agents including glass, collagen, uric acid crystals and antigen–antibody complexes. Thus kinin formation as a result of immune complex activation of Hageman factor may account for some of the clinical features of transfusion reactions found in association with antibodies to plasma proteins. Bradykinin and the anaphylatoxins C3a and C5a all contain a terminal arginine which is cleaved by circulating carboxypeptidase B rendering them biologically inactive. The activity of this enzyme in the plasma would presumably influence the contribution of these various peptides in an acute anaphylactic-type response.

Anaphylactic transfusion reactions associated with anti-IgA antibodies

have received particular attention (Schmidt, Taswell and Gleich, 1969; Vyas et al, 1969). These can be either class-specific antibodies found exclusively in individuals with IgA deficiency or allotype-specific (anti-Am) antibodies in multitransfused patients with normal serum IgA levels (Nadorp et al, 1973). The occurrence of total IgA deficiency has been estimated to be about one in 700 normal individuals. These people occasionally develop circulating anti-IgA antibodies even though they have not received prior transfusions. The associated reactions to plasma are usually mild but occasionally anaphylactic-type reactions occur which can be life-threatening.

The term 'anaphylactic-like' has been used up to now to distinguish these from classical anaphylactic reactions mediated by IgE. The exact significance of tissue-sensitising antibody in blood transfusion reactions is not known but the administration of plasma from 'allergic individuals' does carry the risk of untoward reactions and was in fact one of the earliest recorded examples of transfusion complications. In 1919 Ramirez described the case of a person who developed acute bronchospasm whilst driving in an open horse-drawn carriage several weeks after receiving a transfusion of blood. The donor was subsequently found to be highly allergic to horse hair and thus had passively transferred this sensitivity by his plasma to the previously 'non-allergic' recipient. It is now appreciated that this antibody is of the IgE class and has the capacity of binding firmly to cells of the mast cell/basophil series. Following the incubation of cell-bound IgE with specific antigen a biochemical mechanism is initiated which leads to the release of a number of pharmacological mediators including histamine, slow-reacting substance (SRS-A) and eosinophil chemotactic factor of anaphylaxis (ECF-A) (Austen, 1974; Kay, 1974). Thus the administration of blood from an allergic donor can confer IgE-mediated hypersensitivity to the recipient. This may have particular significance following infusion of plasma from donors with certain forms of drug hypersensitivity. Total levels of serum immunoglobulin E have been measured in multitransfused patients and shown to be elevated. There is no real evidence, however, that these recipients are at any particular risk in developing anaphylactic reactions or other manifestations of hypersensitivity (Cartron et al, 1973).

The occurrence of amyloidosis has been described in association with multiple transfusions (Sharma and Geer, 1970; Prentice et al, 1971). It is appreciated that persistent antigen stimulus in experimental animals leads to amyloidosis and that a similar situation may occur in multitransfused humans. Although the nature of amyloid is not known it has been suggested that it may be derived from the light chains of immunoglobulins.

Purified albumin solution is usually a safe and effective form of therapy but there have been some reports relating to adverse effects of this preparation on the circulation (Torda et al, 1973; Izaka et al, 1974). This has been attributed to the presence of a bradykinin-like activity in certain batches of albumin preparation thought to have been formed during the manufacturing process. This is of little clinical importance in individuals requiring a limited amount of purified albumin since the bradykinin is presumably inactivated by circulating carboxypeptidase B. Individuals requiring large amounts of this preparation, especially those undergoing cardiac bypass surgery and other situations

where the solution is given rapidly, may be at risk in developing hypotension and other haemodynamic changes.

TRANSFER FACTOR

Transfer factor is a dialyzible extract from human leucocytes which has been given to patients with a wide variety of disorders including immunodeficiency states, chronic disseminated infections and certain neoplasms (reviewed by Basten et al, 1975; Grob et al, 1975). Although there is very convincing evidence of the usefulness of transfer factor in the treatment of certain well-defined conditions such as chronic mucocutaneous candidiasis its efficacy still awaits critical analysis by controlled clinical trials. The agent has an estimated molecular size of approximately 5000 daltons and contains a polypeptide and oligonucleotide. These consist of approximately 12 amino acids with three to four RNA bases. Generally speaking transfer factor has proved to be remarkably free from side-effects. There is occasionally pain and erythema at the site of injection which can be accompanied by a transient pyrexia. The administration of large amounts of transfer factor has not been associated with any detectable biochemical abnormalities.

Two patients, one with combined immunodeficiency and the other with the Wiskott–Aldrich syndrome, developed a polyclonal gammopathy following the administration of transfer factor. These gammopathies may have been a manifestation of the underlying disease and emphasise the need to subject the use of transfer factor to a critical clinical appraisal.

PHARMACOLOGICAL AGENTS IN BLOOD PRODUCTS

The potential danger of transfusing plasma from individuals with drug hypersensitivity has been discussed above. The indications for refusing donors tends to vary between blood transfusion centres. In addition to antibiotics it is probably prudent to refuse donations for therapy from individuals taking regular medication with agents such as hypotensive drugs. Aspirin is a difficult problem since many individuals could be unaware that they are taking aspirin contained in proprietary preparations. Therefore, whilst in theory a platelet donation from an individual taking aspirin would be contraindicated due to its capacity to aggregate the platelet, this does not seem in practice to represent a major therapeutic problem since most patients receive pooled platelet preparations from several donors.

It should be emphasised that although a blood donation may be unsuitable for therapy the plasma may still be of use as a source of reagents and this must be taken into account when refusing donors. Such reagents include anti-A and anti-B red cell antigens for use in red cell serology and plasma or serum as a source of tissue culture medium in tests of lymphocyte reactivity.

The practice of introducing drugs into the blood packs prior to transfusion is to be strongly discouraged. For example, when ethacrynic acid was mixed with whole blood this was shown to lead to haemolysis of red cells in vitro in concentrations above 1 mg/ml (Da Costa and White, 1973). All pharmaceutical

agents have the potential capacity of leading to permeability changes across the red cell membrane and, furthermore, the introduction of any agent into blood collection bags carries the risk of bacterial contamination.

The use of phthalic acid esters in the manufacture of plastics used for the storage of blood has given concern in terms of its probably harmful long-term effects. These compounds have been shown to be teratogenic in experimental animals and manufacturers are attempting to provide disposable plastic blood collection bags which do not contain these compounds.

Hypersensitivity to thimerosal, used as a preservative in gamma globulin preparations, has also been described (Mackenzie and Vlahcevic, 1974).

THE INVESTIGATION OF TRANSFUSION REACTIONS

As discussed above transfusion reactions having an 'immunological mechanism' are most likely to occur in individuals who have been sensitised by previous transfusions or pregnancies. In general severe reactions accompanied by symptoms during, or immediately after, infusions are usually due to massive red cell destruction or bacterial contamination. Infected fluids often cause severe rigors with circulatory collapse. Rigor with loin pain, headache, renal impairment and jaundice is suggestive of a haemolytic reaction. An acute anaphylactic reaction (see above) can cause dyspnoea, chest pain, urticaria, pruritus and circulatory collapse. A mild transient pyrexia is often the only symptom of incompatibilities associated with leucocyte and plasma (anti-allotype) antibodies.

It is important to check immediately that the correct blood or blood product has been given to the recipient. The need for scrupulous checking by technicians and doctors has already been stressed and it is also necessary to enquire whether donor blood has been overheated, frozen, stored too long and to determine if it is infected (see below). Such mishandlings of blood will often be apparent by the appearance of free haemoglobin in the donor blood pack.

For a comprehensive investigation of a transfusion reaction the laboratory will require a pre- and post-transfusion sample of the recipient's blood in addition to the samples used for cross-matching. The remainder of the donor blood will also be required even if only the fluid in the giving set is all that is available.

All blood samples should be regrouped for ABO and D antigens and the red cell compatibility tests, using simple agglutination tests, should be repeated to exclude a laboratory error. If there is still no evidence of incompatibility the more sensitive autoanalyser procedure can be employed to detect 'immune' red cell antibodies* in recipient serum. The autoanalyser

* The term 'immune' antibody is red cell serology jargon hallowed by long usage. It is used to describe those red cell antibodies produced as a result of previous transfusions, pregnancy or the injection of foreign red cells and which are therefore apparently distinct from 'naturally-occurring' antibodies for which the antigenic stimulus is unknown. Some 'naturally-occurring' antibodies, apart from those of the ABO blood group system, can give positive reactions in ABO and D antigen compatibility testing as well as mediating red cell transfusion reactions. A common example is 'naturally-occurring' antibodies of the Lewis blood group system.

system has approximately five to ten times the sensitivity of routine agglutination tests. It is advisable to test for the presence of antibodies in both the pre- and post-transfusion samples by the autoanalyser technique since with relatively small amounts of antibody a positive reaction may be detectable only in the pre-transfusion sample and absent in the post-transfusion specimen as a result of adsorption by the transfused red cells. In these situations it is advisable to have a further blood sample 10 to 14 days after the reaction in order to determine whether the antibody has reappeared in the patient's serum. The search for red cell incompatibility should be more strenuous if there is evidence of red cell destruction as shown by haemoglobinaemia or bilirubinaemia. The urine should also be examined for the presence of haemoglobin, urobilinogen and albumin. Serum haptoglobin binds free haemoglobin and so its level will fall following intravascular haemolysis. More sophisticated investigations such as erythrocyte survival studies using ^{51}Cr-labelled red cells may be of value when serological tests are unhelpful but where other clinical and laboratory evidence still points to a haemolytic transfusion reaction.

Often more than one red cell antibody may be present in the serum of a multitransfused individual or following pregnancies. These are often difficult to identify with certainty but it is useful to perform a full red cell genotype on recipient cells since this will exclude the presence of antibodies to the antigens present. To this end it is necessary to use a panel of stock cells which ideally should contain at least the following antigens: C, C^{w}, c, D, E, e, M, N, S, s, P, Lu^{a}, K, k, Le^{a}, Le^{b}, Fy^{a}, Fy^{b}, Jk^{a}, Jk^{b} (Tovey and Gillespie, 1974). If a transfusion reaction associated antibody is identified and confirmed the appropriate genotyped blood will be required if further transfusions of red cells are necessary.

It is essential that a full bacteriological examination be undertaken on the donor blood or blood product and the attending medical personnel should be asked specifically whether drugs or other agents were introduced into the blood pack.

The investigation of reactions in association with white cell, platelet and plasma protein antibodies is usually performed in specialised laboratories. The presence of lymphocytotoxic and leucoagglutinating antibodies can be detected by the methods outlined above. By and large, tests for platelet antibodies are difficult to perform due to the technical problems involved. An antiglobulin consumption test is sometimes used as is direct aggregation of platelets in the presence of platelet specific antibody using an aggregometer. Such tests must be interpreted with caution since many agents are known to aggregate platelets including immune complexes. Antibodies to plasma proteins, in particular allotypic determinants on IgG, require special reagents and techniques for a comprehensive study. This is not usually required for routine investigation of transfusion reactions but the reader is referred to Natvig and Kunkel (1973) for a full account of the identification of human immunoglobulin classes and subclasses and their genetic variants.

It should be emphasised that the technology and approach to the investigations of blood transfusion reactions is a rapidly developing field and therefore it is not possible to give a comprehensive list of investigations to be used as

standard practice in transfusion centres. In practical terms the cause of the more severe reactions, in contrast to milder adverse effects, is almost always determined by the investigations outlined above.

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SECTION C - STUDIES ON CHEMOTAXIS

CHEMOTAXIS OF HUMAN BASOPHIL LEUCOCYTES

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CHEMOTAXIS OF HUMAN BASOPHIL LEUCOCYTES

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SUMMARY

Human peripheral blood basophils from two patients with unusually high basophil counts in association with chronic myelogenous leukaemia migrated *in vitro* toward various chemotactic agents of human origin. These included supernatants from sensitized lymphocytes challenged with specific antigen, diffusates from lung fragments challenged with pollen antigen E, the enzyme plasma kallikrein, and two complement derived agents, C5a and $C\bar{5}\bar{6}\bar{7}$. Thus, chemotaxis *in vitro*, a previously unreported property of human basophils, has been observed, although none of the agents tested produced a selective chemotactic response.

INTRODUCTION

The ability of neutrophils, eosinophils and mononuclear cells to react in chemotaxis has been reported by several workers (Boyden, 1962; Kay & Austen, 1971; Ward, 1968). The capacity of basophil leucocytes to respond to a chemotactic stimulus has been difficult to study since the cell is not readily available in high concentrations. We have had the opportunity to study the chemotactic response of leucocytes of two patients with chronic myelogenous leukaemia who both had an unusually high percentage of circulating basophils. Known chemotactic agents of human origin, including supernatants from lymphocytes challenged with specific antigen (Ward, Remold & David, 1970), diffusates from lung fragments challenged with antigen E (Kay & Austen, 1971), the plasma enzyme kallikrein (Kaplan, Kay & Austen, 1972), a fragment cleaved from the fifth component of complement (C5a) (Ward & Newman, 1969) and the trimolecular complex of the fifth, sixth and seventh components of complement ($C\bar{5}\bar{6}\bar{7}$) (Lachmann, Kay & Thompson, 1970) were examined for their ability to attract *in vitro* the cells of these two patients.

MATERIALS AND METHODS

Chemotactic factors

The preparation of supernatants from sensitized lymphocytes challenged with specific

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antigen, the source of streptokinase-streptodornase (SKSD) and purified protein derivative of tuberculin (PPD) used as antigens, and the method of testing for macrophage migration inhibitory factor (MIF) using guinea-pig macrophages as target cells have been described (Rocklin, Meyers & David, 1970). The lymphocytes from two normal human donors, A and B, were separated from 60 ml of peripheral blood and cultured for 3 days in the presence or absence of antigen. The cell-free supernatants were removed each day, and fresh medium was added. Each sample gave a final pooled volume of between 12 and 14 ml. After extensive dialysis against saline and then water, the supernatants were lyophilized and reconstituted in Hanks' solution. The supernatants from donor A and donor B were concentrated 2·5 and seven times, respectively. The materials were tested for chemotaxis using 0·5-ml volumes.

A diffusate was prepared from human lung passively sensitized with serum from a ragweed sensitive individual and challenged with ragweed antigen E in Tyrode's solution (Kay & Austen, 1971). The 3-ml diffusate from 300 mg of lung fragments contained histamine, slow reacting substance of anaphylaxis (SRS-A) and eosinophil chemotactic factor of anaphylaxis (ECF-A). Volumes of 0·8 ml were used in the chemotactic chambers.

Preparations of highly purified human prekallikrein and Hageman factor fragments were incubated in phosphate buffered saline to yield kallikrein as described (Kaplan *et al.*, 1972; Kaplan & Austen, 1970), and assayed for chemotaxis in volumes of 0·25 ml.

Human C5 was prepared by the method of Nilsson & Müller-Eberhard (1965). C5a was generated by incubating 50 μg of C5, contained in potassium phosphate buffer with added calcium at pH 7·8, with 10 μg of trypsin (Mann Research Laboratories) for 10 min at 30°C, after which the reaction was terminated by adding 5 μg of soybean trypsin inhibitor (Mann Research Laboratories). The amount of soybean trypsin inhibitor (SBTI) used was that which prevented the action of trypsin on benzoyl DL arginine p-nitroanilide at the same ratio of enzyme to inhibitor under conditions previously described (Eriksson, 1965). For chemotaxis, trypsinized or untreated C5 was tested in 0·15-ml volumes each containing 20 μg of protein. As a further control, equivalent amounts of trypsin and SBTI were used. With the cells from patient 2 partially purified C5a was employed. Two millilitres of trypsinized C5, prepared as described above, were applied to a 3×85-cm column of Sephadex G-100, using conditions previously described for the isolation of C3a (Bokisch, Müller-Eberhard & Cochrane, 1969). Three millilitre-fractions were collected. When alternate fractions were tested for chemotaxis of human peripheral blood leucocytes, activity appeared as a single peak shortly after a cytochrome C marker. Two-tenths millilitre from the tube containing the most chemotactic activity was used for basophil chemotaxis studies.

The trimolecular complex, $C\bar{5}\bar{6}7$ was made by incubating purified $C\bar{5}\bar{6}$ with C7, as described in the 'reactive lysis' procedure (Thompson & Lachmann, 1970; Goldman, Ruddy & Austen, 1972). For the chemotactic experiments, 0·005 ml of $C\bar{5}\bar{6}$ in veronal buffered saline at pH 7·5 containing 4·7 haemolytic units per ml and 0·05 ml of a 1 in 500 dilution of C7 in 0·1 M acetate buffer at pH 6·0 containing 60,000 haemolytic units per ml were employed (Goldman *et al.*, 1972).

Chemotactic assay

Chemotaxis was measured by a modification of the Millipore technique previously described (Kay, 1970). Blood was drawn into heparin and the red cells were sedimented with 6% Dextran (Kay & Austen, 1971). The leucocyte-rich supernatant was centrifuged to obtain the white cells which were washed twice in Hanks' solution and resuspended in

Hanks' solution containing 0·5% ovalbumin at a final white cell concentration of 2×10^6 per ml. The final volume in the test compartment was brought to 1 ml. After the incubation period, the Millipores were removed, rinsed in Hanks' solution and immersed for 20 sec in a mixture of 1 part absolute ethanol and 1 part saturated mercuric chloride. After washing for 3 min in running tap water, the Millipores were stained for 5 min in 1% aqueous toluidine blue. Following a further 3 min wash in tap water, the Millipores were dehydrated, cleared and mounted as previously described. Basophils were counted using a high power ($\times 90$) oil immersion objective; the basophil chemotactic count was expressed as the total count of ten fields.

Patients

Patient 1 is a 57-year-old white male. A diagnosis of chronic myelogenous leukaemia was made in April, 1966. At the time of the investigation he was undergoing an acute blastogenic crisis. His total white cell count was 62,000/mm^3 of which 25% were mature basophils, 35% immature basophils, 10% promyelocytes and 30% mononuclear cells. Apart from busulphan, 8 mg/day, he was receiving no other medication. Following venipuncture for chemotactic studies, he was given, intravenously, cytosine arabinoside, daunomycin and vincristine in doses of 2 mg, 1 mg and 1·5 mg/kg of body weight, respectively. Blood was drawn for further chemotactic studies 18 hr later.

Patient 2 is a 68-year-old white female. A diagnosis of chronic myelogenous leukaemia was made in April, 1969. At the time of the investigation her white cell count was 23,000/mm^3 of which 38% were basophils, 45% neutrophils, 12% blast cells, 1% metamyelocytes, 1% myelocytes, 2% lymphocytes and 1% eosinophils. She was receiving 6-mercaptopurine, 50 mg twice a week, and allopurinol, 100 mg twice a day.

RESULTS

The granulocytes from patient 1 consisted entirely of basophils or their precursors and thus it was possible to study basophil chemotaxis using a filter of 3-μm pore size since the only migrating cells were mature or immature basophils. The circulating white cells in Patient 2 were predominantly neutrophils and using a filter of 3-μm pore size, neutrophils preferentially migrated with no observable basophil chemotaxis. With a filter of 8·0-μm pore size basophil chemotaxis could be demonstrated with the cells from patient 2, although there were also high neutrophil counts.

The chemotactic response of human basophils to human lymphocyte supernatants is shown in Table 1. Supernates from lymphocyte donors A and B were tested against the cells from patients 1 and 2, respectively. Donors A and B both had strongly positive delayed-type skin reactions to 5 units of SKSD but reacted weakly to 0·1 ml of intermediate strength (0·001 mg) of PPD. Lymphocytes from donor B produced MIF following culture with SKSD. No MIF activity was detected when cells from donor B were incubated with PPD. MIF was not assayed with cells from donor A. When tested for chemotaxis, basophils from both patients migrated towards the supernatants of lymphocytes of donors A and B following incubation with SKSD. Lymphocyte supernatants from the PPD culture gave little or no chemotaxis. Samples of SKSD, PPD or medium alone or cells incubated with medium alone did not evoke basophil chemotaxis with cells from either patient.

A human lung diffusate containing ECF-A (Kay & Austen, 1971), 600 ng of histamine and

TABLE 1. Chemotactic activity of human lymphocyte supernatants for human basophils

	Lymphocytes + SKSD	Lymphocytes + PPD
Patient 1*		
Basophil chemotaxis	20	0
Patient 2*		
Basophil chemotaxis	39	12
Macrophage migration (%)†	61	91

* Supernatants from the lymphocytes of donors A and B were used to attract cells from patients 1 and 2, respectively.

† Average value from two populations of guinea-pig macrophages.

200 units of SRS-A per ml was chemotactic for the basophils from both patients. The cells from patients 1 and 2 gave chemotactic counts of 18 and 26 respectively, whereas no response was observed to antigen E alone or to diffusate from tissue not antigen challenged.

Neither the fragments derived from active Hageman factor or highly purified prekallikrein were chemotactic for basophils when tested alone (Table 2). When these factors were incubated together bradykinin was generated from heat-inactivated plasma, indicating conversion of prekallikrein to kallikrein, and the mixture was chemotactic for basophils from both patients 1 and 2.

Trypsinized C5, to which SBTI had been added, was chemotactic for basophils from patient 1 whereas no migration was seen with untreated C5 or trypsin and SBTI alone. The basophils of patient 2 migrated toward partially purified C5a but not toward untreated C5 (Table 3).

TABLE 2. Generation of chemotactic activity for human basophils by activation of prekallikrein

	Prekallikrein	Hageman factor fragments	Prekallikrein and Hageman factor fragments
Patient 1			
Basophil chemotaxis	0	0	36
Total bradykinin generated from heat inactivated plasma (μg)	0	0	2·10
Patient 2			
Basophil chemotaxis	N.D.	N.D.	35
Total bradykinin generated from heat-inactivated plasma (μg)	0	0	1·18

N.D. = not done.

TABLE 3. The chemotactic activity of trypsinized C5 for human basophils

	Basophil chemotaxis
Patient 1	
Trypsin treated C5 (with SBTI)	24
Untreated C5	0
Trypsin+SBTI	0
Patient 2	
Partially purified C5a	19
Untreated C5	2

TABLE 4. Generation of chemotactic activity for human basophils following interaction of $C\bar{5}\bar{6}$ with C7

	Basophil chemotaxis		
	$C\bar{5}\bar{6}$	C7	$C\bar{5}\bar{6}+C7$
Patient 1	10	0	20
Patient 2	4	0	20

Preparations of $C\bar{5}\bar{6}$ and C7 had little chemotactic activity when tested alone. When these factors were incubated together to form $C\bar{5}\bar{6}7$, the complex prepared unsensitized sheep red blood cells for lysis by C8 and C9 (Thompson & Lachmann, 1970; Goldman *et al.*, 1972) and was chemotactic for the basophils from both patients (Table 4).

DISCUSSION

The capacity of basophils to respond chemotactically to supernatants from lymphocytes incubated with specific antigen (Table 1), a diffusate from sensitized lung incubated with ragweed antigen E, the plasma enzyme kallikrein (Table 2) and two complement-derived factors, C5a (Table 3) and $C\bar{5}\bar{6}7$ (Table 4), has been demonstrated using cells from two patients with unusually high basophil counts. Although directional basophil migration through the entire thickness of the Millipore was observed, the overall chemotactic counts were low. In previous studies (Kay & Austen, 1971; Kaplan *et al.*, 1972), chemotaxis was expressed as mean cell counts per high power field whereas in the present report the data are presented as the total count of ten high power fields. Of interest is that a few hours after blood was drawn from patient 1, therapy was commenced with several antimitotic agents; the following day his cells did not respond in chemotaxis.

No evidence was found for a selective basophil chemotactic agent since these cells responded to all the agents tested. The preparations of kallikrein, C5a and $C\bar{5}\bar{6}7$ were puri-

fied whereas the lymphocyte supernatants and the lung diffusate contained multiple biological agents which may have influenced the identification of a specific basophil chemotactic agent.

When attempting to relate the *in vitro* observations described with clinical situations, it may be noted that, apart from neoplastic disorders, there have been few reports of conditions associated with an increase in the number of tissue basophils. High basophil counts have been observed in contact allergy (Dvorak & Mihm, 1972), the histological picture being similar to cutaneous basophil hypersensitivity described earlier in the guinea-pig (Dvorak *et al.*, 1970). This phenomenon is thought to be a form of delayed-type hypersensitivity produced under special conditions of immunization. The ability of basophils to migrate towards an anaphylactic diffusate is of interest in view of the recent report that basophil infiltration is a late event in immediate-type skin reactions in certain atopic individuals (Felarca & Lowell, 1971).

The significance of the capacity of kallikrein, C5a and $C\bar{5}\bar{6}\bar{7}$ to attract basophils is not known. It should be noted that with the cells from both patients the *in vitro* conditions were optimal for basophil migration since the basophil was only recognizable granulocyte in patient 1 and in patient 2 a large pore size was used. Our recent data on cell selectivity with the eosinophil suggest that ECF-A, present in a high concentration, will attract the neutrophil when eosinophils are absent or comprise a very small percentage of the total cell population. Thus, it is suggested that a broad specificity of chemotactic factors can be shown when conditions for migration are optimal and when other cells which may be preferentially attracted are absent.

ACKNOWLEDGMENTS

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The authors are indebted to Dr L. Anderson and Dr H. Churchill for preparing the human lymphocyte supernatants and for assaying for MIF activity; to Dr A. Kaplan for supplying human prekallikrein, active Hageman factor fragments and for assaying for bradykinin; to Mrs S. Koethe for her preparation of human C5 and to Dr J. Goldman for supplying $C\bar{5}\bar{6}$ and C7. We would also like to thank Dr W. Maloney for allowing us to study patients under his care.

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A PREALBUMIN ACTIVATOR OF PREKALLIKREIN*

III. APPEARANCE OF CHEMOTACTIC ACTIVITY FOR HUMAN NEUTROPHILS BY THE CONVERSION OF HUMAN PREKALLIKREIN TO KALLIKREIN

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The generation of bradykinin in human plasma is initiated by the activation of Hageman factor (1–3). Activated Hageman factor and a series of fragments derived from activated Hageman factor by digestion with the fibrinolytic enzyme plasmin lead to the subsequent conversion of prekallikrein to kallikrein (4, 5). The smallest of the Hageman factor fragments is stable, has a mol wt of approximately 30,000–35,000, and appears to be the primary activator of prekallikrein having six times greater prekallikrein-activating activity than the parent Hageman factor molecule. Cleavage of the substrate kininogen by kallikrein leads to the elaboration of the permeability factor bradykinin (6).

In order to explore further the potential participation of the kinin-generating system in the inflammatory response, certain of the various components were examined for chemotactic activity. The generation of chemotactic activity specific for human neutrophils is shown to result from the conversion of prekallikrein to kallikrein. Evidence is also presented that both the chemotactic activity and bradykinin-generating activity are dependent upon the integrity of the active site of kallikrein.

Materials and Methods

Bradykinin triacetate (Sandoz Pharmaceuticals, Basel, Switzerland, or New England Nuclear Corp., Cambridge, Mass.) was used as the standard for native bradykinin. Antisera to IgG, IgA, IgM, β-lipoprotein, transferrin, albumin, and whole human serum were purchased from Behring Diagnostics Inc., Woodbury, N. Y. Antiserum to human κ and λ Bence Jones proteins was obtained from Melpar Inc., Falls Church, Va. Hexadimethrine (polybrene) was a gift of Dr. Floyd McIntire of Abbott Laboratories, North Chicago, Ill. Plasma thromboplastin antecedent (PTA)[1]-deficient plasma containing less than 1% of normal PTA activ-

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[1] *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; ECF-A, eosinophil

ity was supplied by Dr. Anthony Britten (Boston, Mass.). Plasma thromboplastin component (PTC)-deficient plasma containing 2% normal PTC activity was obtained from Sera-Tec Biologicals, New Brunswick, N. J. Diisopropyl fluorophosphate (DFP) was a product of Aldrich Chemical Co., Inc., Milwaukee, Wis. Tosyl-L-lysine chloromethyl ketone (TLCK) was obtained from Cyclo Chemical Division of Travenol Laboratory, Los Angeles, Calif. Ampholine carrier ampholytes for use in isoelectric focusing were purchased from Microbiological Associates Inc., Bethesda, Md. Ovalbumin 5X crystalized was a product of Pentex, Kankakee, Ill.

Fractionation Procedures

Serum was drawn for the isolation of active enzymes of the kinin-generating system and was processed as previously described (4). Diethylaminoethyl (DEAE)-cellulose chromatography, carboxymethyl (CM)-cellulose chromatography, Sephadex G-100 gel filtration (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden), and disc gel electrophoresis at pH 9.3 were performed as previously described (4, 5).

Plasma was prepared for the isolation of proenzymes of the kinin-generating system as follows. 100 ml of blood were collected in plastic tubes containing 9 mg of disodium ethylenediaminetetraacetate (EDTA) and 3.6 mg of hexadimethrine in 0.1 ml of 0.15 M saline for each 10 ml of blood drawn. The tubes were immediately centrifuged at 900 *g* for 20 min at 4°C and the plasma was separated using siliconized pipettes and dialyzed against 4 liters 0.01 M PO_4 buffer, pH 7.8. The dialysis bag was opened hourly, the plasma centrifuged at 900 *g* for 10 min at 4°C to remove any precipitated protein, and the supernatant plasma dialyzed for a total of 6 hr. Samples were concentrated by ultrafiltration using a UM-10 membrane (Amicon Corp., Lexington, Mass.) to 5–10 ml and then further concentrated by wall vacuum using Collodion bags No. 100 (Schliecher and Schuell Inc., Keene, N. H.).

Gel Filtration.—A 2.5 × 150 cm column of Sephadex G-150 superfine was equilibrated with 0.02 M tris (hydroxymethyl) aminomethane (Tris) Cl, pH 7.6. Samples of 2–3 ml were applied and the column run by upward flow at 5 ml/hr. 2.5-ml fractions were collected.

A 5.0 × 100 cm column of Sephadex G-200 was equilibrated with 0.01 M PO_4 buffer, 0.15 M NaCl, pH 7.3. Samples of 4–5 ml were applied and the column run by upward flow at 10 ml/hr. 5-ml fractions were collected.

Quaternary Aminoethyl (QAE) Sephadex Chromatography.—A 5 × 30 cm column of QAE Sephadex was equilibrated with 0.01 M PO_4 buffer, pH 7.8. 30-ml samples dialyzed in 0.01 M PO_4 buffer, pH 7.8, were applied to the column. The column was washed with 500 ml of equilibrating buffer and a linear salt gradient of 2 liters of equilibrating buffer and 2 liters of equilibrating buffer containing 0.3 M NaCl was applied. The column was run at 60 ml/hr and 12-ml fractions were collected.

Sulfoethyl (SE) Sephadex Chromatography.—A 5 × 30 cm column of SE Sephadex was equilibrated with 0.01 M PO_4 buffer, pH 6.0. 30-ml samples dialyzed in 0.01 M PO_4 buffer, pH 6.0, were applied. The column was washed with 500 ml of equilibrating buffer and a linear salt gradient of 2 liters of equilibrating buffer and 2 liters of equilibrating buffer containing 0.5 M NaCl was applied. The columns were run at 60 ml/hr and 12-ml fractions were collected.

Isoelectric Focusing in Gels.—Isoelectric focusing in 4% polyacrylamide gels containing from pH 7 to 10 ampholytes was performed as described by Righetti and Drysdale (7). Samples of 200 μl were each electrofocused in a series of gels; the ampholytes of one gel were removed by electrophoresis and the gel was stained with Coomassie blue in 20% ethanol and 7% acetic acid. The others were cut in 5-mm slices and eluted in 0.2 ml of distilled water overnight at 4°C. The pH of each slice was then measured and the eluates assayed.

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chemotactic factor of anaphylaxis; PTA, plasma thromboplastin antecedent; PTC, plasma thromboplastin component; QAE, quaternary aminoethyl; SE, sulfoethyl; TLCK, tosyl-L-lysine chloromethyl ketone.

Preparation and Assay of Components of the Bradykinin-Forming System.—Activated Hageman factor was prepared by sequential fractionation of plasma on QAE Sephadex, Sephadex G-100, CM-cellulose,and elution from disc gels after electrophoresis at pH 9.3 as previously described (5, 8). The prealbumin Hageman factor fragments were prepared by sequential chromatography of serum on DEAE-cellulose, Sephadex G-100, CM-cellulose, and elution from disc gels after electrophoresis at pH 9.3 as previously described (5, 8). Preparation of prekallikrein and kallikrein are described in the Results section.

Bradykinin was routinely determined by bioassay utilizing the isolated guinea pig ileum (9) or by radioimmunoassay (10). Kallikrein, prekallikrein, and the prealbumin Hageman factor fragments were determined as previously described (4). Heat-inactivated plasma utilized as a source of the substrate kininogen was prepared as described by Jonasson and Becker (11).

The shortening of the partial thromboplastin time of PTA-deficient plasma was used as an assay for PTA. Activated PTA was determined by incubating 0.05 ml of PTA source with 0.05 ml cephalin reagent (12) and 0.05 ml of PTA-deficient plasma for 2 min at 37°C. 0.05 ml of 0.05 M $CaCl_2$ was added and the clotting time determined at room temperature. The tubes were tilted each minute and the end point defined as the time interval required for the clot to adhere to the glass tube. Unactivated PTA was determined by incubation of 0.025 ml of PTA source with 0.025 ml purified activated Hageman factor for 5 min at 37°C. 0.05 ml cephalin-kaolin reagent and 0.05 ml of PTA-deficient plasma were then added and the mixture further incubated at 37°C for 2 min. 0.05 ml 0.05 M $CaCl_2$ was added and the clotting time determined.

Measurement of Chemotaxis.—Chemotaxis of human leukocytes was assayed by a modification of the Millipore technique of Boyden as previously described (13). Blood was drawn into plastic syringes and transferred to plastic tubes containing 50 units of heparin/ml of blood. The red blood cells were allowed to settle for 90 min at 37°C, after which the leukocytes were removed and centrifuged for 5 min at 100 *g*. The cells were washed once in Hanks' solution and resuspended in Hanks' solution containing 0.5% ovalbumin at pH 7.3. The cell count was adjusted to 1.5×10^6 leukocytes/ml. Chemotactic experiments were performed using a 3.0 μ pore size; except where stated otherwise, donors of peripheral blood leukocytes were normal volunteers. The 0.5% ovalbumin solution used as a suspending medium in the chemotactic chambers did not contain active kallikrein or a substrate for kallikrein.

An anaphylactic diffusate from passively sensitized human lung challenged with ragweed antigen E containing histamine, slow reacting substance of anaphylaxis (SRS-A), and an eosinophil chemotactic factor of anaphylaxis (ECF-A) was prepared as described (14). In experiments comparing the selective chemotactic activity of kallikrein and ECF-A, Tyrode's solution in 0.5% ovalbumin was used as suspending medium both in the test and cell compartments. Most preparations of kallikrein were dialyzed at 4°C for 6–12 hr against Hanks' solution. Some kallikrein preparations, contained in Tris HCl buffer, were assayed directly, the molarity of the solution in the test compartment being adjusted to that of Hanks' solution.

RESULTS

Chemotactic Activity Associated with Plasma Kallikrein.—Human serum clotted in glass so as to activate Hageman factor is known to possess chemotactic activity (15, 16). In order to assess whether any of the chemotactic activity so obtained was associated with the appearance of serum kallikrein, 5 ml of human serum were fractionated by Sephadex G-200 gel filtration as shown in Fig. 1. Three major peaks of chemotactic activity were observed, the first located between the IgM and IgG peaks, the second along the descending limb of the IgG peak, and the third with low molecular weight moieties eluting subsequent to albumin. Assay of the column for kallikrein activity

indicated a peak which was superimposable upon the second major peak of chemotactic activity. A more highly purified preparation of kallikrein was therefore prepared in order to further assess this association.

Human serum was dialyzed against 0.01 M PO_4 buffer, pH 7.8, and applied to a column of DEAE-cellulose. The effluent is known to contain IgG (17), kallikrein (18, 19), and activated PTA (20). Further fractionation by Sephadex G-150 gel filtration permitted partial separation of these three proteins and the fractions so obtained were assessed for chemotactic activity (Fig. 2). The single peak of optical density at 280 mμ represented primarily IgG as assessed by immunoelectrophoresis using anti-whole human serum. The peak of acti-

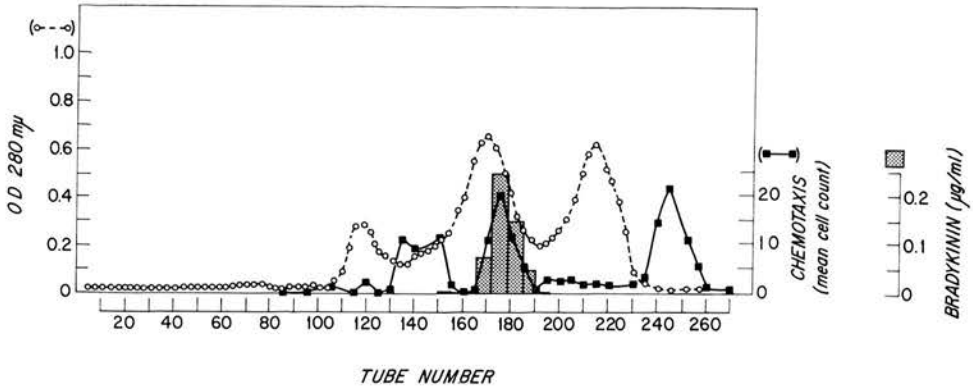

FIG. 1. Chromatography of human serum on Sephadex G-200. After a preliminary assessment for kallikrein activity, the negative fractions were pooled into 100-ml lots, concentrated to 5 ml, and assayed for kinin-generating activity while the positive region extending from tubes No. 165 to 190 was divided into four portions, each concentrated to 5 ml and assayed for bradykinin-generating activity as indicated. Mean cell count refers to the average of the cell counts in five high power fields.

vated PTA was found in the ascending limb of the IgG peak and had an estimated mol wt of 170,000–175,000. The peak of kallikrein activity clearly followed the IgG peak and had an estimated mol wt of 130,000. For chemotactic studies, the column fractions were pooled as follows: 1–40, 41–80, 81–120, 121–150, 151–157, 158–190, 191–220, 221–245, 246–300 and concentrated to 10 ml each so that the peak of activated PTA would be essentially free of kallikrein. The chemotactic activity was found to overlap the peak of kallikrein activity.

When the kallikrein-rich concentrate (Fig. 2) was assessed by disc gel electrophoresis, the Coomassie blue stain revealed a single broad band in the γ-globulin region (Fig. 3). An unstained gel was sliced in 2-mm sections, eluted in 0.5 ml of 0.15 M NaCl, dialyzed against Hanks' solution, and assayed for kallikrein activity and for chemotactic activity; both were located in the first two slices as shown in Fig. 3. In addition, some chemotactic activity was found in slice 7.

The majority of the cells which migrated in response to the various kallikrein preparations in the experiments described above were neutrophils, only an occasional eosinophil being noted. The chemotactic activity of kallikrein for neutrophils was further investigated using peripheral blood leukocytes obtained from a patient who had an eosinophilia of 70% in association with seropositive rheumatoid arthritis. The chemotactic response to kallikrein was compared to that obtained with the anaphylactic diffusate containing ECF-A. As shown in Fig. 4, a dose response was obtained with each chemotactic factor; however while ECF-A attracted primarily eosinophils, the kallikrein preparation attracted only the patient's neutrophils. Bradykinin in concentrations of 1.0, 0.1, and 0.01 μg was not chemotactic for either cell type.

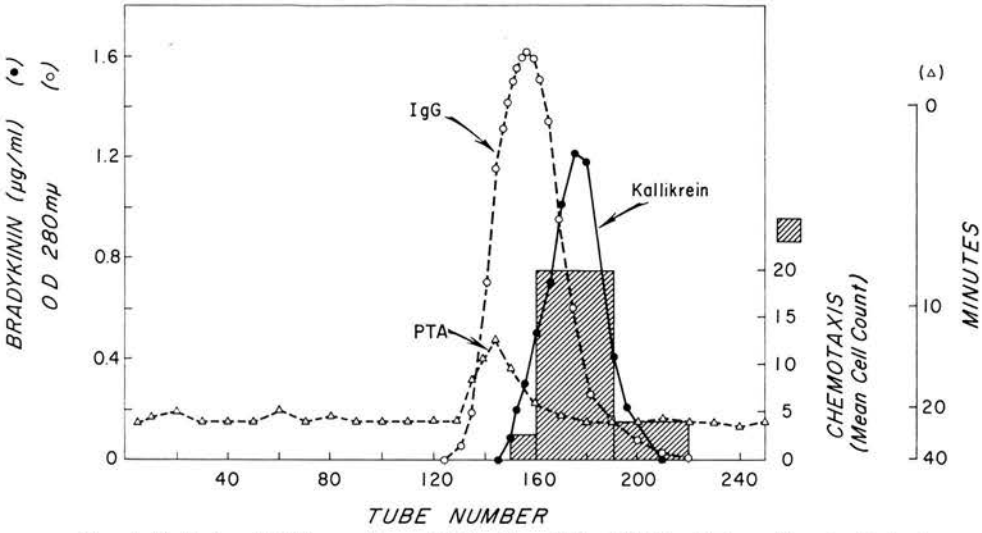

FIG. 2. Sephadex G-150 superfine gel filtration of the DEAE-cellulose effluent obtained with 0.01 M PO_4 buffer, pH 7.8.

Chemotactic Activity Resulting from the Activation of Prekallikrein.—

Preparation and Properties of Human Prekallikrein.—30 ml of dialyzed plasma containing hexadimethrine were applied to a column of QAE Sephadex. The effluent contained IgG, as assessed by immunoelectrophoresis, had no detectable active kallikrein and had only trace quantities of activated PTA. When 5 μl of the effluent were incubated with 10 μl of the purified Hageman factor fragments and then assayed for bradykinin-generating activity after incubation with 0.2 ml of heat-inactivated plasma, 100 ng of bradykinin were generated, indicating that the preparation contained prekallikrein. Incubation of 25 μl of the QAE Sephadex effluent with 25 μl of activated Hageman factor shortened the partial thromboplastin time of PTA-deficient plasma to 2.0 min, indicating the presence of unactivated PTA in the

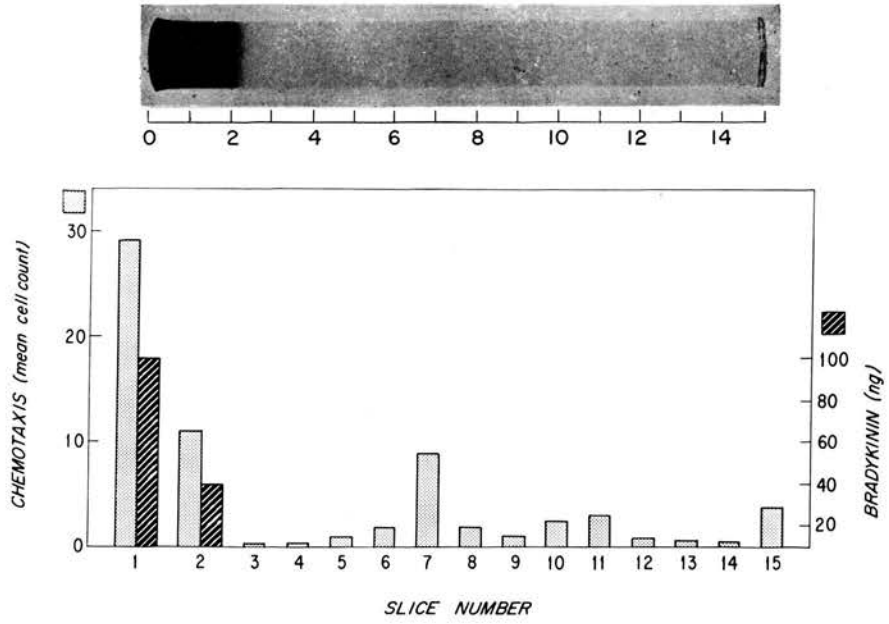

FIG. 3. Disc gel electrophoresis of the kallikrein-rich concentrate obtained from Sephadex G-150. An unstained gel run simultaneously was sliced as indicated into 15 equal sections, eluted, and assayed for kallikrein and for chemotactic activity.

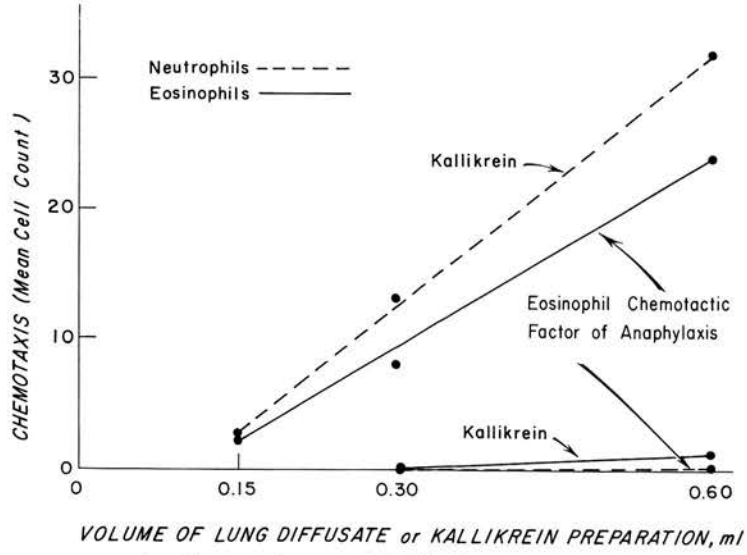

FIG. 4. Comparative chemotactic assay of kallikrein and ECF-A utilizing the peripheral blood leukocytes of a patient who had a 70% eosinophilia.

effluent. The addition of activated Hageman factor alone to the PTA-deficient plasma shortened the partial thromboplastin time from the control time of 28 min to 16 min. Addition of the effluent plus activated Hageman factor mixture in the same amounts as noted above to PTC-deficient plasma gave no shortening of the partial thromboplastin time.

The QAE Sephadex effluent containing IgG, prekallikrein, and unactivated PTA was then subjected to chromatography on SE Sephadex as shown in Fig. 5, resulting in separation of prekallikrein from the majority of IgG and from unactivated PTA. Prekallikrein eluted between 0.17 and 0.19 M NaCl and was

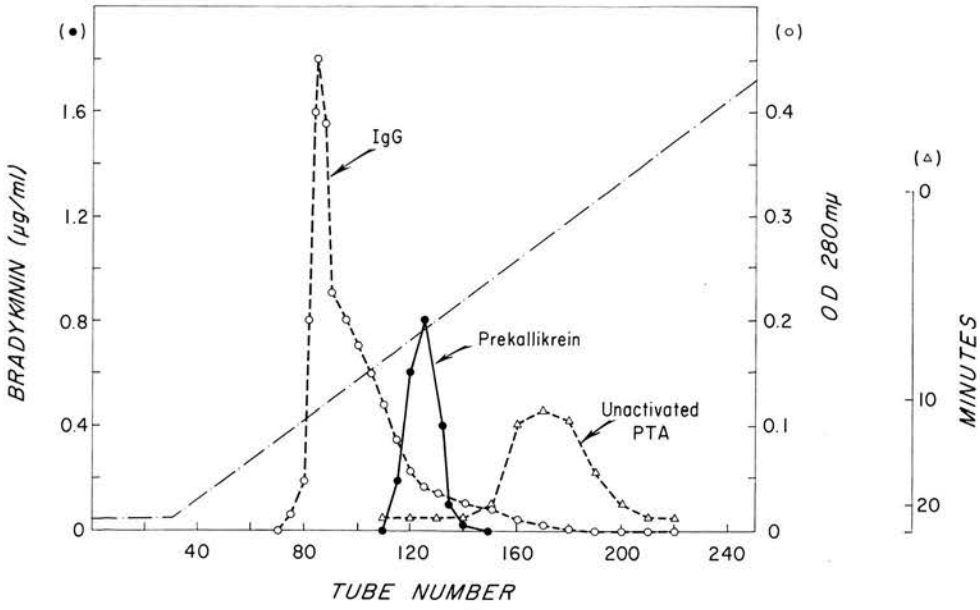

FIG. 5. SE Sephadex chromatography of the QAE Sephadex effluent obtained with 0.01 M PO_4 buffer, pH 7.8.

activated by incubation with the Hageman factor fragments. The prekallikrein peak was then pooled, concentrated, and further fractionated by Sephadex G-150 gel filtration as shown in Fig. 6. When the prekallikrein peak was pooled and concentrated, neither unactivated PTA nor activated PTA was detectable. IgG, however, was still detectable by gel diffusion using anti-IgG or anti-κ chain antisera. This preparation was then examined by disc gel electrophoresis and isoelectric focusing in gels. Analysis of the preparation by disc gel electrophoresis revealed a faint broad band in the γ-globulin region. When an unstained disc gel run simultaneously was sliced in 2-mm slices, eluted, and assayed for prekallikrein, the activity was found only in the first two slices at the top of the gel. When the same preparation was assessed by isoelectric

focusing in polyacrylamide gels, a faint broad band was recognized extending from pH 7.5 to 8.9, shown in Fig. 7. No other discrete bands were visible. When an unstained gel run simultaneously was sliced, eluted, and assayed for prekallikrein, prekallikrein was found between slices 5 and 9, which corresponded to an isoelectric point ranging from pH 8.5 to 8.9, the peak being at pH 8.7 (Fig. 7).

Chemotactic Activity.—500 μl of the Hageman factor fragments were incubated with 250 μl of prekallikrein obtained at the SE Sephadex step (Fig. 5) for 5 min at 37°C, diluted to 1 ml with Hanks' solution, and dialyzed against Hanks' solution for 12 hr. 100 μl of the mixture incubated with 200 μl of heat-inac-

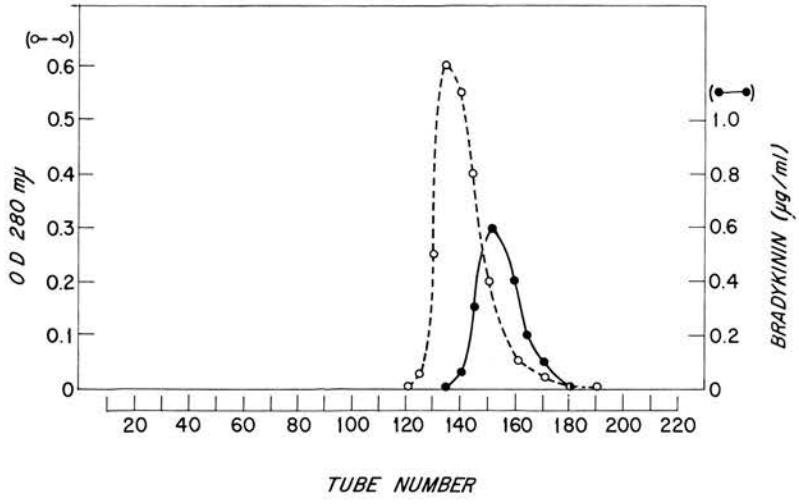

FIG. 6. Sephadex G-150 gel filtration of prekallikrein obtained from SE Sephadex (Fig. 5).

tivated plasma generated 200 ng of bradykinin indicating conversion of the prekallikrein to kallikrein. The same mixture was chemotactic for human neutrophils as shown in Table I, Experiment 1. Controls consisting of 250 μl of prekallikrein incubated with 500 μl of normal saline and 500 μl of the Hageman factor fragments incubated with 250 μl of normal saline, processed identically, did not generate bradykinin from heat-inactivated plasma and had no significant chemotactic activity. Experiment 2 shows a similar result utilizing prekallikrein obtained from the Sephadex G-150 gel filtration step (Fig. 6).

Inhibition of Chemotactic Activity of Kallikrein.—The generation of bradykinin by human kallikrein is dependent upon an enzymatic site which is inhibitable by DFP (19, 21) through the action of this agent upon the hydroxyl group of a serine residue. The effect of DFP as well as an active site histidine inhibitor, TLCK, upon the chemotactic activity and the bradykinin-generating activity

of kallikrein was examined. 300 μl of kallikrein were made 10^{-3} M in DFP or 10^{-3} M in TLCK, the mixture incubated for 15 min at 37°C and dialyzed three times against 500 ml of Hanks' solution for 12 hr. 700 μl of Hanks' solution were then added to each sample and the samples assayed for chemotactic and kinin-generating activity. As a control, an equal quantity of DFP and TLCK

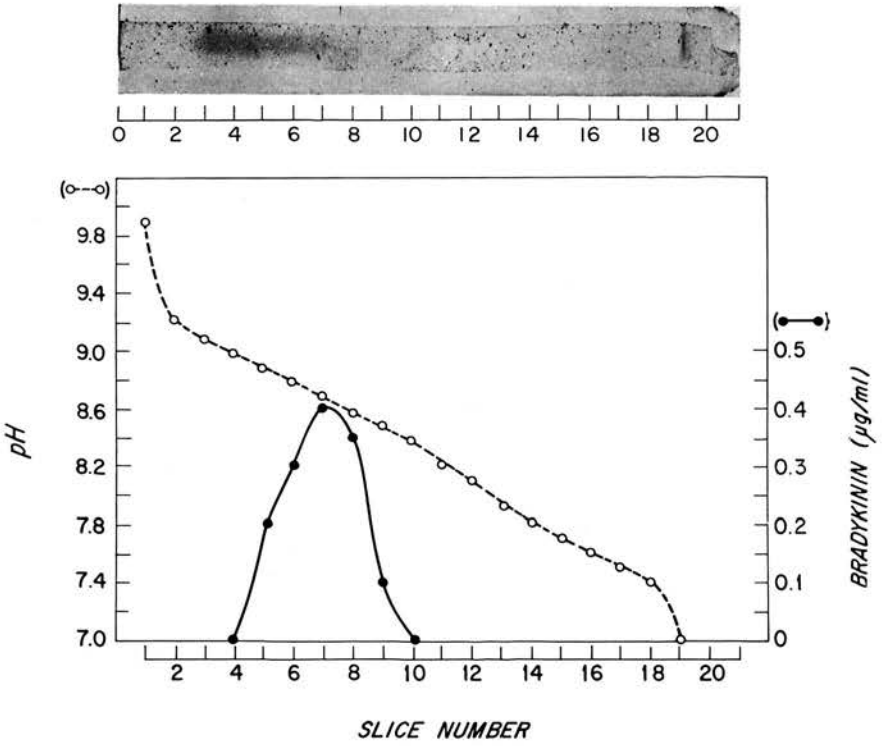

FIG. 7. Isoelectric focusing in 4% polyacrylamide gels of the prekallikrein-rich concentrate obtained from Sephadex G-150 gel filtration (Fig. 6). An unstained gel run simultaneously was sliced into 21 equal sections of 5 mm each and assayed for pH and prekallikrein.

were each added to 100 μl of 0.02 M Tris Cl, pH 7.6, the buffer in which the kallikrein preparation was stored, and dialyzed three times against 500 ml of Hanks' solution for 12 hr. 300 μl of kallikrein were then added, incubated for 15 min at 37°C, the volume brought to 1.0 ml with Hanks' solution, and assayed for chemotactic and kinin-generating activity. As shown in Fig. 8, DFP treatment of kallikrein abolished both kinin-generating and chemotactic activity (*B*), while removal of DFP from the buffer by dialysis before the introduction of kallikrein had no effect upon these two functions (*D*). No effect was seen with 10^{-3} M TLCK (*C*).

A dose response experiment for inhibition of chemotaxis and bradykinin generation by TLCK and DFP is shown in Fig. 9. The experiment was performed as described above except the incubation time of kallikrein with each

TABLE I

Generation of Chemotactic Activity for Human Neutrophils by Activation of Prekallikrein

	Prekallikrein	Hageman factor fragments	Prekallikrein + Hageman factor fragments
Experiment 1*			
Neutrophil chemotaxis (mean cell count)	0	1.5	14.2
Bradykinin generated from heat-inactivated plasma (ng)	0	0	200
Experiment 2‡			
Neutrophil chemotaxis (mean cell count)	0	0	15
Bradykinin generated from heat-inactivated plasma (ng)	0	0	175

* Prekallikrein obtained from SE Sephadex step.

‡ Prekallikrein obtained from Sephadex G-150 step.

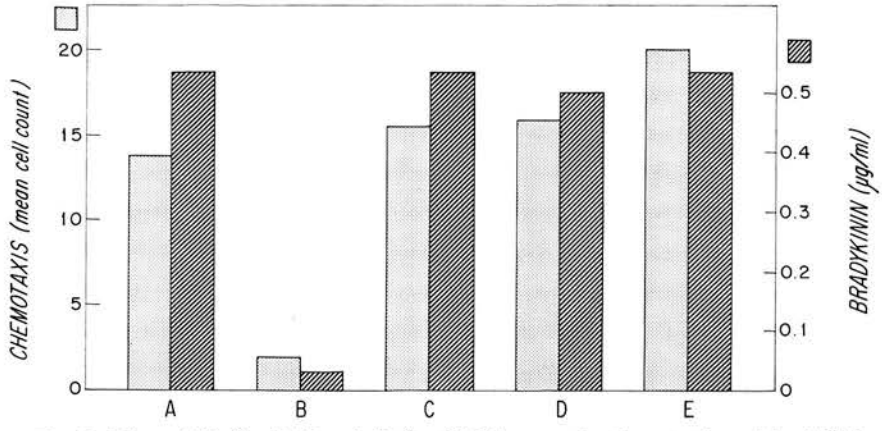

FIG. 8. Effect of 10^{-3} M DFP and 10^{-3} M TLCK upon the chemotactic and bradykinin-generating activity of kallikrein. (*A*) Kallikrein control. (*B*) Kallikrein made 10^{-3} M in DFP. (*C*) Kallikrein made 10^{-3} M in TLCK. (*D*) Kallikrein added to dialyzed DFP solution. (*E*) Kallikrein added to dialyzed TLCK solution.

inhibitor was 30 min. Longer incubation at this temperature resulted in non-specific loss of both kallikrein functions. No inhibition of either activity is obtained with TLCK until a 10^{-2} M concentration is used. A dose response inhibition of both activities was obtained with DFP between 0.25×10^{-4} M and 1.0×10^{-3} M.

In order to determine whether the inhibition of DFP is related to the active site of kallikrein, an experiment was performed in which the action of DFP upon prekallikrein and kallikrein was compared. 1 ml of prekallikrein obtained from plasma by QAE Sephadex, SE Sephadex, and Sephadex G-150 chromatog-

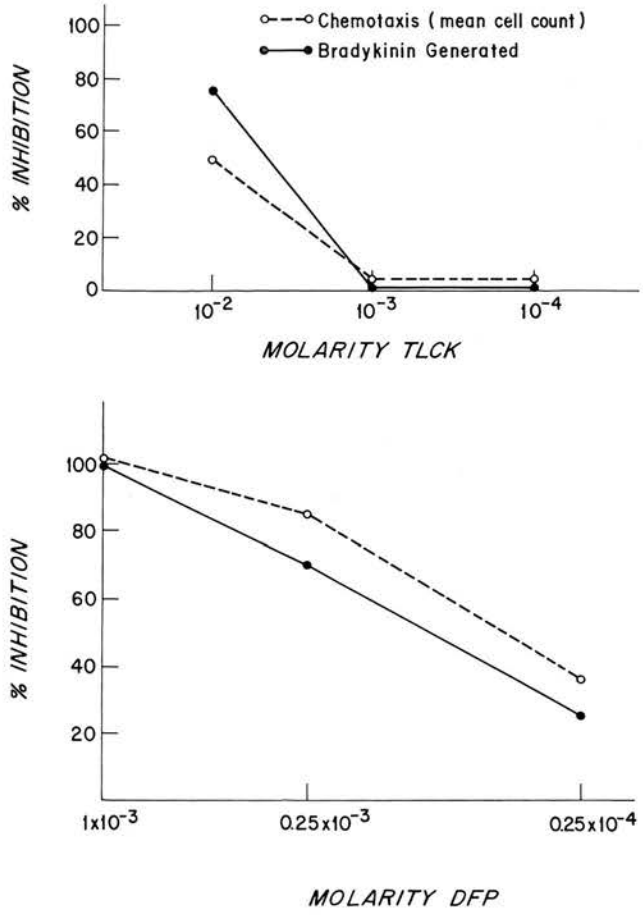

FIG. 9. Dose response inhibition of both chemotactic and bradykinin-generating activity of kallikrein utilizing TLCK and DFP.

raphy was incubated with 100 μl of Hageman factor fragments for 20 min at 37°C. The mixture was then divided in half, one portion was made 10^{-3} M in DFP and both were further incubated at 37°C for 15 min, brought to 1 ml volume with Hanks' solution, and dialyzed three times against 500 ml of Hanks' solution for 12 hr. Two 500 μl aliquots of prekallikrein were first made 10^{-3} M in DFP, incubated at 37°C for 15 min, and dialyzed three times against

500 ml of Hanks' solution for 12 hr. One portion was then activated by addition of 50 μl of the Hageman factor fragments, the mixture incubated for 20 min at 37°C, and both aliquots were then brought to 1 ml volume with Hanks' solution. Controls consisting of prekallikrein or the Hageman factor fragments alone, each brought to the same final concentrations utilized above by the addition of normal saline, were subjected to each incubation and dialysis step. Each preparation was then assayed for bradykinin-generating and chemotactic activity. As shown in Fig. 10, the prekallikrein preparation (*A*), the Hageman factor fragments (*B*), and the mixture of prekallikrein and the Hageman factor fragments which was then exposed to 10^{-3} M DFP (*D*) generated no bradykinin

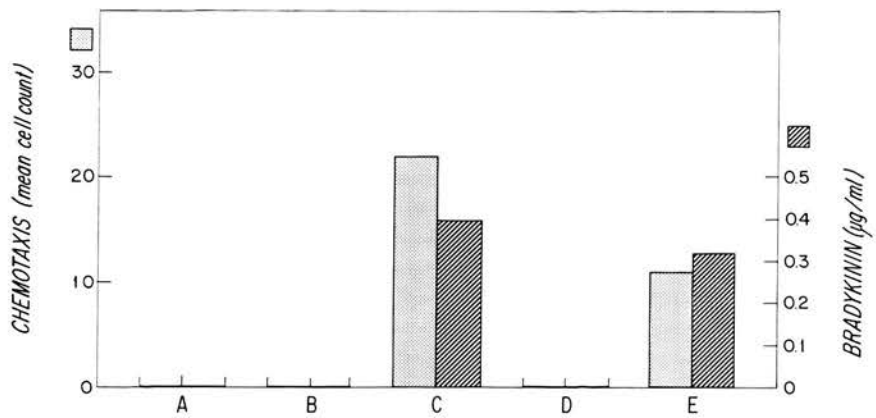

FIG. 10. Comparison of the effect of DFP upon kallikrein and prekallikrein. (*A*) Prekallikrein preparation. (*B*) Hageman factor fragments. (*C*) Prekallikrein activated with the Hageman factor fragments. (*D*) Prekallikrein activated with the Hageman factor fragments and made 10^{-3} M in DFP. (*E*) Prekallikrein made 10^{-3} M in DFP, dialyzed, then activated with the Hageman factor fragments.

from heat-inactivated plasma and possessed no chemotactic activity. Exposure of prekallikrein alone to 10^{-3} M DFP generated no bradykinin from heat-inactivated plasma and possessed no chemotactic activity. Exposure of prekallikrein to 10^{-3} M DFP followed by dialysis to remove the DFP and subsequent conversion to kallikrein by incubation with the Hageman factor fragments (*E*) revealed 80% of the kinin-generating activity and 50% of the chemotactic activity of prekallikrein which was activated without prior exposure to DFP (*C*).

DISCUSSION

When human blood is clotted in glass, thereby activating Hageman factor, the serum obtained has been shown to possess chemotactic activity (15). This activity is partially destroyed by heating for 30 min at 56°C. Since activation

of Hageman factor not only initiates the intrinsic pathway of blood coagulation (22, 23) and the fibrinolytic system (24, 25) but also the kinin-forming sequence, it seemed appropriate to evaluate the components of the kinin-forming system as possible contributors to the chemotactic activity of normal serum. Fractionation of normal serum on Sephadex G-200 revealed several peaks of chemotactic activity of which one coincided with the peak of kallikrein (Fig. 1), the most heat-labile component of the kinin-forming system (26).

The evidence that kallikrein is chemotactic for human neutrophils includes the superposition of kinin-generating and chemotactic activity when partially purified kallikrein is further fractionated by Sephadex G-150 gel filtration (Fig. 2) and by electrophoresis in alkaline disc gels (Fig. 3). Further evidence that chemotactic activity is a function of active kallikrein is provided by the finding that conversion of prekallikrein to kallikrein by the Hageman factor fragments is associated with the appearance of chemotactic activity whereas neither prekallikrein nor the Hageman factor fragments alone exhibited such a property (Figs. 5, 6, Table I). Although it seemed possible that the chemotactic activity observed might be due to a small fragment formed during conversion of prekallikrein to kallikrein, attempts to dissociate such a fragment from kallikrein by rechromatography on G-75 at pH 3.5 failed to reveal chemotactic activity except in association with the kallikrein peak. Furthermore, the capacity of DFP to inhibit both chemotaxis and kinin generation (Figs. 8, 9) is consistent with chemotaxis being an intrinsic function of the kallikrein molecule itself.

Lewis has shown that intradermal injections of relatively high concentrations of bradykinin (1–100 μg/ml) in the rabbit promotes the migration of leukocytes (27). Of the components of the kinin-forming system thus far examined, the Hageman factor fragments, prekallikrein, kallikrein, and bradykinin, only kallikrein was chemotactic. Furthermore, kallikrein attracted neutrophils but not eosinophils from a mixed leukocyte population, even when the suspension contained 70% eosinophils (Fig. 4). In contrast, an eosinophil chemotactic factor released from human lung sensitized with IgE and challenged with specific antigen attracted eosinophils but not neutrophils under the same experimental conditions (14) (Fig. 4).

The activation of Hageman factor by a variety of biologic materials such as collagen (28, 29), sodium urate crystals (30), pyrophosphate crystals (30), L-homocystine crystals (31), and human articular cartilage (32), and the capacity of activated Hageman factor to convert prekallikrein to kallikrein either directly or through its prealbumin fragments (5) offers a nonimmunologic, readily activatable mechanism for the generation of a chemotactic principle independent of the complement system. It is noteworthy that kallikrein is not only chemotactic for human neutrophils but also leads to the generation of the permeability factor bradykinin; two other chemotactic factors, fragments from the third and fifth components of complement both promote leukocyte migra-

tion in vitro (33, 34) and also increase vascular permeability by virtue of their anaphylatoxic activity (35, 36). It may be that a change in vascular permeability plays a role in determining whether or not a chemotactic principle can express this capability in an in vivo situation.

A series of esterases present in rabbit neutrophils, which play an essential role in the chemotactic response, have been described (37–39). That the active site of kallikrein is required for the expression of its chemotactic activity could be explicable either by direct activation of one of these esterases or through a substitution which bypasses an activation step utilized by nonenzymatic chemotactic factors. The relatively high concentration of DFP utilized in the inhibition experiments was necessitated by the requirement to inactivate kallikrein at 37°C within a 15 min period; incubation of kallikrein beyond this interval was associated with a loss in activity which reached 100% in 2 hr. The histidine inhibitor TLCK, however, was inactive at the same concentration and conditions (Fig. 9). The findings that the precursor form of the enzyme, prekallikrein, tolerated a dose of DFP which completely inactivated kallikrein (Fig. 10) is consistent with studies of other serine esterases in which the precursor enzyme was resistant to inactivation (40, 41). Since both the Hageman factor fragments and kallikrein are sensitive to inactivation by DFP (19, 21, 42), the partial loss of activity experienced by the prekallikrein could be attributed to residual DFP despite the extensive dialysis.

SUMMARY

Human plasma kallikrein has been shown to directly and selectively attract human neutrophils from a mixed leukocyte population. The capacity of plasma kallikrein to be chemotactic and to generate the nonapeptide bradykinin was maintained during progressive purification. While neither highly purified prekallikrein nor the prealbumin Hageman factor fragments were chemotactic alone, their interaction so as to convert prekallikrein to kallikrein yielded both chemotactic and kinin-generating activity. Both functions of kallikrein were inhibited by treatment with diisopropyl fluorophosphate, indicating an essential role for the active site of the enzyme in the expression of its chemotactic activity.

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Generation of Chemotactic Activity for Leukocytes by the Action of Thrombin on Human Fibrinogen

THE formation of the fibrin gel during the final stages of blood coagulation results from the action of thrombin on fibrinogen. Thrombin is a limited protease, which cleaves several small peptides from fibrinogen. These fibrinopeptides have been designated A, AP, AY and B, and are recognized on the basis of their electrophoretic mobility[1].

When human blood is clotted on glass so as to activate Hageman factor the serum possesses chemotactic activity for human peripheral blood leukocytes[2]. Following passage of the serum through a column of 'Sephadex G-200' several peaks of chemotactic activity were observed, one of which was subsequently identified as the enzyme kallikrein. Material of low molecular weight accounted for a substantial part of the

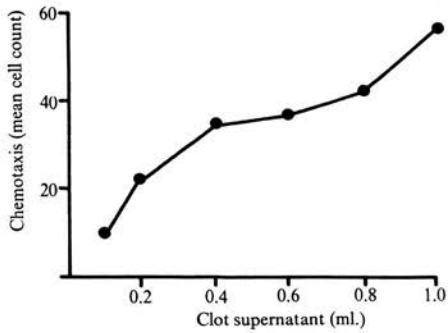

Fig. 1 Chemotaxis of human leukocytes towards a clot supernatant prepared from thrombin and fibrinogen. Thrombin and fibrinogen at equivalent concentrations gave no chemotactic activity.

chemotactic activity of normal serum which we considered may be a consequence, in part, of the release of fibrinopeptides (molecular weights approximately 2,000 each). We therefore prepared clot supernatants by incubating fibrinogen with thrombin and examined their capacity to attract human peripheral blood leukocytes *in vitro*.

Lyophilized human fibrinogen (Kabi, Stockholm) containing 95% of clottable protein was reconstituted in water to a concentration of 14 mg ml.$^{-1}$ of protein and dialysed against phosphate buffered saline (PBS). Bovine thrombin (Parke-Davis, Michigan) was purified by 'Sephadex G-200' chromatography. It eluted as a single peak and was assayed by its ability to clot human plasma in the presence of calcium. One part of thrombin was added to 24 parts of fibrinogen and the concentration of thrombin was adjusted to give a clotting time of approximately 45 s. The clot was left for 5 h at room temperature or overnight at +4° C. The fibrin gel was broken up with scalpel blades and centrifuged at 15,000*g* for 45 min. The supernatant was either assayed immediately or kept frozen at −20° C. Chemotaxis of human leukocytes was assayed by the modification of the 'Millipore' technique of Boyden[3].

Fibrinogen and thrombin, when tested alone, did not attract leukocytes, but the clot supernatant possessed chemotactic activity which was demonstrable in a dose-response fashion (Fig. 1). Most of the migrating cells were neutrophils although occasional eosinophils and monocytes were seen. The activity present in the clot supernatant was unaffected by heating at 60° C for 30 min or 100° C for 5 min. By ultrafiltration with membranes which retain molecules of a certain size and with

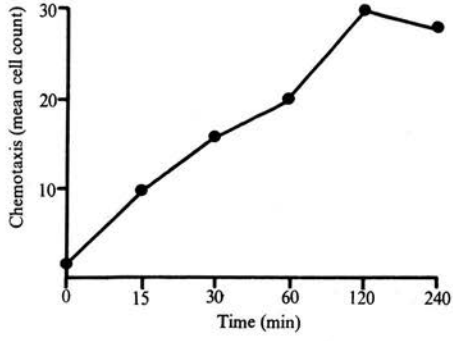

Fig. 2 Chemotactic activity generated by the action of contortrix enzyme on fibrinogen.

chromatography on 'Sephadex G-200' we showed that the activity was associated with low molecular weight material. The supernatants were desalted by passage through a column of 'Sephadex G-25' in 0.1 M pyridine and the material, reconstituted in water, was still active in chemotaxis. By high voltage electrophoresis using conditions previously described[4] the same preparation was shown to contain fibrinopeptides A, AP and B.

Further evidence that the activity was generated by limited proteolysis of fibrinogen was obtained with snake venoms which selectively cleave fibrinopeptides A or B. A purified enzyme from *Agkistrodon rhodostoma* (Arvin, Twyford Laboratories) releases peptides AP, AY and A from fibrinogen and leads to clot formation[4]. When a clot supernatant from fibrinogen treated with 'Arvin' was assayed, no chemotaxis was found. This was not due to an inhibitory effect of the 'Arvin' supernatant as it did not affect the activity of a supernatant prepared from thrombin. When the purified procoagulant enzyme from *Ancistrodon contortrix contortrix* (Sigma, Surrey) was incubated with fibrinogen, chemotactic activity could be demonstrated. Contortrix enzyme selectively cleaves fibrinopeptide B from fibrinogen[5]. The contortrix venom was purified free of fibrinolytic enzymes and their activators by 'Sephadex G-75' chromatography, and its concentration adjusted so that when 0.2 ml. was added to 10 ml. of fibrinogen it gave a clotting time of between 4 and 20 h. At intervals 1.2 ml. of the mixture was removed, heated at 100° C for 5 min to precipitate the fibrinogen and centrifuged to remove particulate matter. The material obtained at 4 h was shown by high voltage paper electrophoresis to contain fibrinopeptide B; other fibrinopeptides were not detected. Duplicate volumes of 0.5 ml. were tested for chemotaxis. The time course of release of chemotactic activity is shown in the experiment depicted in Fig. 2. Synthetic fibrinopeptide A (Schwarz/Mann, Orangeburg, New York) was inactive whereas fibrinopeptide B was chemotactic in a linear dose-response from 25 μg to 200 μg.

Further purification of the clot supernatant is required to confirm the nature of the activity generated from fibrinogen. Further, the contribution of other chemotactic agents released into the clot supernatant under the conditions cited above is still to be determined. Chemotactic activity for human leukocytes can be generated by the action of thrombin or contortrix enzyme on human fibrinogen, however, but not by 'Arvin'. In addition synthetic fibrinopeptide B but not fibrinopeptide A attracts human leukocytes *in vitro*.

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The Identification of Fibrinopeptide B as a Chemotactic Agent Derived from Human Fibrinogen

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Chemotactic activity for human peripheral blood leucocytes was generated by the action of thrombin on human fibrinogen, a reaction known to release low molecular weight fibrinopeptides. Following gel filtration of the clot supernatant most of the activity eluted at the same K_d as synthetic fibrinopeptides. Fibrinopeptides present in the clot supernatant were then identified and separated by high voltage electrophoresis in two dimensions. Chemotactic activity was a property of fibrinopeptide B and not of fibrinopeptides A, AP or AY. Supernatants prepared with contortrix venom, which cleaves the B peptide, were also chemotactic whereas no activity was present in supernatants prepared from Arvin venom which cleaves peptides A, AP and AY. Synthetic fibrinopeptide B and a B analogue, 1-glutamic acid, were found to be chemotactic but not synthetic fibrinopeptide A. Thus chemotaxis of human leucocytes represents a further biological activity of fibrinopeptide B.

In a preliminary study we found that clot supernatants, prepared by the action of thrombin on human fibrinogen, were chemotactic for human peripheral blood leucocytes (Kay *et al*, 1973). This reaction is known to generate the release of low molecular weight (approximately 1500) fibrinopeptides A, AP, AY and B which are recognized on the basis of their electrophoretic mobilities (Blombäck *et al*, 1966). Evidence was provided that low molecular weight material present in the clot supernatant contributed to its chemotactic property (Kay *et al*, 1973).

In the present study we have identified fibrinopeptide B as a chemotactic agent by isolating the peptide from a thrombin clot supernatant using electrophoresis in two dimensions. In addition details are given of studies using snake venoms which cleave selectively either fibrinopeptides A, AP and AY or B. We have also compared the abilities of synthetic fibrinopeptides A, B and a B analogue to attract human peripheral blood leucocytes.

MATERIALS AND METHODS

Preparation of Reagents

Thrombin. Approximately 5000 units of lyophilized bovine thrombin (Parke-Davis,

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Detroit, Michigan) were dissolved in 1 ml of distilled water and centrifuged at 10 000 *g* for 30 min. The material was applied to a column of Sephadex G-200 (95 × 2.5 cm) previously equilibrated with phosphate buffered saline (PBS), pH 7.35, and 2 ml fractions collected. Alternate tubes were assayed for thrombin activity by measuring the clotting time when 0.1 ml of the sample was added to 0.3 ml of human plasma in the presence of calcium. Clotting activity eluted as a single peak corresponding to a molecular size of between 30 000 and 40 000.

Procoagulant enzyme from Ancistrodon contortrix contortrix. One hundred mg of *Ancistrodon contortrix contortrix* venom (Sigma, Kingston, Surrey) were dissolved in 5 ml of borate buffered saline, pH 8.6, and applied to a column of Sephadex G-75 (100 × 2.5 cm) equilibrated in the same buffer. Fractions of 5 ml were collected and each tube assayed for procoagulant and fibrinolytic activity using an automated clot-lysis timer (Medicon (U.K.) Ltd). Procoagulant activity eluted at V_0 and at $K_d = 0.3$. The material eluting at V_0 had no fibrinolytic activity and was pooled and concentrated by ultrafiltration using a UM-10 membrane (Amicon Corp., Lexington, Mass.).

Preparation of Supernatants following Enzymatic Treatments of Human Fibrinogen

Thrombin supernatants. Method 1—Human fibrinogen (Kabi, Stockholm, Sweden) was dissolved in distilled water to a concentration of 1% and dialysed against PBS at 4°C overnight. One part of diluted thrombin was added to 24 parts of fibrinogen giving a clotting time of 45 s. The clot was stored for 5 hr at room temperature or overnight at +4°C. The fibrinogen gel was broken up with scalpel blades and centrifuged at 15 000 *g* for 45 min. Supernatants were either assayed for chemotaxis immediately or frozen at −20°C.

Method 2—To prepare larger quantities for electrophoretic isolation of fibrinopeptides, 10 *g* of human fibrinogen, prepared by the method of Blombäck & Blombäck (1956), was dissolved in 0.3 M ammonium acetate to a concentration of 2%. After dialysis against this same buffer, an equal volume of water was added and the solution clotted as above. The clot supernatant was rotary-evaporated to dryness and lyophilized overnight. To remove residual buffer salts, the sample was heated *in vacuo* for 1–2 hr at 40°C. The residue was washed repeatedly with 20 ml volumes of 0.05 M pyridine until the pH of the extract remained above 5.6 (Blombäck *et al*, 1966). To the pooled and concentrated extracts was added 1/5 vol of 30% TCA. After centrifugation the supernatant was extracted with water-saturated ether until the pH was greater than 4. Pyridine was then added to raise the pH to 6 and the solution concentrated for desalting on a G-25 column in 0.05 M pyridine. The Folin positive peak eluting before the salt peak was concentrated by rotary evaporation. This material containing the fibrinopeptides was then suitable for electrophoresis as described below.

Arvin supernatants. Arvin (*Agkistrodon rhodostoma* venom, Twyford Laboratories, London) was diluted in PBS to give a clotting time of 10 min when added to 24 parts of 1% fibrinogen (prepared by Method 1).

Contortrix supernatants. The contortrix venom was diluted in PBS so that 1 vol when added to 24 vol of 1% fibrinogen (prepared by Method 1) released fibrinopeptide B within 2 hr (as detected by electrophoresis) but gave no coagulation at 4 hr.

Gel-filtration of clot supernatants. Ten to 15 ml of fibrinogen clot supernatants were desalted on Sephadex G-25 as described above. Samples of 1–2 ml were applied to a 104 × 2.5 cm

column of Sephadex G-75 equilibrated with 0.05 M pyridine. From alternate 8 ml fractions 1 ml was taken, lyophilized and redissolved in 2 ml of PBS. These solutions were tested for chemotaxis either undiluted or diluted 1 in 4.

Electrophoresis. Vertical electrophoresis was performed as described by Ambler (1963) using a pyridine–acetic acid–water buffer (25 : 1 : 225 by vol for pH 6.5 and 1 : 10 : 89 for pH 3.5). The coolants used were toluene for pH 6.5 and white spirit for pH 3.5. Salt-free samples prepared as above, were applied to Whatman No. 3MM paper (57 cm × 45 cm) and a potential gradient of 60 V/cm applied. Synthetic fibrinopeptides A and B and amino acids were used as markers (Milstein & Milstein, 1968). After separation in the first dimension for 1.5 hr at pH 6.5, strips were cut from either side of the paper and sprayed with Sakaguchi reagent (Leggett-Bailey, 1967). Sakaguchi positive spots were marked and the strips immersed in 0.2% ninhydrin (in acetone) and dried at 60°C. Paper containing fibrinopeptides located by these methods was cut and sewn onto a second sheet of paper and separated in the second dimension for 2.5 hr at pH 3.5. The fibrinopeptides were again located by Sakaguchi and ninhydrin reagents by cutting strips from either side of the paper. Fibrinopeptides AP and AY were located as Sakaguchi and ninhydrin positive spots migrating in positions compatible with their known structure (Blombäck *et al*, 1966; Herzig *et al*, 1970). Each fibrinopeptide was eluted in 0.1 N ammonia and individually passed through columns of Sephadex G-25 in 0.05 M pyridine. The peak of Folin positive material from each column run was pooled, lyophilized and the residue dissolved in PBS. Each peptide was quantitated by the Sakaguchi reaction (Shainoff & Page, 1960) using an arginine-standard curve, diluted with PBS and tested for chemotaxis.

Measurement of chemotaxis. Chemotaxis of human leucocytes was assayed by a modification of the Millipore technique of Boyden as previously described (Kay, 1970). The principle of the technique is as follows. Two compartments are divided by a Millipore filter. In the upper or cell compartment a suspension of cells rich in human peripheral blood leucocytes is placed. In the lower, or test compartment, the agent being tested for chemotaxis is introduced. The chemotactic agent creates a gradient between the cell and test compartments thereby enabling the cells to migrate through the thickness of the filter. Following an incubation period the filter is removed, fixed and stained and the number of cells which have migrated through the entire thickness of the filter are counted. The results are expressed as the mean cell count of five high power (× 40) microscopic fields. The leucocyte-rich preparation was prepared as follows. Blood from normal human volunteers was drawn into plastic syringes and transferred to plastic tubes containing 20 units of heparin and 0.1 ml of 6% Dextran per ml of blood. The red blood cells were allowed to settle at 37°C for 30 min after which the leucocyte plasma was removed and centrifuged for 3 min at 100 *g*. The cells were washed once in Hanks' solution and resuspended in Hanks' solution containing 0.5% ovalbumin at pH 7.2. The cell count was adjusted to 2×10^6 leucocytes per ml. Chemotactic experiments were performed using an 8.0 μm pore size and a 90 min incubation period.

RESULTS

Chemotactic Activity Generated by the Action of Thrombin on Fibrinogen

Supernatants prepared by centrifugation of the clot formed by the action of thrombin on

TABLE I. The generation of chemotactic activity for human leucocytes by the action of thrombin on human fibrinogen. In experiments 1–4 and experiment 5 the dilutions of clot supernatants with Tyrode's solution were 4:1 and 2:3 respectively.

Experiment	*Chemotaxis (mean cell count)*		
	Fibrinogen	*Thrombin*	*Clot supernatant*
1	0	0	24
2	0	0	12
3	0	0	>50
4	8	0	24
5	0	0	30

human fibrinogen were found to be chemotactic for human peripheral blood leucocytes (Table I). This was demonstrable in a dose–response fashion (Fig 1). Fibrinogen and thrombin when tested alone were inactive (Table I). When clot supernatants were heated at 56°C for 30 min there was a slight loss of chemotactic activity (Fig 1).

In order to determine the nature of the chemotactic substance(s) generated by the interaction of thrombin with fibrinogen, a concentrated clot supernatant was applied to a column of Sephadex G-75. The major peak of chemotactic activity eluted at a distribution coefficient (K_d) of 0.72. This peak was concentrated and re-applied to the same column and alternate fractions tested for chemotaxis at two doses. Chemotactic activity eluted in the same position and was demonstrable in a dose–response fashion. When synthetic fibrinopeptides were applied to the column, they eluted with the same K_d as the major chemotactic peak.

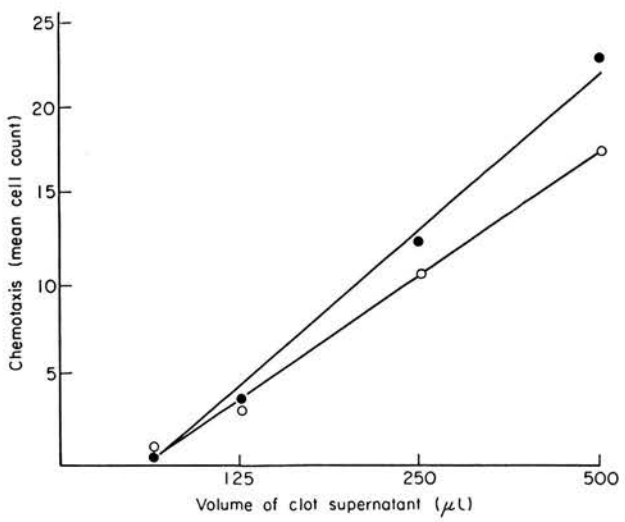

FIG 1. Chemotaxis of heated (○) and unheated (●) clot supernatants prepared by the action of thrombin on human fibrinogen.

The clot supernatant was then prepared for high voltage electrophoresis. The material applied to paper was active in chemotaxis indicating that the chemotactic agent(s) was soluble in TCA. Fibrinopeptide A was identified by comparing its mobility with a synthetic marker and by its positive reactivity with Sakaguchi and ninhydrin reagents (Fig 2). The A peptide was inactive in chemotaxis at all doses (Fig 3). Fibrinopeptide B gives a negative ninhydrin reaction but like the A peptide can be located with Sakaguchi reagent. Its mobility was also determined with a synthetic marker (Fig 2). Chemotaxis or fibrinopeptide B was demonstrable in a dose–response fashion between 0.03 μmol/ml and 0.3 μmol/ml (Fig 3).

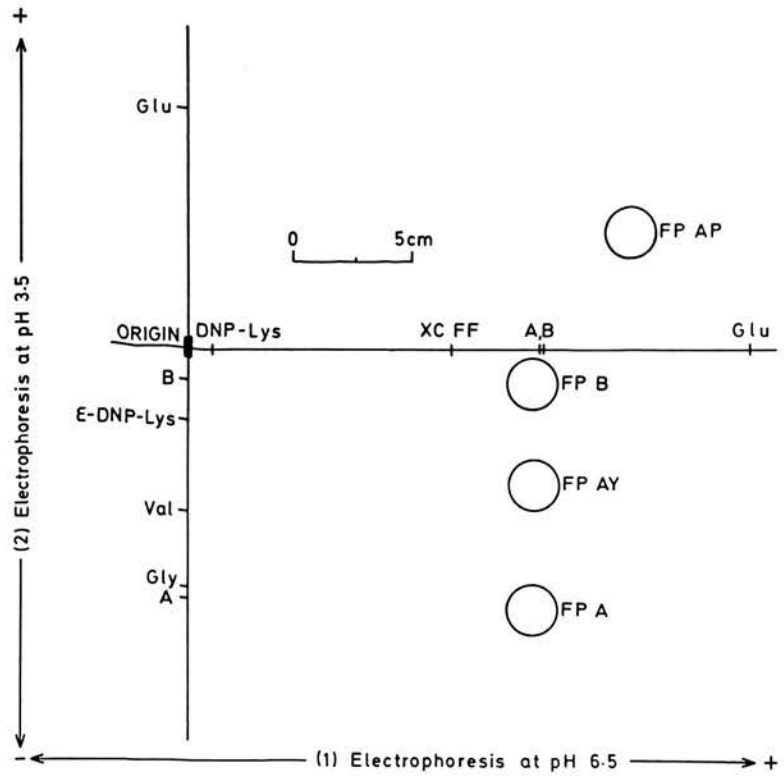

FIG 2. The separation of fibrinopeptides (FP) by high voltage electrophoresis in two dimensions. The origin represents the point where the uncharged marker, taurine, migrated at the pHs indicated. A, Gly, Val, ε-DNP lys, B, Glu, XCFF and B (along the axes) indicate respectively the positions reached by synthetic fibrinopeptide A, glycine, valine, epsilon-dinitro-phenol lysine, synthetic fibrinopeptide B, glutamic acid, and xylene cyanol FF.

The peptides AP and AY were present in smaller and variable amounts. There was no evidence that they had chemotactic activity. Both neutrophils and eosinophils migrated towards fibrinopeptide B in similar proportions to those present in the cell compartment prior to migration.

Chemotactic Activity Generated by the Action of Snake Venoms on Fibrinogen

Further evidence that fibrinopeptide B is chemotactic was obtained using purified snake

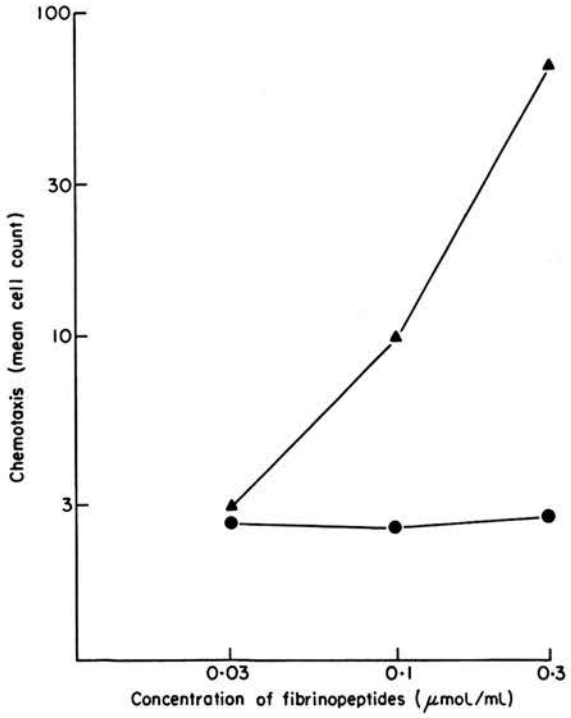

FIG 3. Chemotaxis of fibrinopeptide A (●) and fibrinopeptide B (▲) separated by high voltage electrophoresis prepared as in Fig 2. The points represent the mean result of five experiments.

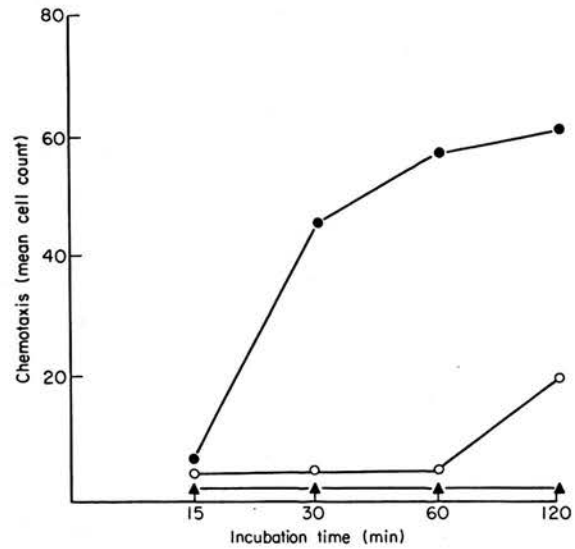

FIG 4. Chemotaxis of clot supernatants prepared by the action of contortrix (●), thrombin (○) or Arvin (▲) on fibrinogen.

venoms which selectively cleave either fibrinopeptides A, AP and AY or fibrinopeptide B from fibrinogen. Arvin cleaves the A peptides and contortrix the B peptide.

Five ml volumes of 1% fibrinogen in PBS were incubated at time intervals from 15 min to 4 hr with thrombin, Arvin and the procoagulant enzyme from contortrix. The reactions were terminated by heating the mixtures at 85°C for 5 min, and the insoluble material removed by centrifugation. In these experiments the dose of thrombin was adjusted to give the same coagulation time as Arvin (10 min). No coagulation was observed with contortrix at the termination of the experiment indicating that the A peptide was not released. Leucocytes migrated towards the supernatants prepared with contortrix and thrombin but not with Arvin (Fig 4). When treated in a similar fashion alone, neither fibrinogen, contortrix, Arvin or thrombin evoked chemotaxis. Arvin had no inhibitory effect on the chemotactic activity

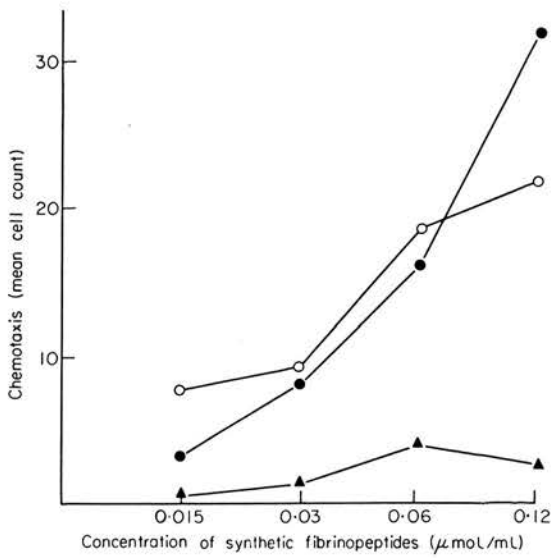

FIG 5. Chemotaxis of synthetic fibrinopeptides B (●), [1-Glu] B (○) and A (▲).

of supernatants prepared from the action of thrombin on fibrinogen. Electrophoresis was used to identify the fibrinopeptides released by each enzyme in the supernatants tested for chemotaxis. With Arvin only the A peptides were identified, and with contortrix only fibrinopeptide B. Electrophoresis of the supernatant obtained with thrombin indicated that both fibrinopeptide A and B were released.

The Chemotactic Activity of Synthetic Fibrinopeptides

Synthetic fibrinopeptides were tested for their capacity to attract human peripheral blood leucocytes. Fibrinopeptides A, B and the [1-Glu] B analogue were tested at concentrations between 0.015 μmol/ml and 0.12 μmol/ml. Chemotaxis was observed in a dose–response fashion with fibrinopeptides B and [1-Glu] B but not with fibrinopeptide A (Fig 5).

DISCUSSION

Chemotactic activity for human leucocytes was generated by the action of thrombin on human fibrinogen (Table I). The activity present in the clot supernatant was relatively resistant to heat at 56°C and was demonstrable in a dose–response fashion (Fig 1). Following gel-filtration of this material, a major peak of chemotaxis was observed with the same K_d as synthetic fibrinopeptide markers indicating that this activity was associated with material of molecular size approximately 1500. Fibrinopeptides were separated and identified by high voltage electrophoresis in two dimensions (Fig 2). Only fibrinopeptide B possessed chemotactic activity for human peripheral blood leucocytes, no activity being observed with fibrinopeptides A, AP or AY (Fig 3). In two experiments fibrinopeptide B gave weak or negligible chemotaxis. This has been attributed to variations in the response of the target cells. In these experiments low chemotactic counts with fibrinopeptide B were accompanied by a low response to the normal serum control.

When the purified procoagulant enzyme from contortrix was incubated with fibrinogen, chemotactic activity could be demonstrated in a dose–response fashion (Fig 4). Contortrix enzyme selectively cleaves fibrinopeptide B from fibrinogen (Herzig *et al*, 1970). Purified Arvin releases, AP, AY and A from fibrinogen and leads to clot formation (Ewart *et al*, 1970). The clot supernatant from Arvin was inactive in chemotaxis (Fig 4).

Further evidence that fibrinopeptide B and not fibrinopeptide A was chemotactic was obtained using synthetic human fibrinopeptides (Fig 5). Synthetic B was chemotactic in a dose–response fashion.

Chemotaxis, therefore, joins other biological activities associated with fibrinopeptide B such as the potentiation of bradykinin-induced contraction of the isolated oestrous rat uterus (Gladner *et al*, 1968) and prolonged rhythmic vaso-constriction (Colman *et al*, 1967).

It is probable that the biological activity associated with fibrinopeptide B is located at its C-terminus since this part of the molecule is exposed following the action of thrombin and other enzymes which cleave B from the β polypeptide chain of fibrinogen. The parent molecule, with the N terminus exposed, was inactive in chemotaxis. Furthermore, in experiments using a synthetic fibrinopeptide B analogue in which glutamic acid was substituted for pyrrolidone carboxylic acid at the N terminus, there was no effect on the chemotactic activity (Fig 5). It would be of interest to determine the species specificity of the chemotactic activity of fibrinopeptides since these differ markedly in their amino-acid composition (Blombäck *et al*, 1965a).

The biological significance of the chemotactic activity of fibrinopeptide B is unknown but it may play a part in the initial accumulation of leucocytes *in vivo* around the fibrin clot. Fibrinopeptide B is released more slowly than the A peptide following the action of thrombin on fibrinogen (Blombäck & Vestermark, 1957) a situation which may favour a chemotactic gradient.

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THE GENERATION OF CHEMOTACTIC ACTIVITY FOR HUMAN LEUKOCYTES BY THE ACTION OF PLASMIN ON HUMAN FIBRINOGEN

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ABSTRACT Chemotactic activity for human peripheral blood leukocytes was generated by the action of plasmin on human fibrinogen. When plasmin digestion was stopped at time intervals up to 24 hours, a small amount of activity was present at 15 and 30 min. corresponding to the transient appearance of fragment Y. The activity contained in the 24-hour digest was considerably higher and eluted from Sephadex G-75 with molecules of approximately 30,000 daltons. When purified X, Y, D and E were assayed individually for chemotaxis only fragment Y was active but in relatively high concentrations. Thus the chemotactic activity generated by the action of plasmin on fibrinogen was mainly associated with one or more lower molecular weight polypeptides and to a lesser extent with the Y fragment.

INTRODUCTION

A number of studies have shown that plasmic degradation of human fibrinogen yields four major fragments designated X, Y, D and E in addition to smaller peptide material (1,2,3). Fibrin/fibrinogen degradation products (FDP) appear in body fluids in association with various clinical states (4,5,6,7) and have a number of biological activities (8,9,10,11). In a previous report we have shown that chemotactic activity can be generated by the action of thrombin on human fibrinogen (12). This was subsequently identified as a property of fibrinopeptide B (13). In the present study we have examined the capacity of plasmin digestion products of fibrinogen to attract human peripheral blood leukocytes.

MATERIALS AND METHODS

Plasmin Digestion of Human Fibrinogen. One gm of human fibrinogen (KABI,

Grade L) was dissolved in 100 ml of water and dialysed for 18 hours at 4°C against phosphate buffered saline (PBS), pH 7.2, containing 50 units of penicillin and 5 µg of streptomycin per ml. To 100 ml of fibrinogen diluted to a concentration of 5 mg/ml was added 0.25 ml of streptokinase (Streptase, Behring, 10,000 units/ml) and 2 ml of plasminogen (KABI, 5 CU/ml). The reaction was stopped at time intervals by the addition of one volume of Trasylol (Bayer, 10,000 units/ml) to 40 volumes of the reaction mixture. The preparation was assayed for chemotaxis immediately or following storage at -80°C. Control mixtures of fibrinogen alone, and plasminogen and streptokinase alone were prepared in the same way with the addition of Trasylol at zero time and 24 hours. The digests were analysed by polyacryamide disc gel electrophoresis (14). For trichloracetic acid (TCA) precipitation one fifth of the volume of 30% TCA was added to the digest. After centrifugation the supernatant was extracted with ether, concentrated and desalted on a Sephadex G-25 column in 0.05 M pyridine as previously described (13).

Gel Filtration. One millilitre of a 10 times concentrated 24-hour plasmin digest of fibrinogen was applied to a column of Sephadex G-75 (100 x 2.5 cm) equilibrated in PBS with streptomycin and penicillin. The separation was performed at 4°C and 5 ml fractions collected. Markers with the following molecular weight were also filtered on the column - Blue Dextran, 200,000; ovalbumin, 45,000; vitamin B_{12}, 1,357, and sodium chloride, 58.

Preparation of Purified Fibrinogen Digestion Products. Fragments X, Y, D and E were prepared by Sephadex G-200 and ion exchange chromatography on CM-52 (Whatman) (3). The preparations were concentrated by ultrafiltration on a UM-10 membrane (Amicon) and dialysed against PBS with added antibiotics as described above. The fragments gave single bands on disc gel electrophoresis which using the appropriate markers also corresponded to their previously described monomeric molecular weights (3).

Measurement of Chemotaxis. Chemotaxis was assayed by a modification of the Millipore technique of Boyden (13). The number of cells migrating through an 8.0 µm filter were counted and the results expressed as the mean cell count of five high power (x 40) fields or as the percentage of a normal human serum pool used as the chemotactic control (15).

RESULTS

A 24-hour plasmin digest of human fibrinogen was found to be chemotactic for peripheral blood leukocytes in a dose-dependent fashion (Fig. 1).

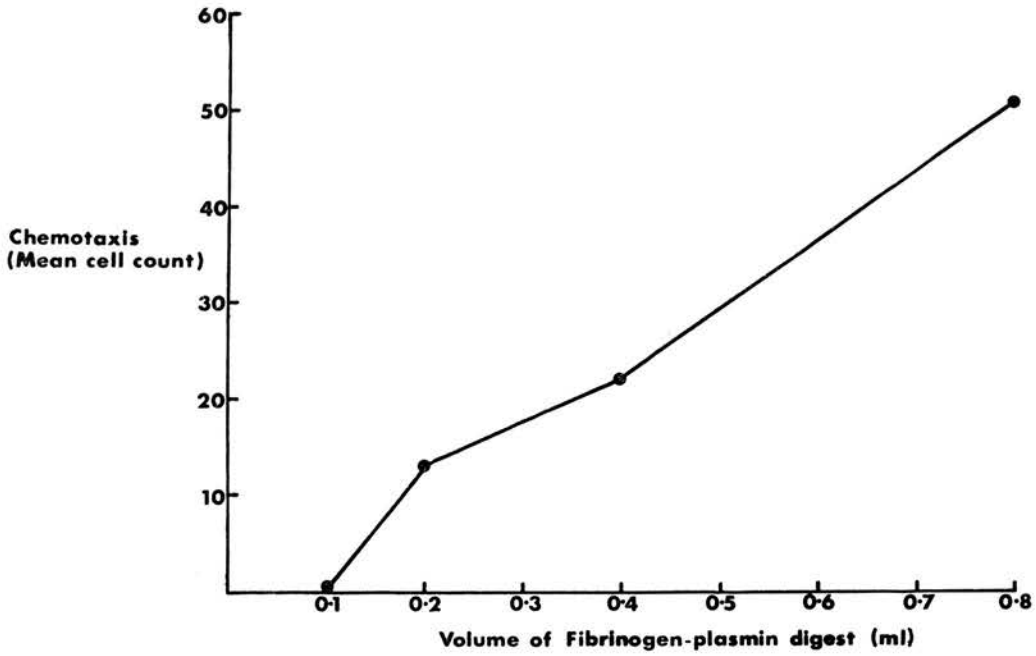

FIG. 1

Chemotaxis of a 24-hour plasmin/fibrinogen digest. The volume of digest was made up to 0.8 ml with PBS.

No activity was detected with fibrinogen or plasmin alone incubated under the same conditions. Trasylol was also devoid of chemotactic activity and did not inhibit or potentiate the activity of normal human serum used as the positive chemotactic control. Following precipitation of a 24-hour fibrinogen/plasmin digest with TCA, little activity was found in the desalted supernatant, suggesting that the activity was not associated with small peptides.

In Fig. 2 three experiments are depicted in which fibrinogen was digested with plasmin for time intervals up to 24 hours and the chemotactic activity and patterns on disc gel electrophoresis compared at intervals. In two out of three studies a small peak of activity was seen at 15 and 30 min. corresponding to the transient appearance of fragment Y. The major chemotactic activity, however, was in the 24-hour digest.

When a preparation of a 24-hour fibrinogen digest was passed over a column of Sephadex G-75, the major peak of activity eluted with material of an apparent molecular size of approximately 30,000 daltons (Fig. 3)

The chemotactic activity of purified fragments X, Y, D and E are shown in Fig. 4. Significant activity was detected only with fragment Y and was demonstrable at concentrations of 0.5 mg and 1.0 mg per ml.

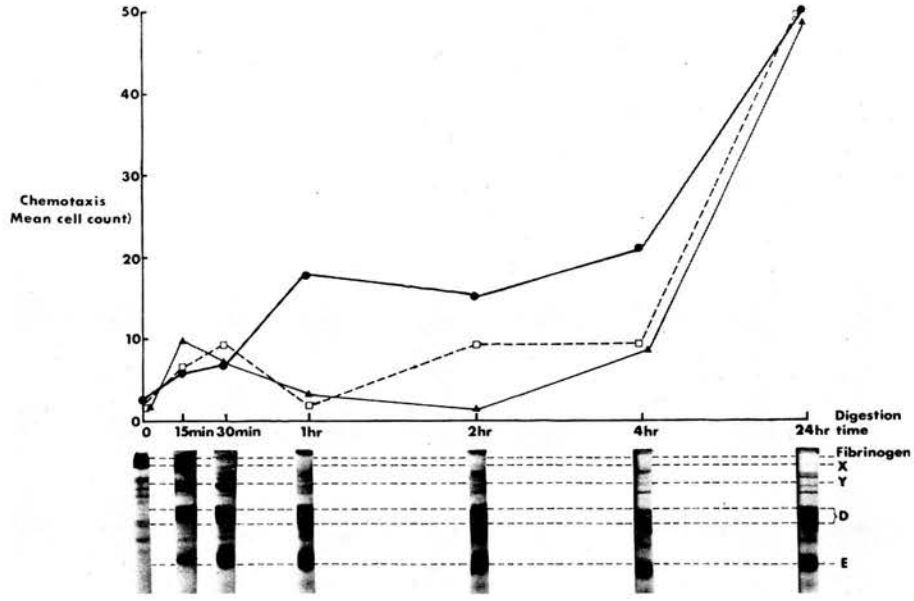

FIG. 2

Disc gel electrophoresis and chemotaxis of plasmin/fibrinogen digests at various time intervals. The chemotactic results of three experiments are depicted along with the positions of fibrinogen and fragments X, Y, D and E on the disc gels.

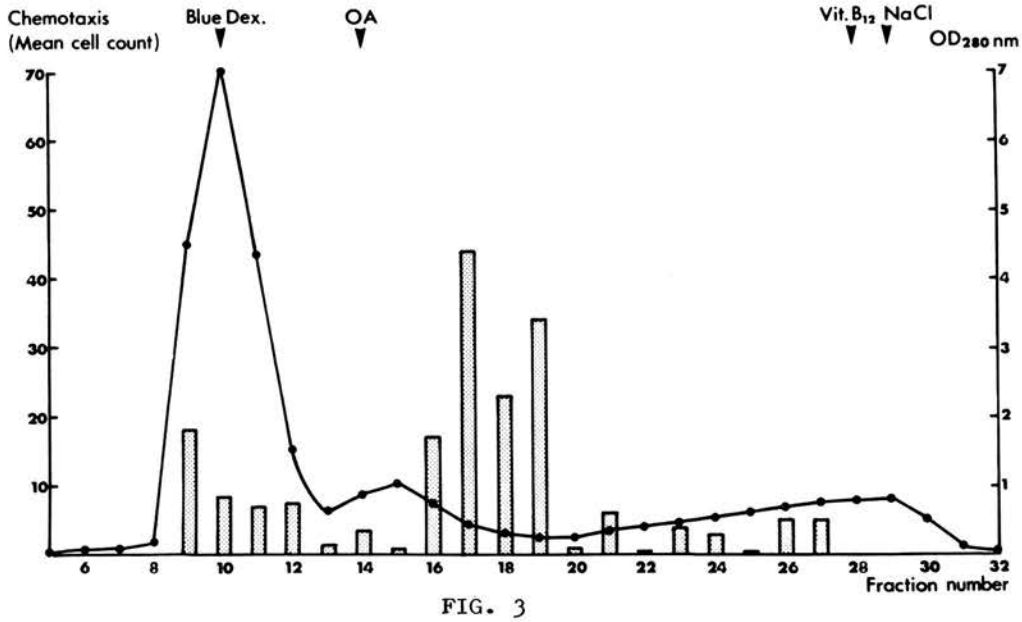

FIG. 3

Sephadex G-75 chromatography of a 24-hour plasmin/fibrinogen digest. The optical density (●--●)of fractions was measured at 280 nm. Chemotaxis is indicated by vertical bars and the elution of molecular weight markers is indicated by arrows. Molecular markers are Blue Dextran (Blue Dex.), ovalbumin (OA), vitamin B_{12} (Vit. B_{12}) and sodium chloride (NaCl).

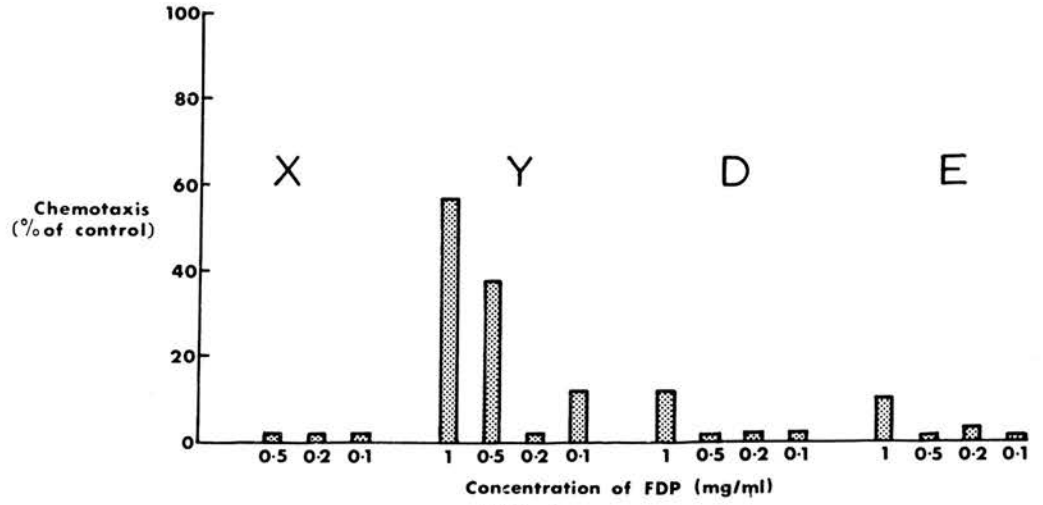

FIG. 4

Chemotaxis of purified fragments X, Y, D and E.

DISCUSSION

Chemotactic activity for human leukocytes was generated by plasmin digestion of fibrinogen (Fig. 1). When the reaction was stopped after 15 or 30 minutes, some activity was found in these digests (Fig. 2). A chemotactic stimulus in these preparations was probably fragment Y since in separate experiments this fragment was isolated in a pure form and shown to be active at the concentration estimated for the 15 or 30 minute digests (Fig. 4).

At 24 hours, when Y was no longer detectable, there was considerably more chemotactic activity. When a sample of the 24-hour digest was applied to a column of Sephadex G-75 the major peak of chemotactic activity eluted with molecules having an apparent molecular weight of approximately 30,000 daltons. The nature of this material is unknown but the activity was clearly generated by the action of plasmin on fibrinogen whereas neither of these agents alone were chemotactic. It is possible that the activity rests in unidentified band(s) which appeared during the plasmin digest. Thus on disc gel several bands were observed which migrated further than fragment E (ca. 50,000 molecular weight). These bands first appeared at 30 min. (Fig. 2) and may have contained the later chemotactic material. The failure to demonstrate greater activity with the earlier digests may have been a result of inhibition of leukocyte migration by the larger fragments or possibly a requirement for further digestion. Furlan and Beck described three polypeptide fragments in addition to D and E which survive prolonged plasmin digestion (3). These have disc gel electrophoretic mobilities corresponding to molecular sizes of 25,000, 14,000

and 11,000. Whether these contain the chemotactic property described herein is unknown.

Though fibrinopeptide B is cleaved from the β-chain of fibrinogen in the latter stages of plasmin digestion (17), little activity was found in the Sephadex G-75 column fractions corresponding to the molecular weight of this peptide (ca. 1,500). However it was estimated that the amount of B peptide in the fibrinogen digest applied to the column was approximately 0.3 μmol/ml if 100% of this peptide was cleaved by plasmin. Thus the concentrations in the fraction would be less than the minimum concentration previously reported to stimulate leukocyte migration (0.1 μmol/ml) (13).

Barnhart reported that fragment D evoked migration of canine granulocytes in vivo using a skin window technique (18). Under the conditions described in this report fragment D was not chemotactic (Fig. 4). There are difficulties in attributing chemotactic properties to agents which cause cell migration following contact with abraded skin as this may lead to the activation of Hageman factor and subsequent generation of a number of recognised chemotactic factors (13,15).

Our results suggest that the main chemotactic effect of plasmin digestion lies in one or more small peptides produced after 24 hours digestion. Using rabbit leukocytes, Stecher and Sorkin found only weak chemotactic activity in a 24-hour human plasmin/fibrinogen digest (17). In the present investigation human leukocytes were used as target cells; the use of a homologous system may thus explain this apparent discrepancy.

Other biological activity associated with fibrin/fibrinogen digestion products include anticoagulant activity (8,9) and ability to alter platelet function (9), potentiation of smooth muscle contraction (10) and increased capillary permeability (11). These biological activities together with the capacity of plasmin digestion products to attract human leukocytes may be of significance in a variety of pathological conditions such as disseminated intravascular coagulation (4), pulmonary embolism (5), transplantation rejection (6) and glomerulonephritis (7) in which high levels of FDP's have been detected in the blood and urine.

ACKNOWLEDGEMENTS

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Chemotaxis for Human Monocytes by Fibrinogen-derived Peptides

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SUMMARY. Chemotactic activity for human peripheral blood monocytes was generated by the action of thrombin on human fibrinogen, a reaction known to release low molecular fibrinopeptides. Supernatants prepared by incubating Contortrix venom with fibrinogen, which cleaves the B peptide, were also chemotactic, whereas no activity was present in supernatants from Arvin venom, which cleaves peptides A, AP and AY. Thus fibrinopeptide B was chemotactic for the monocyte in addition to the neutrophil, as previously reported.

Monocyte chemotactic activity was also generated by the action of plasmin on human fibrinogen and shown to be associated with the D and E fragments but not with a mixture of fragments X and Y. When plasmin digestion was stopped at time intervals up to 24 h, monocyte chemotactic activity corresponded with the appearance of the D and E fragments. The monocyte chemotactic activity, contained in a 24 h digest, eluted from Sephadex G-75 at V_0, corresponding to the expected position of the D and E fragments whereas neutrophil chemotactic activity eluted with molecules of molecular size of approximately 30 000 daltons. Thus fragments D and E derived from plasmin digestion of fibrinogen attract the monocyte whereas only the small uncharacterized peptides were chemotactic for the neutrophil. These different profiles of chemotactic activity for the neutrophil and the monocyte in terms of plasmin digestion products of fibrinogen may be of significance in the events leading to the accumulation of these cells *in vivo* during fibrin deposition.

We have recently shown that a number of peptides derived from fibrinogen are chemotactic for human peripheral blood neutrophils. These include fibrinopeptide (FP) B, released from fibrinogen together with FPs A, AP and AY following the action of thrombin (Kay *et al*, 1974), and fragment Y and uncharacterized lower molecular weight polypeptides (LWP) cleaved from fibrinogen during plasmin digestion (McKenzie *et al*, 1975).

During inflammatory reactions associated with fibrin deposition, such as various types of glomerulonephritis and clot formation, the initial accumulation of neutrophils is followed by the appearance of mononuclear cells. In the present study we report on the capacity of fibrinogen-derived peptides to attract human monocytes in chemotaxis and have shown a

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difference in the chemotactic properties of these peptides in terms of their capacity to attract either the monocyte or the neutrophil.

MATERIALS AND METHODS

Plasmin Digestion of Human Fibrinogen

One gram of human fibrinogen (KABI Grade L) was dissolved in 100 ml of water and dialysed for 18 h at 4°C against phosphate buffered saline (PBS), pH 7.0. To 100 ml of fibrinogen diluted to a concentration of 5 mg/ml was added 0.25 ml of streptokinase (Streptase, Behring, 10 000 units/ml) and 2 ml of human plasminogen (KABI, 5 CU/ml). The reaction was stopped at time intervals by the addition of one volume of Trasylol (Aprotinin, Bayer, 10 000 units/ml) to 40 volumes of the reaction mixture. The preparation was assayed for chemotaxis immediately or following storage at −40°C. Control mixtures of fibrinogen alone, and plasminogen and streptokinase alone were prepared in the same way with the addition of Trasylol at zero time and 24 h. The digests were analysed by polyacrylamide disc gel electrophoresis (Weber & Osborn, 1969).

Clot Supernatants

Supernatants prepared by the action of purified bovine thrombin, Arvin (*Agkistrodon rhodostoma* venom) and Contortrix (*Ancistrodon contortrix contortrix*) on fibrinogen were prepared as previously described (Kay *et al*, 1974) and quantitated by the Sakaguchi reaction (Shainoff & Page, 1960) using an arginine-standard curve.

Gel Filtration

Two millilitres of a 10 times concentrated 24 h plasmin digest of fibrinogen was applied to a column of Sephadex G-75 (100 × 2.5 cm) equilibrated in PBS. The separation was performed at 4°C and 7 ml fractions collected. Markers with the following molecular weight were also filtered on the column: Blue Dextran, 200 000; ovalbumin, 45 000; vitamin B_{12}, 1357, and glucose 58.

Preparation of Purified Fibrinogen Digestion Products

Fragments X+Y, D and E were prepared by Sephadex G-200 and ion exchange chromatography on CM-52 (Whatman) (Furlan & Beck, 1972). The preparations were concentrated by ultrafiltration on a UM-10 membrane (Amicon) and dialysed against PBS. The D and E fragments gave single bands on disc gel electrophoresis which, using the appropriate markers, corresponded to their previously described monomeric molecular weights (Furlan & Beck, 1972). The X and Y fragments were not clearly separated and were tested together in chemotaxis.

Measurement of Chemotaxis

(i) *Human monocytes.* Human mononuclear cells, free of granulocytes, were prepared by layering whole heparinized blood onto a Ficol-hypaque gradient as described (Böyum, 1968). The separated mononuclear cells were washed twice in PBS, pH 7.0, and resuspended in Hanks' solution, pH 7.0, to a concentration of 1.5×10^6 monocytes per millilitre. Monocyte

chemotaxis was assayed by measuring the distance that the migrating front of cells had travelled into Millipore filters of 8.0 μm pore size following a 90 min incubation period (Zigmond & Hirsh, 1973). Results are expressed as an average of five high power fields, each test being done in either duplicate or triplicate.

(ii) *Human neutrophils.* Chemotaxis was assayed by a modification of the Millipore technique of Boyden (Kay, 1970; Kay *et al*, 1974). The number of cells migrating through an 8.0 μm filter was counted and the results expressed as the mean cell count of five high power ($\times$ 40) fields.

RESULTS

Supernatants prepared by centrifugation of mixtures of thrombin, Contortrix or Arvin with fibrinogen were tested for monocyte chemotactic activity as shown in Fig 1. The reactions were terminated at time intervals by heating the mixtures at 85°C for 5 min and removing the insoluble material by centrifugation. The dose of thrombin was adjusted to give the same

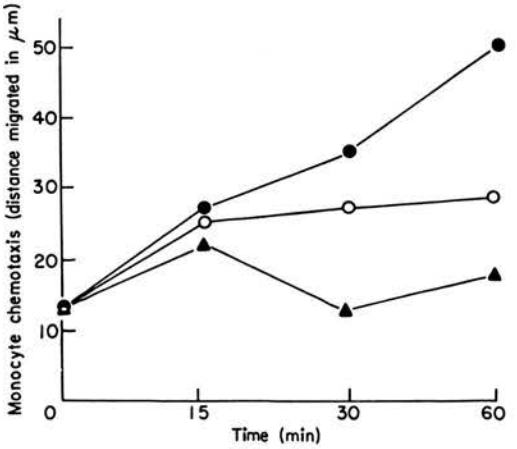

FIG 1. Time course of the appearance of monocyte chemotaxis of clot supernatants prepared by the action of Contortrix (●), thrombin (○) or Arvin (▲) on fibrinogen.

coagulation time as Arvin (10 min). No coagulation was observed with Contortrix at the end of the experiment, indicating that fibrinopeptide A was not released. Monocytes migrated towards the supernatant prepared with Contortrix and thrombin but not with Arvin. When treated in a similar fashion alone, neither fibrinogen, Contortrix, Arvin nor thrombin evoked monocyte or neutrophil chemotaxis. These experiments were repeated three times and gave essentially similar results. Electrophoresis was used to identify the peptides released by each enzyme in the heated supernatants tested for chemotaxis (Kay *et al*, 1974). With Arvin only the A peptides (A, AY and AP) were identified whereas only fibrinopeptide B was cleaved by the action of Contortrix on fibrinogen. Electrophoresis of the supernatant obtained with thrombin indicated that fibrinopeptides A, AP, AY and B were released.

The Contortrix and thrombin supernatants taken after 60 min incubation (Fig 1) were then

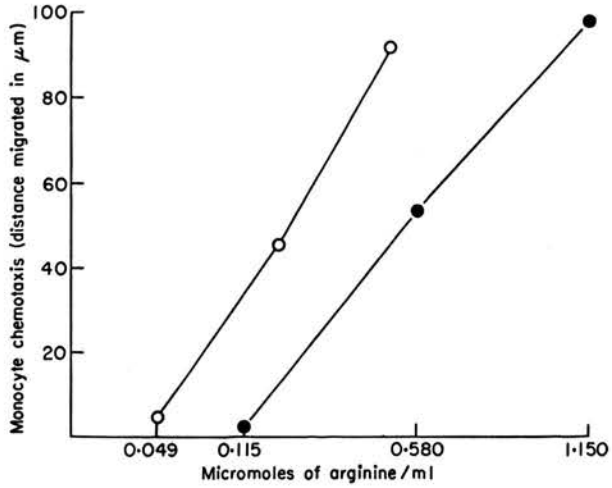

FIG 2. Monocyte chemotaxis of Contortrix (○) and thrombin (●) clot supernatants prepared from 60 min incubation times as shown in Fig 1.

tested for monocyte chemotactic activity which was demonstrable in a dose–response fashion as shown in Fig 2. These same supernatants (Contortrix and thrombin) were also chemotactic for neutrophils (thus confirming our previous report: Kay *et al*, 1974) whereas the Arvin supernatant was inactive. Therefore fibrinopeptide B was chemotactic for both the monocyte and the neutrophil whereas FPs A, AP and AY did not chemoattract these cell types (Table I).

Monocyte chemotaxis by the products of plasmin digestion of fibrinogen is shown in Fig 3. Both the D and E fragments were chemotactic, whereas no activity could be demonstrated with a mixture of the X and Y peptides. Neither the D, E nor X+Y fragments were chemotactic for human neutrophils using the same concentrations as shown in Fig 3.

When fibrinogen was digested with plasmin for time intervals up to 24 h, monocyte chemotactic activity corresponded to the appearance of the D and E fragments and was

TABLE I. The chemotactic activities of neutrophils and monocytes for fibrinogen-derived peptides

	Molecular sizes	*Neutrophils*	*Monocytes*
Fibrinopeptides A, AP and AY	1500 each	−	−
Fibrinopeptide B	1500	+	+
Fragment X+Y	250 000 and 155 000 respectively	−	−
Fragment D	82 000	−	+
Fragment E	45 000	−	+
'Small uncharacterized peptides' derived from plasmin digestion of fibrinogen	Approx. 30 000	+	−

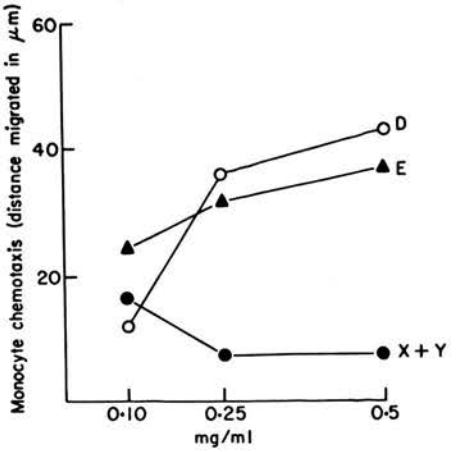

FIG 3. Monocyte chemotaxis of purified fragments D and E and the mixture of X and Y. The results represent the mean values of four experiments.

clearly demonstrable at 15 min and increased to a maximum by 60 min when the distance in migration was maintained for the duration of the experiment (Fig 4). This experiment was repeated twice with essentially similar results.

When a 24 h plasmin digest of fibrinogen was applied to a column of G-75, chemotactic activity for monocytes eluted with the void volume which would be compatible for the elution profile of fragments D and E. In contrast, neutrophil chemotactic activity eluted with molecules of lower molecular size (approximately 30 000 daltons) suggesting that activity for

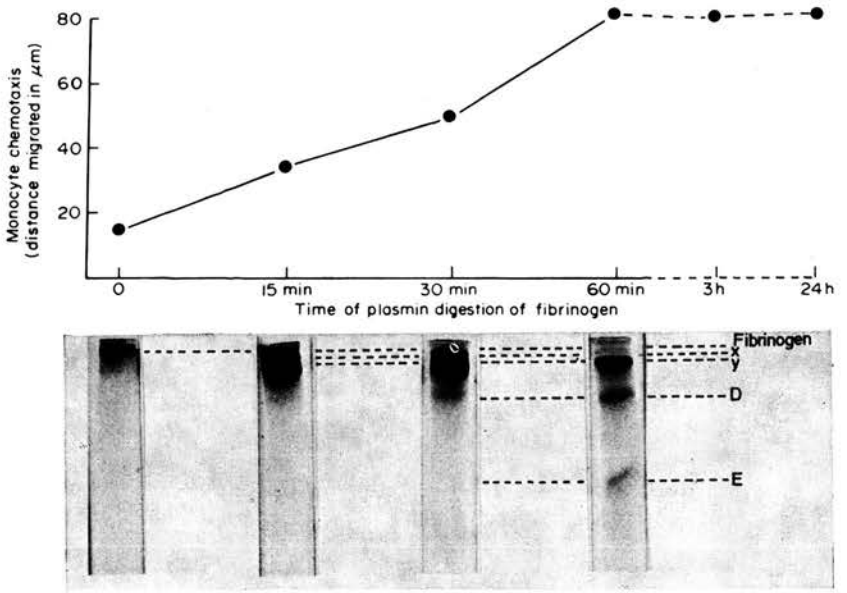

FIG 4. Disc gel electrophoresis and monocyte chemotaxis of plasmin/fibrinogen digestions at various time intervals.

this cell-type was associated with the uncharacterized low molecular weight peptides (LWP). The capacity of the peptides derived from the action of thrombin and snake venoms on fibrinogen (the fibrinopeptides), and plasmin digestion of fibrinogen (FDPs), to attract either the monocyte or the neutrophil is summarized in Table I.

DISCUSSION

Chemotactic activity for human monocytes was generated by the action of thrombin and Contortrix on fibrinogen (Fig 1). Thrombin cleaves fibrinopeptides A, AY, AP and B (Blombäck *et al*, 1966) whereas Contortrix selectively cleaves the B peptide (Herzig *et al*, 1970). In contrast, Arvin releases FPs A, AP and AY (Ewart *et al*, 1970) and clot supernatants prepared from this enzyme had no chemotactic activity. These experiments suggest that the B peptide is chemotactic for blood monocytes as well as the neutrophil as previously reported (Kay *et al*, 1974).

Evidence is provided that plasmin digestion products of fibrinogen are also chemotactic for monocytes and that the majority of activity is associated with the D and E fragments (Figs 3

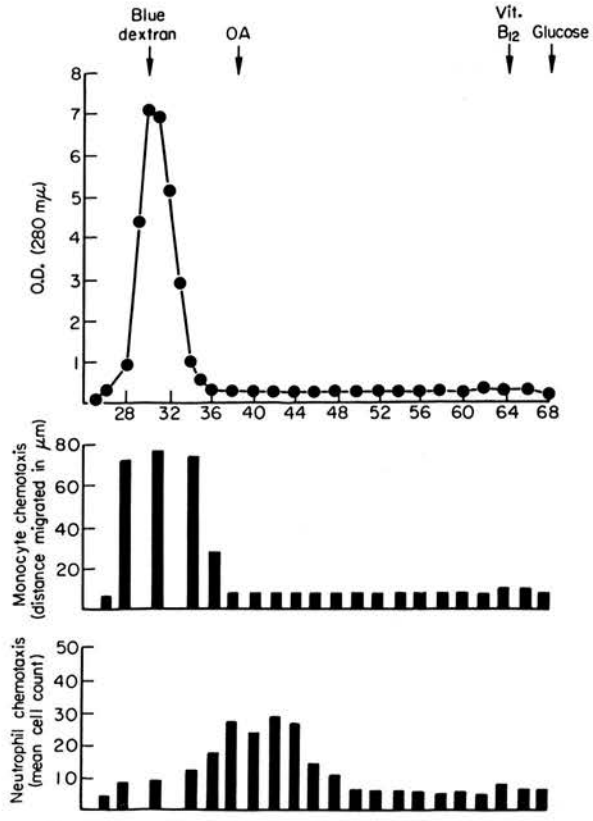

FIG 5. Sephadex G-75 chromatography of a 24 h plasmin/fibrinogen digest showing the peaks of monocyte and neutrophil chemotactic activities. Chemotaxis is indicated by vertical bars and the elution of molecular weight markers by the arrows. Molecular markers were Blue Dextran, ovalbumin (OA), vitamin B_{12} (Vit. B_{12}) and glucose.

and 4). In a previous report (McKenzie *et al*, 1975) we showed that the D and E fragments did not attract the neutrophil and the preparations of D and E prepared for this study were also inactive in this respect. In that same report (McKenzie *et al*, 1975) we were able to separate the X and Y fragments and demonstrated that the Y fragment was chemotactic for the neutrophil, but only at very high concentrations (> 1 mg/ml). In the present study, using the same procedures (Sephadex G-200 and CM 52) and further separation techniques such as recycling on Sephadex G-200 and heparin agarose chromatography, it was not possible to separate fully the X and Y fragments. The reasons for this are not clear but are probably related to variations in the batches of fibrinogen. It was apparent, however, that a mixture of X and Y on a milligram basis had no activity for monocytes or neutrophils whereas the D and E fragments attracted the monocyte at comparable concentrations.

In contrast to the neutrophil, the lower molecular weight unidentified polypeptides (LWP) produced by the action of plasmin on fibrinogen had no activity for human monocytes (Fig 5).

A characteristic feature of clot formation *in vivo* is the initial accumulation of neutrophils. During resolution of the clot, cells of the monocyte/macrophage series predominate. The difference in *in vitro* activity of plasmin digestion products of fibrinogen for neutrophils and monocytes may be of significance in the changing cellular response during clot formation and resolution. The observations reported here may also be of importance in the understanding of tissue damage mediated by the neutrophil and possibly the macrophage.

ACKNOWLEDGMENTS

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Neutrophil Accumulation *in Vivo* following the Administration of Chemotactic Factors

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Summary. Intravenously administered ^{51}Cr-labelled homologous neutrophils accumulated at guinea-pig skin sites prepared by intradermal injection of factors derived from complement-activated serum, possessing *in vitro* chemotactic activity. There was a strong correlation between the *in vitro* potency of Sephadex G-100 fractions of the activated serum and their ability to evoke neutrophil accumulation *in vivo*. These experiments suggest that agents which are chemotactic for neutrophils *in vitro* also induce the localization of this cell *in vivo*.

Chemotaxis may be defined as unidirectional migration of cells towards a concentration gradient of a chemical stimulus. The term is usually reserved for *in vitro* studies, in particular the technique of Boyden in which cells traverse through a micropore filter towards a chemotactic stimulus (Boyden, 1962). It is thought that the capacity of cells to respond in chemotaxis is an essential prerequisite for the defence against certain micro-organisms and conditions have been described in man in which a defect of chemotaxis *in vitro* is associated with recurrent infections (Miller *et al*, 1971). There is no direct evidence that chemotaxis as defined above occurs *in vivo*, although the specific accumulation of radiolabelled homologous leucocytes at carageenin-induced inflammatory sites in the rat has recently been demonstrated (Perper *et al*, 1974a). Subsequently the same workers demonstrated the drastic effect of complement depletion on cellular infiltration in the same model (Perper *et al*, 1974b). In the present study we provide evidence that intradermal injection of factors derived from complement-activated serum, which are chemotactic for neutrophils *in vitro*, evoke the local accumulation of the same cell in guinea-pig skin.

MATERIALS AND METHODS

Preparation of Labelled Neutrophils

Albino Dunkin-Hartley strain guinea-pigs, 300–400 g, of either sex, were injected intraperitoneally with 20 ml of 0.9% w/v saline containing 20 mg of glycogen (ex oyster pure AR, Koch Light Laboratories, Colnbrook, Bucks., England). 3–4 h later peritoneal leucocytes were harvested by lavage with a further 20 ml of saline. At that time 93–98% of the leucocytes were neutrophils. Contaminating erythrocytes were removed by briefly

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suspending the cells in 0.5% trisodium citrate. After washing twice in saline the white cells were resuspended to a concentration of $2–3 \times 10^7$/ml. To each millilitre of this suspension was added 100 μCi [^{51}Cr]sodium chromate (1 mCi/ml, 100–400 mCi/mg, Radiochemical Centre, Amersham, England) and the mixture incubated at 37°C for 45 min. The cells were then washed in 10 ml volumes of saline until the total radioactivity associated with the supernatant was less than 10% of that associated with the neutrophil-rich pellet. The leucocytes were finally resuspended to a concentration of 2.5×10^7 cells/ml. The viability of the white cells, as judged by trypan blue dye exclusion, was 95–99%.

Chemotactic Factors

Complement-activated serum was prepared by incubating fresh normal guinea-pig serum with 20 mg/ml of particulate inulin (Schwarz-Mann, Orangeburg, N.Y.) at 37°C for 30 min, with frequent inversion to allow mixing. The inulin was removed by centrifugation and the serum heated at 56°C for 30 min. The heated serum (CAS) was stored in 1 ml portions at −85°C until use. Control serum (CS) was prepared in an identical fashion but in the absence of inulin.

Fractionation of CAS

Two ml amounts of CAS were fractionated by gel filtration on a 90 × 1 cm column of Sephadex G-100, in phosphate buffered saline, pH 7.2. Alternate fractions were tested for chemotaxis by the micropore technique. Fractions were then pooled (Fig 3), concentrated approximately four times using a Diaflo UM-05 ultrafilter (Amicon, Lexington, Massachusetts) and 0.1 ml amounts of the pooled concentrates tested for neutrophil accumulation *in vivo*.

Neutrophil Accumulation in Vivo

^{51}Cr-labelled leucocytes were administered intravenously in 0.75 ml volumes, into the ear of guinea-pigs. 5 min later eight dorsal skin sites, previously shaved and marked to a diameter of 15 mm, were injected intradermally with 0.1 ml volumes of test solutions. At time intervals the animals were killed, skin sites removed, dissected free of subcutaneous tissue, placed in 2 ml plastic tubes, and assayed directly for radioactivity using a standard γ-counter (Tracerlabs Gamma/Guard 150).

The radioactivity in peripheral blood samples was measured as follows. 10 ml of heparinized blood were centrifuged at 400 ***g*** for 5 min. 0.5 ml of supernatant plasma was removed for counting and the remainder discarded. The cell pellet was resuspended to a volume of 10 ml in 0.5% trisodium citrate for 2–3 min, and then recentrifuged. 0.5 ml of red cell lysate was retained for counting and the remainder again discarded. The white cell pellet was resuspended to a volume of 10 ml and 0.5 ml of this suspension counted.

Measurement of Chemotaxis

Chemotaxis was measured by a modification of the micropore technique of Boyden as previously described (Kay, 1970). Filters of 8 μm pore size (Millipore Corporation, Bedford, Massachusetts) and an incubation time of 2 h at 37°C were used throughout. Cell counts were expressed as the mean of five high-power (×40) fields.

RESULTS

The radioactivity present in skin sites excised 18 h after the intradermal injection of various doses of either CAS, CS or saline is shown in Fig 1. ^{51}Cr accumulated at sites pretreated with CAS in a dose-dependent manner. Low levels of activity were present in the saline-treated sites, but these were below the amount found in any of the sites pretreated with CAS. At dilutions of 1:4 and 1:8 the amount of radioactivity in the CS-treated sites was similar to that in saline treated sites. Higher levels were present in sites treated with undiluted CS. The experiment was repeated three times with essentially identical results.

CS also evoked neutrophil chemotaxis *in vitro* but it was approximately nine times less active than CAS, the mean cell count for 0.1 ml of CAS and CS being 104 and 12 respectively.

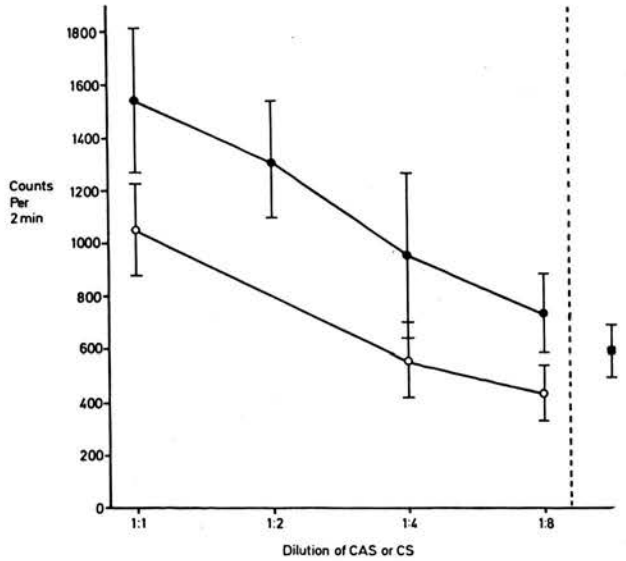

FIG 1. ^{51}Cr in guinea-pig skin sites 18 h after the intradermal administration of CAS (●), CS (○) and saline (■). Each point represents the mean results from three animals pretreated intravenously with the same ^{51}Cr-neutrophil preparation (2×10^7 cells; $1{\cdot}2 \times 10^6$ counts/2 min/10^7 cells) ± SEM.

TABLE I. Distribution of ^{51}Cr in peripheral blood elements after the intravenous administration of a radiolabelled neutrophil preparation (2×10^7 cells; 1.2×10^6 counts per 2 min/10^7 cells).

Sample	% *Total counts/2 min/0.5 ml*			
	1	2	3	*Mean*
Whole blood	100	100	100	100
Plasma	24	8	6	13
Red cell lysate	7	8	10	8
White cell pellet	69	84	84	79

In order to show that the label remained principally with the leucocytes the distribution of ^{51}Cr between blood elements was determined 18 h after injection (Table I). Relatively little activity was present in the plasma or red cell lysates, the majority being present in the white cell pellet.

The time course of accumulation of ^{51}Cr in skin sites treated with undiluted CAS or saline following the intravenous injection of ^{51}Cr-labelled neutrophils is shown in Fig 2. In CAS-treated sites maximal accumulation of ^{51}Cr occurred at 8 h, the levels decreasing thereafter. Since CS contained chemotactic properties and evoked some cell accumulation *in vivo* (Fig 3) saline was used as the control in this experiment. The activity in the saline treated sites was relatively high after 1 h but decreased thereafter. This closely paralleled the disappearance of ^{51}Cr from the peripheral blood as shown in Fig 2.

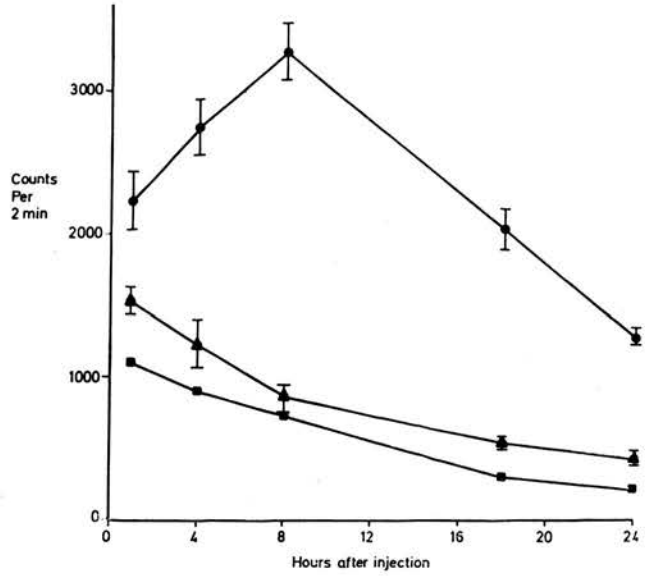

FIG 2. Time course of the appearance of ^{51}Cr in guinea-pig skin sites injected intradermally with CAS (●) or saline (▲). Each point represents the mean of four CAS-treated and four saline-treated sites in two animals pretreated intravenously with the same ^{51}Cr-neutrophil preparation (2.5×10^7 cells; 0.8×10^6 counts/2 min/10^7 cells) ± SEM. The mean levels of ^{51}Cr in 0.1 ml peripheral blood samples from the same animals are also shown (■).

The relationship between the *in vitro* chemotactic activity of Sephadex G-100 fractions of CAS and their capacity to evoke the accumulation of radiolabelled cells following intradermal injection is shown in Fig 3. The major peaks of activity were in pools 1 and 4 as assessed by the micropore technique. The same pools were associated with ^{51}Cr-neutrophil accumulation *in vivo*. Pools 2, 3 and 5 had relatively little chemotactic activity *in vitro* or neutrophil-accumulating properties *in vivo*. Thus there was a strong association between *in vivo* ^{51}Cr accumulation following intradermal injections of G-100 fractions of CAS and the chemotactic activity of these fractions *in vitro*.

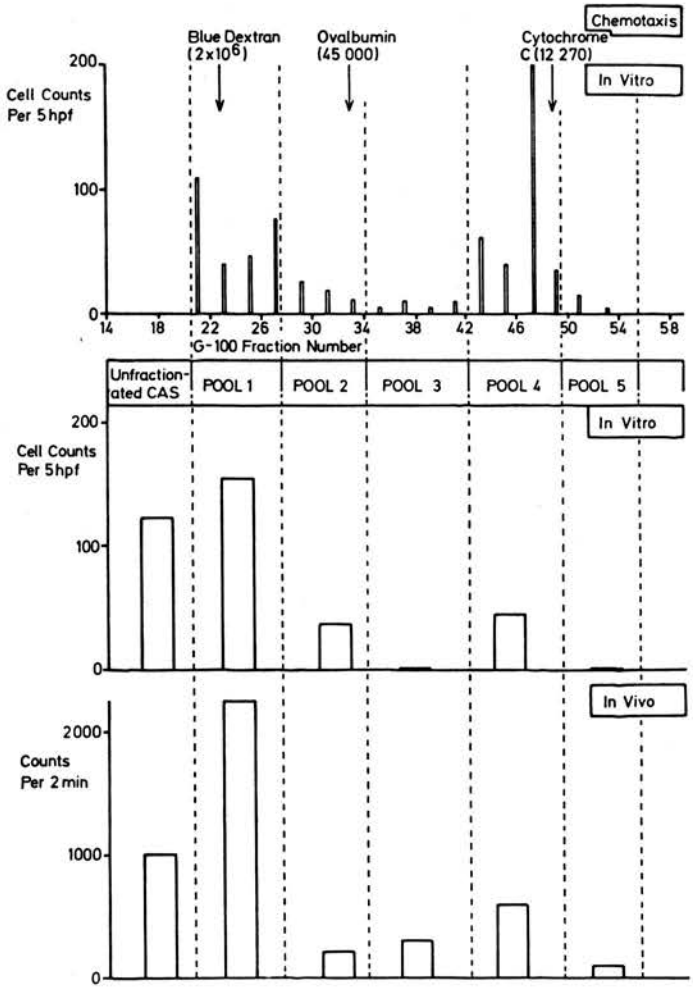

FIG 3. The *in vitro* and *in vivo* chemotactic activity of fractions of CAS obtained by gel filtration on Sephadex G-100.

DISCUSSION

These experiments indicate that the *in vivo* administration of preparations chemotactic *in vitro* leads to cell accumulation at the site of injection. Preparations which were not chemotactic in the micropore technique evoked relatively little *in vivo* cell accumulation. Therefore it is probable that agents chemotactic *in vitro* play a part in the recruitment of cells but whether they do this by a direct action or by increasing the 'stickiness' of the vasculature or other mechanisms is yet to be determined.

The use, in this study, of radiolabelled cells had considerable advantages over histological techniques, both in terms of simplicity and accuracy. It was important to determine, however, that the label remained with the leucocyte fraction and this is shown in Table I. Thus in three animals a mean of 80% of the total radioactivity was associated with the leucocyte fraction, only small amounts being recovered in the plasma or red cell lystates.

In both the *in vitro* an *in vivo* systems, control serum (CS) possessed some neutrophilotactic activity. Normal serum clotted on glass is known to possess a number of chemotactic agents due to the initiation of Hageman factor (XII)-dependent pathways (Kay & Kaplan, 1975). This activity was enhanced in inulin-activated serum (CAS) due presumably to the additional presence of complement-derived chemotactic factors (Fig 1). For establishing the baseline parameters of the *in vivo* technique and for comparing the relative abilities of agents which stimulate neutrophil chemotaxis *in vitro* to evoke the accumulation of the same cell *in vivo* the more active CAS was used in further experiments (Figs 2 and 3).

In time course studies maximal labelling of CAS-treated sites was observed at 8 h, the levels decreasing thereafter (Fig 2). The appearance and disappearance of radioactivity in saline-treated sites paralleled the levels present in the peripheral blood.

Following the passage of CAS through a column of Sephadex G-100 two major peaks of chemotactic activity were observed (Fig 3) as previously described (Kay, 1970). The higher molecular weight material is as yet unidentified whereas the material with an apparent molecular weight of approximately 13 000 daltons has been identified as C5a (Kay *et al*, 1973). The similar *in vivo* accumulation and *in vitro* chemotactic patterns of Sephadex G-100 fractions of CAS suggest that directional migration of neutrophils towards complement derived factors may be operative in the mechanisms by which this cell accumulates at tissue sites *in vivo*. This has also been suggested by the recent observations of Perper *et al* (1974a, b) on the migration of ^{51}Cr-labelled leucocytes to sites of carageenin-induced inflammation in the rat, and its inhibition following complement depletion with cobra venom factor.

We suggest that the technique described represents a simple, reproducible, quantitative model for measuring cell accumulation *in vivo*, and may therefore be a potentially useful tool for studying the mechanisms of cell kinetics in inflammatory sites. It may also provide further insight into the role of chemotaxis *in vivo*.

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MONOCYTE CHEMOTAXIS IN BRONCHIAL CARCINOMA AND CIGARETTE SMOKERS

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Summary.—Chemotaxis of blood monocytes was measured in 31 patients with bronchial carcinoma and 19 cigarette smokers. Thirteen patients with metastatic bronchial carcinoma had significantly less ($P < 0{\cdot}005$) chemotactic response than matched controls. Those with disease confined to the chest, or with recurrent or operable bronchial carcinoma, had no significant depression of monocyte chemotaxis. There was also no significant difference in monocyte chemotaxis between cigarette smokers and matched controls. These results support the concept that in human cancer there is a defect in monocyte chemotaxis, but in bronchial carcinoma significant depression was only apparent in those with advanced disease.

THE role of cells of the mononuclear phagocytic series in immune surveillance has been suggested by a number of workers (Hibbs, Lambert and Remington, 1972; Alexander, 1976). For example, an inhibitor of macrophage chemotaxis produced by various transplanted tumours in mice has been described (Snyderman and Pike, 1976) and in man the capacity of peripheral blood monocytes to respond by chemotaxis *in vitro* was depressed in patients with genito-urinary neoplasms (Hausman *et al.*, 1975), malignant melanoma (Rubin, Cosimi and Goetzl, 1976) and other human cancers (Boetcher and Leonard, 1974). We have studied monocyte chemotaxis from 31 patients with bronchial carcinoma at various clinical stages, and also the monocyte chemotactic response of cigarette smokers who are known to be at risk for developing bronchial neoplasms.

PATIENTS AND CONTROLS

Patients with bronchial carcinoma were classified according to the stage of their disease. Those with small tumours deemed suitable for surgical resection were termed *operable*. Disease which reappeared locally at the site of a surgical resection was termed *recurrent*. Disease *confined to the chest* had spread locally from the primary site in the bronchus to involve surrounding lung, local lymph nodes and chest wall. The *metastatic* group had deposits of tumour outside the chest, commonly in liver or bone, as demonstrated clinically, or by radionuclide scanning.

Controls for the cancer groups were all convalescent, hospitalized patients who had sustained either myocardial infarctions or respiratory infections and in whom there was no evidence of malignant disease. Controls for cigarette smokers were all healthy, non-smoking volunteers.

MATERIALS AND METHODS

Human peripheral-blood monocytes were separated on a Ficoll–Triosil gradient as previously described (Böyum, 1968). Chemotaxis was quantified either by the "leading front" method using Millipore filters (Millipore Co., Wembley) of 8 μm pore size (Zigmond and Hirsch, 1973) or by the method of Snyderman *et al.*, (1972) employing

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Nucleopore filters and polycarbonate "Boyden chambers" Neuroprobe, Bethesda, Maryland, U.S.A.). The only modification was that the suspending medium for monocytes and for dilutions of chemoattractant was Medium 199 containing 30 mM Hepes buffer. The chemoattractant was either human serum in which the complement system had been activated with purified cobra venom factor (CVF) (Ballow and Cochrane, 1969) or solutions of casein (British Drug Houses). Casein was used in the early part of the study, but its chemoattracting properties often deteriorated after a few days, even under a variety of storage conditions. The experiments with casein reported here are with freshly prepared material. In contrast, serum activated with CVF was divided into portions after preparation and stored at $-80°C$ until use.

The chemotactic responses from patients with bronchial carcinoma or cigarette smokers were compared with age- and sex-matched controls and each pair was performed on the same day under the same experimental conditions. A three-point dose-response of chemoattractant was performed for each experiment. Optimal monocyte migration of cells, either from patients or smokers, was achieved with 0·5 mg/ml of fresh casein or 2·5% CVF-activated serum. Each assay was performed in duplicate, and measurements from each filter were the pooled results from 10 random high-power fields. The test and control samples were analysed by the Wilcoxon test of paired differences. The variation between duplicate filters was $\pm 15\%$ as previously described (Turnbull and Kay, 1976; Turnbull, Evans and Kay, 1977).

RESULTS

Bronchial carcinoma

The clinical staging and histology of the 31 patients with bronchial carcinoma are shown in Table I. Apart from one individual, all patients were male, and were matched with controls within 10 years of their age. The monocyte chemotactic response of patients with metastatic disease, and their respective controls, are shown in Table I, together with the histology and the treatment being received either at the time of sampling or before the chemotactic assay. There was a significantly greater depression in the monocyte chemotactic response in the metastatic group ($P < 0·005$) than in their respective controls. No statistical difference was observed with patients with disease confined to the chest, recurrent cancer or operable disease (Table III, Fig.). It is unlikely that the observed effect with the metastatic group was a result of medication. Three of the 13 were receiving antibiotics and one had treatment with prednisolone, but most of the patients were receiving no treatment at the time of the chemotactic test. The two patients receiving prednisolone in the group with disease confined to the chest had higher chemotactic responses than the control, whereas the one in the metastatic group having corticosteroids had a lower chemotactic response.

TABLE I.—*Clinical Staging and Predominant Histology of the* 31 *Patients Studied with Bronchial Carcinoma*

Clinical stage	
Metastatic	13
Confined to chest	12
Recurrent	2
Operable	4
Histology	
Anaplastic	6
Squamous	10
Oat cell	5
Adenocarcinoma	1
Unknown	9

In the Fig. the results are expressed as the percent migration of each patient's monocytes as compared to their respective matched contol. With the patients as a whole there was a wide scatter; however, the metastatic group responded significantly less in monocyte chemotaxis. The one patient who gave a high response had a pulmonary infection with a white-cell count of $17{,}000/\mu l$. There was no significant difference between patients and controls in the other groups, although with operable and recurrent cancer the numbers were very small.

Cigarette smokers

The monocyte chemotactic response of 19 male cigarette smokers, compared with

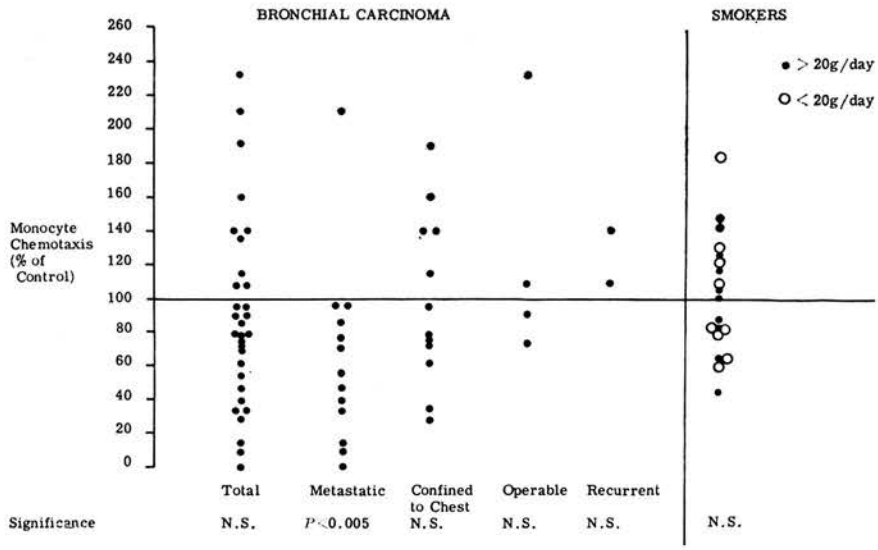

FIG.—The monocyte chemotactic response of patients with various clinical stages of bronchial carcinoma, and cigarette smokers, expressed as the percent migration of each patient's monocytes compared to its matched control. NS = not significant.

TABLE II.—*The Histology, Treatment and Monocyte Chemotactic Response of Patients with Metastatic Bronchial Carcinoma. Controls were Matched for Age and Sex*

Pt. No.	Age (Years) Pt.	Age (Years) Control	Histology	Prior treatment (interval between end of treatment and chemotaxis test	Medication at time of test	Monocyte chemotaxis (distance migrated in μm) Pt.	Monocyte chemotaxis (distance migrated in μm) Control
1	67	69	Unknown	—	—	19·7	26·8
2	62	61	Anaplastic	—	—	35·9	17·2
3	55	59	Oat cell	PR (22 months)	Prednisolone	7·6	13·3
4	66	61	Unknown	—	Ampicillin	2·0	16·0
5	54	60	Unknown	—	—	8·0	17·0
6	85	75	Unknown	—	—	2·0	18·0
7	73	73	Unknown	—	Ampicillin	0·5	17·0
8	78	71	Unknown	PR (3 years)	—	8·3	23·7
9	56	55	Oat cell	—	—	26·4	33·5
10	61	55	Oat cell	—	—	13·4	33·5
11	53	53	Anaplastic	PR (1 day)	—	53·6	59·0
12	69	65	Squamous	—	Ampicillin	15·0	17·6
13	65	65	Squamous	—	—	46·4	49·7

PR = palliative radiotherapy. The chemoattractant was complement-activated serum (2·5%) in Patients 1 to 10 and casein (0·5 mg/ml) in Patients 11 to 13.

non-smoking controls, is shown in Table IV and the Fig. There was no significant difference between the two groups as a whole, nor when the smokers were divided into those who smoked more or less than 20 g per day.

DISCUSSION

Our results support previous findings on depressed monocyte chemotactic responses in various human cancers (Hausman *et al.*, 1975; Rubin *et al.*, 1976; Boetcher and Leonard, 1974). In the present study on bronchial carcinoma, only those patients with metastatic disease showed a significant depression (Table II, Fig.). Although this may have been a non-specific effect due to general debilitation it was unlikely to be the result of treatment. Many of the patients were receiving no medication at the time of sampling and had not received prior chemotherapy or radiotherapy

TABLE III.—*The Histology, Treatment and Monocyte Chemotactic Response of Patients with Bronchial Carcinoma that was Confined to the Chest, Recurrent or Operable. Controls were Matched for Age and Sex*

Pt. No.	Age (years) Pt.	Control	Histology	Prior treatment (interval since end of treatment until chemotaxis test)	Medication at time of test	Monocyte chemotaxis (distance migrated in μm) Pt.	Control
				Confined to chest			
1	61	69	Anaplastic	PR (20 weeks)	Ampicillin, Prednisolone	24·0	12·7
2	68	65	Squamous	—	Oxytetracycline	13·9	18·4
3	62	66	Anaplastic	—	Ampicillin	14·0	50·2
4	61	63	Squamous	—	—	16·1	17·2
5	60	62	Anaplastic	PR (24 weeks)	—	17·1	48·1
6	64	65	Unknown	—	Prednisolone	20·7	12·9
7	51	51	Oat cell	—	—	21·1	15·2
8♀	54	55	Adenocarcinoma	—	—	25·9	33·5
9	67	58	Oat cell	—	—	33·0	23·6
10	80	80	Squamous	PR (23 days)	—	37·8	59·8
11	68	65	Unknown	Chemotherapy (16 weeks)	—	36·4	46·9
12	57	57	Squamous	Chemotherapy (6 weeks)	—	58·6	50·2
				Recurrent			
13	82	81	Unknown	—	Oxytetracycline	42·5	30·6
14	53	55	Squamous	—	—	20·0	18·4
				Operable			
15	65	61	Squamous	—	—	18·8	17·2
16	50	48	Squamous	—	—	8·0	11·0
17	57	54	Anaplastic	Post-surgery (3 weeks)	Ampicillin	28·0	31·0
18	68	65	Squamous	—	Ampicillin	28·8	12·3

PR = palliative radiotherapy. The chemoattractant was casein (0·5 mg/ml) in Patients and controls 9 to 12 and 18. In the others it was complement-activated serum (2·5%).

TABLE IV.—*The Monocyte Chemotactic Response of* 19 *Male Cigarette Smokers Compared with Non-smoking Male Controls*

Age (years) Smoker	Control	Tobacco smoked/day (g)	No. of years smoking	Monocyte chemotaxis (mean cell count) Smoker	Control
56	63	60	41	49	76
62	51	60	45	45	53
49	42	53	31	65	61
42	33	40	17	76	53
40	55	36	19	66	74
52	64	30	38	116	90
49	31	30	35	17	38
58	44	26	40	*46	31
64	60	23	47	57	48
27	22	21	8	82	81
35	34	20	16	43	70
48	54	20	35	76	95
32	28	20	14	37	28
25	27	20	8	84	76
47	34	20	23	84	66
28	23	16	10	134	72
54	55	15	36	65	100
29	29	29	5	75	91
53	49	14	28	50	59

The chemoattractant was 2·5% CVF-activated serum, with the exception of the pair indicated (*) in which the concentration was 1·25%.

(Tables II and III). In a similar study on malignant melanoma (Rubin *et al.*, 1976), only patients with advanced disease had a monocyte defect.

The inhibitor of macrophage chemotaxis produced by various transplanted neoplasms in the peritoneal cavity of mice was partially identified as a protein of mol. wt. 6000–10,000 (Snyderman and Pike, 1976). A similar inhibitor is possibly elaborated from human neoplasms and if it is related to tumour mass this may account for the effect observed in the metastatic group in the present study.

A recent leading article in the *Lancet* (1976), discussing the possible role of macrophages in tumour surveillance, emphasized the present difficulties in relating *in vitro* data from man and experimental animals to the clinical situation. Nevertheless, if tumour-derived material with inhibitory effects on monocyte function can be demonstrated, this may provide some additional evidence to support the concept that tumour products overcome possible tumoricidal effects of mononuclear phagocytes. Experiments currently in progress suggest that extracts of human tumours may inhibit the chemotactic response of normal blood monocytes (Abell, C. and Kay, A. B., unpublished).

Cigarette smokers showed no difference from controls in their monocyte migratory capacity, indicating that monocyte chemotaxis will not be useful in detecting individuals at risk for developing bronchial carcinoma (Table IV). Studies with human alveolar macrophages obtained from smoking and non-smoking volunteers demonstrated both an increase in the number of cells recovered by bronchial lavage and of the chemotactic response of these cells from smokers when compared to controls (Warr and Martin, 1974). This suggests that cigarette smoke probably has an initial non-specific "macrophage-activating effect" analogous to the influx of macrophages into tissues treated with various irritants such as mineral oil and glycogen.

The chemotactic activity of human serum activated with cobra venom factor is due almost entirely to the fragment cleaved from the 5th component of complement (C5a) liberated as a result of activation of the alternate pathway. When CVF-activated serum is placed on either side of the micropore chamber, migration is either minimal or absent, suggesting that this agent evokes chemotaxis, (i.e. directional migration) rather than random migration (Kay, unpublished).

There are difficulties in employing the chemotactic assay for clinical studies. The reasons include possible deterioration of the chemoattractant during storage, variations in an individual's cell response with time, and failure to reproduce this biological assay exactly on each occasion. In the present study these difficulties were largely overcome by matching each patient or smoker with a control individual for age and sex, withdrawing blood from each pair at the same time and performing the test under identical conditions. Comparison of these matched pairs by the Wilcoxon test of paired differences allowed statistical analysis.

Thus the present study suggests that defects in monocyte chemotaxis are only apparent at advanced stages of bronchial carcinoma, and not in those with relatively confined disease or in those individuals who are at risk for developing bronchial neoplasms.

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Annotation

CHEMOTAXIS AND HAEMOSTASIS

The micropore technique of Boyden is a sensitive and reproducible *in vitro* model for studying chemotaxis. It has been used for the identification of chemotactic factors and the response of the target cells both in health and disease (Boyden, 1962; Baum, 1975). The Boyden chambers consist essentially of two compartments divided by a micropore filter. A suspension of leucocytes is placed in the upper compartment and the chemotactic agent is introduced into the lower part of the chamber. Following an incubation period the filter is removed, fixed and stained and chemotaxis is 'quantitated' either by counting the number of cells which have traversed the entire thickness of the micropore or by measuring the distance the 'leading front' of cells has migrated from the origin (Zigmond & Hirsch, 1973). Further refinements to the technique include a method in which leucocytes are labelled with ^{51}chromium and chemotaxis is expressed in terms of radioactivity of a second filter placed on the side to which the cells are migrating (Gallin *et al*, 1973).

The majority of early studies which employed this technique related almost exclusively to complement-derived chemotactic factors. Those identified included fragments cleaved from the third and fifth components (C3a, C5a) and the trimolecular complex of the fifth, sixth and seventh component ($C\overline{567}$) (Ward, 1967; Ward & Newman, 1969; Ward *et al*, 1966; Lachmann *et al*, 1970).

It was apparent, however, that a certain amount of activity was invariably present in normal serum which had not undergone prior complement activation. Thus normal serum, under the appropriate conditions, was chemotactic for the neutrophil (Kay, 1969), the monocyte (Wilkinson *et al*, 1969) and to a lesser extent the eosinophil (Kay, 1970).

Initial investigations on the chemotactic activity of normal human serum indicated that many factors were involved since multiple peaks of chemotactic activity could be demonstrated following chromatography on Sephadex G-200 (Kaplan *et al*, 1972). One of these peaks superimposed the position in which the enzymes kallikrein (mol wt 108 000) and a plasminogen activator (mol wt 90 000) eluted. Although these molecules have a similar molecular size it was possible, by further purification and separation, to show that chemotactic activity was associated with both enzymes (Kaplan *et al*, 1973). Thus kallikrein, in addition to cleaving bradykinin from its substrate kininogen, was a chemo-attractant as was this plasminogen activator.

It should be emphasized that a number of fibrinolytic pathways have been described with presumed plasminogen activator activity. The activity referred to in this annotation relates exclusively to Hageman factor (factor XII)-dependent plasminogen-converting activity. It would therefore appear to be distinct from the Hageman factor-independent fibrinolytic pathway present in whole plasma which has a requirement for C3 (Schreiber & Austen, 1974), and from the plasminogen activator derived from human vascular endothelium which has an estimated molecular size of 65 000 daltons (Aoki & von Kaulla, 1971).

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More direct evidence of the chemotactic properties of these enzymes was obtained by incubating highly purified Hageman factor (HF) fragments with their respective proenzymes. Thus a mixture of purified HF fragments and prekallikrein led to the elaboration of both chemotactic activity and cleavage of bradykinin from kininogen. Similarly, plasminogen-converting and chemotactic activity were generated by the action of activated HF on the plasminogen proactivator. Of particular interest was the fact that both the enzymatic and chemotactic principle of the plasminogen activator and kallikrein were inhibited by the serine esterase inhibitor diisopropyl fluorophosphate (DFP). The activity of these enzymes was therefore dependent upon the integrity of their active sites since neither enzyme in its precursor form was DFP sensitive.

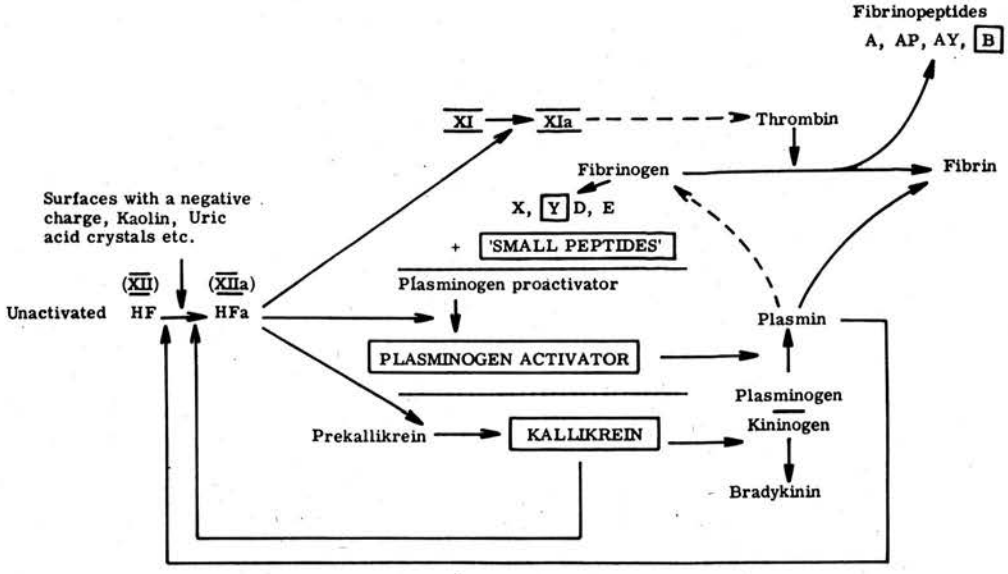

FIG 1. Hageman factor-dependent pathways and chemotaxis. The chemotactic factors associated with the haemostatic mechanisms are depicted by the 'closed boxes'.

Further insight into the chemotactic properties of kallikrein and plasminogen activator was provided by studies on prekallikrein (Fletcher factor) deficient plasma (Weiss *et al*, 1974). Plasma from these individuals have defects in coagulation, fibrinolysis, kinin-generation and chemotaxis all of which can be corrected by the addition of purified prekallikrein. The correction of these defects by prekallikrein is attributed to a further property of kallikrein, namely its capacity to activate HF so providing a positive feedback for HF-dependent pathways (Cochrane *et al*, 1972). These pathways are activated by the action of HF on its three known substrates, factor XI, prekallikrein and the plasminogen proactivator (Fig 1). Using kaolin as the HF activator, restoration of chemotactic activity in Fletcher factor deficient plasma could be achieved by activated HF as well as prekallikrein. This suggested that the contribution of kallikrein to the chemotactic activity of kaolin-activated serum was small and that under these conditions the plasminogen activator and other unidentified HF-dependent chemotactic factors were probably providing the majority of the activity.

These studies on the chemotactic activity of kallikrein and the plasminogen activator used unseparated human peripheral blood leucocytes as target cells. Whereas unseparated cell populations are acceptable for measuring the chemotactic response of the neutrophil, eosinophil and the basophil, study of monocyte chemotaxis requires prior separation of the mononuclear cells. This can be conveniently achieved by layering a leucocyte mixture onto Hypaque-Ficoll. Following centrifugation, the less dense mononuclear cells can be separated in a viable form. These cells also responded in chemotaxis to kallikrein and plasminogen activator and there was a similar inhibition of the active site by DFP (Gallin & Kaplan, 1974). α_2-Macroglobulin inhibited both the enzymatic effect of kallikrein and plasminogen activator in terms of their actions on kininogen and plasminogen and also the ability of these enzymes to respond in monocyte chemotaxis. The C1 inhibitor prevented both the enzymatic action of kallikrein on kininogen and its chemotatic principle but had no effect on the expression of the plasminogen activator either as a chemotactic agent or its action on plasminogen. Therefore these naturally occurring protein inhibitors (α_2-macroglobulin and C1 inhibitor) affect the enzymatic principle in a similar fashion to their chemotactic activity.

Using unseparated leucocytes, neutrophil migration alone was observed when a mixture of the plasminogen activator and kallikrein was the chemo-attractant. However, these enzymes attracted the basophil when the cell source were blood leucocytes from individuals with basophilic leukaemia (Kay & Austen, 1972). In contrast, the eosinophil did not respond in chemotaxis to these agents when leucocytes from patients with a peripheral blood eosinophilia were used as target cells (Kay & Austen, 1971).

The action of Hageman factor on factor XI (plasma thromboplastin antecedent—PTA) leads to the formation of thrombin via the intrinsic coagulation pathway. Clot supernatants prepared by the action of purified thrombin on fibrinogen are chemotactic for neutrophils, and to a lesser extent for eosinophils, while neither thrombin nor fibrinogen alone possesses this activity (Kay *et al*, 1973). Thrombin is known to be a limited protease which cleaves several small peptides from fibrinogen. These fibrinopeptides have been designated A, AP, AY and B and are recognized on the basis of their electrophoretic mobility (Blombäck *et al*, 1966). Evidence has been provided that the chemotactic activity is a property of fibrinopeptide B and not of fibrinopeptides A, AP or AY (Kay *et al*, 1974). This was shown by progressive purification of thrombin-induced clot supernatants using high voltage electrophoresis in two dimensions. Supernatants prepared by the action of the snake venom *Contortrix* which cleaves the B peptide, were also chemotactic whereas no activity was present in supernatants prepared from the *Arvin* venom which cleaves peptides A, AP and AY. Furthermore, synthetic fibrinopeptide B and a B analogue, L-glutatamic acid, were chemotactic but not synthetic fibrinopeptide A. Thus chemotaxis joins other biological activities associated with fibrinopeptide B such as the potentiation of bradykinin-induced constriction of the isolated oestrous rat uterus (Gladner *et al*, 1968) and prolonged rhythmic vaso-constriction (Colman *et al*, 1967). It is probable that the biological activity associated with fibrinopeptide B is located at its C-terminus since this part of the molecule is exposed following the action of thrombin and other enzymes which cleave B from the β polypeptide chain of fibrinogen.

Other peptides derived from fibrinogen have been examined for possible chemotactic activity since fibrin/fibrinogen degradation products (FDP) appear in body fluids in associa-

tion with various clinical states and have a number of biological activities. Chemotactic activity for human neutrophils and to a lesser extent eosinophils could be generated by the action of plasmin on human fibrinogen (McKenzie *et al*, 1975). Previous studies have shown that plasmin degradation of human fibrinogen yields four major fragments designated X, Y, D and E in addition to smaller peptide material (Furlan & Beck, 1972). When plasmin digestion was stopped at time intervals up to 24 h, a small amount of activity was apparent at 15 and 30 min corresponding to the transient appearance of fragment Y. Considerably more chemotactic activity was present in the 24 h digest and by gel-filtration this was shown to be associated with relatively small molecules having a molecular size of approximately 30 000 daltons. When purified X, Y, D and E were assayed individually for chemotaxis fragment Y was active but only at relatively high concentrations. Therefore the chemotactic activity generated by the action of plasmin on fibrinogen was mainly associated with one or more lower molecular weight polypeptides and to a lesser extent with the Y fragment. Recent studies have shown that plasmin digestion products of fibrinogen and fibrinopeptide B have chemotactic activity for human monocytes but the active FDP fragment(s) are yet to be identified (Richardson & Kay, unpublished observations). The response of these various cell types to the chemotactic agents is shown in Table I.

TABLE I. The response of various cell types to chemotactic factors associated with haemostasis

	Neutrophils	*Monocytes*	*Eosinophils*	*Basophils*
Fibrinopeptide B	Yes	Yes	Yes	?
Products of plasmin digestion of fibrinogen	Yes	Yes	(Yes)	?
Kallikrein	Yes	Yes	No	Yes
Plasminogen activator	Yes	Yes	?	?

Thus there is a formidable list of chemotactic factors associated with coagulation, kinin formation and fibrinolysis (Fig 1). The biological significance of these observations is unknown but the accumulation of various cell types in inflammatory conditions associated with HF-dependent pathways is well recognized. Thus, following the formation of fibrin there is an initial accumulation of neutrophils whereas during the resolution the mononuclear cell predominates. These factors may also act together since synergism between chemotactic factors has been shown for neutrophils (Wilkinson *et al*, 1969), monocytes and eosinophils (Kay *et al*, 1973). The association of the kinins and related peptides to various disease states is also appreciated. For instance, the accumulation of neutrophils in the joint fluids of gout arthritis may in part be mediated by kallikrein formed as a consequence of uric acid crystal-induced HF activation (Kellermeyer & Breckenridge, 1965).

Many chemotactic agents possess other biological activities. C3a and C5a are anaphylatoxins (Cochrane & Müller-Eberhard, 1968), $C\overline{567}$ act in the phenomenon of 'reactive lysis' (Thompson & Lachmann, 1970) and various activities have been attributed to fibrinopeptide B (see above). It is clear that the full complexity of these cascade-enzyme systems is still to be

appreciated. However, a consideration of the relationship between the leucocyte, coagulation, kinin-formation and fibrinolysis may help our understanding of the significance of these biochemical pathways in health and disease.

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SECTION D - CLINICAL STUDIES

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COMPLEMENT COMPONENTS AND IgE IN BRONCHIAL ASTHMA

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Summary Levels of circulating IgE, total hæmolytic complement, and components C1q, C4, C3, C3PA, C6, and C7 were measured in controls and in 93 asthmatics. In selected patients C2 assays were also performed. C4 levels were significantly higher than normal in child asthmatics but significantly lower in adults with asthma. IgE was significantly higher in both adults and children with asthma, and C7 was raised in adult asthmatics. When patients developing the disease in adult life were compared with those whose asthma started in childhood, the levels in the adult group were similar, whatever the duration of the disease. In the childhood group, levels were initially high with short duration of disease but fell to that of the adult-onset group as the duration of asthma increased. This effect was not found with IgE levels or complement components other than C4. When the levels were compared with various clinical features associated with asthma, significant differences were found only in the levels of C4 and IgE. High C4 levels were found in association with the symptoms of "allergic disease" whereas low C4 levels were associated with features of "non-allergic disease". A similar but far less conclusive pattern was found with IgE levels. When 6 asthmatics having all the chosen clinical features of allergic disease were compared with 18 having all the features of non-allergic asthma, the allergic group had significantly higher levels of C4 than the non-allergic patients, whereas the levels of C2 and IgE were not significantly different. These

results suggest that measurements of C4 may be of use in the classification of asthma since they correlated better with the clinical features studied than did levels of circulating IgE. Since C4 is extremely sensitive to the action of activated C1, these findings also raise the possibility that there is sequential activation of the classical complement pathway in non-allergic disease, possibly due to recurrent infection, even though levels of C1q and C2 were not significantly different from the allergic group.

Introduction

THE IgE-mediated release of pharmacological mediators from human lung, which in turn constrict bronchial smooth muscle, is generally accepted as a major mechanism in the pathogenesis of "allergic asthma". However, many asthmatics either have an inconclusive history of allergy or have no evidence of allergen-induced bronchospasm.

Studies with in-vitro laboratory models have demonstrated a mechanism independent of IgE which leads to the elaboration of biologically active peptides and subsequent histamine release from mast cells [1] and human leucocytes.[2] These effector molecules, or anaphylatoxins, are fragments derived from the third and fifth components of complement (C3a and C5a). Several workers have suggested that the complement system may participate in the pathogenesis of asthma. Deposition of C3 on the bronchial basement membrane was found at necropsy in three of eighteen asthmatics,[3] and decreased arteriovenous levels of whole complement have been recorded after antigen infusion in a sensitive patient.[4] There is also evidence of a requirement for complement components in the antigen-induced release of histamine from passively sensitised lung.[5] Some asthmatics have been found to have tissue-sensitising antibody of the IgG class [6] which may have required complement components in the expression of its action.

We have looked for altered serum-complement profiles in the various forms of asthma and for changes in profiles with the duration of the disease. Levels of complement components and IgE were analysed in relation to the presence of "asthma-associated" clinical features. Recognition of altered complement profiles in asthma, especially of the fourth component, may be of value both in the classification and pathogenesis of certain forms of asthma.

Patients and Methods

Patients and Controls

93 asthmatics (70 adults and 23 children) have been investigated, together with age and sex matched adults and children as controls. Blood from childhood controls was taken for clinical purposes during convalescence from virus meningitis (1 case), tonsillitis (3 cases), or otitis media (1 case), or from inpatients subsequently found to have enuresis for which no organic cause was found (8 cases), hydrocephalus (1 case), dermatitis herpetiformis (1 case), epilepsy (2 cases), ingestion of 10 ml. of 'Tedral' suspension (1 case), "non-accidental" injury (1 case), congenital pulmonary-valve stenosis (1 case), and urinary-tract infection (3 cases). None of the child controls had asthma or other respiratory diseases or a history of other allergies. The age range in asthmatics and controls was from 2 to 72 years. The asthmatics had generalised airway obstruction which reversed by 20% or more of the initial forced expiratory volume either spontaneously or in response to therapy. None of the adult controls had an "allergic history" or were on long-term medication. Sera were collected at outpatient clinics, divided into portions, and stored at −70°C or in liquid nitrogen.

Clinical Features

Individual clinical features were assessed before the laboratory results were known. The pattern of wheeze was taken from the history, and graded as either "episodic" or "chronic" in character. Similarly the association of infection with exacerbations of asthma was assessed as being "usual" or "infrequent". Patients were judged to have "allergen-induced bronchospasm" if they gave both a clear history of symptoms associated with exposure to an inhalant or ingestant and reacted to the appropriate allergen with a positive skin-prick test. Patients with "other allergies" had a history of either allergic rhinitis, atopic or infantile eczema, or urticaria that was clearly antigen induced. All patients were skin tested to a wide variety of allergens which included extracts of mixed grass pollens, the mite *Dermatophagoides pteronyssinus, Aspergillus fumigatus*, and house dust. All asthmatics on treatment with corticosteroids had received their medication for 6 weeks or more. Patients who could not be confidently graded in terms of the above clinical features were excluded from the relevant analysis.

Measurement of IgE

Levels of IgE were measured by solid-phase radioimmunoassay using the 'Phadebas' kit (Pharmacia) in which the anti-IgE was raised in the rabbit. The IgE of the standard sera in the kit was checked against W.H.O. standard 68/341 and both had virtually identical values. Twenty of the sera giving high values were measured by

radial immunodiffusion (Meloy). Both methods gave similar results.

Measurement of Serum-complement Component Levels

The hæmolytic complement (CH_{50}) was measured by the method of Mayer.[7] Levels of complement components C1q, C4, C3, and the C3 proactivator (C3PA) were measured by single radial diffusion using monospecific antisera raised in the rabbit. Anti C1q, C4, and C3 were prepared as described[8]; anti-C3PA was purchased from Beringwerke. Serum levels of C6 were measured using C6-deficient rabbit serum and C7 by the reactive lysis method.[9] Levels of hæmolytically active C2 were measured by effective molecular titrations.[10]

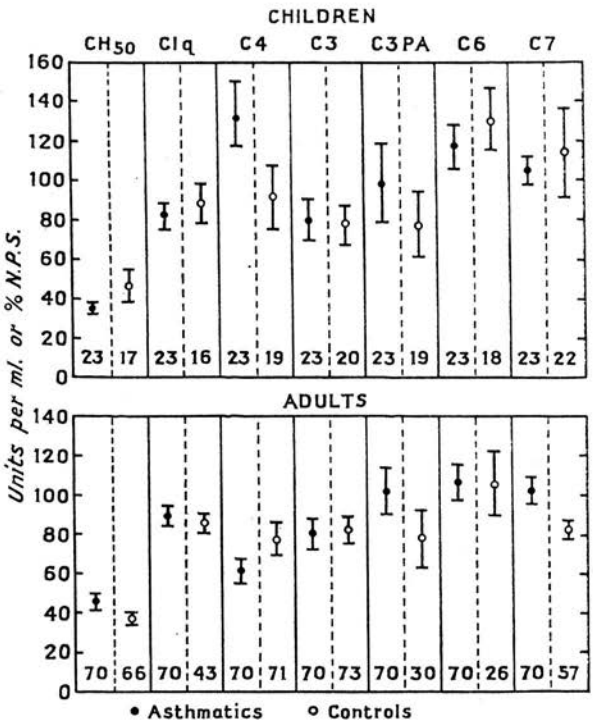

Fig. 1—Serum-complement levels in children and adults with asthma and controls.

The values are expressed as units per ml. for the CH_{50} and % N.P.S. for the individual components. The bars represent the mean and 1·96 standard errors about the mean. The number of individuals studied is indicated at the foot of each block. N.P.S. = normal pooled serum.

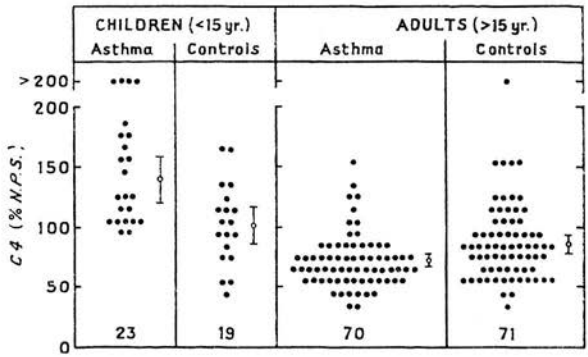

Fig. 2—Levels of circulating C4 in asthmatic adults and children and controls.

Bars represent the mean and 1·96 standard errors about the mean.

Statistical Analyses

The P values were calculated either by Student's t test (where the variances were found to be equal) or by the equation $d = \frac{\text{the difference of the means}}{\text{the sum of the standard errors}}$ in which P was calculated from the normal distribution curve (where the variances were not equal).

Results

Adult and Childhood Asthma

There was no significant difference between the levels of circulating C1q, C3, C3PA, C6, and C7 when adult and child asthmatics were compared with their respective normal groups (fig. 1). Adult asthmatics had a higher CH_{50} than adult controls ($P<0{\cdot}002$), but differences between child asthmatics and their controls were not significant ($P<0{\cdot}10$). The levels of C4 were significantly higher in child asthmatics than in controls ($P<0{\cdot}05$) or adult asthmatics ($P<0{\cdot}001$) (fig. 2). In controls there was no significant change in C4 levels with age. In adults there was a small but significant difference ($P<0{\cdot}01$) in the range of C4 levels between asthmatics and controls, the asthmatics (in contradistinction to asthmatic children) having lower levels (figs. 1 and 2). Only a few adult asthmatics had values which extended into the range of childhood diseases (fig. 2). It was not possible to exclude the effect of treatment with corticosteroids on the slightly low C4 levels in adult asthmatics since the C4 level of the 28 adult

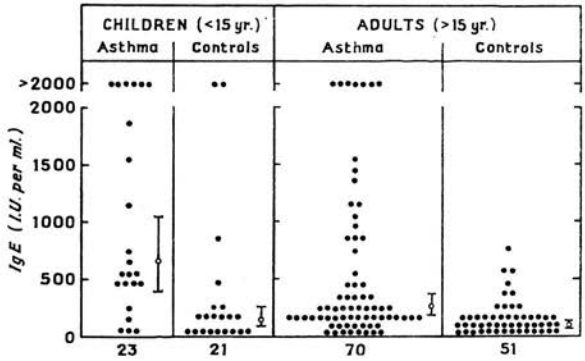

Fig. 3—Circulating IgE levels in asthmatic children and adults compared with their controls.

The bars represent the mean and 1·96 standard errors about the mean on a log distribution. I.U. =international units.

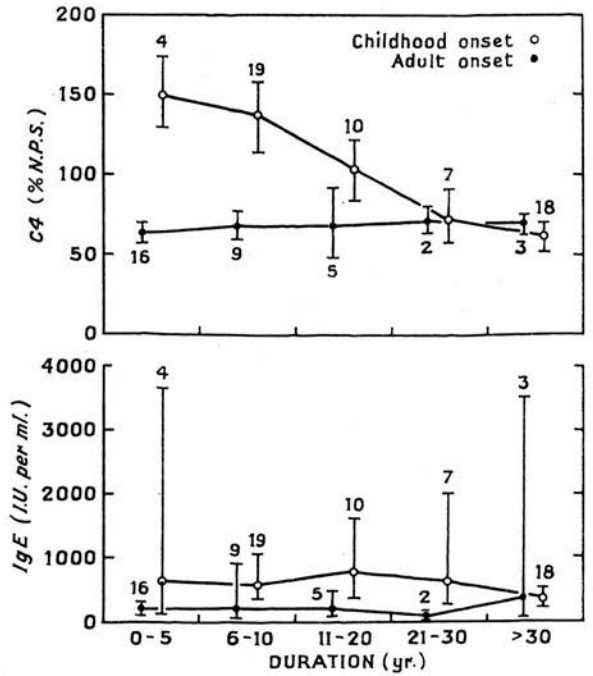

Fig. 4—Effect of duration of disease on the levels of C4 and IgE between child and adult onset asthma.

The bars represent the mean and 1·96 standard errors about the mean as described in figs. 2 and 3.

asthmatics not on treatment with corticosteroids was not significantly below the normal adult mean ($P>0.10$). There are other reasons, however, to believe that this is not a corticosteroid effect (see below).

The mean C7 level was approximately 20% higher in asthmatic adults than in adult controls ($P<0.001$); 14% had C7 levels above the highest values of normal individuals (fig. 1). These effects were not attributable to treatment with corticosteroids since those not on steroids also had high levels. There was no significant difference in the C7 levels between normal and asthmatic children.

Most asthmatics had IgE levels within the normal range, although some had very high levels (fig. 3). The distribution of IgE levels was different between asthmatic children and adults in that fewer asthmatic children had low values. Differences in IgE levels were not associated with any particular form of treatment.

There was no significant difference in the levels of the CH_{50} and of the individual circulating complement components or of IgE levels between male and female controls, or between asthmatic males and females.

Duration of Disease

Differences in the levels of circulating C4 and IgE between child and adult onset asthmatics with duration of disease are shown in fig. 4. In the short-duration group (asthma less than 5 years) child-onset asthmatics had considerably higher mean C4 levels than did adult-onset asthmatics ($P<0.001$). In adult-onset asthma the C4 levels were lower than normal adults, and these values were depressed throughout the duration of the disease. When the same analyses were applied to patients not on corticosteroids a similar significant difference was found. With CH_{50} and C3 levels there was a similar changing pattern in terms of the mean values but the differences were not significant. No changing patterns were found with levels of C1q, C3PA, C6, and C7. When the same analysis was made for levels of IgE (fig. 4) there were no significant differences between childhood and adult onset asthmatics and the levels remain virtually unchanged whatever the duration of the disease.

Association with Clinical Features

The associations between various clinical features and levels of C4 and IgE are shown in fig. 5. The levels of C4 were lower in asthmatics with a chronic

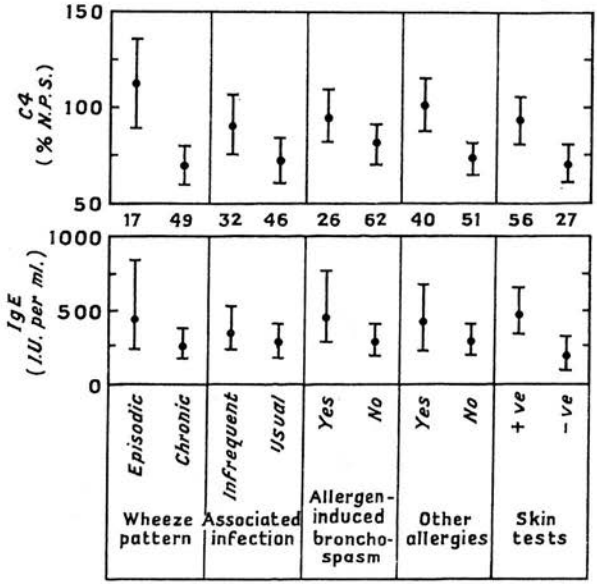

Fig. 5—The levels of C4 and IgE compared in terms of individual clinical features.

The bar represents the mean and 1·96 standard errors about the mean on a linear distribution for C4 and a log distribution for IgE.

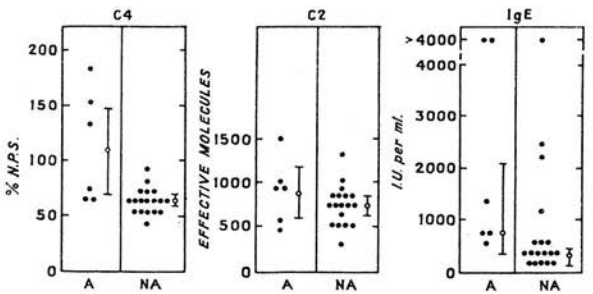

Fig. 6—Comparison of levels of C4, C2, and IgE between 6 asthmatics with all the features of allergic (A) disease and 18 with all the features of non-allergic asthma (NA).

The bars represent the mean and 1·96 standard errors about the mean on a linear distribution for C4 and C2 and log distribution for IgE.

wheeze pattern as compared with an episodic wheeze pattern ($P<0.01$), lower in those who had no other allergies ($P<0.01$) and lower in patients with negative skin tests ($P<0.05$). There were also differences in C4 levels when the association of attacks with infection and a history of allergen-induced bronchospasm was compared, but these were not significant. In contrast, the association between IgE levels and clinical features was far less striking. Although higher mean values of IgE were associated with an episodic wheeze pattern, allergen-induced bronchospasm, a history of other allergies and skin test positivity, the differences, with the exception of skin tests, were not significant. There was no association between the clinical features studied and changes in the levels of the CH_{50}, C1q, C3, C3PA, C6, and C7.

Of the 93 asthmatics, only 6 had all the chosen features of allergic disease, and 18 had all the features of non-allergic asthma (fig. 5). There was no significant difference between these two selected groups in terms of the levels of the CH_{50}, C1q, C3, C3PA, C6, and C7. However, as shown in fig. 6 the allergic group had a significantly higher mean C4 than the non-allergic asthmatics ($P<0.05$), but there were no significant differences between the levels of C2 and IgE. Thus in these selected groups the levels of C4 also showed greater differences than IgE. It was not possible to provide evidence that the low C4 in non-allergic asthmatics was associated with activation of the classical pathway of complement activation since, although the mean C2 levels were lower in those asthmatics, they were not significantly different from the allergic group.

Discussion

Bronchial asthma is very difficult to define. The classification into intrinsic (non-allergic) and extrinsic (allergic) is of limited value since most patients have features associated with both groups. Although early reports on raised levels of IgE in some patients with bronchial asthma have been confirmed,[11,12] we have been unable to show that circulating levels of this immunoglobulin distinguish between the various forms of the disease.[11] Thus for the reasons stated we have measured complement components in addition to IgE and in the first analyses have compared the values in childhood and adult asthmatics with their respective controls (fig. 1). Low levels of C4

were observed in adult asthmatics. Although this might have been associated with treatment with corticosteroids, it is relevant that in laboratory animals cortisol at a dose of 5 mg. per kg. per day significantly increased the level of C4 and other components, depressed levels being found only with much larger doses. Moreover, when the asthma had begun in childhood C4 concentrations fell progressively with duration of disease (fig. 4); this also happened in those not receiving corticosteroids, although the numbers were smaller. In contrast, raised levels of C4 were found in childhood asthma, but none of these patients were on steroid treatment. The significance of or reasons for the high levels of C4 is unknown. It is presumably a result of increased synthesis and/or decreased metabolism.

C7 was raised in adult asthmatics but the significance is yet to be determined. This has also been found in chronic liver disease, and it has been suggested that C7 is secreted by connective-tissue cells during fibrosis and repair.[14]

The raised CH_{50} in adult asthmatics is of little clinical significance as levels of whole complement are high in a variety of disease states.[15]

A further limitation on the value of the terms "extrinsic" and "intrinsic" asthma is suggested by our findings [16] that adults whose disease originated in childhood showed a changing pattern in terms of allergy-associated clinical features. Thus, as the duration of the disease increased, the wheeze pattern tended to change from episodic to chronic in nature and the features studied became less clearly associated with allergy.

When individual clinical features associated with allergic or non-allergic asthma were correlated with complement components and IgE, the only significant differences were found in the levels of C4. Thus raised levels of C4 were more consistently found with allergic features than were raised IgE values. Conversely, low levels of C4 were usually associated with the features of non-allergic disease whereas many of these patients had raised levels of IgE.

To provide evidence that the lower C4 levels in non-allergic asthma were a result of sequential activation of the complement pathway, effective molecular titrations of C2 were performed in two groups of patients with all the selected features of allergic or non-allergic disease. Although the differences in C2 were not significant it does not exclude an activation

mechanism. In contrast to C2, C4 is exquisitely sensitive to the action of a few molecules of activated C1.[17] A striking change in C4 can occur with comparatively little alteration in the levels of C1 and C2. If C1 activation did occur this may be attributable to natural antibodies combining with infective agents, although levels of C4 in a number of patients with recurrent respiratory infection were normal.[18] Natural antibodies against *Corynebacterium parvum* have been demonstrated in normal human serum and the resultant complexes have been shown to activate the classical complement pathway.[19] Further evidence of complement activation in chronic asthma is being sought by measuring levels of the C1 inhibitor, the anaphylatoxin inactivator, and the presence of circulating immune complexes.

The reduced C4 levels in non-allergic asthma could also be a result of decreased synthesis. C4 synthesis by cells of the macrophage/monocyte series has been described although the source of macrophages was from the peritoneal cavity.[20] Whether alveolar macrophages can synthesise C4 is yet to be determined.

Thus, although the mechanism of alterations in the levels of C4 in asthmatics with allergic and non-allergic associated disease has yet to be determined, measurements of this component in the circulation may prove to be relevant to the pathogenesis of the disease and perhaps of some value in its classification.

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COMPLEMENT COMPONENTS AND IgE IN PATIENTS WITH ASTHMA AND ASPIRIN IDIOSYNCRASY

BY

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Complement components and IgE in patients with asthma and aspirin idiosyncrasy

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Delaney, J. C. and Kay, A. B. (1976). *Thorax*, **31**, 425–427. **Complement components and IgE in patients with asthma and aspirin idiosyncrasy.** Levels of circulating IgE, total haemolytic complement, and components C4 and C3 were measured in 16 asthmatics with aspirin idiosyncrasy and in control subjects. IgE levels were mostly within normal limits. No differences were found between the complement profiles—in particular the C4 levels—in the two groups. As low levels of C4 have been found in patients with intrinsic asthma, these results would suggest a fundamental difference between asthmatics with aspirin idiosyncrasy and others with intrinsic asthma.

The development of increased airways obstruction soon after aspirin ingestion is a well recognized clinical entity (Samter and Beers, 1967). It is seen more commonly in females and typically it is associated with the presence of vasomotor rhinitis and nasal polyposis. The same effect may be produced by other simple analgesics, eg, paracetamol (Smith, 1971). The underlying pathogenetic mechanism is unknown. The sharp increase in airways obstruction soon after ingestion suggests an allergic reaction. Extensive studies, however, have failed to find an immunological mechanism (Giraldo, Blumenthal, and Spinks, 1969; Girard, Hildenbrandt, and Favre, 1969), and IgE levels have been within normal limits (Henderson, Swedlund *et al.*, 1971). Asthmatics with aspirin idiosyncrasy are considered to form a subgroup of intrinsic asthma.

It has been suggested that the complement system may be activated directly by aspirin in sensitive subjects (Yurchak, Wicher, and Arbesman, 1970) and that this may lead to the elaboration of anaphylatoxins which in turn release histamine and other mediators of the allergic response by a mechanism independent of IgE.

Measurements of complement components in asthma have recently revealed high levels of C4 in the serum of allergic asthmatics and low levels in the serum of non-allergic asthmatics (Kay *et al.*, 1974).

In the present study, serum IgE measurements were undertaken to confirm that, in general, normal levels occur in asthmatics with aspirin idiosyncrasy. Measurements of serum complement components were undertaken to determine if there was any alteration in the complement profile, especially C4, in this condition. Absence of an altered profile would distinguish this subgroup of asthma from others in the intrinsic group.

PATIENTS AND METHODS

PATIENTS Sixteen patients with asthma and aspirin idiosyncrasy were investigated. The diagnosis of aspirin idiosyncrasy had been suggested by the history and was confirmed by oral challenge with paracetamol and small doses of soluble aspirin. The clinical features of those patients are outlined in the Table. Nasal speculum examination revealed the presence of polyps in all the patients. Skin testing by the prick-method revealed four patients with positive reactions to one or more common allergens.

Age- and sex-matched adults attending the general medical clinic were used as controls. None of these patients had allergic disease, asthma or other respiratory disorders. The age-range in asthmatics was 21 to 67 years (mean 46·6 years) and in the controls it was 25 to 67 years (mean 47·5 years). Sera were collected at outpatient clinics, randomly numbered, and stored at −70°C. Measurements of IgE and complement components were made by one of us (ABK) without knowledge of the origin of the samples.

TABLE

CLINICAL FEATURES OF PATIENTS WITH ASTHMA AND ASPIRIN IDIOSYNCRASY

Patient	Age	Sex	Skin Tests	Nasal Polypectomy (No.)	Asthma (years)	Steroid Therapy (daily dose)
MB	63	F	Positive DP A	4	9	Nil
SM	39	F	Positive DP A	3	16	Pred 5 mg
BP	40	M	Positive DP	7	34	Nil
PR	51	F	Positive DP	0	3	Pred 7·5 mg
PC	32	F	Negative	4	14	Becotide
LT	63	F	Negative	8	30	Pred 5 mg
DJ	54	F	Negative	6	29	Pred 5 mg
JS	50	F	Negative	5	16	Nil
JD	64	M	Negative	1	2	Pred 10 mg
LT	61	F	Negative	3	20	Pred 5 mg
SA	35	F	Negative	0	4	Pred 5 mg + Bextasol
HC	50	F	Negative	3	4	Pred 5 mg
JA	36	M	Negative	13	22	Nil
SR	44	F	Negative	3	10	Pred 2·5 mg
SK	21	M	Negative	3	4	Pred 5 mg
LK	43	F	Negative	1	12	Pred 5 mg

DP = *Dermatophagoides pterynissinus* (house-dust mite).
A = aspergillus.
G = grass-pollen.
Pred = prednisolone

METHODS The total haemolytic complement (CH_{50}) was measured by the method of Mayer (1961). Sheep cells were optimally sensitized by antisera prepared in the rabbit. Increasing dilutions of serum were added to constant volumes of sheep cells and the degree of haemolysis (Y) was measured by spectrometry at OD 414. The total haemolytic complement giving 50% haemolysis (CH_{50}) was calculated as $1-1/Y$.

Levels of C3 and C4 were measured by radial immunodiffusion using monospecific antisera raised in the rabbit (Lachmann, Hobart, and Aston, 1973).

Levels of IgE were measured by solid phase radioimmunoassay using the Phadebas Kit (Pharmacia) in which the anti IgE was raised in the rabbit. The IgE of the standard sera in the kit was checked against WHO standard 68/341 and both had virtually identical values.

RESULTS

The complement profiles and IgE values for the groups with aspirin idiosyncrasy and for the control groups are outlined in the Figure.

The mean complement components for the two groups were as follows:

Asthmatics: $CH_{50}=132$, C4=119, C3=136
Controls: $CH_{50}=148$, C4=125, C3=129

(The values are expressed as a percentage of the normal pooled serum.)

There was no significant difference in the complement profiles between the two groups.

The IgE levels were mostly in the lower range in both groups. The mean IgE in the asthmatic groups was 281 IU per ml and in the controls it was 150 IU per ml. The two asthmatic patients with raised IgE levels had positive skin tests.

DISCUSSION

Asthmatic patients with aspirin idiosyncrasy form a subgroup of chronic (intrinsic) asthma. Recently, chronic asthmatics have been shown to have lower levels of circulating C4 than episodic (extrinsic) patients. In the present study, however, there was no significant difference in the levels of complement components between asthmatic patients with aspirin idiosyncrasy and controls. Apart from the two atopic patients the finding of normal IgE values is in agreement with previous reports. Measurements of complement components have not previously been recorded in this condition. The normal profile does not exclude direct activation of the complement system by aspirin and, in this respect, measurements of complement levels during acute episodes provoked by aspirin would be helpful.

The absence of an altered complement profile in asthmatics with aspirin idiosyncrasy would support the belief that patients with this syndrome form a subgroup quite separate from others in the intrinsic asthma group.

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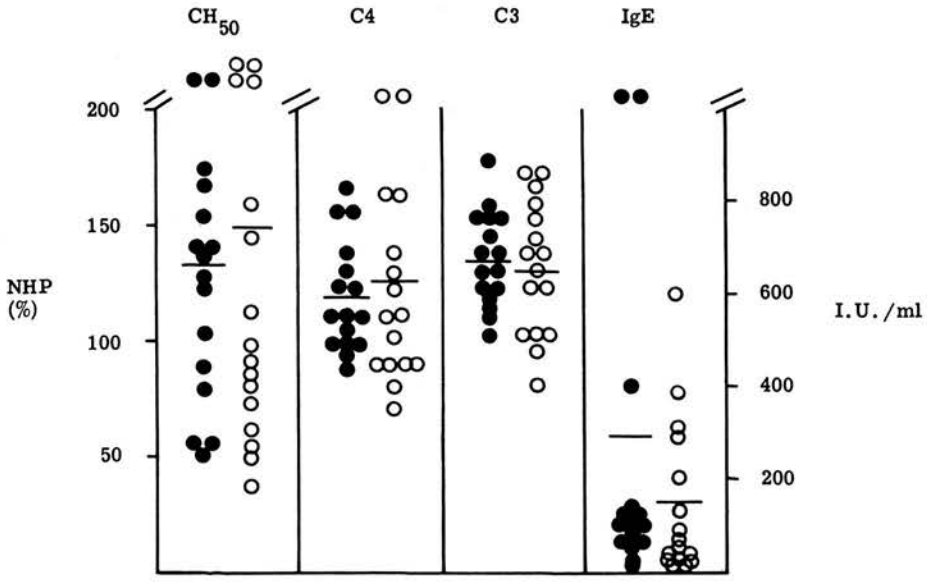

FIGURE *Complement and IgE levels in patients with asthma and aspirin idiosyncrasy (●) and age- and sex-matched controls (○). The bars represent the mean values. There was no statistical difference between patients and controls as assessed by the Student* t *test. NHP=normal human pool (serum).*

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COMPLEMENT AND BRONCHIAL ASTHMA IN CHILDHOOD AND ADULTS

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SUMMARY :

To differenciate the various clinical forms of bronchial asthma, the level of circulating IgE and of some components of the complement were measured in the patients serum : adults and children. A significant difference is observed—in patients as compared to healthy subjects—in the C4 component : in children the level is increased, on the contrary in adults it is decreased. Some clinical aspects which enable the distinction between extrinsic and intrinsic asthma, correlate better with the C4 level than with the IgE level for which no significant difference has been shown in the various forms of the disease.

Key words : C4; Bronchial asthma; IgE; Allergy; Complement.

RÉSUMÉ : **Complément et asthme bronchique de l'enfant et de l'adulte**

Afin de différencier les diverses formes cliniques de l'asthme bronchique, le taux d'IgE circulante et de certains composants du complément ont été mesurés dans le sérum de malades : enfants et adultes. Une différence significative est observée, chez les malades par rapport aux sujets normaux, pour le composé C4 : chez les enfants le taux est augmenté, par contre pour les adultes ce taux est diminué. Certains aspects cliniques qui permettent de distinguer entre l'asthme extrinsèque et intrinsèque corrèlent mieux avec le taux de C4 qu'avec le taux d'IgE, pour lequel aucune différence significative n'a pu être mise en évidence dans les diverses formes de la maladie.

Mots clés : C4; Asthme bronchique; IgE; Allergie; Complément.

INTRODUCTION

Inhalation of specific allergen is widely accepted as the initiating factor which leads to bronchoconstriction in allergic asthmatics. Pharmacological agents such as histamine and slow reacting substance are thought to be released from lung mast cells sensitized by IgE and these in turn constrict bronchial smooth muscle. However many asthmatics, including children, often have either an inconclusive history of allergy or no evidence of allergen-induced bronchospasm. Studies *in vitro* have shown that peptides derived from sequential activation of the complement system evoke histamine release from mast cells [1] or human leucocytes [2]. These effector molecules (or anaphylatoxins) are fragments derived from the third and fifth components of complement and they are referred to as C3a and C5a. Their action does not require prior sensitization of mast cells by IgE or equivalent tissue-sensitizing antibody. Several workers have suggested that the complement system may participate in the pathogenesis of asthma. For example, deposition of C3 on the bronchial basement membrane was found at autopsy in three of eighteen asthmatics [3], and decreased arteriovenous levels of whole complement have been recorded after antigen infusion in a sensitive patient [4]. One *in vitro* system has been described which apparently

Tirés à part : Dr A. B. KAY, à l'adresse ci-dessus.

required complement components for the antigen-induced release of histamine from passively sensitized lung [5]. In addition, some asthmatics have been found to have a tissue sensitizing antibody of the IgG class [6] which may exert its effect through complement components for the full expression of its action.

In order to extend these studies we have measured the serum complement profiles in various forms of bronchial asthma. We have also determined whether alterations in serum complement levels could be related to the duration of disease, a number of « asthma-associated » clinical features and concentrations of IgE [7].

PATIENTS STUDIED AND RESULTS

Ninety-three asthmatics were investigated; 70 adults and 23 below the age of 15. All measurements were compared with age and sex matched controls.

There was no significant difference between the levels of circulating C1q, C3, C3PA, C6 and C7 when adult and child asthmatics were compared with their respective normal groups (Table I). Adult asthmatics had a higher CH_{50} than adult controls

TABLE I. — *Serum-complement levels in children and adults with asthma and controls. The values are expressed as units per ml for the CH_{50} and percent normal pooled serum (N.P.S.) for the individual components. The mean values and 1.96 standard errors are recorded* (NS : not significant; p : values were calculated by the student's t test).

	Children (<15 yrs)			Adults (>15 yrs)		
	Asthma	Controls	Significance	Asthma	Controls	Significance
CH_{50}	36 ± 2	46 ± 7	NS	45 ± 5	38 ± 2	p <0.002
C1q	84 ± 6	90 ± 10	NS	90 ± 6	88 ± 4	NS
C4	131 ± 15	92 ± 19	p <0.05	62 ± 4	78 ± 10	p <0.01
C3	80 ± 11	78 ± 8	NS	80 ± 8	82 ± 6	NS+
C3PA	96 ± 19	78 ± 15	NS	111 ± 12	80 ± 13	NS
C6	118 ± 10	130 ± 12	NS	108 ± 6	106 ± 14	NS
C7	105 ± 6	115 ± 20	NS	102 ± 4	84 ± 4	p <0.001

($p < 0.002$), but differences between child asthmatics and their controls were not significant ($p < 0.10$). The levels of C4 were significantly higher in child asthmatics than in controls ($p < 0.05$) or adult asthmatics ($p < 0.001$). In controls there was no significant change in C4 levels with age. In adults there was a small but significant difference ($p < 0.01$) in the range of C4 levels between asthmatics and controls, the asthmatics (in contradistinction to asthmatic children) having lower levels. Only a few adult asthmatics had values which extended into the range of childhood diseases. It was not possible to exclude the effect of treatment with corticosteroids on the slightly low C4 levels in adult asthmatics since the C4 level of the 28 adult asthmatics not on treatment with corticosteroids was not significantly below the normal adult mean ($p > 0.10$). There are other reasons, however, to believe that this is not a corticosteroid effect (see below). The mean C7 level was approximatively 20 % higher in asthmatic adults than in adult controls ($p < 0.001$); 14 % had C7 levels above the highest values of normal individuals (Table I). These effects were not attributable to treatment with corticosteroids since those not on steroids also had high levels. There was no

significant difference in the C7 levels between normal and asthmatic children. Most asthmatics had IgE levels within the normal range, although some had very high levels. The distribution of IgE levels was different between asthmatic children and adults in that fewer asthmatic children had low values. Differences in IgE levels were not associated with any particular form of treatment. There was no significant difference in the levels of the CH_{50} and of the individual circulating complement components or of IgE levels between male and female controls, or between asthmatic males and females. Differences in the levels of circulating C4 and IgE between child and adult onset asthmatics with duration of disease are shown in Tables II and III. In the short-duration group (asthma less than 5 years) child-onset asthmatics had considerably

TABLE II. — *Levels of C4 (% NPS) in patients whose symptoms of bronchial asthma began either in childhood or adult life compared with the years before the onset of symptoms. The mean and 1.96 standard error are given.*

Years Since Onset of Symptoms	Onset in Childhood (No. of Patients)	Adult Onset (No. of Patients)
0-5	150 ± 26 (4)	52 ± 8 (16)
6-10	140 ± 25 (19)	56 ± 10 (9)
11-20	105 ± 23 (10)	57 ± 23 (5)
21-30	70 ± 20 (7)	60 ± 11 (2)
>30	62 ± 8 (18)	61 ± 7 (3)

TABLE III. — *Levels of IgE (I.U per ml) in patients whose symptoms of bronchial asthma began either in childhood or adult life compared with the number of years before the onset of symptoms. The numbers of patients is the same as in table II. The range represents 1.96 standard errors on a logarithmic distribution.*

Years Since Onset of Symptoms	Onset in Childhood Mean IgE Value (Range)	Adult Onset Mean IgE Value (Range)
0-5	600 (75-3,600)	100 (40-180)
6-10	550 (200-1,050)	100 (20-950)
11-20	750 (400-1,800)	100 (40-420)
21-30	600 (200-2,000)	50 (10-60)
>30	400 (200-600)	400 (20-3,300)

higher mean C4 levels than did adult-onset asthmatics ($p < 0.001$). In adult-onset asthma the C4 levels were lower than normal adults, and these values were depressed throughout the duration of the disease. When the same analyses were applied to patients not on corticosteroids, a similar significant difference was found. With CH_{50} and C3 levels there was a similar changing pattern in terms of the mean values but the differences were not significant. No changing patterns were found with levels of C1q, C3PA, C6 and C7. When the same analysis was made for levels of IgE (Table III) there were no significant differences between childhood and adult-onset asthmatics and the levels remain virtually unchanged whatever the duration of the disease.

The association between various clinical features and levels of C4 and IgE are shown in Table IV. The levels of C4 were lower in asthmatics with a chronic wheeze pattern as compared with an episodic wheeze pattern ($p < 0.01$), lower in those who had no other allergies ($p < 0.01$) and lower in patients with negative skin tests ($p < 0.05$). There were also differences in C4 levels when the association of attacks

TABLE IV. — *The levels of C4, IgE, eosinophil numbers, β-glucuronidase and sputum histamine when compared to asthma associated clinical features. The mean and 1.96 standard errors are given* (NS : not significant; p : values were calculated by the student's t test).

Clinical Manifestations (No. of Patients)			C4 (% NPS)	IgE (I.U. per ml)	Eosinophils (per µl)	β-glucuronidase (units/100 ml)	Sputum Histamine (µg/gram dry residue)
Wheeze Pattern	Chronic	(17)	112 ± 22	400 (320-800)	290 ± 60	610 ± 180	42 ± 15
			p <0.01	NS	NS	NS	NS
	Episodic	(49)	69 ± 8	250 (170-400)	260 ± 50	615 ± 90	43 ± 12
Association of infection with attacks	Infrequent	(32)	85 ± 22	580 (230-520)	270 ± 65	700 ± 80	45 ± 21
			NS	NS	NS	NS	NS
	Usual	(46)	73 ± 12	250 (120-400)	295 ± 110	615 ± 50	41 ± 19
Bronchospasm induced by allergen	Yes	(26)	91 ± 19	410 (260-760)	350 ± 100	617 ± 110	36 ± 12
			NS	NS	NS	NS	NS
	No	(62)	80 ± 10	260 (200-400)	300 ± 100	618 ± 60	48 ± 15
History of other allergies	Yes	(40)	100 ± 15	400 (200-700)	300 ± 90	590 ± 60	50 ± 23
			p <0.01	NS	NS	NS	NS
	No	(51)	74 ± 10	260 (210-400)	305 ± 95	612 ± 55	41 ± 12
Skin Tests	Positive	(56)	90 ± 9	450 (300-700)	310 ± 270	613 ± 70	43 ± 20
			p <0.05	p <0.01	NS	NS	NS
	Negative	(27)	69 ± 8	170 (100-260)	285 ± 110	590 ± 65	42 ± 18

with infection and a history of allergen-induced bronchospasm was compared, but these were not significant. In contrast, the association between IgE levels and clinical features was far less striking. Although higher mean values of IgE were associated with an episodic wheeze pattern, allergen-induced bronchospasm, a history of other allergies and skin test positivity, the differences, with the exception of skin tests, were not significant. There was no association between the clinical features studied and changes in the levels of the CH_{50}, C1q, C3, C3PA and C7.

Levels of circulating eosinophils and concentrations of serum β-glucuronidase and sputum histamine were also related to clinical features. No significant differences were found when the same analyses were applied (Table IV).

GENERAL DISCUSSION

Bronchial asthma is very difficult to define. The classification into intrinsic (non-allergic) and extrinsic (allergic) is of limited value since most patients have features associated with both groups. Although early reports on raised levels of IgE in some patients with bronchial asthma have been confirmed [8, 9], we have been unable to show that circulating levels of this immunoglobulin distinguish between the various forms of the disease [8]. Thus for the reasons stated we have measured complement components in addition to IgE and in the first analyses have compared the values in childhood and adult asthmatics with their respective controls (Table I). Low levels of C4 were observed in adult asthmatics. Although this might have been associated with treatment with corticosteroids, it is relevant that in laboratory animals cortisol at a dose of 5 mg per kg per day significantly increased the level of C4 and other components, depressed levels being found only with much larger doses [10]. Moreover,

when the asthma had begun in childhood, C4 concentrations fell progressively with duration of disease (Table II); this also happened in those not receiving corticosteroids, although the numbers were smaller. In contrast, raised levels of C4 were found in childhood asthma, but none of these patients were on steroid treatment. The significance of, or reasons for, the high levels of C4 is unknown. It is presumably a result of increased synthesis and/or decreased metabolism. C7 was raised in adult asthmatics but the significance is yet to be determined. This has also been found in chronic liver disease, and it has been suggested that C7 is secreted by connective-tissue cells during fibrosis and repair [11]. The raised CH_{50} in adult asthmatics is of little clinical significance as levels of whole complement are high in a variety of disease states [12]. A further limitation on the value of the terms « extrinsic » and « intrinsic » asthma is suggested by our findings that adults whose disease originated in childhood showed a changing pattern in terms of allergy-associated clinical features. Thus, as the duration of the disease increased, the wheeze pattern tended to change from episodic to chronic in nature and the features studied became less clearly associated with allergy. When individual clinical features associated with allergic or non-allergic asthma were correlated with complement components and IgE, the only significant differences were found in the levels of C4. Thus raised levels of C4 were more consistently found with allergic features than were raised IgE values. Conversely, low levels of C4 were usually associated with the features of non-allergic disease whereas many of these patients had raised levels of IgE. To provide evidence that the lower C4 levels in non-allergic asthma were a result of sequential activation of the complement pathway, effective molecular titrations of C2 were performed in two groups of patients with all the selected features of allergic or non-allergic disease. Although the differences in C2 were not significant it does not exclude an activation mechanism. In contrast to C2, C4 is exquisitely sensitive to the action of a few molecules of activated C1 [13]. A striking change in C4 can occur with comparatively little alteration in the levels of C1 and C2. If C1 activation did occur, this may be attributable to natural antibodies combining with infective agents, although levels of C4 in a number of patients with recurrent respiratory infection were normal. Natural antibodies against *Corynebacterium parvum* have been demonstrated in normal human serum and the resultant complexes have been shown to activate the classical complement pathway [14]. The reduced C4 levels in non-allergic asthma could also be a result of decreased synthesis. C4 synthesis by cells of the macrophage/monocyte series has been described although the source of macrophages was from the peritoneal cavity [15]. Whether alveolar macrophages can synthesise C4 is yet to be determined.

Thus, although the mechanism of alterations in the levels of C4 in asthmatics with allergic and non-allergic associated disease has yet to be determined, measurements of this component in the circulation may prove to be relevant to the pathogenesis of the disease and perhaps of some value in its classification.

CONCLUDING COMMENTS

1. C4 levels were higher in childhood asthma than controls.
2. C4 was slightly but statistically significantly lower in adult asthma than controls.

3. Some of the clinical features which distinguish « extrinsic » from « intrinsic » asthma seemed to correlate better with C4 than either IgE concentrations, eosinophil counts, β-glucuronidase levels or the amounts of sputum histamine.

4. With asthmatics who develop their disease in childhood, C4 levels are initially high but with duration of the disease the concentrations become similar to those with chronic or intrinsic asthma.

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Immunoglobulins and complement in pleural effusions associated with bronchogenic carcinoma

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Immunoglobulins and complement in pleural effusions associated with bronchogenic carcinoma

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From the South-East Scotland Regional Blood Transfusion Service, Royal Infirmary, Edinburgh[1], *the University Department of Clinical Chemistry, Royal Infirmary, Edinburgh*,[2] *and the University Department of Respiratory Diseases, City Hospital, Edinburgh*[3]

SYNOPSIS Levels of IgG, IgA, IgM, the total haemolytic complement (CH_{50}), and the individual components C1q, C3, C4, C6, and C7 were measured in 29 pleural effusions. Of these, 18 were associated with carcinoma of the bronchus and 11 were non-malignant effusions including empyemas.

The level of IgG was significantly lower in the malignant group when compared with non-malignant effusions. The usefulness of measurements of IgG with respect to malignant effusions associated with carcinoma of the bronchus requires an expanded study to show whether it has any real diagnostic value. There were no significant differences in other immunoglobulins, the CH_{50}, and individual complement components between the two groups.

The identification of total haemolytic activity in the majority of effusions in both groups indicates that all nine components of the classical pathway of complement, including macromolecules such as C1, can be present in pleural fluids.

There have been relatively few reports on measurements of immunoglobulins and complement in pleural fluid. Hunder *et al* (1972) found that the total haemolytic complement (CH_{50}), C4, and C3 concentrations were lower in the pleural fluids from patients with rheumatoid arthritis and systemic lupus erythematosus than in fluids from patients with malignant and other diseases. Hirsch *et al* (1971) reported the presence of immunoglobulins IgG, IgA, and IgM in nine pleural fluids in amounts comparable to those concentrations present in the serum.

Since there have been a number of reports which suggest that cancer patients have circulating antigen-antibody complexes which in turn may lead to activation of complement, we have measured immunoglobulins and individual complement components in 18 malignant pleural fluids and compared these with 11 non-malignant effusions, including empyemas.

Material and methods

PLEURAL EFFUSIONS

Samples were centrifuged at 1500 *g* to remove particulate matter, divided into portions, and kept at −80°C. The effusions were processed and stored on the same day as the pleural aspiration and were thawed only once before each assay.

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TOTAL PROTEIN

This was measured by a standard AutoAnalyzer (Technicon Instruments Corporation Ltd, Basingstoke) method (N14b) which uses the biuret reaction. The between-batch precision (± 1 SD) was 1·0 g/l.

IgG, IgA, AND IgM

These were measured by an automated nephelometric procedure on an AutoAnalyzer II (method no. 12). The method incorporates 4% polyethylene glycol in the antiserum diluent. Samples were prediluted manually (1:50 for IgA and IgM, and 1:200 for IgG) rather than on the AutoAnalyzer. The between-batch precision (± 1 SD) in the range up to 300 IU/ml was 8·0 IU/ml for IgG, 12·6 IU/ml for IgA, and 7·0 IU/ml for IgM.

COMPLEMENT

The haemolytic complement (CH_{50}) was measured by the method of Mayer (1961). Levels of complement components C1q and C3 were measured by single radial diffusion using monospecific antisera

raised in the rabbit. Levels of C4 and C6 were measured using C4-deficient guinea-pig and C6-deficient rabbit sera respectively (Lachmann *et al*, 1973), and C7 was measured by the reactive lysis method (Thompson and Lachmann, 1970). Immunoelectrophoresis was performed as described (Scheidegger, 1955) using a rabbit antiserum to human plasma proteins purchased from Wellcome Reagents Ltd.

Results

When the electrophoretic pattern of malignant and non-malignant pleural effusion, including empyemas, was compared to the pattern of normal serum there were no major differences in the number of positions of the principal protein bands.

The amount of Ig Gper milligram total protein in the malignant effusions was significantly ($P < 0.01$) lower than the IgG content of the nonmalignant group (fig 1). The differences in IgA and IgM per milligram of total protein and in total protein concentration itself between the two groups were not significant.

Total haemolytic complement was measurable in the majority of pleural effusions but there was no significant difference in levels between the malignant and nonmalignant fluids (fig 2). The individual components C1q, C4, C3, C6, and C7 were also detectable in the majority of effusions but no significant differences were found between the two groups (fig 2).

Discussion

In the report by Hirsch *et al* (1971), three out of the nine pleural effusions were from patients with

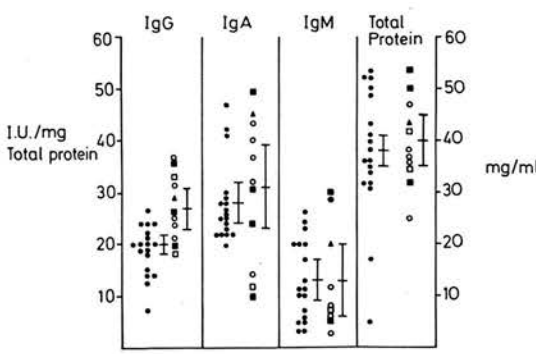

Fig 1 *Immunoglobulins and total protein in malignant and nonmalignant pleural effusions. Malignant effusions* (●), *empyema* (○), *tuberculosis* (■), *pneumonia* (□), *pulmonary infarction* (▲).

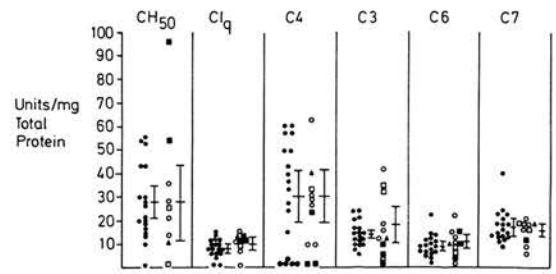

Fig 2 *Total haemolytic complement* (CH_{50}) *and the individual complement components C1q, C4, C3, C6, and C7 in malignant and nonmalignant pleural effusions. Malignant effusions* (●), *empyema* (○), *tuberculosis* (■), *pneumonia* (□), *pulmonary infarction* (▲).

carcinoma of the lung, but in this small series the levels of IgG, IgA, and IgM were not markedly depressed when compared with nine nonmalignant fluids. Our findings show that the amount of IgG per milligram of total protein in malignant effusions was significantly lower than the IgG content of the nonmalignant fluids, but there was considerable overlap between the two groups (fig 1). This observation may, however, have relevance when very low IgG concentrations are present although further studies are necessary to evaluate the real diagnostic value of these findings. Immunoelectrophoresis alone does not appear to have diagnostic value as the patterns were similar with each disease state.

The reasons for the lower concentration of IgG in the malignant group is unknown. One explanation is that IgG is an inadvertent substrate for plasmin as a result of local fibrin formation and activation of the fibrinolytic pathway. Disseminated intravascular coagulation (DIC) in association with disseminated carcinoma is well recognized. Although DIC was not a complication in any of the patients studied here it is possible that there was low-grade conversion of plasminogen by a plasminogen activator in the pleural space of the patients with carcinoma. Levels of fibrin degradation products were higher in pleural fluids from patients with carcinoma than in nonmalignant fluids (J. D. Cash, personal communication).

Although we did not have the opportunity to measure plasma concentrations of immunoglobulins at the time of pleural aspirations, it is unlikely that our findings merely reflect low circulating IgG levels. In a report by Hughes (1971) IgG levels in cancer patients, including those with bronchogenic carcinoma, were significantly increased when compared with those in matched controls.

Measurements of the CH_{50} and individual components in body fluids other than plasma have

been of value diagnostically. The complement levels in joint effusions in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) have been found to be lower than in 'non-rheumatoid' arthritis fluids from patients with osteoarthritis or gout (Pekin and Zvaifler, 1964; Franco and Schur, 1971). Similarly, the CH_{50}, C1q, C3, and C4 were lower in pleural fluids secondary to SLE and RA than in malignant pleural effusions (Hunder *et al*, 1972). These findings are thought to be a consequence of complement activation by antigen-antibody complexes. Since there is evidence in both man and experimental animals for the presence of immune complexes in cancer (Baldwin *et al*, 1974), in this study we have measured complement in malignant effusions and have included the terminal components C6 and C7. We have compared our malignant group with exudates associated with pneumonia, tuberculosis, and pulmonary infarction and with empyemas. Although we found no significant difference between our two groups in terms of complement measurements (fig 2), the observation that many of these components were present adds to knowledge of the composition of pleural fluids.

The mechanism by which fluid accumulates within the pleural cavity is incompletely understood. Leckie and Tothill (1965) found that radiolabelled albumin can diffuse freely out of the pleural space, and it seems likely that most proteins in pleural effusions derive from the circulation as a result of the inflammatory process rather than from local production. It is reasonable to suppose that macromolecules, such as C1 (molecular size 900 000), can diffuse into the pleura. The majority of fluids from the malignant and nonmalignant groups had total complement activity (the CH_{50}) which requires C1 and the other components of the 'classical pathway'.

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MEDIATORS OF IMMEDIATE-TYPE HYPERSENSITIVITY IN SPUTUM FROM PATIENTS WITH CHRONIC BRONCHITIS AND ASTHMA

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Summary Mediators of immediate-type hypersensitivity were studied in the sputum of patients with chronic bronchitis. The same mediators were also measured in early-onset, skin-test-positive asthmatics, in late-onset, skin-test-negative asthmatics, and in patients with bronchial carcinoma, bronchiectasis, and pneumonia. Sputum eosinophilia was a feature of bronchitics and asthmatics, whereas raised blood eosinophil levels were found only in the early-onset, skin-test-positive asthmatics. Histamine and IgE were present in considerable amounts in the sputum of bronchitics and early-onset, skin-test-positive asthmatics. Smaller amounts were found in the other groups. The sputum in all the groups contained material giving an "S.R.S (slow-reacting substance) like" induced contraction of the guineapig ileum. "Classical" S.R.S.-A., determined by arylsulphatase IIB susceptibility, was present only in bronchitics and both types of asthmatics. Since the bronchitics were, in general, skin-test negative and had normal concentrations of circulating IgE and eosinophils, it is suggested that the findings in the sputum indicate an element of local immediate-type (type I) hypersensitivity in bronchitis although its significance for pathogenesis is not known.

Introduction

DURING a study of the ætiology of exacerbations in patients with "chronic bronchitis" a common finding was intermittent eosinophilia in the sputum. This feature suggested that these patients might have an element of local, rather than general, immediate-type (type I) hypersensitivity, because most of them were skin-test negative to a variety of common allergens and had no blood eosinophilia. We therefore measured the concentrations in the sputum of a number of agents associated with type-I hypersensitivity—histamine, slow-reacting

substance of anaphylaxis (S.R.S.-A.), and IgE. We compared the results with those for sputum of patients with asthma and other pulmonary diseases.

Patients

Chronic Bronchitis

These 26 patients had the following features: (1) persistent cough and sputum for at least 3 months in at least 2 consecutive years, (2) at least one period of illness which had kept the patient from normal activities for at least 3 weeks in 2 successive years, (3) reversibility of airways obstruction of not greater than 20% of the initial forced expiratory volume in 1 s ($F.E.V._1$) after one or two inhalations of salbutamol, (4) no evidence of pulmonary tuberculosis, bronchiectasis, or bronchial carcinoma. They had been or were heavy smokers and were skin ("prick") test negative, with 2 exceptions, to common allergens.

10 of the 26 were given prednisolone (20 mg a day for 2 weeks) with no improvement in the peak expiratory-flow rate. 9 of these 10 patients underwent the tests shown in the table. The values obtained were consistent with the diagnosis of chronic bronchitis (except for patient no. 7, who had no airways obstruction but had the other characteristics described above).

Bronchial Asthma

All had generalised airways obstruction which was reversible spontaneously or as a result of treatment, with an increase of 30% or more of the initial $F.E.V._1$. 18 of the 26 had never smoked.

The asthmatics were divided into two groups. One group was skin-test positive and all had had symptoms before the age of 35. The patients in the other group were skin-test negative and in all of them the first symptoms had started after the age of 35. (In the figures the patient's age is also recorded as greater or less than 35 years, but this refers to the time of sampling.) All except 4 of the 15 asthmatics who were more than 35 years old at the time of sampling (whether skin-test positive or negative) were receiving oral corticosteroids.

Bronchial Carcinoma

The diagnosis in all these patients had been proved histologically. They had no history of chronic bronchitis and at the time of sampling had no clinical infection.

Bronchiectasis

The diagnosis was made either by bronchography or on the presence of persistent localised coarse crepitations on at least 3 occasions during a period of at least 2 years. All the patients had produced copious amounts of purulent sputum for a number of years.

Pneumonia

The patients with pneumonia had had a typical acute infective episode with no history of previous respiratory disease.

Materials and Methods

Eosinophil-counts in sputum were expressed as a percentage of the first 400 intact cells counted in a smear. Glass beads were then added to the sputum and the sample shaken vigorously for 5–10 min, centrifuged, divided and stored at −80°C.

Histamine and slow-reacting substance (S.R.S.-A.)were measured on the isolated guineapig ileum[1,2] and arylsulphatase digestion was carried out.[2]

IgE was measured with a 'Phadebas' kit (Pharmacia) and the levels of IgG, IgA, and IgM were estimated by the Mancini technique, monospecific antisera being used. To relate sputum IgE to protein content values were expressed as international units (I.U.) per g of total immunoglobulin protein per g of sputum.

Because of the size of the samples all the measurements could not be made in all the patients.

The P values were calculated by the equation

$$d = \frac{\text{the difference of the means}}{\text{the sum of the standard errors}}$$

in which P was calculated from the normal distribution curve.

Results

4 out of 23 patients with chronic bronchitis had a sputum eosinophilia of 20% or more (fig. 1). 17 had

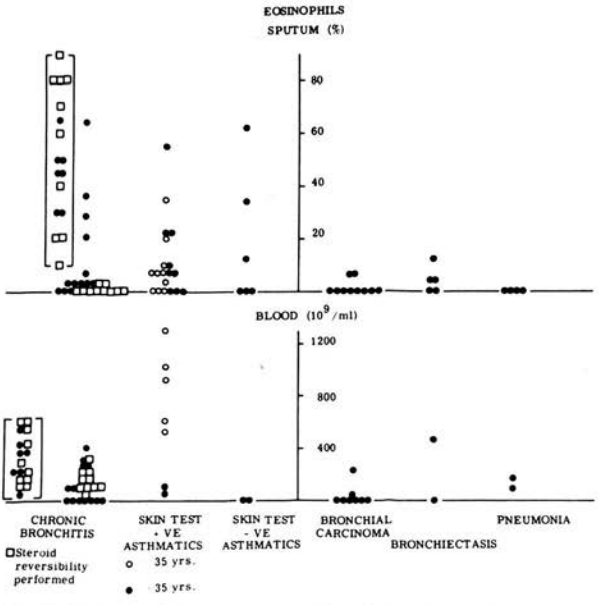

Fig. 1—Eosinophils in sputum and blood of patients with chronic bronchitis, asthma, and other pulmonary diseases.

Values given in parentheses are the highest recorded in those bronchitics studied monthly for one year.

monthly counts and the highest recorded percentage is also shown. Over a period of 12 months all had values more than 10% in at least one of the specimens; in 12

it was over 40%, though the number of specimens containing eosinophils varied from patient to patient and many were negative. The blood eosinophil-count at the time of sampling was normal (i.e., less than 640×10^9 cells/l). In the skin-test-positive asthmatics, on the other hand, most of the patients tested had raised sputum and blood eosinophil counts. 3 out of the 6 skin-test-negative asthmatics also had a sputum eosinophilia. Blood counts could be done only on 2, but 4 of the 6 patients were on long-term corticosteroid therapy. In general, sputum and blood eosinophil-counts in the patients with bronchial carcinoma, bronchiectasis, and pneumonia were low.

The sputum histamine values are shown in fig. 2. Only patients with chronic bronchitis (11 out of 26) and the skin-test-positive asthmatics (12 out of 20) had values of 0·75 mg/g sputum or more. There was no sig-

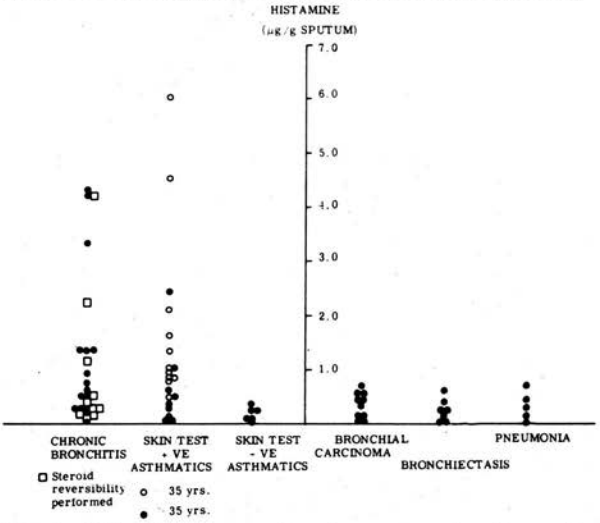

Fig. 2—Histamine concentrations in sputum of patients with chronic bronchitis, asthma, and other pulmonary diseases.

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nificant difference in the sputum histamine values between the chronic bronchitics and the skin-test-positive asthmatics. But when these groups were each compared with skin-test-negative asthmatics and patients with bronchial carcinoma, bronchiectasis, and pneumonia, the differences were highly significant ($P<0.002$ to <0.005).

Nearly all the samples tested showed some "S.R.S.-like" activity and there was no apparent difference between any of the groups (fig. 3). To establish whether the ileal-contracting activity was associated with "classical" S.R.S.-A., samples from each of the 6 groups were treated with an excess of arylsulphatase IIB (fig. 4). Spu-

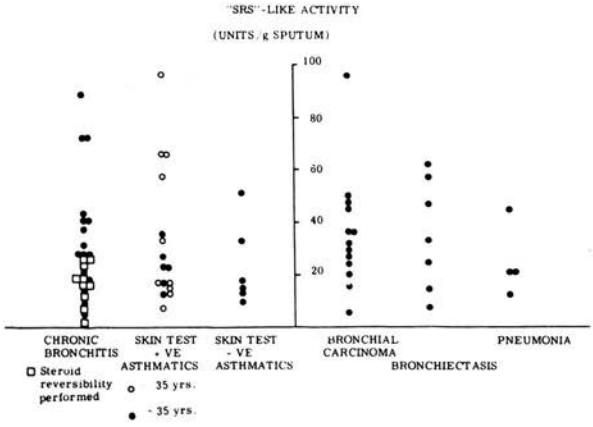

Fig. 3—"S.R.S.-like" activity in sputum of patients with chronic bronchitis, asthma, and other pulmonary diseases.

tum samples from patients with bronchial carcinoma, bronchiectasis, and pneumonia were unaffected by this treatment, indicating that the substance was not "classical" S.R.S.-A. On the other hand, samples from chronic bronchitics and asthmatics were all susceptible to treatment by this enzyme, indicating "classical" S.R.S.-A. Activity was completely destroyed in all but 5 samples and even in these there was partial inactivation. Unlike the other bronchitics and asthmatics these 5 patients had respiratory infections at the time samples were tested.

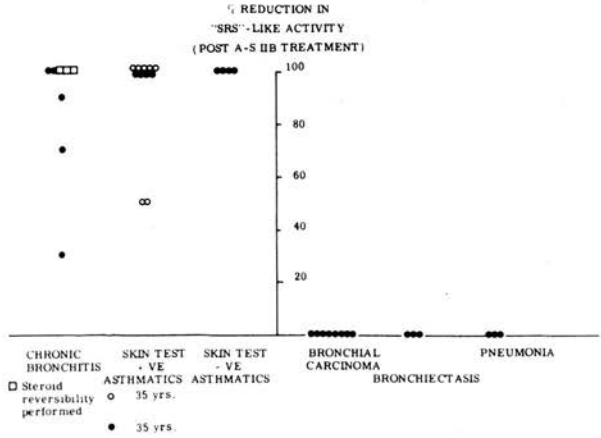

Fig. 4—Percentage reduction in S.R.S.-like activity after incubation of sputum samples with arylsulphatase IIB.

IgE was measured in the serum and sputum (fig. 5). Serum-IgE concentrations were greatly raised in 5 of the 18 skin-test-positive asthmatics and in 1 of the 6 skin-

LUNG-FUNCTION TESTS AND DEGREE OF REVERSIBILITY TO IPRATOPIUM BROMIDE AND SALBUTAMOL IN THE 10 PATIENTS SUBJECTED TO THE "STEROID-REVERSIBILITY" INVESTIGATION

Patient no.	Age	R.V./T.L.C. (%)	Tco_{SB} (mmol min^{-1} k Pa^{-1})	F.E.V.$_1$(l)	F.V.C.(l)	Reversibility (%)		
						Ipratopium bromide	Salbutamol	Prednisolone
1	69	61·0 (38·0)	8·1 (9·3)	1·10 (2·55)	2·76 (3·65)	–6	7·8	0
2	67	76·0 (40·0)	N.D.	0·38 (1·85)	0·90 (2·95)	26	4·4	0
3	50	36·0 (34·0)	8·5 (9·6)	1·94 (3·30)	4·00 (4·30)	11	0·2	0
4	54	72·0 (35·0)	9·8 (8·2)	0·55 (2·88)	2·42 (3·73)	27	17	0
5	53	44·0 (35·0)	7·8 (8·3)	2·30 (2·70)	3·55 (4·00)	17	1·6	0
6	67	66·0 (38·0)	0·5 (7·8)	0·80 (2·10)	2·35 (2·84)	–6	7·7	0
7	70	33·0 (41·0)	7·4 (8·4)	2·50 (2·25)	3·40 (3·10)	0	0	0
8	57	45·0 (37·0)	3·6 (7·4)	0·92 (2·55)	2·35 (3·35)	20	17·5	0
9	65	48·0 (38·0)	4·5 (8·8)	1·9 (2·2)	2·8 (3·6)	11	6	0
10	63	N.D.	N.D.	1·3 (2·35)	3·4 (3·3)	N.D.	0	0

R.V./T.L.C. = ratio of residual volume to total lung capacity; $Tco_{S.B.}$=single-breath carbon-monoxide transfer factor; F.E.V.$_1$ = forced expiratory volume in one second. F.V.C.=forced vital capacity. The predicted values for the patient's age, weight and height are in parentheses. N.D. = not done.

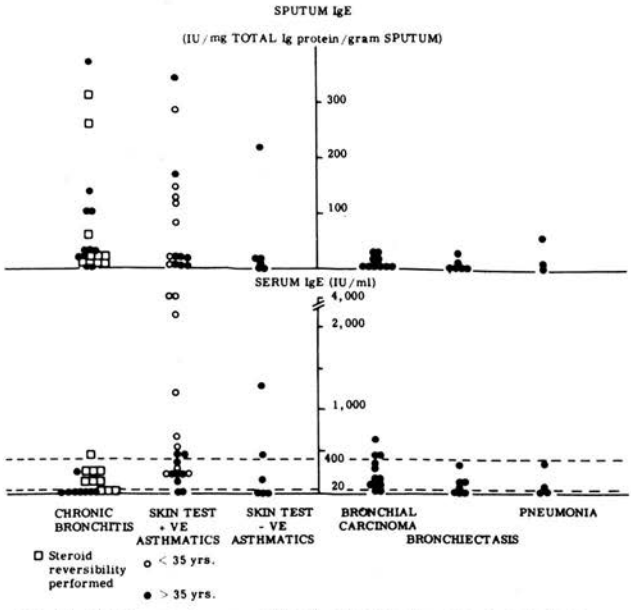

Fig. 5—IgE in sputum and blood of patients with chronic bronchitis, asthma and other pulmonary diseases.

test-negative asthmatics, but in none of the other patients. In contrast, appreciable amounts of IgE were detected in the sputum from 7 of the 22 chronic bronchitics and from 7 of the 15 skin-test-positive asthmatics. Apart from 1 skin-test-negative asthmatic, the values for the other groups were low.

In general, the younger skin-test-positive asthmatics (below 35 y) tended to have higher blood eosinophil values (fig. 1), sputum histamine concentrations (fig. 2), and serum-IgE (fig. 5) than those in the older age-group; this difference did not hold for the other measurements.

Discussion

The bronchitics were carefully selected to exclude those with asthmatic features. They fulfilled the Medical Research Council criteria,[3] were heavy smokers, and those assessed were unresponsive to corticosteroids. The presence of sputum mediators, accompanied by normal blood eosinophil and IgE values, and the negative skin-tests suggest a local type-I reaction in at least some chronic bronchitics for at least some of the time. The significance of these findings for pathogenesis is unknown, as is the nature of the allergen, although the H_1 antigen of *Hæmophilus influenzæ* has been suggested.[4]

The detection of S.R.S.-A. is of particular interest because it is primarily associated with type-I reactions and may have other actions besides its effect on smooth muscle.[5,6] The "S.R.S.-like", but arylsulphatase resistant, activity in sputum from the other patient groups may be due to to prostaglandins.[7]

The findings in the asthmatics support the view that mediators of hypersensitivity may participate in "extrinsic" disease and that in "intrinsic" asthma S.R.S.-A. may be released by an IgE-independent mechanism, though the numbers in the "intrinsic" group were too small for firm conclusions to be drawn.

This work was supported by an anonymous gift to the department of respiratory diseases, University of Edinburgh. We thank Dr Valentine U. McHardy for allowing us to study patients in a Medical Research Council study on exacerbations in chronic bronchitis (of which she is the coordinator for Edinburgh); Mrs Georgia Clark for technical help; and the following for their assistance in collecting samples and for useful discussions: Dr Catherine Anderson, Dr Margaret Calder, Dr J. D. Cash, Dr L. J. Clancy, Dr G. K. Crompton, Dr A. C. Douglas, Dr Joyce M. Duncan, Dr A. J. Dyson, Dr D. A. Ellis, Dr D. C. Flenley, Dr J. P. Galloway, Dr I. W. B. Grant, Dr A. Gray, Dr J. M. Hopkin, Dr Charlotte P. Kent, Dr R. J. E. Leggett, Dr Elizabeth M. Lumb, Dr C. R. McGavin, Dr G. J. R. McHardy, Dr J. G. McVie, Miss Sylvia Merchant, Dr W. M. Middleton, Dr Catherine Ng, Dr I. Paterson, Dr M. Sudlow, Prof. Margaret Turner-Warwick, Dr W. A. Whitelaw, and Dr Agnes P. Wood. Part of this work was the subject of a B.SC. (Hons.) thesis (L.S.T.), department of bacteriology, University of Edinburgh.

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INTERACTION BETWEEN ISOPRENALINE AND AMINOPHYLLINE IN ASTHMA

BY

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Interaction between isoprenaline and aminophylline in asthma

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From the University Department of Respiratory Diseases, City Hospital, Edinburgh, the University Department of Statistics, Edinburgh, and the South-East Scotland Regional Blood Transfusion Service, Royal Infirmary, Edinburgh

Campbell, I. A., Middleton, W. G., McHardy, G. J. R., Shotter, Margaret V., McKenzie, R., and Kay, A. B. (1977). *Thorax*, **32**, 424–428. **Interaction between isoprenaline and aminophylline in asthma.** Using a factorially designed study, 38 patients with bronchial asthma received a single dose of either isoprenaline by inhalation (9), aminophylline intravenously (10), isoprenaline and aminophylline (11), or placebo (8). The maximum expiratory flow (V̇ max), the maximum expiratory flow at 50% of vital capacity (V̇ max_{50}), and the concentrations of plasma cyclic AMP were measured at time intervals up to two hours. The combination of isoprenaline and aminophylline acted synergistically in terms of the percent increase in V̇ max_{50}. However, this was statistically significant only at 20 minutes. Plasma cyclic AMP concentration rose with a similar time course of response to the changes in small airways. The elevations in plasma cyclic AMP observed with the drug combination were higher than those for the individual drugs at 10, 20, 30, and 60 minutes but these differences were not statistically significant. These observations support the concept that changes in bronchial smooth muscle tone are mediated by concentrations of cyclic nucleotides and that combinations of isoprenaline and aminophylline, rather than the administration of each drug separately, may have therapeutic advantages in the treatment of bronchial asthma.

It is common practice to use beta-adrenergic stimulants and methyl xanthines in the treatment of bronchial asthma. The studies of Robison *et al.* (1971) suggest that the beta-adrenergic effects of catecholamines are mediated through 3′5′ cyclic adenosine monophosphate (cyclic AMP), the intracellular 'second messenger'. Methyl xanthines inhibit phosphodiesterase, the enzyme which inactivates cyclic AMP (Butcher and Sutherland, 1962), and it is proposed (Leading article, 1970) that methyl xanthines and beta-adrenergic agents may produce bronchodilatation by increasing the level of cyclic AMP in bronchial and bronchiolar smooth muscle.

Drugs which individually act at different points in the metabolic pathway of a compound in such a way as to increase the amount of that compound may, when used together, produce an increase greater than the sum of the increases produced by each drug alone, that is, they may interact synergistically (Veldstra, 1956). There is evidence *in vitro* for synergy between beta-adrenergic agents and methyl xanthines (Rall and West, 1963; Lichtenstein and Margolis, 1968; Kaliner *et al.*, 1971). The present study was designed to establish whether such interaction could be demonstrated *in vivo* in man. We studied the effects of inhaled isoprenaline and intravenous aminophylline on the airways and on heart rate. The level of cyclic AMP in plasma was also measured as it has been described as a convenient index of effects of drugs on tissue levels of cyclic AMP (Ball *et al.*, 1972; Karlberg *et al.*, 1974; Wehman *et al.*, 1974).

Patients and methods

Thirty-eight patients were studied. All patients had airways obstruction and, at some time in the past, had shown a 20% or more increase in

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FEV_1 after inhaled isoprenaline or salbutamol. At the time of study they were symptomatically well and were therefore considered to be in a stable state. Informed consent was obtained from each patient, and the protocol was approved by the Ethical Committee of the City Hospital, Edinburgh.

DESIGN AND DOSAGE

A factorial design permitting the detection of drug interaction (Armitage, 1971) was used. Patients were allocated in random order to one of four treatments:

1 Placebo inhaler plus placebo injection (P), 8 patients;
2 Isoprenaline inhaler plus placebo injection (I), 9 patients;
3 Placebo inhaler plus aminophylline injection (A), 10 patients;
or
4 Isoprenaline inhaler plus aminophylline injection (I+A), 11 patients.

The placebo inhaler delivered inactive propellant only and the placebo injection consisted of 10 ml of normal saline. The inhalers and ampoules were prepared by the hospital pharmacist in such a way that active and placebo preparations were indistinguishable. Two 'puffs' were taken from the isoprenaline inhaler (0·16–0·18 mg ex valve) and the intravenous dose of aminophylline was 250 mg in 10 ml. The treatments were given in a double-blind fashion.

METHODS

Maximum expiratory flow volume (MEFV) curves were recorded at the mouth with a Fleisch pneumotachograph and an integrator, displayed on a storage oscilloscope (Tektronix 5100), and photographed on Polaroid film. The system was calibrated for volume with a Gaensler spirometer, which had previously been calibrated by water displacement, and for flow using a rotameter and vacuum cleaner. Calibration was performed before, during, and at the end of each experiment. The maximum expiratory flow rate ($\dot{V}$ max) and the maximum expiratory flow rate at 50% of forced vital capacity ($\dot{V}$ max_{50}) were measured from two acceptable records of the MEFV curve and the greater value of each index was used for analysis. Obvious differences in the forced vital capacity or distortion by coughing were the usual reasons for considering the records unacceptable. Heart rate was measured from lead II of the electrocardiogram (ECG) recorded for 30 seconds on a Sharp MT23 machine. Plasma cyclic AMP was measured by the competitive protein binding assay of Gilman (1970) using the materials supplied by the Radiochemical Centre, Amersham. Blood was drawn into ethylenediaminetetracetate (EDTA) to a final concentration of 0·005 M and the plasma was separated and stored at −80° C. The samples were thawed once and the proteinaceous material removed by precipitation with 67% ethanol. In order to show that the drugs or their metabolites did not influence the assay 8 picomoles of cyclic AMP were added to 1 ml aliquots of the seven plasma samples from a patient who received the combination of isoprenaline and aminophylline. Values obtained gave the expected increase above aliquots to which no cyclic AMP had been added.

PROCEDURE

The patients were instructed not to use any bronchodilator preparations for at least six hours before the investigation. A Medicut cannula was inserted into a forearm vein. The patient remained at rest in an armchair for at least 15 minutes and then the ECG was recorded for 30 seconds. Five millilitres of blood were drawn and two MEFV curves recorded with the patient in the sitting position. Two puffs of isoprenaline or of a placebo inhaler were then taken by the patient during the first half minute of a five-minute infusion of either aminophylline or saline. During the last 30 seconds of the infusion the ECG was recorded. Blood was taken immediately after the end of infusion, that is, at time five minutes, and two MEFV curves were recorded. Measurements and samples were repeated 10, 20, 30, 60, and 120 minutes from the start of the infusion.

One patient in the placebo group, one in the A group, and two in the I+A group fainted during or immediately after the injection. All four insisted on continuing the investigation and their results are included in the analysis.

STATISTICAL ANALYSIS

The data obtained were subjected to an analysis of variance for a factorial experiment (Armitage, 1971). This analysis provided an assessment of the effects of isoprenaline, aminophylline, and of the combination of the two drugs. An interaction term was derived at each time and its standard error was calculated. If the interaction term was larger than twice its own standard error (that is, was significantly different from zero), the drugs had interacted synergistically.

Results

The mean pre-treatment values of $\dot{V}$ max_{50},

Table *Mean pre-treatment values (±SE) of $\dot{V}$ max, $\dot{V}$ max$_{50}$, heart rate, and plasma cyclic AMP*

	Placebo	*Isoprenaline*	*Aminophylline*	*Isoprenaline + aminophylline*
No. of patients	8	9	10	11
$\dot{V}$ max$_{50}$ (l/sec)	1·32 (±0·25)	1·02 (±0·27)	1·53 (±0·22)	1·13 (±0·11)
$\dot{V}$ max (l/sec)	4·36 (±0·63)	3·82 (±0·52)	4·42 (±0·45)	3·85 (±0·2)
Heart rate per min	75 (±4)	80 (±3)	83 (±5)	71 (±4)
Cyclic AMP (nanomoles/l)	11·8 (±2·15)	11·3 (±1·5)	11·7 (±1·7)	9·7 (±1·8)

$\dot{V}$ max, heart rate, and plasma cyclic AMP for each group are shown in the Table. The differences between groups were not statistically significant.

The means of the percentage increases in $\dot{V}$ max$_{50}$ produced by each treatment are shown in Figure 1. The placebo group remained much the same over the two hours. The combination I+A produced increases which were larger than those produced by either drug alone, but at 60 minutes and 120 minutes the differences between A and I+A were not significant at the 5% level. Although I+A was significantly different from the other three groups at all other times, it was only at 20 minutes that the interaction term was greater than twice its standard error. At 5, 10, and 30 minutes the suggestion that the combination was synergistic was not significant at the 5% level.

The same trend towards a synergistic effect on $\dot{V}$ max was apparent up to 30 minutes after treatment (Fig. 2), but this trend did not achieve statistical significance. At 60 and 120 minutes the effects of I+A were greater than those of I alone ($P<0·05$) but were much the same as A alone.

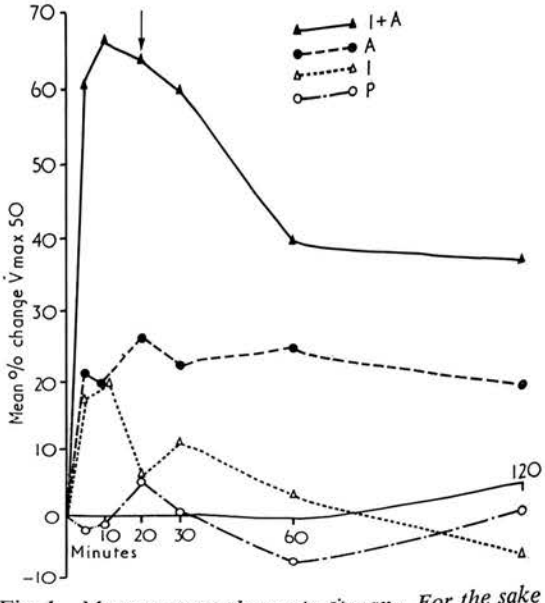

Fig. 1 *Mean percent change in $\dot{V}$ max$_{50}$. For the sake of clarity standard errors are not shown. Significant differences at each time are stated in the text, and the arrow indicates the time at which interaction between I and A was found.*

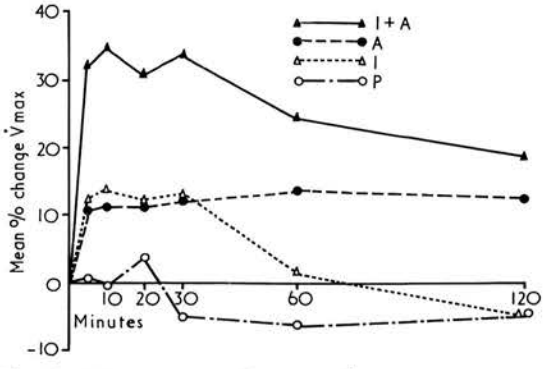

Fig. 2 *Mean percent change in $\dot{V}$ max*

The effects on heart rate are shown in Figure 3. Neither synergy nor addition occurred. I, A, and I+A produced increases which were not statistically distinguishable from each other. At five

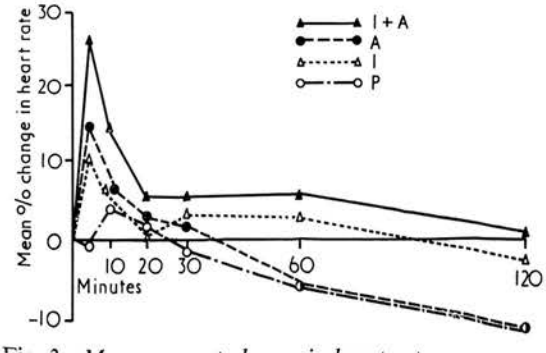

Fig. 3 *Mean percent change in heart rate.*

minutes A and I+A were different from placebo ($P<0.05$) but the changes resulting from I alone were at no time significantly different from placebo.

I, A, and I+A produced similar changes in plasma cyclic AMP (Fig. 4), and these changes paralleled the time course of the changes in the airways. At 10, 20, 30, and 60 minutes the combination I+A produced changes greater than those produced by each drug alone but the differences were not statistically significant.

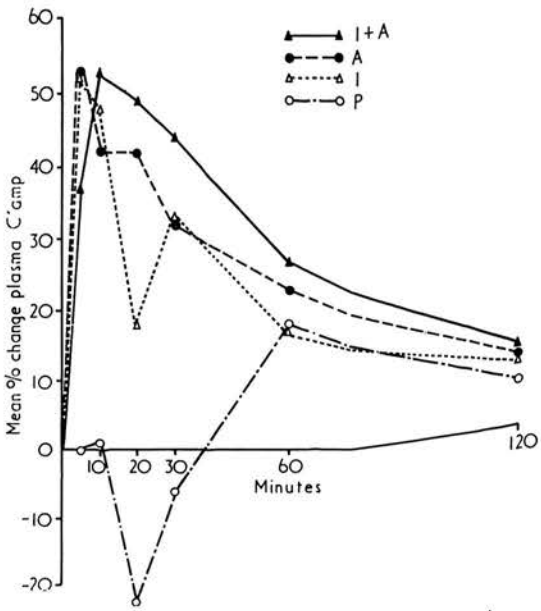

Fig. 4 *Mean percent change in concentration of cyclic AMP in plasma.*

Discussion

Synergy between two drugs is said to occur if the effect produced when they are given together is greater than the sum of their effects when given individually. It is generally accepted that a displacement to the left of the dose-response curve of one drug by another drug indicates synergistic interaction. To be conclusive this shift should be significantly greater than the response to the second drug alone. An alternative method for investigating interaction, which avoids the necessity of establishing dose response curves, is to use single, fixed doses of the drugs in a factorially designed experiment (Armitage, 1971; Pearson *et al.*, 1976), and we have used this latter method.

Hume and Rhys Jones (1961) observed an additive effect of isoprenaline and aminophylline on FEV_1 in six patients. Pihlajamäki *et al.* (1972) examined the effects of glyphylline and isoprenaline singly and in combination, and Cohen and Elizabeth (1974) studied ephedrine and theophylline, but none of these studies was designed in a way which would allow the observed effects to be analysed quantitatively for synergy. Using a factorial design, we have now shown a synergistic bronchodilator effect between isoprenaline and aminophylline on $\dot{V}\ max_{50}$ but not on $\dot{V}$ max. While it is possible that reducing variation by studying larger numbers of patients or by using a within-patient comparison of the drugs might have resulted in the demonstration of synergistic interaction on $\dot{V}$ max it is also possible that the different findings for $\dot{V}$ max and $\dot{V}\ max_{50}$ represent a true difference of the effects of the drugs at different sites in the airways, because it has been argued that changes in maximum expiratory flow rate at 50% forced vital capacity reflect changes in calibre of the smaller airways (McFadden and Linden, 1972).

It would seem that plasma levels of cyclic AMP reflect only broadly changes occurring at tissue level for although the time courses of the plasma cyclic AMP changes were similar to those of the airways we have found no difference between the effect of I, A, and I+A on plasma cyclic AMP. This is not entirely surprising when one considers that the level of plasma cyclic AMP is only 1% of the level in tissues (Karlberg *et al.*, 1974) and that the plasma concentration of cyclic AMP is thought to be the result of a leakage of cyclic AMP from cells into the extracellular space (Broadus *et al.*, 1971). Nevertheless the marked response in plasma cyclic AMP 10 minutes after the administration of drugs used in treating acute asthma may have important therapeutic implications. In status asthmaticus the response to beta-adrenergic stimulants is sometimes impaired or absent, and it has been suggested that alpha-adrenergic blocking drugs can restore beta-responsiveness (Palmer *et al.*, 1974; Patel and Kerr, 1975). Furthermore, some patients with chronic asthma appear to have lost their ability to respond to beta-adrenergic stimulants, and Ellul-Micallef and Fenech (1975) have shown that this responsiveness can be restored by administering corticosteroids. The measurement of the responses of plasma cyclic AMP to beta-adrenergic stimulants might therefore provide an objective means of deciding when beta-responsiveness is no longer present in a clinical situation in which tests of ventilatory function are either too insensitive or too impractical to be a useful index of response.

Neither synergy nor addition was found in the action of the drugs on heart rate. At rest heart rate is controlled predominantly by the parasympathetic nervous system (Robinson *et al.*, 1953; Robinson *et al.*, 1966) and reflex parasympathetic changes might be expected to redress changes induced by isoprenaline and aminophylline, thereby making it difficult to show interaction of these drugs on heart rate. However, both A and I+A produced a significant rise in heart rate five minutes after treatment. In status asthmaticus such a rise might be unacceptable unless oxygen was available to counteract the possible worsening of hypoxaemia that might result.

Our demonstration of the synergistic interaction between a beta-adrenergic stimulant and a methyl xanthine on the airways of man *in vivo* supports the concept that cyclic AMP may participate in the events leading to bronchodilatation and provides a rationale for their combined use in the treatment of asthma.

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Leucocyte Function in a Case of Chronic Benign Neutropenia of Infancy Associated with Circulating Leucoagglutinins

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SUMMARY. A case of chronic benign neutropenia is described in association with circulating leucoagglutinins. IgG and IgM leucoagglutinins demonstrated by Sephadex G-200 chromatography agglutinated neutrophils and monocyte enriched leucocyte preparations. Total IgG, IgA, IgM and IgE, total haemolytic complement, C3 and C4 complement components were all within normal limits. Lymphocyte populations and transformation were normal apart from a relative increase in the number of B lymphocytes. *In vitro* studies showed that the patient's monocytes responded in chemotaxis, phagocytosis and intracellular killing of *S. aureus* in a comparable fashion to that of control neutrophils. Although the patient's serum also agglutinated monocytes these findings do not exclude the possibility that leucoagglutinins may have aetiological significance in this disease. The study also emphasizes the versatility of the monocyte in benign neutropenia.

There have been several reports in the literature of a chronic benign form of neutropenia in infants and Stahlie (1956), reviewing the features of 16 cases, found leucoagglutinins to be absent from three sera examined. A similar benign syndrome has been described in adults and termed 'chronic idiopathic neutropenia' (Kyle & Linman, 1968).

We describe a further case of benign neutropenia in infancy associated with circulating leucocyte antibodies. Immunological investigations suggested that monocytes compensated for the lack of neutrophils.

CASE REPORT

The patient, born in October 1973, was the only child of first cousin Pakistani parents. The mother was receiving phenobarbitone for grand mal seizures associated with her first pregnancy. The child had a normal, full term delivery and was of normal height and weight. In March 1974 she was investigated for diarrhoea thought to be associated with feeding problems and at this time the neutropenia was noted at a routine blood examination. Apart from three transient respiratory infections which responded to antibiotics the child has remained clinically well.

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MATERIALS AND METHODS

Leucoagglutination. Leucoagglutination was performed as essentially described by Kissmeyer Nielsen & Thorsby (1970). Leucocytes were prepared from defibrinated blood by sedimentation of the red blood cells with 'Plasmagel' (Roger Bellon, Paris). Two microlitres of the serum under investigation were incubated with 3000–6000 leucocytes for 1 h after which the white cells were stained with toluidine blue prior to microscopic evaluation of agglutination.

For the investigation of monocyte agglutination the same technique was used but a monocyte enriched cell population (purity 40%) was prepared using a modification of the method of Böyum (1968).

Sephadex G-200. Half a millilitre of the patient's serum taken on day 40 (Fig 1) was applied to a column of Sephadex G-200 (95 × 3.5 cm) equilibrated in phosphate buffered saline. The separation was performed at 4°C and 2 ml fractions collected. The fractions were pooled as indicated (Fig 2) and concentrated to 2 ml by ultrafiltration on a UM10 (Amicon) membrane. Fractions were tested for leucoagglutination and for their contents of IgG or IgM.

Skin window technique. This was performed according to the method of Rebuck & Crowley (1955).

Chemotaxis. Chemotaxis was assayed by measuring the distance, in microns, that the migrating front of cells had travelled into Millipore filters of 3.0 μm pore size following a 90 min incubation period (Zigmond & Hirsch, 1973). The results were expressed as the average distance migrated in five fields examined.

Phagocytosis and bactericidal activities. Phagocytosis and bacterial killing of *S. aureus* was measured as described (McCrae & Raeburn, 1972) using three times washed separated leucocytes and 10% AB serum as a source of opsonin.

Immunoglobulins. IgG, IgA and IgM estimations were performed by single radial immunodiffusion on plates purchased from Hyland, Thetford. IgE was measured by solid-phase radioimmunoassay using the 'Phadebas' kit (Pharmacia, London).

Complement. The total haemolytic complement (CH_{50}) was measured by the method of Mayer (1961). The third and fourth components of complement (C3 and C4) were measured by single radial immunodiffusion using monospecific antibody prepared in the rabbit.

Lymphocyte studies. Populations of T cells were estimated by the rosette technique using sheep erythrocytes (Er) (Jondal *et al*, 1972) and B cells by complement coated sheep cells (EACr) according to the method of Bianco *et al* (1970).

Lymphocyte transformation following stimulation by optimal doses of phytohaemagglutinin (1.5 μg/ml), Concanavalin A (16 μg/ml) or pooled mitomycin treated allogeneic cells (800 000/ml) was estimated by the following method. Lymphocytes were separated from defibrinated blood using a Ficoll-Triosil gradient (Böyum, 1968). The separated cells were washed twice and suspended at 2×10^5/ml in the final culture medium TC199 containing bicarbonate (0.2%), penicillin (100 units/ml) and streptomycin (100 μg/ml), 30 mM Hepes and 20% pooled heat inactivated human serum. Tritiated thymidine (TRA306, Amersham) was added to give 1 μCi/ml. After thorough mixing, 4 ml aliquots were taken into culture tubes (Nunclon 1410.1), the mitogens added and then triplicate 1 ml cultures set up for each treatment. Incubation was at 37°C in a 5% CO_2/air humidified atmosphere, the mitogen

cultures being processed at 72 h and those containing allogeneic cells being terminated at 120 h.

RESULTS

Total and differential leucocyte counts for a 4 month period are shown in Fig 1 and Table I. A transient increase in the neutrophil count occurred between days 80 and 85. The relative lymphocyte counts were persistently elevated but the monocyte numbers were generally within normal limits. Platelets were present in plentiful numbers on all occasions. The bone marrow showed normal cellularity and erythropoiesis. Megakaryocytes were present. There was a marked increase in the numbers of promyelocytes and myelocytes but few cells developed beyond this stage.

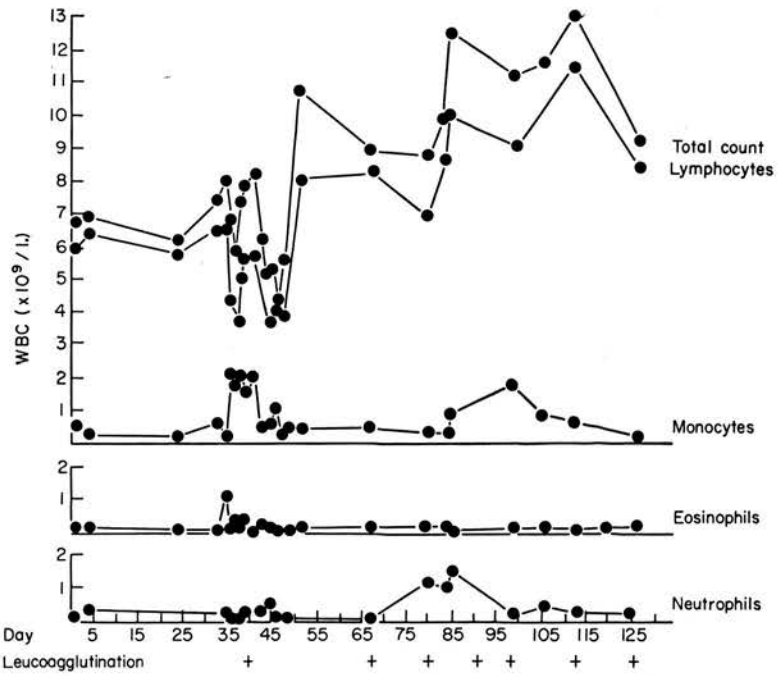

FIG 1. Patient's total and differential WBC. (+), Leukoagglutination test days (all samples positive).

The child was group O Rhesus positive and her serum agglutinated the leucocytes of 12 normal O Rhesus positive individuals. Leucoagglutinins were detected on every sample tested as indicated in Fig 1 and were positive even during the small transient rise in the neutrophil count between days 80 and 85. A homologous and an autologous control serum was negative on each occasion. The agglutinating titres were variable depending on the source of target cells; they were usually 1 in 2 but sometimes 1 in 40. No cytolysis of the agglutinated cells was observed following the addition of sources of complement from humans, guinea-pig and the rabbit. The leucoagglutinating activity in the serum was unchanged following heating at 56°C for 30 min. When the child's serum was passed over a column of Sephadex G-200

TABLE I. Total and differential white cell counts (× 10^9/l.) on selected study days

Day	Total WBC	Differential WBC			
		Neutrophils	*Eosinophils*	*Monocytes*	*Lymphocytes*
5	6.9	0.207	0.069	0.207	6.42
55	5.5	0.0	0.055	0.495	4.95
85	9.8	0.980	0.098	0.294	8.43
105	11.5	0.460	0.115	0.920	10.00
125	9.1	0.182	0.182	0.364	8.37

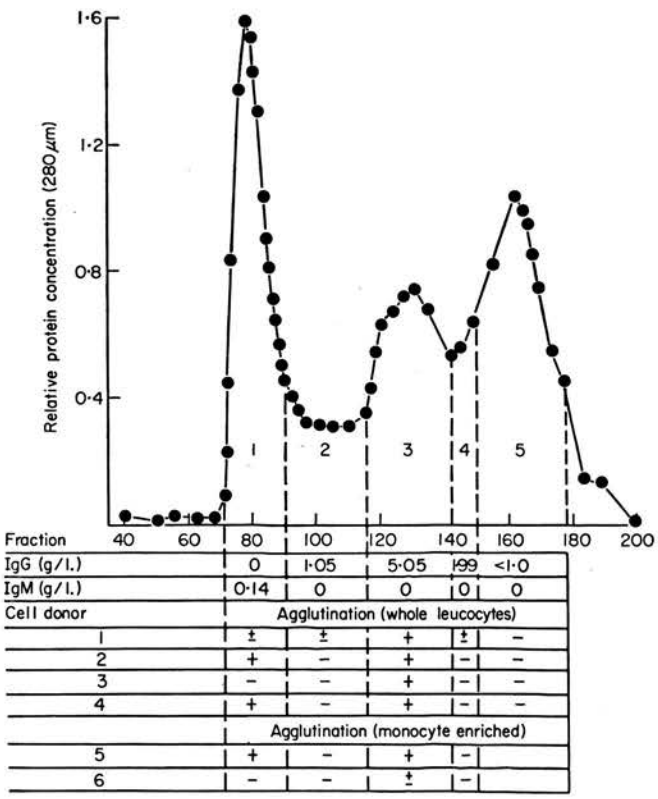

FIG 2. Sephadex G-200 chromatography of patient's serum showing IgM (peak 1) and IgG (peak 3) and the capacity of these antibodies to agglutinate whole leucocytes and monocyte enriched preparations.

leucoagglutinin activity was associated with both the IgM (peak 1) and IgG (peak 3) fractions (Fig 2). By microscopy it could be seen that most of the agglutinating leucocytes were neutrophils. However, when separated blood monocytes containing lymphocytes but virtually free of neutrophils were used as target cells these were also agglutinated by the IgM and IgG fractions. The sera did not agglutinate lymphocytes from normal donors.

TABLE II. Phagocytosis and bactericidal killing by peripheral blood leucocytes

	Sex	*Age (yr)*	*Phagocytosis (% at 15 min)*	*Phagocytosis (% at 30 min)*	*Bactericidal index*
Patient	F	1½	68 and 80	84	10.5 and 6.0
Controls	M	2	—	99	7.2
	F	4	—	97	2.4
	M	4	—	97	10.0
	M	6	—	90	8.1
	F	8	—	98	6.3
	F	10	—	97	15.6
Controls	M	4	91	—	—
	M	7	94	—	—
	F	11	93	—	—
	M	2	85	—	—

TABLE III. Immunoglobulins and complement in patient's serum

		Patient	*Normal values for children*
Serum immunoglobulins (g/l.)	IgG	12	4.0 –14.0
	IgA	1.10	0.2 – 1.2
	IgM	1.54	0.3 – 1.5
	IgE	0.041*	0.024– 0.12
Complement levels†	CH_{50}	30%	20– 40%
	C_4	125%	40–170%
	C_3	136%	40–135%

* mg/l.

† Complement values are expressed as the percentage of a pool of 50 samples of normal human serum (NHP). The normal range was obtained by comparing the values of the 50 individual samples to the NHP and is expressed as ±2 SD.

With the skin window technique neither neutrophils nor monocytes were seen following adequate abrasion at time intervals up to 24 h. However, in chemotaxis, *in vitro*, the child's monocytes migrated as well as neutrophils from a control at a time when the patient had virtually no neutrophils. The migration distance (in microns) of the patient's unseparated leucocytes to doses of 0.5 and 1.0 mg/ml of casein was 74 and 90 respectively. The migrating

cells were all monocytes. In contrast, unseparated leucocytes from a matched control migrated distances of 51 and 80 μm to the same doses of casein but these cells were virtually all neutrophils.

Measurements of phagocytosis and intracellular killing of *Staphylococcus aureus* by the patient's cells are shown in Table II. The patient's per cent phagocytosis of *S. aureus* in 15 min was 68 and 80 on two separate occasions (mean in four healthy child controls: 91). The patient's value after 30 min phagocytosis was 84 (mean in six healthy child controls: 96). The bactericidal indices of 10.5 and 6.0 were comparable to the controls.

The level of IgM was slightly elevated whereas levels of IgG, IgA, IgE, the CH_{50}, C3 and C4 were all within the normal ranges (Table III).

TABLE IV. Subpopulations and transformation of the patient's lymphocytes; normal adult values shown in parentheses

Lymphocyte populations				
T cells (Er)	1858 mm^3	(700–3000),	50%	(54–86)
B cells (EACr)	1152 mm^3	(200–400),	31%	(5–25)
Transformation (cpm of ^{3}H-thymidine/10^6 cells)				
	(dose)	(time)		
PHA	1.5 μg/ml	72 h	927 158	(255 000–580 000)
Con A	16 μg/ml	72 h	154 075	(70 000–350 000)
Frozen mitomycin treated pooled allogeneic cells	800 000/ml	120 h	8 005	(6000–30 000)

When compared to normal adult ranges the per cent of T cells was slightly low although when expressed as the absolute counts they fell within the normal range. The number of B cells was markedly elevated. The response to PHA was above the normal adult value whereas normal counts were obtained following stimulation by Con A or pooled mitomycin treated allogeneic cells (Table IV).

The sera from the mother or father contained no leucoagglutinins and they both had normal white cell and differential counts. The probably HL-A genotypes of the family were, mother, HL-A 11, 12/W32, 'X'; father, HL-A 1, W15/'X', HL-A5 and the patient, HL-A 11, HL-A 12/'X', HL-A 5 ('X' being an unidentified antigen(s)).

DISCUSSION

The association of circulating neutrophil and monocyte antibodies in a patient with benign neutropenia and maturation arrest of the granulocyte series is described. Although antibodies were directed against monocytes in addition to neutrophils, these observations did not exclude the possibility that leucoagglutinins had a causal significance. It is extremely difficult to obtain a preparation of leucocytes entirely free of neutrophils. The observed agglutination with the monocyte rich preparation may have been due to small numbers of contaminating neutrophils which, following agglutination, led to non-specific clumping of monocytes. Another possibility is that the antigen is present on both these cell types but is expressed

relatively weakly by the monocyte. Therefore a monocytopenia would only be seen in the presence of relatively high amounts of circulating agglutinins.

The patient had other unique features in that although neutrophils were virtually absent she appeared to compensate for this lack as assessed by *in vitro* chemotaxis, phagocytosis and bacterial killing. The poor cellular response in both neutrophils and monocytes in the skin window technique emphasizes the differences between chemotaxis *in vitro* and *in vivo*. It has been suggested that an initial neutrophil infiltration into an inflammatory site is a prerequisite for subsequent monocyte infiltration (Ward, 1968). Therefore although monocytes from this patient could pass through micropore filters *in vitro* the full expression of their migratory properties in the skin may require the presence of neutrophils.

The slightly raised level of IgM and increased numbers of B lymphocytes were possibly related to the production of leucoagglutinins. Although we were only able to give normal adult values in the lymphocyte studies it is unlikely that the B cell count was in the normal range for a child of this age.

There is no explanation for the presence of the leucoagglutinins; they are presumably antibodies of either the IgM and IgG class (Fig 2). It was thought that this abnormal finding may have been related to the consanguinity of the parents. However, the mother's and the father's HL-A genotypes were distinct. This does not preclude them, however, for being histocompatible at the LD loci.

The capacity of this patient's monocytes to respond in chemotaxis, phagocytosis and intracellular killing point to the versatility of the monocyte in benign neutropenia and may help explain the remarkable resistance to bacterial infection characteristic of this condition (Zuelzer & Bajoghli, 1964). This is further supported by our patient who remains well and fits the diagnostic criteria of chronic benign neutropenia of infancy.

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The Effect of Transfer Factor on Neutrophil Function in Chronic Mucocutaneous Candidiasis

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SUMMARY. Chronic mucocutaneous candidiasis with hypoparathyroidism in a 6-year-old boy is described. In addition to defects of *in vivo* and *in vitro* correlates of delayed-type hypersensitivity to *Candida albicans* the child also had abnormalities of neutrophil function in terms of their capacity to respond by chemotaxis to a known attractant and to kill suspensions of *C. albicans*. Dialysable transfer factor was given on six occasions at intervals of between 26 and 45 days. Neutrophil chemotaxis (optimal conditions) was restored following each of the six injections, neutrophil chemotaxis (sub-optimal conditions) following five of the six injections and candidicidal capacity following four of the six injections. The effects of transfer factor were transient requiring repeated injections. The Candida delayed-type hypersensitivity skin test was restored to normal but lymphocyte transformation to Candida extract was not consistently positive following treatment. There was a slight clinical improvement following therapy. These abnormalities of neutrophil and lymphocyte function point to the complexity of chronic mucocutaneous candidiasis. The improvement in neutrophil chemotaxis and candidicidal capacity following treatment suggests that transfer factor may be a heterogeneous group of molecules, some of which affect granulocytes and restore defects in their function.

Idiopathic chronic mucocutaneous candidiasis (CMC) is a disorder characterized by recurrent or persistent infections of the skin, nails and mucous membranes with *Candida albicans* (Kirkpatrick *et al*, 1971). The onset is usually in infancy or early childhood and the extent of infection is variable but only rarely systemic. Approximately one third to one half of the patients have an associated defect in endocrine function which may not appear until many years later (Kirkpatrick *et al*, 1971; Kirkpatrick & Smith, 1974).

There is a heterogeneous pattern of defects in cell mediated immunity (CMI) as shown by skin tests and *in vitro* assays of lymphocyte function (Valdimarsson *et al*, 1973). Two cases of CMC have been described in association with defects in chemotaxis. In one patient the monocyte response was impaired and this was corrected by the administration of transfer factor (Snyderman *et al*, 1973). In another case an impairment of neutrophil chemotaxis was found

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but the patient's neutrophils were normal in terms of their capacity to kill *Staph. aureus* and *E. coli* (Clark *et al*, 1973).

In this report we describe a case of CMC in association with defects of neutrophil chemotaxis and Candida killing, both of which were corrected by transfer factor.

CASE REPORT

K.H., born in August 1968, developed at the age of 10 months a *C. albicans* infection of the mouth, scalp, toenails and fingernails with intermittent involvement of the interdigital skin. Investigations at 4 years showed a normal blood picture, a serum iron of 14.3 μmol/l. (normal range 14.3–36 μmol/l.) and a positive delayed-type hypersensitivity skin reaction to Candida extract. His neutrophils could reduce nitroblue tetrazolium (NBT). At this time the serum calcium and phosphate concentrations were normal. A course of oral iron was given without apparent improvement in his clinical condition.

At 4½ years of age he became hypocalcaemic at which time the serum calcium was 1.44 mmol/l., phosphate 3.1 mmol/l. and alkaline phosphatase 100 units/l. The Ellsworth–Howard parathormone stimulation test supported the diagnosis of hypoparathyroidism. There were no clinical signs of hypocalcaemia and no corneal or lenticular abnormalities were found. A skeletal survey was normal. Laboratory tests of other endocrine functions were normal. Vitamin D therapy (calciferol 1.25 mg daily) corrected the biochemical defect (present serum calcium 2.4 mmol/l., phosphate 2.0 mmol/l.) and oral nystatin resulted in a slight improvement of his mouth lesions.

Measles at the age of 6 months and mumps and chickenpox at the age of 4 years were without complications. The patient had had a number of staphylococcal skin infections requiring incision but he did not suffer from systemic pyogenic infections.

In 1974, at the age of 6 years, his immunological function was fully investigated. Therapy with dialysable transfer factor (TF) was begun in 1975 and was administered subcutaneously at intervals of approximately 4 weeks. The first dose was 1 unit, the second to fifth doses were 2 units and the sixth dose 5 units (total 14 units). Since commencing TF therapy the oral lesions have improved and the nail lesions are slowly regressing.

METHODS

Lymphocyte Studies

Peripheral blood T lymphocytes were estimated by the rosette technique using sheep erythrocytes (Kaplan & Clark, 1974) and B cells by rosette formation with complement coated sheep cells (Kaplan & Clark, 1974; Jondal *et al*, 1972). Lymphocyte transformation in response to optimal concentrations of the mitogens phytohaemagglutinin (PHA) and Concanavalin A (Con A), Candida antigen and pooled mitomycin-C treated allogeneic cells (MLR) were determined by the incorporation of [^{3}H]thymidine into DNA as previously described (Kay *et al*, 1976). Preservative-free Candida extract was obtained from HAL-Allergenen, Haarlem, Holland, and from Dr D. W. R. McKenzie, London School of Hygiene and Tropical Medicine. Lymphocytes from a donor responsive to Candida were cultured with the patient's serum to detect any serum inhibitor to transformation.

Skin Tests

Delayed-type hypersensitivity skin tests were performed using streptokinase-streptodornase (SKSD) (5 units, Varidase Lederle), mumps antigen (1 complement fixing unit Eli Lilly Ltd) and Candida extract (0.02 ml 1% extract, Allergenen).

Neutrophil Function

Neutrophil enzymatic activity was assessed by the leucocyte alkaline phosphatase (LAP) score, NBT test (Gordon *et al*, 1973), and myeloperoxidase (MPO) stain (Quaglino & Flemans, 1958). Neutrophil phagocytosis and killing of *Staph. aureus* (Type 42b) was performed as previously described (Raeburn, 1972). Neutrophil candidicidal activity was assessed essentially as described by Lehrer & Cline (1969), as modified by Goldman & Th'ng (1973). Stained smears of the incubation mixtures were examined to count the percentage of neutrophils containing Candida (% phagocytosis) and the mean number of organisms per neutrophil (phagocytic index).

Neutrophil chemotaxis *in vitro* was assayed by the leading front method using Millipore filters of 3 μ pore size and a solution of casein (0.5 mg/ml, sub-optimal dose; 1.0 mg/ml, optimal dose) as the chemoattractant (Wilkinson, 1974).

Immunoglobulins and Complement

IgG, IgA and IgM estimations were performed by single radial immunodiffusion on plates purchased from Hyland, Thetford. IgE was measured by solid phase radioimmunoassay using the 'Phadebas' kit (Pharmacia, London). Total haemolytic complement was measured by the method of Mayer (1961). The individual complement components C3 and C4 were measured by single radial immunodiffusion using monospecific antibody prepared in the rabbit (Lachmann *et al*, 1973).

Preparation of Transfer Factor (TF)

Material was prepared from donors highly sensitive to Candida extract (> 30 mm delayed-type skin reaction). Buffy coat cells were obtained by leucapheresis, contaminating red cells being removed by hydroxyethyl starch sedimentation at 37°C. The leucocyte supernatant was washed in sterile pyrogen free saline and submitted to 10 cycles of freezing and thawing. The leucocyte extract was vacuum dialysed against distilled water. The dialysate was freeze-dried, reconstituted in saline, filtered through a 0.22 μ Millipore filter and stored at -40°C. One unit was defined as the amount of material prepared from 1×10^9 leucocytes.

RESULTS

Lymphocyte Function

Peripheral blood T and B lymphocyte subpopulations and lymphocyte transformation to PHA, Con A and allogeneic cells were assessed on several occasions before and after treatment, and were consistently normal, with no changes noted in relation to TF therapy. There was no response to Candida extract initially, but a slight response was observed after the first dose of TF; the response was unequivocally positive 4 d after the fourth dose of TF but has not

been consistently positive thereafter (Fig 1). No specific inhibition of lymphocyte stimulation by Candida extract could be detected when the patient's pre-treatment serum was incubated with normal responsive lymphocytes.

Skin Tests

Delayed-type hypersensitivity reactions to Candida, mumps antigen and SKSD were all negative initially. The reaction to Candida extract was positive 3 d after the third dose of TF (7 mm induration) and has since remained positive (Fig 1).

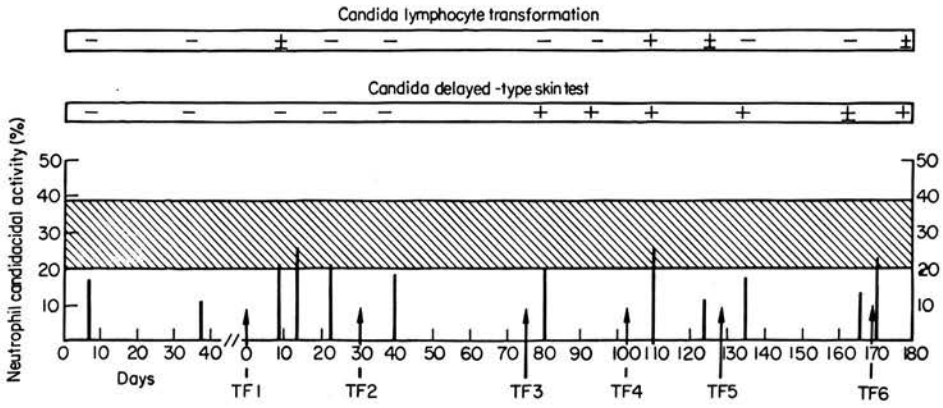

FIG 1. Effect of transfer factor on Candida killing by neutrophils, lymphocyte transformation by Candida and Candida delayed-type skin test. Hatched area represents normal range for neutrophil candidicidal activity. TF = injection of transfer factor.

Neutrophil Function

Peripheral blood neutrophils were normal in number and morphology. The neutrophil LAP score, MPO stain, and spontaneous and stimulated NBT tests were normal. Neutrophil phagocytosis and killing of *Staph. aureus* was normal and phagocytosis of Candida by neutrophils was also repeatedly normal. The results of neutrophil candidicidal capacity and chemotaxis are summarized in Figs 1 and 2. Prior to TF therapy, candidicidal capacity was low (17.1% and 11.5%; normal range 21–38%). The capacity of neutrophils to kill Candida was restored to the normal range after four of the six treatments with TF but this effect was transient.

Neutrophil chemotaxis was also impaired prior to TF therapy both with the optimal dose of casein (40 μm migration; normal range 41–75 μm) and the sub-optimal dose (9 μm migration; normal range 27–59 μm). These impairments were corrected following TF therapy but the effect was short-lived since chemotaxis was abnormal 2–3 weeks after the third and fourth doses. The candidicidal defect in the patient's neutrophils was not corrected by incubation with normal plasma suggesting that there was probably an intrinsic neutrophil defect.

Immunoglobulins and Complement

Serum levels of IgG, IgA, IgM and IgE were essentially normal. Serum total haemolytic complement (CH_{50}), C4 and C3 were also within normal limits.

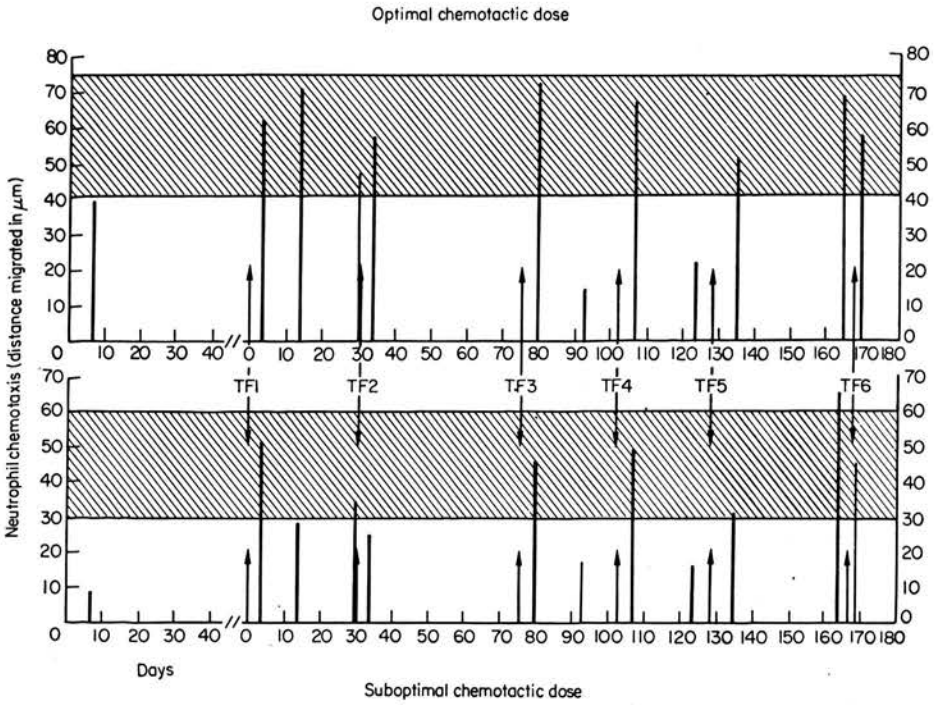

FIG 2. Effect of transfer factor on neutrophil chemotactic response to optimal and sub-optimal doses of chemoattractant (casein). Normal ranges shown by hatched areas. TF = injection of transfer factor.

DISCUSSION

Other authors have demonstrated that the clinical response of CMC to TF therapy is usually transient, lasting a few weeks or months and necessitating repeated doses of TF to maintain improvement (Pabst & Swanson, 1972; Grob *et al*, 1975). In our patient optimal neutrophil chemotaxis was restored on six occasions, sub-optimal chemotaxis on five of six occasions, and candidicidal capacity on four of six occasions. These effects were not permanent and repeated injections of TF were required. When TF did not fully restore neutrophil function the preparation may not have been fully active. This emphasizes the necessity for a reliable *in vitro* assay of the potency of TF prior to its *in vivo* use.

Dermal reactivity and lymphocyte *in vitro* transformation to Candida antigen were observed after the third and fourth treatments respectively. Skin conversion was permanent but lymphocyte transformation was not consistently restored. There is no correlation between the pattern of skin reactivity or lymphocyte transformation to *Candida albicans* and the restoration of neutrophil function by TF, emphasizing the complexity of the underlying defect in CMC and demonstrating the imperfect correlation between the *in vivo* skin test and *in vitro* lymphocyte transformation to the same antigen.

Our findings suggest that TF therapy can transiently affect neutrophil function in addition to the known effects on lymphocytes. It is generally assumed that TF is derived from blood lymphocytes or monocytes and that a single family of informational molecules (RNA-

oligopeptide) is responsible for the transfer of reactivity (Grob *et al*, 1975; Lawrence, 1969). However, no critical studies have been published which demonstrate conclusively the cell type from which TF is derived. It is possible, therefore, that dialysable TF is a mixture of factors, some of which may be derived from granulocytes and which are capable of affecting granulocyte function in the recipient.

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Stroke

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INTER-REACTION BETWEEN COAGULATION AND OTHER BIOLOGICAL SYSTEMS

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INTRODUCTION

The deposition of fibrin and its consequent lysis are complex biological events. Not only are numerous factors and co-factors involved but the haemostatic mechanism is also intimately involved with other cascade-enzyme systems such as complement and kinin formation. In addition, the generation of fibrin and its removal is associated with the elaboration of various biologically active peptides such as chemotactic factors and agents which potentiate the effect of other vaso-active principles. Some of these inter-relationships will be described and their possible in vivo significance discussed.

The Initiation of the Intrinsic Coagulation Pathway

Hageman factor (HF - factor XII) is a remarkable protein. It is an enzyme which can be activated by contact with a variety of negatively charged materials such as glass and collagen. In its activated form (XIIa) HF has at least three major substrates (Kaplan & Austen, 1972; Kaplan & Austen, 1975). One is factor XI, which is converted to XIa, proceeding to thrombin formation via the intrinsic coagulation pathway (Fig. 1). Hageman factor can also convert a circulating plasminogen proactivator to its active form (which in turn converts plasminogen to plasmin) and prekallikrein to kallikrein which cleaves bradykinin from its substrate kininogen. In addition to charged surfaces,

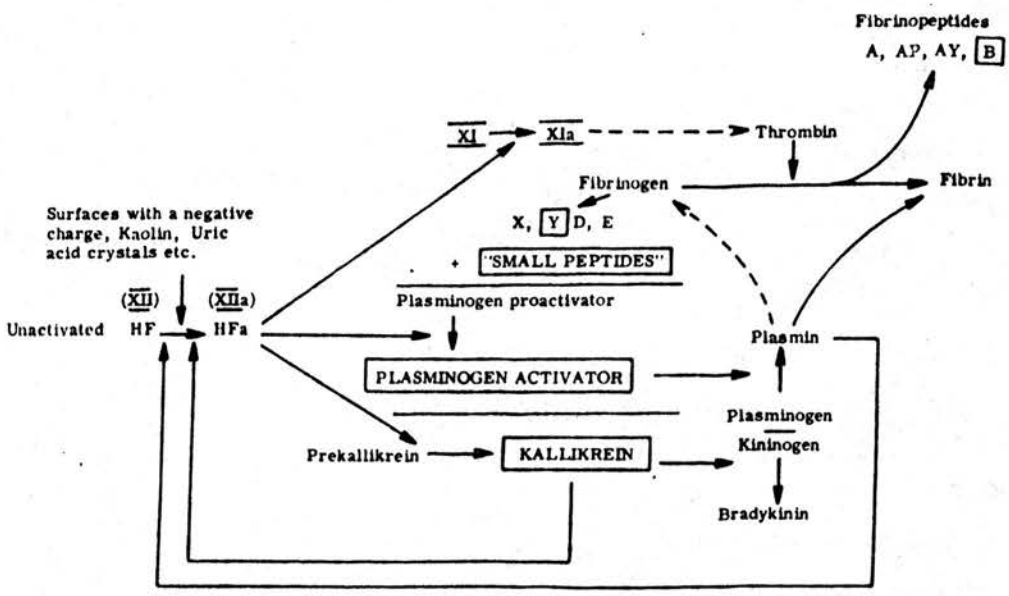

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Figure 1. Hageman factor-dependent pathways and chemotaxis for human neutrophils. The neutrophil chemotactic factors associated with the haemostatic mechanisms are depicted by the "closed boxes". Differences between neutrophil and monocyte chemotaxis in relation to these peptides is shown in Table 1.

the formation of activated Hageman factor is also dependent on, and accelerated by Kallikrein (Cochrane et al, 1972) suggesting that in coagulation, as in other cascade-enzyme systems such as complement, there are positive feedback mechanisms at various stages during the enzymatic sequence. Thus the initiation of the intrinsic coagulation pathway is intimately involved with two other major protein systems, plasmin generation and kinin formation. Their associated biological properties are discussed below.

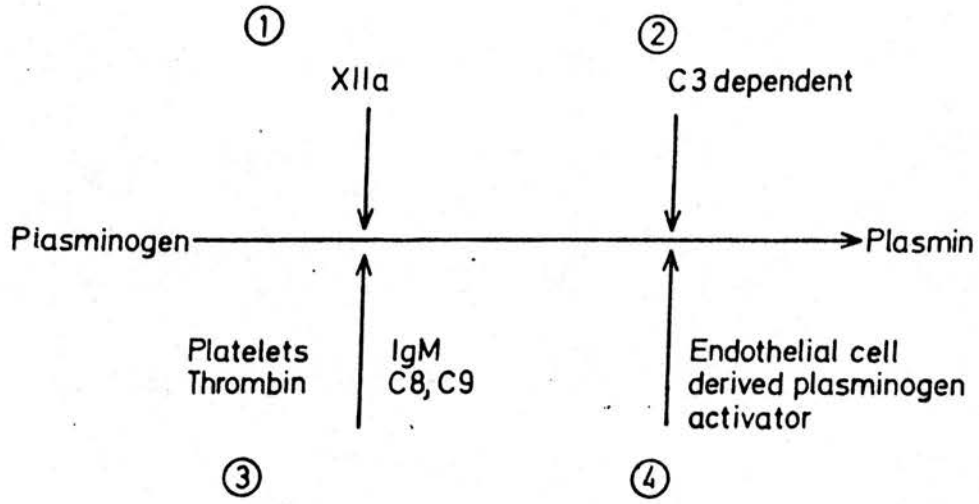

Figure 2. Diagrammatic representation of the various plasminogen activator systems. (1) Hageman factor-dependent (Kaplan & Austen, 1972). (2) Hageman factor-independent, C3-dependent (Schreiber & Austen, 1974). (3) Platelets, IgM, thrombin and complement (Taylor & Müller-Eberhard, 1970). (4) Endothelial cell-derived (Aoki & von Kaulla, 1971).

Plasmin Generation and Its Substrates

A number of pathways have been described which convert plasminogen to plasmin in vitro (Fig. 2). The substance or substances involved are referred to as "plasminogen activators". There are probably several and which is of greatest in vivo significance is yet to be determined. The Hageman factor-dependent plasminogen activation pathway has been mentioned above. In addition, a Hageman factor-independent fibrinolytic system has been described with an apparent requirement for the third component of complement (C3) (Schreiber & Austen, 1974). Plasma deficient in C3 could not sustain fibrinolysis under the experimental conditions described. Furthermore, a particularly complex system has been reported which involves the platelet, thrombin, IgM and the terminal complement components, in particular C8 (Taylor & Müller-Eberhard, 1970). These

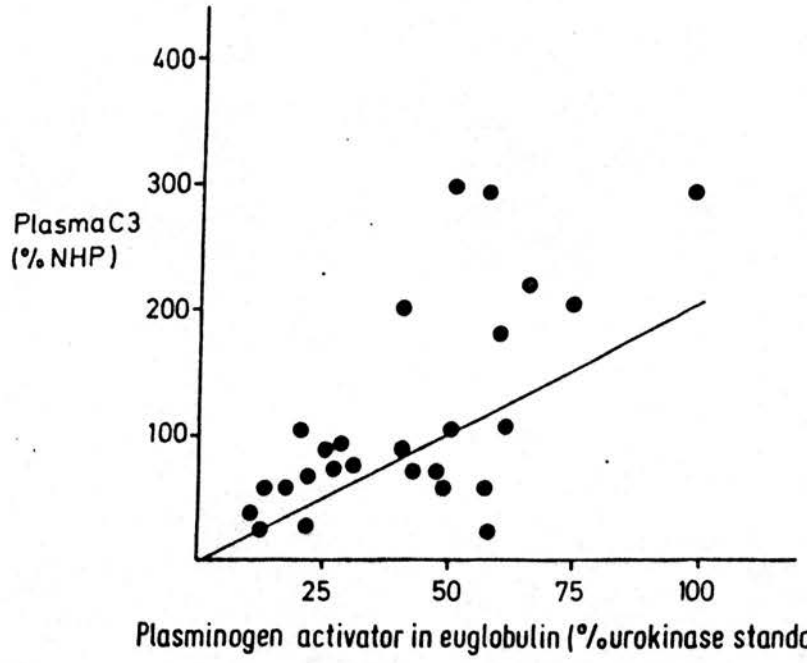

Figure 3. Correlation between plasma C3 concentrations, expressed as a per cent of a normal human pool (NHP) and the plasminogen activator activity in the euglobulin portion of plasma, measured on fibrin plates and expressed as the per cent of a standard of urokinase. ($r = 0.60$)

workers suggested that a plasminogen activator is generated on the surface of the platelet following the action of all these agents. Finally a plasminogen activator derived from human vascular endothelium has been identified which differs both in molecular size and stability to other putative plasminogen activators (Aoki & von Kaulla, 1971).

Preliminary work has been undertaken in an attempt to determine the importance of complement in fibrinolysis by determining whether there was a correlation between circulating plasminogen activator levels and C3 (Kay, A.B. - unpublished observation). The concentration of C3 was plotted against the plasminogen activator activity in the euglobulin fraction of plasma from a number of patients and a statistically significant correlation was found (Fig. 3).

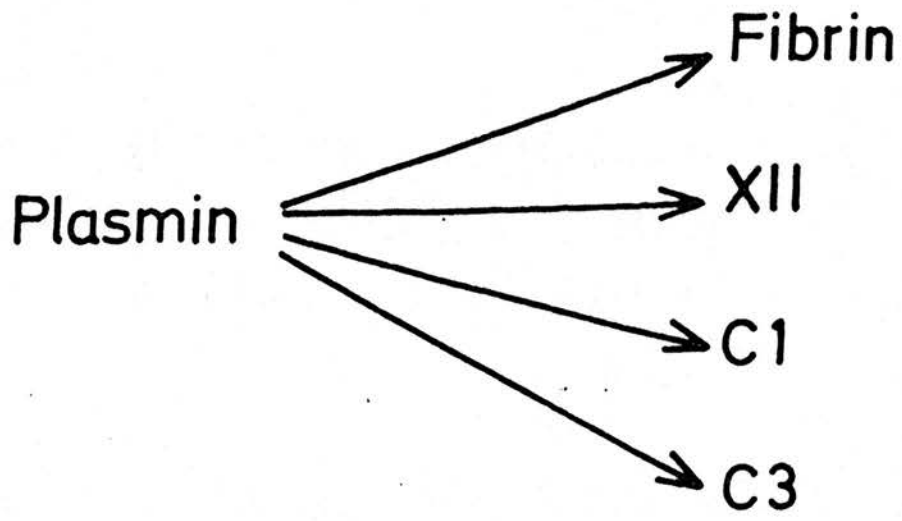

Figure 4. Various substrates of plasmin.

The possibility that C3 may be an inadvertent substrate for plasmin was considered. This would appear unlikely from previous studies in which it was shown that patients on fibrinolytic therapy did not show changes in total haemolytic complement or concentrations of C3 (Tomar & Kolchins, 1972).

Like Hageman factor, plasmin is known to have a number of substrates. These include fibrin, fibrinogen, activated Hageman factor, C1 and C3 (Fig. 4). Plasmin digestion of fibrin (and fibrinogen) leads to the generation of various "fibrin/fibrinogen degradation products" (FDP) many of which have biological activity (see below). The action of plasmin on XIIa leads to the formation of Hageman factor fragments of low molecular weight. The low molecular weight fragments are far more efficient at converting prekallikrein to kallikrein and the Hageman factor-dependent plasminogen proactivator to its active form. In contrast, activated Hageman factor, which has not been acted on by plasmin, has greater activity for factor XI (Kaplan & Austen, 1970).

Plasmin is also said to activate the first component of complement (Ratnoff & Naff, 1967) whose natural substrates

are C4 and C2. Some doubt has been cast on the significance of this form of "triggering" of the classical complement pathway. A fragment can be cleaved from C3 by plasmin which has chemotactic activity for a number of cell types including neutrophils, eosinophils and monocytes (Ward, 1967).

Biological Activities of Coagulation-Associated Peptides

Of the "non-coagulation" biological activities associated with haemostasis, chemotaxis has probably received the most attention (reviewed by Kay & Kaplan, 1975) (Fig. 1).

Initial investigations on the chemotactic activity of normal human serum indicated that many factors were involved since multiple peaks of chemotactic activity could be demonstrated following chromatography on Sephadex G-200 (Kaplan et al, 1972). One of these peaks superimposed the position in which the enzymes kallikrein (mol. wt. 108,000) and the Hageman factor-dependent plasminogen activator (mol. wt. 90,000) eluted. Although these molecules have a similar molecular size it was possible, by further purification and separation, to show that chemotactic activity was associated with both enzymes. Thus kallikrein, in addition to cleaving bradykinin from its substrate kininogen, was a chemoattractant as was this plasminogen activator. More direct evidence of the chemotactic properties of these enzymes was obtained by incubating highly purified HF fragments with their respective proenzymes. Thus a mixture of purified HF fragments and prekallikrein led to the elaboration of both chemotactic activity and cleavage of bradykinin from kininogen. Similarly plasminogen-converting chemotactic activity was generated by the action of activated Hageman factor on the plasminogen proactivator. Of particular interest was the fact that both the enzymatic and chemotactic principle of the plasminogen activator and kallikrein were inhibited by the serine esterase inhibitor, diisopropyl fluorophosphate (DFP). The activity of these enzymes was therefore dependent upon the integrity of their active sites since neither enzyme in its precursor form was DFP sensitive.

Further insight into the chemotactic properties of kallikrein and plasminogen activator was provided by studies on prekallikrein (Fletcher factor) deficient plasma (Weiss et al, 1974). Plasma from these individuals have defects in coagulation, fibrinolysis, kinin-generation and chemotaxis all of which can be corrected by the addition of purified prekallikrein. The correction of these defects by prekallikrein is attributed to a further property of kallikrein, namely its capacity to activate HF so providing a positive feedback for HF-dependent pathways (Cochrane et al, 1972). These pathways are activated by the action of HF on its three known substrates, factor XI, prekallikrein and the plasminogen proactivator (Fig. 1). Using kaolin as the HF activator, restoration of chemotactic activity in Fletcher factor deficient plasma could be achieved by activated HF as well as prekallikrein. This suggested that the contribution of kallikrein to the chemotactic activity of kaolin-activated serum was small and that under these conditions the plasminogen activator and other unidentified HF-dependent chemotactic factors were probably providing most of the activity.

These studies on the chemotactic activity of kallikrein and the plasminogen activator used unseparated human peripheral blood leucocytes as target cells. Whereas unseparated cell populations are acceptable for measuring the chemotactic response of the neutrophil, study of monocyte chemotaxis requires prior separation of the mononuclear cells. This can be conveniently achieved by layering a leucocyte mixture onto Hypaque-Ficoll. Following centrifugation, the less dense mononuclear cells can be separated in a viable form. These cells also responded in chemotaxis to kallikrein and plasminogen activator and there was a similar inhibition of the active site by DFP (Gallin & Kaplan, 1974). Alpha-2-macroglobulin inhibited both the enzymatic effect of kallikrein and plasminogen activator in terms of their actions on kininogen and plasminogen and also the ability of these enzymes to respond in monocyte chemotaxis.

The C1 inhibitor prevented both the enzymatic action of kallikrein on kininogen and its chemotactic principle but had no effect on the expression of the plasminogen activator either as a chemotactic agent or its action on plasminogen. Therefore these naturally occurring protein inhibitors (alpha-2-macroglobulin and C1 inhibitor) affect the enzymatic principle in a similar fashion to their chemotactic activity.

The action of Hageman factor on factor XI (plasma thromboplastin antecedent - PTA) leads to the formation of thrombin via the intrinsic coagulation pathway. Clot supernatants prepared by the action of purified thrombin on fibrinogen are chemotactic for neutrophils, and to a lesser extent for eosinophils, while neither thrombin nor fibrinogen alone possesses this activity (Kay et al, 1973). Thrombin is known to be a limited protease which cleaves several small peptides from fibrinogen. These fibrinopeptides have been designated A, AP, AY and B and are recognised on the basis of their electrophoretic mobility (Blombäck et al, 1966). Evidence has been provided that the chemotactic activity is a property of fibrinopeptide B and not of fibrinopeptides A, AP, or AY (Kay et al, 1974). This was shown by progressive purification of thrombin-induced clot supernatants using high voltage electrophoresis in two dimensions. Supernatants prepared by the action of the snake venom Contortrix which cleaves the B peptide, were also chemotactic whereas no activity was present in supernatants prepared from the Arvin venom which cleaves peptides A, AP and AY. Furthermore, synthetic fibrinopeptide B and a B analogue, 1-glutamic acid, were chemotactic but not synthetic fibrinopeptide A. More recent studies have shown that fibrinopeptide B has chemotactic activity for human monocytes (Richardson et al, 1976).

Other biological activities are associated with fibrinopeptide B. These include potentiation of bradykinin-induced constriction of the isolated oestrous rat uterus (Gladner et al, 1968) and prolonged rhythmic vaso-constriction (Colman et al, 1967). It is probable that the

biological activity associated with fibrinopeptide B is located at its C-terminus since this part of the molecule is exposed following the action of thrombin and other enzymes which cleave B from the β polypeptide chain of fibrinogen.

Other peptides derived from fibrinogen have been examined for possible chemotactic activity since fibrin/fibrinogen degradation products (FDP) appear in body fluids in association with various clinical states and have a number of biological activities. Chemotactic activity for human neutrophils and to a lesser extent eosinophils could be generated by the action of plasmin on human fibrinogen (McKenzie et al, 1975). Previous studies have shown that plasmin degradation of human fibrinogen yields four major fragments designated X, Y, D and E in addition to smaller peptide material (Furlan & Beck, 1972). When plasmin digestion was stopped at time intervals up to 24 hours, a small amount of activity was apparent at 15 and 30 minutes corresponding to the transient appearance of fragment Y. Considerably more chemotactic activity was present in the 24 hour digest and by gel-filtration this was shown to be associated with relatively small molecules having a molecular size of approximately 30,000 daltons. When purified X, Y, D and E were assayed individually for chemotaxis, fragment Y was active but only at relatively high concentrations. Therefore the chemotactic activity generated by the action of plasmin on fibrinogen was mainly associated with one or more lower molecular weight polypeptides and to a lesser extent with the Y fragment.

Monocyte chemotactic activity was also generated by the action of plasmin on human fibrinogen and shown to be associated with the D and E fragments but not with a mixture of fragments X and Y (Richardson et al, 1976). When plasmin digestion was stopped at time intervals up to 24 hours, monocyte chemotactic activity corresponded with the appearance of the D and E fragments. The monocyte chemotactic activity contained in a 24 hour digest, eluted from Sephadex G-75 at V_o corresponding to the expected position of the D and E

fragments whereas neutrophil chemotactic activity eluted with molecules of molecular size of approximately 30,000 daltons. Thus fragments D and E derived from plasmin digestion of fibrinogen attract the monocyte whereas only the small uncharacterised peptides were chemotactic for the neutrophil. These different profiles of chemotactic activity for the neutrophil and the monocyte in terms of plasmin digestion products of fibrinogen may be of significance in the events leading to the accumulation of these cells in vivo during fibrin deposition.

Thus there is a formidable list of chemotactic factors associated with coagulation, kinin formation and fibrinolysis (Table 1). The biological significance of these observations

Table 1. The chemotactic activities of leucocytes for fibrinogen-derived peptides and Hageman factor-dependent enzymes

	Molecular Sizes	Neutrophils	Monocytes
Fibrinopeptides A, AP and AY	1,500 each	-	-
Fibrinopeptide B	1,500	+	+
Fragment X + Y	250,000 and 155,000 respectively	-	-
Fragment D	82,000	-	+
Fragment E	45,000	-	+
"Small uncharacterised peptides" derived from plasmin digestion of fibrinogen	Approx. 30,000	+	-
Kallikrein	108,000	+	+
Plasminogen activator	90,000	+	+

is unknown but the accumulation of various cell types in inflammatory conditions associated with HF-dependent pathways is well recognised. For instance, following the formation of fibrin there is an initial accumulation of neutrophils whereas during the resolution the mononuclear cell predominates. These factors may also act together since synergism between chemotactic factors has been shown for neutrophils (Wilkinson et al, 1969), monocytes and eosinophils (Kay et al, 1973). The association of the kinins and related peptides to various disease states is also appreciated. For instance, the accumulation of neutrophils in the joint fluids of gout arthritis may in part be mediated by kallikrein formed as a consequence of uric acid crystal-induced HF activation (Kellermeyer & Breckenridge, 1965).

Many chemotactic agents possess other biological activities. C3a and C5a are anaphylatoxins (Cochrane & Müller-Eberhard, 1968), $\overline{C567}$ act in the phenomenon of "reactive lysis" (Thompson & Lachmann, 1970) and various activities have been attributed to fibrinopeptide B (see above). It is clear that the full complexity of these cascade-enzyme systems is still to be appreciated. However, a consideration of the relationship between the leucocyte, coagulation, kinin-formation and fibrinolysis may help our understanding of the significance of these biochemical pathways in health and disease.

ACKNOWLEDGEMENT

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SECTION E - METHODOLOGY

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HUMAN TRANSFER FACTOR PREPARED BY DIALYSIS, ULTRAFILTRATION AND GEL CHROMATOGRAPHY: BIOLOGICAL ACTIVITY IN LOCAL TRANSFER OF SKIN SENSITIVITY

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Human transfer factor (TF) was prepared by a variety of methods including dialysis using cellophane tubing, ultrafiltration through a membrane of known pore size, Sephadex G-25 chromatography or combinations of some of these methods. In general the various preparations when injected locally into human skin gave greater delayed-type responses than antigen (PPD or Candida) alone. The combination of either vacuum dialysis, or ultrafiltration, with G-25 chromatography gave as good or better TF activity when compared with unchromatographed materials. Since ultrafiltration and concentration is a rapid procedure and eliminates the need for freeze-drying, in contrast to vacuum dialysis against water, these results indicate that ultrafiltration and G-25 chromatography provide a convenient method for preparing large batches of relatively pure TF from leucocyte extracts.

INTRODUCTION

Human transfer factor (TF) for both experimental work and clinical use is usually prepared using cellophane dialysis (Visking) tubing. Transfer factor can also be prepared by ultrafiltration or gel filtration, but a critical comparison of biological activity prepared by the three different methods is difficult due largely to the lack of a suitable in vitro assay. Furthermore, this lack of a simple and reliable in vitro assay is a major hindrance to proper characterisation and standardisation of TF for clinical trials. A number of laboratories have found the lymphocyte transformation assay, described by Ascher et al. (1974) to be unreliable mainly because of inconsistencies in reproducibility (Arala-Chaves et al., 1974; Ahern, 1975; Neidhart and LoBuglio, 1975; Sanderson, 1975).

The local transfer of intradermal sensitivity by TF was first described by Lawrence and Zweiman (1968) and was applied more recently to the characterisation of TF, from a PPD-positive donor, prepared by Sephadex G-25 chromatography (Neidhart et al., 1973).

In the present report the local transfer of skin sensitivity has been used

as the assay for comparing the activity of human TF prepared by cellophane tubing dialysis alone, ultrafiltration and G-25 gel chromatography.

MATERIALS AND METHODS

Selection of leucocyte donors

Two volunteers, one highly skin-test sensitive to PPD and the other to Candida extract were selected as leucocyte donors for the preparation of TF. Both were negative for HB_sAg by haemagglutination inhibition.

Preparation of transfer factor

Four hundred ml of blood were drawn into a double plasmapheresis pack (Fenwal, Thetford, Norfold) containing 75 ml acid—citrate—dextrose and centrifuged at 2000 *g* for 2 min at 22° C. The buffy-coat was slightly disturbed to ensure a maximal leucocyte yield and the leucocyte-rich plasma together with a minimal amount of red cells was expressed into the transfer pack. The red cells were reinfused and the process repeated. Both packs of leucocyte-rich plasma were then concentrated by centrifugation at 3,500 *g* for 10 min at 22° C following which the supernatant plasma was removed. The buffy-coat concentrates were allowed to stand for 1 h at 37° C to allow disaggregation of cells. The concentrates were pooled and mixed with 6% Dextran 70 (30% by volume) and allowed to stand for 45 min at 37° C to sediment red blood cells. The leucocytes were removed, centrifuged at 400 *g* for 10 min at 22° C, washed once in heparin/saline (Fenwal 'saline' with 10 units of preservative-free heparin (Evans Medical, Liverpool) per ml) and resuspended in the same buffer and total white cells counted. The leucocytes were then finally centrifuged at 1,500 *g* for 10 min and resuspended in 0.01 M $NH_4H\ CO_3$. A leucocyte extract was prepared by freezing and thawing from —70° C to 37° C, the procedure being repeated 10 times. No DNase was added. The extract was divided into three aliquots for the preparation of TF by three methods (fig. 1). One 'unit' of TF was arbitrarily defined as the material prepared from 10^9 leucocytes.

Dialysis by vacuum

One portion of leucocyte extract (B, fig. 1) was placed in 8/32 inch Visking dialysis tubing (previously boiled). The tubing was then placed in a 500 ml Büchner flask containing 200 ml of pyrogen-free distilled water. One end of the tubing was fitted over a plastic tube ('biotip') which in turn was connected to a syringe barrel and covered with a sterile pad as shown in fig. 2. The vacuum was then applied (22 inches of Hg) for 20 h at 4° C, the tubing removed and the dialysate 'shell-frozen' in liquid nitrogen and freeze-dried. The material was reconstituted in 20 ml of saline (Fenwal) and divided into two portions. One was used directly for in vivo tests (sample 5, fig. 1)

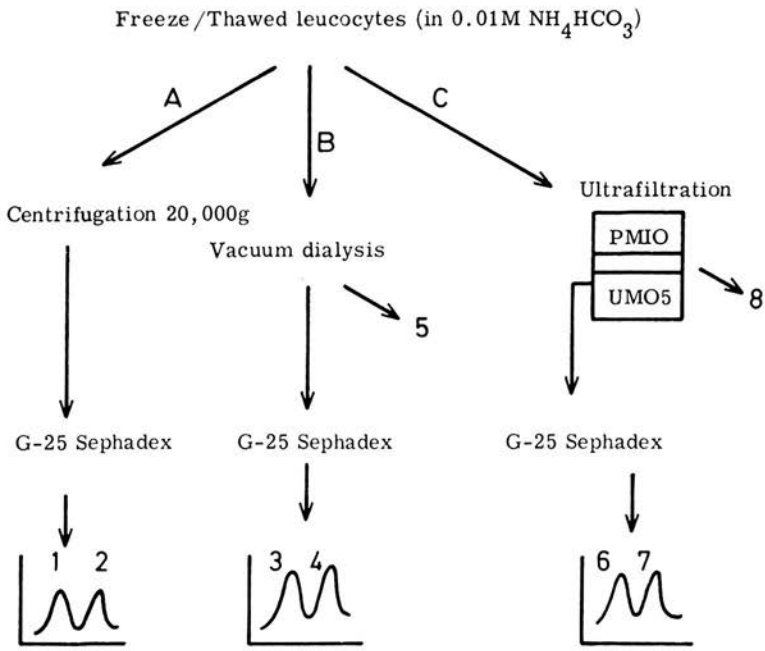

Fig. 1. The preparation of freeze/thawed leucocytes (aliquots A, B and C) for the production of transfer factor by three different methods.

and the other applied to a column of G-25 under the conditions described below.

Dialysis by ultra filtration

The second aliquot (C, fig. 1) was passed through a PM10 membrane in

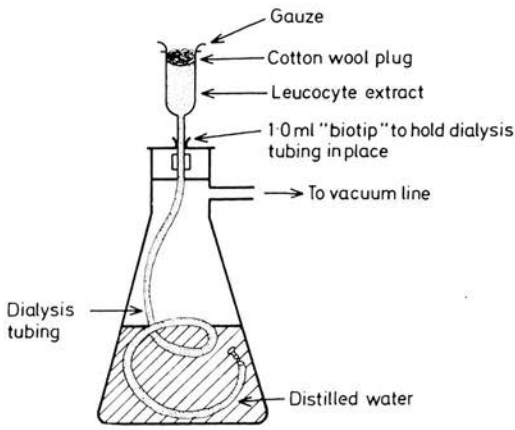

Fig. 2. The preparation of transfer factor from leucocyte extract by vacuum dialysis.

an Amicon Cell 50 at a pressure of 25 pounds per cm^2. The ultrafiltrate was then concentrated in the same cell using a UM05 membrane. The concentrate was divided into two. One portion was freeze-dried, reconstituted in saline (sample 8, fig. 1) as described above and tested for in vivo activity. The second was applied to a column of Sephadex G-25 as described below.

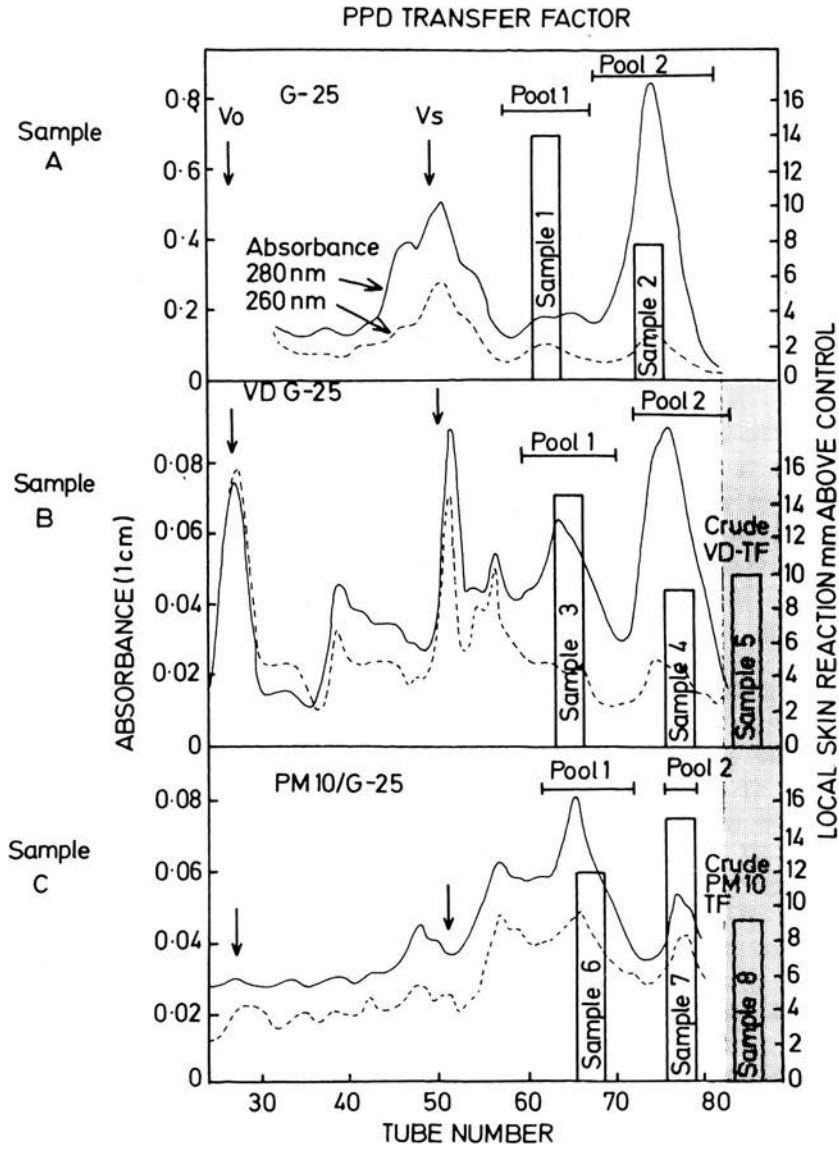

Fig. 3. Sephadex G-25 chromatography of leucocyte extracts prepared by three different methods (fig. 1) from PPD sensitive donors. Unfractionated (crude) material is indicated on the right hand side of the histograms for samples B and C respectively.

Gel chromatography

Prior to application to columns of G-25 Sephadex all samples where necessary were centrifuged to remove particulate matter. Two ml of the various samples were applied to 2.5 × 100 cm columns of Sephadex G-25 in 0.01 M NH_4HCO_3, pH 7.75 and 7.5 ml fraction volumes collected. Glucose was added to the sample, and detected using 'Clinistix', to estimate the bed volume (Vs). Material eluted after this point was identified by estimation of

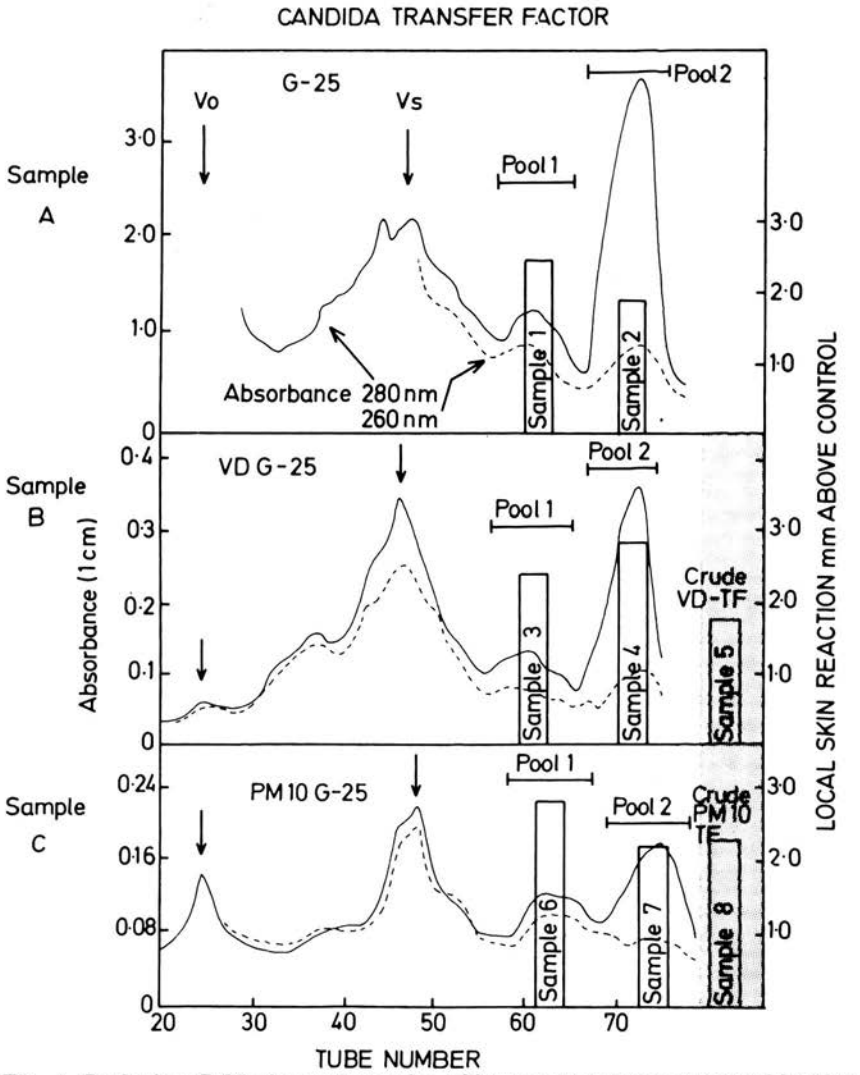

Fig. 4. Sephadex G-25 chromatography of leucocyte extracts prepared by three different methods (fig. 1) from Candida-sensitive donors. Unfractionated (crude) material is indicated on the right hand side of the histograms for samples B and C respectively.

the absorbance at 260 and 280 nm according to the method of Neidhart et al. (1973) and Zuckermann et al. (1974). The two peaks of activity at OD 260 nm, eluting after Vs, were pooled as shown in figs. 3 and 4.

Sample A (fig. 1) was applied directly to the column after centrifugation to remove particulate matter. The vacuum dialysed (VD-TF) and ultrafiltered (PM10-TF) preparations were also subjected to G-25 gel filtration and the peaks appearing after Vs were designated pool 1 and pool 2 (figs. 3 and 4). Thus the two peaks (pool 1 and pool 2) for aliquot A are referred to as samples 1 and 2; and the G-25 peaks from B and C as samples 3 and 4, and samples 6 and 7 respectively (fig. 1). All the G-25 pools and the crude Vd-TF were concentrated by freeze drying and reconstitution in saline (Fenwal).

The G-25 pools were reconstituted to the same volume as the original starting material for skin testing: i.e. 1 unit of crude TF was diluted to a volume of 50 ml and a G-25 pool derived from 1 unit was reconstituted to a volume of 50 ml.

All preparations were sterilised by filtration through Millipore filters (0.22 μ pore size) and stored at $-40°$ C.

Local transfer of intradermal hypersensitivity

The method of Lawrence and Zweiman (1968) was modified as follows. 0.05 ml of 10^{-3} units of TF in saline were injected intradermally into the ventral surface of the forearm. Twenty-four h later the test antigen was injected intradermally at the same site. Antigen doses were 5 TU of PPD (Evans) or 0.02 ml of Candida extract (Allergenen). Delayed hypersensitivity skin reactions were read at 24 and 48 h. Results were assessed by two independent observers. The diameter of induration in 2 directions was measured and the results averaged. Erythema without induration or swelling was not considered to be significant. All test sites were on one forearm and the control site (saline followed by antigen) was on the other forearm.

The measurement of TF activity was the increase in skin reaction above the control, expressed in mm.

Intradermal injection of TF alone caused no reaction, either immediate or delayed.

RESULTS

The elution profiles of the various G-25 columns are shown in fig. 3 (PPD-sensitive donor) and fig. 4 (Candida-sensitive donor). The local skin reactions above control values for selected responders (see below) are indicated for the various pools. Individual data are given in table 1 and table 2 (for Candida).

Local transfer of PPD sensitivity

Eight preparations were tested in 7 subjects of whom 4 were found to give a substantial response. Since the increment of increased skin reaction above

TABLE 1

Effect of local PPD transfer factor on delayed-type skin reactions to PPD.

Sample (see fig. 1)		1	2	3	4	5	6	7	8	
Subject		G-25		VD/G-25		VD	PM10/G-25		Crude PM 10	Control (antigen) m.m.
		Pool 1	Pool 2	Pool 1	Pool 2		Pool 1	Pool 2		
P.F.	Test site, m.m.	8.3	6.5	8.3	8.8	8.3	8.3	9.5	7.5	4.0
	Increment, m.m.	4.3	2.5	4.3	4.8	4.3	4.3	5.5	3.5	
J.L.	Test site	21.0	13.8	22.5	13.3	13.5	17.5	22.0	13.5	0.0
	Increment	21.0	13.8	22.5	13.3	13.5	17.5	22.0	13.5	
L.C.	Test site	7.5	ND	6.0	6.8	6.0	5.3	7.8	ND	5.5
	Increment	2.0	ND	0.5	1.3	0.5	−0.3	2.3	ND	
G.McN	Test site	13.0	ND	14.0	17.0	11.0	13.5	11.8	ND	12.3
	Increment	0.8	ND	1.8	4.8	−0.6	1.3	−0.5	ND	
S.W.	Test site	6.8	ND	6.5	7.8	5.8	7.5	7.8	ND	6.8
	Increment	0.0	ND	−0.3	1.0	−0.5	0.8	1.0	ND	
R.B.	Test site	12.5	ND	6.8	11.0	7.5	10.5	14.0	ND	6.0
	Increment	6.5	ND	0.8	5.0	1.8	4.5	8.0	ND	
S.P.	Test site	11.3	ND	7.3	12.0	8.3	12.8	13.3	ND	7.8
	Increment	3.5	ND	−0.5	4.3	0.5	5.0	5.5	ND	
Mean Increment		5.4	(8.2)	4.2	4.9	2.8	4.7	6.3	(8.5)	

TABLE 2

Effect of local Candida transfer factor on delayed-type skin reactions to Candida

Sample (see fig. 1)		G-25	VD	VD/G-25		Crude VD-TF	PM10/G-25		Crude PM10 TF	Control site m.m.
		Pool 1	Pool 2	Pool 1	Pool 2		Pool 1	Pool 2		
S.W.	Test site, m.m.	9.0	8.8	10.0	10.3	9.5	10.3	9.8	9.8	7.5
	Increment, m.m.	1.5	1.3	2.5	2.8	2.0	2.8	2.3	2.3	
M.M.	Test site	7.5	7.8	7.5	8.3	8.3	8.8	8.0	8.3	5.8
	Increment	1.8	2.0	1.8	2.8	2.5	3.0	2.3	2.3	
J.H.	Test site	9.0	8.8	10.0	10.3	9.5	10.3	9.8	9.8	5.5
	Increment	3.5	3.3	4.5	4.8	4.0	4.8	4.3	5.3	
B.C.	Test site	5.5	9.5	4.8	5.3	4.5	7.8	6.3	6.0	5.3
	Increment	0.3	0.0	−0.5	0.0	−0.8	2.5	1.0	0.8	
J.L.	Test site	12.5	12.0	10.5	12.0	9.5	11.5	11.0	11.0	11.5
	Increment	1.0	0.5	−1.0	0.5	−2.0	0.0	−0.5	−0.5	
Mean Increment		1.6	1.4	1.5	2.2	1.2	2.6	1.9	1.8	

control was similar at 24 h and 48 h only the 24 h data are given (table 1).

The results in two responsive recipients (P.F. & J.L.) were averaged as shown in fig. 3 and are illustrated against the corresponding chromatograms. In general activity could be demonstrated with all preparations but some recipients (G. McN and S.W.) gave minimal or no increase in induration (table 1). However, these two individuals gave vigorous skin responses to PPD alone. VD-TF Pool 1 (Sample 3) and PM10-TF Pools 1 and 2 (Samples 6 and 7) were in general more active than the corresponding crude preparations (which had not been subjected to G-25 chromatography). In general all samples gave similar increments in skin reactivity with the exception of Samples 2 and 8 although the latter are the mean results of two good responders.

Local transfer of Candida sensitivity

The eight preparations were tested in 5 subjects of whom 3 were found to be reasonably good responders. As with PPD, only the 24 h results are shown (table 2, fig. 4). The results in three responsive recipients (S.W, M.M. & J.M.) were averaged as shown in fig. 4. The increase in skin reactions conferred by Candida/TF is noticeably less than that conferred by PPD/TF. In general the mean increments with all samples were essentially similar. However, the good responders (S.W, M.M. and J.M.) gave higher increments (fig. 4).

Again the TF prepared by G-25 gel infiltration alone showed more activity in pool 1 than in pool 2.

These data and those from preliminary tests on other subjects showed that 6/13 were 'responders' in the PPD system and 5/11 were 'responders' in the Candida system.

DISCUSSION

When the mean skin test increments of all the samples (1—8) in all volunteers are compared, little difference in activity is apparent (as shown by the mean increments, tables 1 and 2). However, when the results in the best responders (P.F. and J.L. for PPD: S.W, M.M, and J.H. for Candida) are averaged and compared, in general the peaks from the G-25 columns gave greater activity than the starting material (aliquots B and C, figs. 3 and 4). This might indicate the presence of inhibitor(s) in the crude unfractionated preparations as had previously been suggested by Neidhart et al. (1973).

When crude leucocyte extract was subjected only to G-25 filtration, Pool 1 was slightly more active than Pool 2 thus agreeing with the results of Neidhart et al. (1973) who also used the local transfer of skin reactivity as the assay.

The molecular nature of TF remains unknown although there is some evidence that it may be an oligopeptide-oligonucleotide, perhaps an antigen fragment linked to RNA (Gottlieb and Schwartz, 1972).

It has been previously demonstrated that when human TF is eluted from G-25 with low ionic strength (0.01 M) phosphate buffer the major biological activity resides in the peak immediately following Vs (Neidhart et al., 1973) i.e. the active material is adsorbed and therefore the ionic strength of the buffer is probably of critical importance in determining the pattern of elution. Thus the G-25 Sephadex procedure probably functions by ion exchange and not gel filtration in this application.

Crude leucocyte extract would contain a variety of membrane-associated antigens. Most of these are probably eliminated by dialysis or ultrafiltration and therefore G-25 chromatography alone is probably unsatisfactory because there is no means of ensuring that all antigenic material is eluted before Vs.

An important limitation of our results is that significant TF activity may have been eluted in the peaks appearing before Vs, which were not tested. However, the need to test all preparations simultaneously in one volunteer limited the number of fractions that could be assayed.

PM-10 filtration and VM-05 concentration is the quickest and most convenient of the three 'single-stage' methods of preparing human TF. Ultrafiltration followed by G-25 chromatography yields active fractions which are at least as active as ultrafiltered material. Therefore, this may be the method of choice for preparing batches of partially purified human TF for clinical use.

Although local transfer of intradermal sensitivity by TF is simple and reflects in vivo activity, there is still a need for a simple, reliable and sensitive in vitro assay. In evaluating this technique we made the following observations:

a) About 50% of the suitable volunteer recipients were responders to TF in the local transfer test: 4/6 responded with an increased skin reaction to PPD and 3/5 responded with an increased skin reaction to Candida extract. However, even in these individuals the increment obtained with some of the preparations was slight. Hence it is necessary to test a batch of TF in at least five, and preferably more, suitable recipients.

b) Each skin test was painful — usually more painful than a venepuncture — despite the use of isotonic solutions and preservative-free test antigens.

c) Neither the injection of a small volume intradermally nor the reading of the delayed hypersensitivity skin reaction can be performed with great accuracy — thus the need to average results from a group of subjects and to have more than one observer.

d) Local transfer of PPD sensitivity was in general a more sensitive system than transfer of Candida sensitivity, although this may well have been a function of the particular combinations of donors and recipients in our study.

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CHAPTER 47

Tests of immune function

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SHEENA M. WOOD & A. B. KAY

This chapter is concerned with the investigations required for assessment of the nature and extent of defects of immunity. In most cases the nature of the illness is self-evident and only the extent of immunodeficiency needs to be defined, together with an assessment as to whether the defects are primary or secondary.

Before embarking on time-consuming laboratory investigations it is important to be satisfied that 'non-immunological' causes have been ruled out. Immunological investigations are most likely to be helpful in cases of unusual or recurrent infections.

The cardinal feature of T lymphocyte dysfunction is the occurrence of severe infection with viral pathogens or 'unusual' organisms. For instance, measles, chickenpox, and candidiasis may take an unusually severe course and uncommon pathogens such as cytomegalovirus and pneumocystis carinii may also be involved. In addition severe illness may follow routine vaccination with live viruses. T cell deficiency may be associated with failure to thrive, chronic diarrhoea, chronic candidiasis, and graft-versus-host disease following blood transfusion.

Failure of B lymphocyte function with low or absent immunoglobulins (and antibodies) results in repeated infections with the more common bacterial pathogens. These include staphylococci, streptococci, pneumococci and haemophilus. The most common sites of infection are the lung, middle ear, sinuses and meninges. In contrast to T cell deficiency, B cell deficient individuals tolerate viral infections, including vaccination.

Neutropenia or deficiency of granulocyte function also results in repeated bacterial infections. Deficient phagocyte function as in splenic agenesis or splenectomy in childhood may be associated with overwhelming septicaemia with organisms such as pneumococci or meningococci. Adults with splenectomy also have an increased incidence of septicaemia.

Deficiency of complement components are rare but may result in either repeated bacterial infections or a lupus-like syndrome, or no apparent ill health depending on which factor(s) is involved.

GENERAL INVESTIGATIONS

Simple laboratory investigations such as full blood counts and chest X-rays may give valuable information. For instance, lymphopaenia is common in primary immunodeficiency although a normal lymphocyte count does not exclude the diagnosis. In contrast, a very high lymphocyte count, or abnormal type (as in the lymphoproliferative disorders) often results in secondary immunodeficiency. Neutrophil numbers may be unusually low in the presence of pyogenic infections and apart from obvious causes such as cytotoxic drug therapy or agranulocytosis following drug sensitization, may be the result of antibodies to neutrophils. Examination of neutrophil numbers over a period of time is necessary for the diagnosis of cyclical neutropenia. Morphologically abnormal granulocytes may be found, e.g. in the giant lysosomal granules of the Chediak–Higashi syndrome.

A lateral chest X-ray of the mediastinum in infants or neonates may indicate the absence of the thymus gland and with an abdominal X-ray the absence of a splenic shadow may indicate agenesis of that organ. Histopathology of lymphoid tissue is also a useful adjunct to the diagnosis of immunodeficiency. Bone marrow, rectal mucosa and lymph nodes are the most accessible tissues for study. Lymph node biopsy will reveal absent lymphoid follicles and germinal centres in B cell deficiency diseases whereas there will be a paucity of lymphocytes in the para-cortical areas in T cell deficiency. Guidelines for morphological assessment have been suggested by the W.H.O. [1]. Lymph node biopsy is not to be undertaken lightly as a matter of routine in the diagnosis of immunodeficiencies as the information obtained is somewhat limited and there is serious risk of subsequent infection.

Where there is a poor response to apparently adequate chemotherapy it is important to determine the antibiotic sensitivities of the suspected organism. Unusual organisms requiring specialized sampling and culture techniques may be involved.

GENERAL TECHNICAL CONSIDERATIONS

Many tests for evaluation of the immune system are complex and time-consuming. There will inevitably be a compromise between desirability and practicality. Tests must therefore be informative, discriminatory and clinically relevant. In the remaining sections of this chapter we will outline the currently available technology with emphasis on its clinical relevance.

Many of the serological techniques have been well established in other specialities for diagnosing and assessing disease, e.g. antistreptolysin-O titre, organ specific autoantibodies, viral antibody levels. Their use in clinical immunology is one of different emphasis and they will not be considered in any detail.

It is most important that the sample collection, cell separation and culture methods are standardized and that normal values are obtained by each laboratory by their own technology. Because of the complexity of many tests, it is necessary to include a normal control with each batch of test samples so that negative results due to methodological errors are minimized. A single abnormal *in vitro* result must not be considered in isolation. If there is a discrepancy between the *in vitro* and *in vivo* test results then the former must be interpreted with caution and, if necessary, repeated. There are always occasions when it is impossible to interpret the results obtained in the light of the clinical findings and one must accept the limitations of present day technology. Where there is genuine doubt one should be guided primarily by the patient's clinical condition and not by an abnormal laboratory result.

The investigation of the immune system may be conveniently considered as follows:

(a) investigation of T lymphocyte function;
(b) investigation of B lymphocyte function;
(c) investigation of phagocyte function;
(d) investigation of the complement system.

INVESTIGATION OF T LYMPHOCYTE FUNCTION

Full investigation requires both a numerical and functional assessment. The enumeration of T lymphocytes is usually performed with peripheral blood samples, although some additional information may be obtained from studies on other human material such as lymph nodes, spleen, thoracic duct lymph and synovial fluid. Surface marker methods are used to identify circulating T and B lymphocytes since they are morphologically indistinguishable. The functional assessment of T lymphocytes *in vitro* is complex and technically demanding, involving tissue culture, and the interpretation of results is often difficult. Some of the tests in current use require large volumes of blood and there is a need for reliable micromethods; this is especially important in the assessment of children and those patients with lymphopaenia. There are limitations in testing only one 'compartment' of the immune system, however convenient, and the absence of circulating T cells may indicate sequestration elsewhere and not an absolute deficiency.

The *in vivo* assessment of T cell function is technically undemanding. However, tests such as delayed hypersensitivity (DH) skin tests reflect many aspects of cell mediated immunity, and even non-immunologic effector systems. Thus the interpretation of abnormal results may be difficult.

Quantitation of T lymphocytes

The spontaneous sheep red cell rosette (E rosette) and anti-T cell sera are the most common methods used for identifying T cells. The E-rosette method is technically simple and consistent results may be obtained by any one laboratory. However, widely differing normal ranges have been reported by slight variations in technology [2, 3]. Approximately 70 per cent of blood lymphocytes are identified as T cells by this method. With certain technical modifications a subpopulation of T cells may be identified which are thought to have 'high affinity' receptors for sheep red cells [4].

Antisera specific for T cells have been produced by a number of workers [3, 5] and their use requires a cytotoxic assay or a fluorescent method. Even so, different results may be obtained with the same antiserum using both of these methods. For this reason enumeration of T cells using specific antisera has had limited application in routine clinical work.

It is essential that lymphocyte subpopulations are reported not only as a *percentage* of circulating lymphocytes but also in *absolute numbers*. In this way, variations in total lymphocyte numbers are accounted for. It should be appreciated that separation methods may result in selective losses of certain sub-populations [6, 7]. The use of T and B cell markers has been shown to be of value in the classification of a number of lympho-proliferative diseases and immunodeficiencies [8, 9]. Thus, T cells may be virtually absent from the circulation of patients with chronic lymphatic leukaemia [10] whereas in acute leukaemia, cells may be devoid of surface markers or identified as T cell derived [11]. Patients with immunodeficiencies have variable findings and it is important to correlate assessment of T cell *function* with T cell *numbers* since discrepancies may indicate blocking or inhibitory factors. Many diseases in which there is evidence of depressed cell mediated immunity (CMI) by functional analysis, e.g. lepromatous leprosy, certain cancers, can also be shown to have low circulating T cell numbers [4, 12].

Much of this information cannot be translated clinically in terms of prognosis and better treatment and the value of many of the tests must await clarification.

Method for estimation of T lymphocytes by 'E' rosettes (after Kaplan & Clark) [13]*

Principle

Lymphocytes which have surface receptors for sheep red cells are believed to be thymus-dependent (T cells). Lymphocytes with these receptors will bind a cluster of sheep red cells, forming an erythrocyte rosette (Er). Normally the binding of sheep red cells (E) to T lymphocytes is relatively weak, treatment of E with AET stabilizes the binding, giving a more reproducible test with maximal identification of T cells (per cent $E_{(AET)}r$ + per cent EACr approximate to 100 per cent). Similar reproducibility and maximization can be achieved using papain-treated sheep red cells [14].

Materials

(1) Sheep red cells (approximately 50 per cent solution in Alsever's).

(2) AET solution (0·402 g of S2-aminoethylisothiouronium-hydrobromide in 10 ml of distilled water, adjusted to pH 9·0 with 4 N sodium hydroxide).

(3) Fetal calf serum, heat-inactivated (Wellcome, Beckenham, Kent, U.K.).

(4) Medium 199 (Gibco-Biocult Ltd., Paisley, Scotland).

(5) Latex suspension (1/100 dilution, size 1·011 μ (Dow-Latex, Serva, Heidelberg, W. Germany).

(6) May-Grunwald and Giemsa stains.

(7) Liquid cover glass (Trycolac, Aerosol Marketing & Chemical Co. Ltd., London).

Procedure

Preparation of AET treated sheep cells

(1) Two millilitres of the sheep red cell suspension is washed three times in 10 ml of saline.

(2) To 1 volume of washed packed sheep red cells (approximately 1 ml) add 4 volumes of AET solution.

(3) Mix and incubate at 37° C for 15 min with repeated mixing.

(4) Wash three times in saline and once in medium 199 and resuspend in 80 per cent medium 199 (7 ml)/20 per cent fetal calf serum (2 ml) to give a 10 per cent suspension of $E_{(AET)}$; store at 4° C. The suspension can be used for up to 1 week.

* A recent publication indicates that it may be possible to estimate T lymphocytes on smears using a modification of the non-specific esterase stain (KULENKAMPFF J., JANOSSY G. & GREAVES M.F. (1977) Acid esterase in human lymphoid cells and leukaemic blasts. A marker for T lymphocytes. *Brit. J. Haemat.* **36**, 231).

Lymphocyte preparation

(1) Mononuclear cells are prepared as for lymphocyte transformation (see p. 47.5).

(2) Phagocytic cells are identified by incubation of the cell suspension with latex particles. Usually 3 to 4 drops of 1/100 latex suspension is added to 1 ml at approximately 2–4 million mononuclear cells per ml. The mixture is incubated at 37° C for 30 min with frequent mixing and then washed in medium 199 and adjusted to give 2 million cells per ml.

Rosetting procedure

(1) To 0·5 ml of lymphocyte suspension (2×10^6/ml) 0·25 ml of fetal calf serum is added and 6–8 drops of $E_{(AET)}$ to give a minimum ratio of 8 red cells to 1 lymphocyte and mix thoroughly.

(2) Centrifuge gently at 25 *g* for 5 min.

(3) Incubate overnight at 4° C for maximal rosette formation.

(4) The cell pellet is resuspended by gentle aspiration with a Pasteur pipette.

(5) Slides are prepared immediately after resuspension as follows. Capillarity is used to draw the cell suspension into the Pasteur pipette, from which the cell suspension is deposited on a glass slide by surface tension; an area of about three-quarters of the slide surface is covered. The slide is air-dried with a fan and then fixed in methanol for a minimum of 2 min. Staining is done with May-Grunwald-Giemsa in the usual way (see NBT test, p. 47.18) and the slides may be preserved by spraying with liquid cover-glass (Trycolac) after drying, which aids resolution.

Examination

Lymphocytes having three or more red cells bound are counted as rosettes. All non-rosetting lymphocytes are identified and counted. Contaminating cells should be excluded from the count and are identified morphologically mainly as polymorphonuclear cells and monocytes, most of which have phagocytosed latex particles.

Lymphocyte transformation

The stimulation of lymphocytes by certain agents results in a sequence of events called *lymphocyte transformation* with the production of blast-like cells which synthesize DNA *de novo*. The extent of transformation can be quantified either morphologically or by assessing the incorporation of radioactive

DNA precursors into the cells. Stimulating substances can be divided into three main groups:

(1) Polyclonal mitogens, e.g. phytohaemagglutinin (PHA), Concanavalin A (Con A), poke-weed mitogen (PWM);

(2) Antigen specific stimulation, e.g. purified protein derivative (PPD) from *M. tuberculosis*, Candida antigen, streptokinase–streptodornase (SK–SD);

(3) Stimulation by allogeneic lymphocyte mixed lymphocyte reaction (MLR).

The major responding cells in the above systems are T lymphocytes and *in vitro* lymphocyte transformation is thought to reflect T cell function and CMI [15, 16]. 'Non-specific' transformation by mitogens can be used as an indicator of overall T cell function since a high percentage of cells are 'triggered' normally and a low level of transformation usually correlates well with depressed CMI. A significant degree of transformation (i.e. at least three times greater than the spontaneous incorporation) to a given antigen usually indicates prior sensitivity to that antigen.

Transformation tests are technically demanding and are subject to many variables. It is essential to include a normal control or even better a 'reference pool of responders' (see below) with each experiment. If responses are significantly different from control values serial tests are necessary for confirmation.

Some patients (e.g. cancer-bearing subjects) may have inhibitors present in their serum which will be detected if cultures are done with autologous as well as with 'standard' sera. Conversely, if cultures are always performed in autologous serum some negative results may be due to the presence of inhibitors and not to depression of CMI *per se* [17, 18].

Method for lymphocyte transformation (microculture technique) (see also Chapter 26)

Principle

This micro-culture technique provides a standardized test system for measuring lymphocyte responses *in vitro* to polyclonal mitogens, antigens and in the mixed lymphocyte reactions (MLR). Low numbers of lymphocytes are required, and all stimulants, with the exception of allogeneic lymphocytes for the MLR reaction, may be pre-dispensed on the plates and stored in liquid nitrogen vapour. Lymphocyte transformation is assessed by tritiated thymidine incorporation during the final 18 hours of culture. A pool of normal lymphocytes, stored in liquid nitrogen vapour, is used as an internal control for technical reproducibility of responses, and mitomycin-treated pooled lymphocytes are stored in liquid nitrogen vapour for use as MLR stimulators.

Materials

(1) Ficoll/Triosil mixture (specific gravity 1·077).

(2) TC medium 199 (Gibco-Biocult, 25 mmol/l HEPES-buffered, single strength), containing 100 units penicillin and 100 μg of streptomycin per ml (Difco, Detroit, Michigan, USA).

(3) Pooled human serum (from ten untransfused healthy males), heat-inactivated and filter sterilized.

(4) Tissue culture plates (LINBRO—flat-bottom, Flow Labs., Irvine, Ayrshire, Scotland, cat. no. 77-020-62).

(5) Plate sealers (Mylar) and roller (Flow Labs., cat. nos. 77-021-02 and 77-021-10).

(6) Phytohaemagglutinin (PHA-P, Wellcome).

(7) Concanavalin A (Con A, 3× recrystallized, Miles Laboratories Ltd., Slough, Berks., UK).

(8) Candida antigen (10 per cent *Candida albicans* antigen suspension, preservative-free, HAL-allergenen, Haarlem, Holland).

(9) PPD (freeze-dried, Central Vet. Labs., Weybridge, Surrey, UK).

(10) Pooled human lymphocytes: (a) untreated (pooled responders); (b) mitomycin-treated (pooled stimulators).

(11) Tritiated thymidine (TRA 306, specific activity 2 Ci/mM, Radiochemical Centre, Amersham, Bucks., UK).

(12) Mitomycin C (Kyowa Co., Japan),

(13) Plastic straws and accessories for freezing lymphocytes (Instruments de Médecine Vétérinaire, Societé Anonyme, L'Aigle, France).

(14) Dimethylsulphoxide (DMSO).

(15) Scintillation fluid (NE233, Nuclear Enterprises Ltd., Edinburgh, Scotland).

(16) Scintillation vials (Packard, Caversham, Berks., UK).

(17) Heparin (preservative free) (Evans Medical, Liverpool, UK).

Procedure

(1) *Separation of lymphocytes* (see also Chapter 23) Lymphocytes are separated from heparinized venous blood samples by layering the blood over Ficoll/Triosil mixture in sterile centrifuge tubes and centrifuging at 400–500 *g* for 20 min as described [19]. The band of mononuclear cells is recovered from the Ficoll/Triosil–plasma interface and washed three times in

TC 199 (approximately 100 *g* to remove platelets), resuspended in TC 199 and the lymphocytes counted.

(2) *Preparation of lymphocyte pools*

Lymphocyte pools are prepared by combining, in equal proportions, the lymphocytes obtained from ten healthy individuals. When used as a control responding population, the response of the pool to a stimulant is equal to the mean response of the individuals within the pool to that stimulant. When used as a stimulator of MLR, the pool presents a diversity of antigenic determinants to the responding population. Both pooled responder and pooled stimulator cells may be prepared in advance by freezing at a controlled rate in liquid nitrogen vapour.

To block DNA-synthesis in pooled stimulators, lymphocyte suspensions in TC 199 are incubated with mitomycin C (25 μg/ml) at 37° C for 30 min. The lymphocytes are then centrifuged, washed three times in TC 199, resuspended and counted.

(3) *Lymphocyte freezing*

Lymphocytes are suspended in tissue culture medium containing 10 per cent dimethyl sulphoxide and 10 per cent autologous or 'pooled' serum. Cells at any concentration up to approximately 1×10^7/ml are dispensed into plastic straws and sealed with plastic balls or polyvinyl alcohol powder.

Freezing is carried out in a controlled manner, usually 5 C° per min to the eutectic point, then 1 C° per min until −25° C, 5 C° per min until −50° C, 5–10 C° per min until −100° C, and then rapidly to −196° C. Final storage is in liquid nitrogen vapour at a temperature of at least −130° C.

When required the straws are thawed rapidly by dilution of their contents in 10 ml of TC 199 at 37° C, followed by two washes in TC 199 at 400 *g* to remove all the cryoprotective agent (DMSO is toxic above freezing temperature). The cells can then be suspended at the right concentration in the appropriate TC medium for use.

(4) *Tissue culture medium*

Tissue culture medium is made up by supplementing TC 199 with penicillin and streptomycin and pooled human serum (20 per cent v/v).

(5) *Preparation of microplates*

The 96 wells in the microplates are arranged in 8 rows (A–H) of 12 wells. Lymphocyte cultures are performed in triplicates: thus four triplicates (four different responding lymphocyte populations) can be applied per row, and different stimulants can be added to the different rows. Final culture volumes are 0·25 ml, and stimulants and lymphocytes are each added in 0·125 ml volumes of tissue culture medium at *twice* their final concentration. All operations are carried out in a laminar flow hood.

When plates are prepared for storage in liquid nitrogen vapour, pooled stimulating lymphocytes (mitomycin-treated) are not added. These should be added when the plates have been thawed out before adding the responding cells. In our hands, techniques for freezing cells in microplates have not proved satisfactory largely because of the problems of removal of cryoprotective agent from the wells.

	Row	*Stimulant*	*Concentration* (in 0·125 ml aliquots)
Process 72 hours	A	None (Baseline responding cells, 72 hr)	(Medium alone)
	B	PHA	3 μg/ml
	C	Con A	32 μg/ml
Process 120 hours	D	None (Baseline responding cells, 120 hr)	(Medium alone)
	E	PPD	20 μg/ml
	F	Candida antigen	40 μg/ml
	G	Pooled stimulating lymphocytes (mitomycin treated)	$1{\cdot}5 \times 10^6$/ml
	H	Pooled stimulating lymphocytes (baseline stimulating cells, 120 hours) (wells 1–3 only)	(0·125 ml) + Medium (0·125 ml)

(6) *Cultures*

Lymphocyte suspensions (3 ml at $0{\cdot}5 \times 10^6$/ml) are added to rows A–G in 0·125 ml aliquots. Three different individuals' lymphocytes (wells 1–3, 4–6 and 7–9) and pooled responding lymphocytes (wells 10–12) may be added to each plate. The plates are then sealed (Mylar) and incubated in a humidified atmosphere of 5 per cent CO_2 in air at 37° C. At 18 hours before harvesting of cultures, 1 μCi of tritiated thymidine is added in 4 μl volumes (diluted TRA 306, 1/4 in sterile saline) to each well to be harvested. It is convenient to deliver the isotope through the seal directly into each well using a 50 μl Hamilton syringe fitted with a 1 μl automatic dispenser.

Rows A–C are harvested after three days, and the remaining rows after 5 days, at the times when these

cultures reach their peak DNA-synthesis. The seal can be removed from rows A–C by cutting the sealing sheet between rows C and D using a scalpel. Cultures are harvested using an automatic culture processor (e.g. Sam 21a, Cryotech, Abingdon, Oxon., U.K.). The lymphocytes from each culture are trapped in filter paper discs and washed free of isotope which has not been incorporated. The filters are then dried at 50° C, placed in scintillation vials with 10 ml of scintillation fluid and the amount of tritium-labelled DNA in each sample is determined by counting for 1 min each in a scintillation counter. Results are expressed as mean counts per minute (cpm) per triplicate.

Comments

When applying this culture system to the investigation of patients' lymphocyte responses, a normal range of responses for healthy individuals should be established. It should be noted that the responses of healthy individuals may show a wide range of values, and the responses of an individual can vary from day to day [20].

Plates should be stored in liquid nitrogen vapour to prevent the stimulants losing their activity. Aseptic techniques should be observed for all operations to prevent contamination of cultures. When adding cell suspensions these should be well mixed to ensure even cell dispersion for good triplicate reproducibility. Accurate dilution of lymphocyte suspensions to the required cell number is essential, as responses are related to total cell numbers. Equally, stimulant concentrations may be highly dose-dependent and stimulants must be dispensed accurately.

Lymphokine production

As an integral part of the lymphocyte transformation response, a variety of soluble factors are produced which are thought to have important short-range effects in amplifying the response to antigens. These soluble lymphocyte-derived mediators are sometimes referred to as *lymphokines* (see Chapter 27). Assessment of the effects of lymphokines can be used as a correlate of cell mediated immunity [21]. One of the factors released from sensitized lymphocytes challenged with specific antigen inhibits the migration of macrophages from capillary tubes and is termed macrophage-migration inhibitory factor (MIF).

The elaboration of MIF has been widely used to indicate sensitization to a variety of antigens [22]. The most reliable system involves the use of a two-stage culture—firstly, lymphocytes/leucocytes are incubated in the presence of the antigen under test and secondly, cell-free supernatants are assayed using guinea-pig macrophages as target cells. MIF tests are technically difficult with associated problems of reproducibility. The test has been shown to work best with particulate rather than soluble antigens. As with antigen transformation tests, MIF production can be used as a test of sensitization to a number of antigens such as thyroglobulin and tumour antigens, or as a test of competence of CMI where prior sensitization is assumed or known.

Analysis of MIF and lymphocyte transformation to Candida antigens in patients with chronic mucocutaneous candidiasis has revealed that some are unable to transform to the antigen and others can transform but are unable to synthesize or release MIF [23]. Overall T cell immunity in many of these patients as assessed by PHA or MLR responses shows no impairment and these patients appear to have only the one defect in CMI.

Many other lymphokines have been described including a factor selectively chemotactic for monocytes. It is still not clear whether the various lymphokines are chemically distinct or a family of closely related glycoproteins. In practical terms, however, we find measurement of lymphocyte derived monocyte chemotactic factor to be a convenient method of assessing lymphokine production.

Method for production and assay of lymphocyte derived monocyte chemotactic factor (LD-MCF) (after Snyderman *et al.* [24])

Principle

LD-MCF has a specific effect on cells of the monocyte/macrophage series, and is released *in vitro* by lymphocytes when stimulated either by specific antigen or following mitogen stimulation. The assay may be used to detect either defective lymphocyte or monocyte function.

Materials

(See Lymphocyte transformation, p. 47.5 and Chemotaxis, p. 47.20)

(1) Sterile tissue culture tubes (Nunclon, Roskilde, Denmark, Cat. No. 1410-1).

Procedure

Production of LD-MCF

(1) Lymphocytes are separated as described for

lymphocyte transformation (see p. 47.5) and resuspended in TC 199 (Gibco-Biocult) (N.B. do not add serum) containing penicillin and streptomycin to a concentration of 3×10^6 lymphocytes per ml.

(2) For each stimulant used (mitogen or antigen) two sets of triplicate 1 ml cell suspensions are prepared in sterile tissue culture tubes.

(3) The stimulant is added at the optimal concentration (see Lymphocyte transformation, p. 47.7) to one set, while the second serves as the unstimulated control.

(4) The cultures are incubated at 37° C in a humidified atmosphere of 5 per cent CO_2 in air for the appropriate time (3 days for mitogens, 4–5 days for antigens).

(5) An identical amount of the stimulant is then added to the unstimulated controls before all the cultures are sedimented (400 *g* for 25 min).

(6) The supernatants are removed to Tek lab. tubes (Med. Lab. Ltd., Sacriston, Co. Durham, U.K.) to be tested for chemotactic activity (below). If necessary, the supernatants may be stored at 4° C for up to 5 days.

Monocyte chemotaxis to LD-MCF

In the LD-MCF assay lymphocyte culture supernatants (1·0 ml) are placed in Tek lab. tubes in triplicate. Negative controls (TC 199 alone or with lymphocyte stimulant) and positive controls (casein, 1·0 mg/ml in TC 199) may be included. However, stimulation of LD-MCF production is measured by the difference between migration of a monocyte suspension towards supernatants from stimulated lymphocyte cultures and supernatants from unstimulated lymphocyte cultures. Details of the monocyte chemotactic assay are described elsewhere (p. 47.21).

Comments

Unstimulated lymphocyte culture supernatants always show a greater level of chemotactic activity for monocytes than TC 199 alone. Distances migrated by monocytes in LD-MCF assays are much less than distances migrated towards casein (a known monocyte chemoattractant) and it may be necessary to increase incubation times to 90 min to amplify differences between unstimulated and stimulated supernatants.

T lymphocyte induced cytotoxicity

Antigen sensitized T lymphocytes have been shown to kill antigen-bearing target cells *in vitro*. Target cells of various sources have been used, including tumour cells and allogeneic lymphocytes and it would appear that the mechanism of cell damage is analogous to that seen in graft rejection [25]. Whereas the quantitation of lymphocyte transformation gives an indication of the *recognition phase* of cell mediated immunity, T cell cytotoxicity estimates the efficiency of one aspect of the *effector stage*. As yet, this technique has been used mainly as a research tool and details of the methodology will not be given herein. T lymphocytes transformed by PHA show similar cytotoxic activity [26]. A number of laboratories have used this test to assess one particular aspect of cell mediated immunity. The clinical significance of these tests remains uncertain and Chapter 36 discusses the scope and limitations of the methods.

Delayed hypersensitivity skin test

This is one of the oldest and simplest tests of CMI and probably detects pre-existing cellular immune function. The reaction to a 'panel' of antigens such as tuberculin, mumps antigen, Candida, trichophyton, or SK–SD is assessed 48 hours after intradermal injection at a convenient site, usually the volar surface of the forearm [8]. In cases of absent CMI all responses are negative. In young children, however, the interpretation is less satisfactory since it presupposes prior antigenic exposure. Furthermore there may be poor skin expression of the inflammatory response in this age group. Negative results are difficult to interpret. For example, the antigenic material may be inactive or administered incorrectly, the patient may be overwhelmed by infection and unable to respond, or may not have had prior exposure to the antigen.

The skin test response to antigen is complex and may involve many aspects of the immune response including antigen recognition as well as T cell activation. Thus a negative test can mean that a defect exists at one of several levels, as is seen in the disease complex of chronic mucocutaneous candidiasis [23].

In some circumstances active sensitization by agents such as dinitrochlorobenzene (DNCB) which results in primary sensitization in over 90 per cent of normal individuals may be of value. Evidence of sensitization is obtained by measuring the response to a second application of the chemical approximately 21 days later. This test has the advantage that

it is independent of other antigen exposure but has the drawback of giving, in some individuals, rather vigorous and painful responses.

An older method of assessing CMI which is not now generally used is skin graft rejection. Normally skin from an unrelated donor is rejected between 12–20 days. One of the dangers of this type of investigation is that the individual becomes sensitized to the donor histocompatibility antigens which may thus prejudice future treatment by materials such as transfer factor and bone marrow.

Method for delayed hypersensitivity skin tests

Principle

The test is based on recall of T lymphocyte immunologic memory and thus overall T lymphocyte function is assessed. The postulated sequence of events leading to the appearance of the delayed hypersensitivity type skin test is that circulating T lymphocytes come into contact with antigen (mainly held by skin macrophages) and pre-sensitized cells present are stimulated to lymphokine production and blast cell transformation. The lymphokines encourage the 'trapping' of circulating mononuclear cells at the site of antigen and the activation of non-sensitized 'bystander' cells into the reaction. A cascade effect is produced with increasing localization of mononuclear cells which is clinically manifest as induration. Local persistence of antigen favours the production of a more vigorous response and *vice versa*. Thus, subcutaneous (instead of intradermal) injection of antigen results in its rapid removal before a local response is apparent. The presence of oedema of the skin will result in false negative results due to increased lymphatic clearance of antigen.

Materials

(1) Disposable razor.
(2) Isopropyl alcohol.
(3) Sterile gauze swabs.
(4) Skin pencil or ballpoint pen.
(5) Sterile disposable tuberculin-type syringes with 0·01 ml graduations.
(6) Sterile disposable needles (26 gauge) (Plastipak no. 5625, Tuberculin syringes already have 26 gauge needle attached) (Becton Dickinson Ltd., Wembley, Middlesex, UK).
(7) 'Control' intradermal solution (Bencard, Brentford, Middlesex, UK).
(8) Sterile antigen solutions. The antigens used depend on the choice available and the expected exposure of the population to be tested. Commonly used antigens include:

(a) Streptokinase–streptodornase ('Varidase', Lederle Labs., Gosport, Hants, UK);
(b) Candida extract (1 per cent preservative-free; HAL-Allergenen, Haarlem, Holland, or Hollister–Stier, USA);
(c) Mumps (Eli–Lilly, Indianapolis, USA);
(d) PPD (various sources available);
(e) Trichophyton extract (1 per cent preservative-free; HAL-Allergenen or Hollister–Stier).

Histoplasmin and coccidioidin may also be used in the appropriate geographical areas.

Dosage used:

It is conventional to use 0·1 ml volumes for injection, but this is not critical provided the same volume is used on all occasions. Some antigen solutions, e.g. Candida, trichophyton, are rather painful and 0·05 ml is a convenient volume to use especially in children. It is our practice to use 0·05 ml volumes of the following materials:

(1) 'Control' intradermal solution (Bencard).
(2) 1 per cent Candida extract, preservative-free (HAL-Allergenen).
(3) Streptokinase–streptodornase (100 u/ml/25 u/ml) (Varidase).
(4) Mumps skin test antigen (Eli-Lilly).
(5) PPD 1/100 (1000 u/ml).
(6) 1 per cent Trichophyton (preservative-free) (HAL-Allergenen).

The 'control' solution is antigen-free and may become 'positive' in individuals with dermographism.

Method

The volar aspect of the forearm is the most convenient site for testing.

(1) Prepare the arm by shaving and cleaning with isopropyl alcohol, finally drying thoroughly with a sterile swab.

(2) Mark out the area to be tested with the code numbers of the antigens to be tested with a skin pencil or suitable non-washable marker (e.g. ballpoint pen).

(3) Inject the appropriate antigen solution using a disposable syringe. The skin is stretched taut with the free hand and then, with the needle of the syringe at a very shallow angle with the bevel uppermost, the needle is introduced intradermally. Care should be taken not to inject subcutaneously causing bleeding,

or not to penetrate enough resulting in an exit puncture and leakage of antigen solution over the skin. Once the needle is in place the solution is gently injected and the skin should blanch and a small bleb is raised. Some of the antigen solutions are painful on injection and the subject should be warned in advance to obviate inadvertent movements of the arm. When the needle is withdrawn, ideally there should be no bleeding at the site of injection indicating that dermal vessels have not been punctured. There may occasionally be very slight blood leakage with good technique but persistent oozing suggests that the injection has been subcutaneous and not intradermal; a false negative result may thus be obtained.

(4) The test sites are examined 48 hours later. If problems are expected in distinguishing Arthus reactions, the tests can be read at 6 and 24 hours.

(5) Both erythema and induration are measured in mm. The response is not always symmetrical and irregular induration can be measured in two directions at right angles and the result averaged. A positive response is conventionally assessed as one giving ≥ 5 mm induration. Responses can be graded with 3–4 mm = ±; 5–8 mm = +; 9–11 mm = ++; 12 mm or more = +++.

Comments

There are no normal values as such available for skin test responses and there is very little information as to the percentage of normal population responding to the various antigens. It is for this reason that a 'battery' of antigens is used with the expectation that at least one positive result indicates normal cell mediated immunity.

It is conventional to measure a positive response as one with 5 mm or more of induration, although again information is lacking as to the normal range of induration in those giving a positive response.

INVESTIGATION OF B LYMPHOCYTE FUNCTION

Quantitation of B lymphocytes

Plasma cells are immunoglobulin secreting cells derived from B lymphocytes and can be identified morphologically. The majority reside in lymphoid organs but accurate quantitation of these cells in tissue sections is difficult. B lymphocytes are operationally defined by the detection of surface immunoglobulin [3]. Surface immunoglobulins are synthesized by the cells carrying them and must be distinguished from adsorbed immunoglobulin [27]. B lymphocytes (or a significant sub-population of them) may also be detected by the presence of Fc receptors [28] and a receptor for the third component of complement (C3) [29]. Between 10 and 25 per cent of circulating lymphocytes have such surface markers. Some information is available on other lymphoid tissues such as spleen, lymph nodes and tonsils [2, 5, 10, 27].

There are considerable technical problems with all of the marker methods currently used. Surface membrane immunoglobulin (SIg), although the most specific, is technically most exacting, since complexes and aggregates in the test antisera bind to Fc receptors giving false positive results [27]. It is possible to use broad spectrum antisera to enumerate the total number of B cells, and specific antisera to identify the various immunoglobulin classes. Occasionally, cells may carry more than one SIg, e.g. some 'double myelomas' giving rise to discrepancies. Anti-lymphocyte antibodies or immune complexes in serum may also be adsorbed on to lymphocytes so giving 'false' high results as sometimes seen in systemic lupus erythematosus [27]. Trypsinization will remove some of the surface layers of the cells, including immune complexes and adsorbed material, enabling endogenous immunoglobulins resynthesized by the 'true' B cells *in vitro* to be recognized. The lymphocytes may also be allowed to 'cap' and endocytose complexes. These manoeuvres should be considered where abnormally high values for SIg are obtained. Such problems are particularly liable to occur where an indirect technique is used employing two or more antisera [27].

Lymphocyte Fc and C3 receptors may be detected by rosetting methods with appropriately treated red blood cells. However, this is also a characteristic of the monocyte. Therefore it is necessary to identify the monocytes either by their capacity to phagocytose latex particles, or to remove them prior to testing by carbonyl iron or plastic/nylon adherence. Neither of these methods is entirely satisfactory since not all monocytes may be phagocytic under the conditions used and some B cells may be removed by the adherence methods [3]. Some of the earlier results in the literature enumerating B cells in various diseases may have been erroneous due to insufficient attention to such details.

Even in health, it is apparent that not all B cells

possess all three markers (i.e. SIg, C3 and Fc) and single marker bearing cells or double marker bearing cells can sometimes be identified. In some diseases these discrepancies may be even more noticeable and are possibly important to the disease pathogenesis. For instance, some agammaglobulinaemia patients with no SIg bearing cells have relatively normal levels of Fc or C3 bearing cells [2, 5, 10].

The percentage of B lymphocytes bearing SIg of different classes in normal people bears little relation to the serum immunoglobulin levels of the same class (although the relative proportions of these lymphocytes are remarkably constant) since presumably SIg bearing cells are not actively secreting immunoglobulin in any quantity. In Bruton-type agammaglobulinaemia SIg bearing cells are generally very low in numbers, or entirely absent. However some patients with the 'common variable' form of hypogammaglobulinaemia have normal numbers of circulating SIg bearing cells but abnormally low serum immunoglobulin. The converse has also been demonstrated. Most patients with isolated IgA deficiency have normal populations of circulating lymphocytes with IgA [2, 5, 10, 27].

Such information gives interesting insight into the immunobiology of B cells and their defects and provides a rational basis for classification of immunodeficiency diseases. However, clinically this is of rather limited practical importance at the present time.

As with T cells it is important that B cells be enumerated both as percentages and as absolute numbers.

Method for determination of B cell numbers

Principle

B lymphocytes have membrane receptors for C3. The technique depends on the capacity of the B lymphocytes to form rosettes with complement-coated red cell intermediates. The indicator cells are sheep red blood cells (E) treated with erythrocyte antibody (A) and human complement (C). Alternatively B lymphocytes can be identified by the demonstration of immunoglobulin on their surface membrane, usually shown by a fluorescent-labelled anti-human immunoglobulin serum (direct) or by an indirect (sandwich) technique using an unlabelled anti-human immunoglobulin serum raised in a particular animal species followed by a fluorescent-labelled anti-species serum.

B lymphocytes by rosettes (EAC) (After Bianco *et al.* [29])

Materials

(1) Sheep red blood cells (50 per cent solution in Alsever's).

(2) IgM anti-sheep red blood cell serum.

(3) Fresh frozen human AB serum.

(4) May-Grunwald-Giemsa stain.

Procedure

Preparation of indicator cells (EAC)

(1) 0·5 ml of sheep red blood cells are washed three times with 10 ml of saline and finally suspended in 5 ml of saline.

(2) 0·1 ml of rabbit IgM anti-sheep red blood cell serum is added (sub-haemagglutinating dose) and the mixture is incubated at 37° C for 20 min with mixing.

(3) Wash three times in saline and resuspend in 10 ml of saline.

(4) Add 0·3 ml of fresh frozen human AB serum (as a source of complement) and incubate for 20 min at 37° C with frequent mixing.

(5) Wash three times in saline and resuspend in 5 ml of saline (EAC suspension). The suspension must be freshly prepared on the day of the test. Wash once more in saline if more than an hour has elapsed between preparing the suspension and its use.

Lymphocyte preparation

(1) Mononuclear cells are prepared as for lymphocyte transformation (see p. 47.5).

(2) Phagocytic cells are identified as outlined under T lymphocytes (see p. 47.4).

Rosetting procedure

(1) To 0·5 ml of latex-treated lymphocyte suspension (approximately 2×10^6/ml) add 8–10 drops of the EAC suspension (8:1 ratio).

(2) Mix the suspension and sediment by gentle centrifugation at about 25 *g* for 5 min. Incubate at 37° C for 40 min.

(3) Resuspend by aspiration until no clumps are apparent.

(4) Slides are prepared and fixed as for T lymphocyte enumeration (see p. 47.4).

Examination of rosettes

Essentially this is the same as for E rosettes but it should be noted that polymorphs and monocytes can also form EAC rosettes. However, B cell EAC

rosettes are small with a single layer of adherent red blood cells. The use of an IgM fraction of the anti-erythrocyte serum excludes rosette formation due to Fc receptors.

B lymphocytes by surface immunoglobulin [27]

Materials

(1) Goat anti-human immunoglobulin (polyvalent for immunoglobulins G, A, M and D).

(2) Rabbit anti-goat immunoglobulin, fluorescein isothiocyanate (FITC) labelled.

(3) Cold fluorescent antibody (FA) buffer (Difco) with 0·2 per cent azide.

(4) FA mounting medium (buffered glycerol) (Difco).

(5) Clean glass microscope slides and coverslips.

(6) Clear cosmetic nail varnish.

(7) 1 per cent buffered paraformaldehyde.

Procedure

Staining

(1) $5–10 \times 10^6$ latex-treated lymphocytes (see detection of T lymphocytes p. 47.4) are washed at 4° C with FA buffer containing 0·2 per cent azide.

(2) Remove the supernatant thoroughly and add 5–7 drops of a 1/6 dilution of goat anti-human immunoglobulin and resuspend. Incubate at 4° C for 30 min.

(3) Wash three times with 10 ml of cold FA buffer with azide and remove the supernatant thoroughly.

(4) Add 5–7 drops of a 1/6 dilution of FITC rabbit anti-goat immunoglobulin serum, resuspend and incubate for 30 min at 4° C.

(5) Wash three times in 10 ml of cold FA buffer with azide and discard the supernatant.

(6) Resuspend the cells in 2–3 drops of FA mounting medium. Alternatively fixation can be carried out here by adding 200 μl of 1 per cent paraformaldehyde to the cell pellet for 5 min at room temperature. The cells are then washed twice and mounted in FA mounting medium.

(7) Seal the edges of the coverslip with clear nail varnish (prepare duplicate slides).

Slides can be examined immediately or stored in the dark at 4° C.

Examination and counting

(1) Examine with a phase-contrast/fluorescence microscope with a high-power oil-immersion objective. Count the number of lymphocytes in the field by phase-contrast, eliminating red cells and phagocytes (latex particles ingested) from the count.

(2) Examine the same field under incident ultraviolet illumination and count the number of fluorescing lymphocytes.

(3) At least 200 lymphocytes are counted per duplicate slide.

Comment

Phagocytes often show fluorescence due to surface-adsorbed immunoglobulins and should be excluded by identification under phase-contrast illumination. Since antisera vary in strength, optimal dilutions should be determined by each laboratory.

Immunoglobulin estimation

It is technically easier, and in some respects more clinically meaningful, to measure the product of B cells, i.e. immunoglobulins (or antibodies), than to estimate the numbers of B cells in the circulation, although both measurements give complementary information.

Simple electrophoresis of serum proteins is of very limited value although it has some use as a screening test to indicate very low or high levels of gammaglobulins. A variety of changes in the relative proportions of the various bands seen on electrophoresis is seen in a number of disease states, but these changes are in no way diagnostic.

Immunoelectrophoresis with specific or polyvalent antisera gives qualitative information on the main serum immunoglobulin classes, IgG, IgA and IgM, and a single immunoglobulin class deficiency may be identified, e.g. IgA deficiency; excess immunoglobulin production, or production of abnormal immunoglobulins, as in myelomas, can also be identified. This test can therefore also have limited use as a preliminary screen.

Of far more practical value is the measurement of concentrations of serum immunoglobulins. Methods for quantitating immunoglobulins require the use of specific antisera and the test material is compared with a standard of known concentration (standards which are now available from the W.H.O. may be used to prepare local laboratory standards). Methods such as single radial diffusion (e.g. Mancini technique [30]) may be used. These are technically simple and commercial kits are available. False results will be obtained if the immunoglobulins are aggregated or dissociated, and appropriate standards must be used on all occasions. There are a variety of other methods available which have the advantage of

sensitivity such as immunoelectrodiffusion (Laurell rocket technique), radioimmunoassay or nephelometry, but these tests are technically more complex (see Chapter 19). Immunoglobulin levels in serum vary both with age, environment and sex and also in the same individual over a period of time. Values must be interpreted accordingly [8]. Measurements of IgG in neonates must take into account the placental passage of maternal IgG. This may delay the clinical presentation of an agammaglobinaemic child. A 'physiological' hypogammaglobulinaemia may also be seen between 3 and 5 months of age before the infant's own IgG production increases as maternal IgG is being lost. IgA and IgM of fetal origin are usually present in low levels at birth. High levels of either may be an indication of intrauterine infection. Failure to produce *salivary* IgA by 6–8 weeks should suggest a defect. *Serum* IgA in normal infants may still be low at this time. The concentration of serum immunoglobulins cannot be used as the sole criterion for the diagnosis of primary immunodeficiency since selective deficiency of a single Ig class, e.g. IgA, can occur occasionally in apparently healthy individuals [8]. Low levels may also be seen in protein losing conditions or in hypercatabolism since the serum level of a protein reflects the sum of synthesis, distribution, breakdown and loss. Estimation of serum IgD is in general of little clinical value except in the identification of IgD-myeloma. Syndrome classification solely on the basis of the pattern of immunoglobulin levels has, so far, been of limited value [31].

Absent or very low levels of serum immunoglobulins usually indicate a deficiency of the B cell compartment either alone, or in combination with T cell deficiency as in severe combined immunodeficiency. X-linked agammaglobulinaemia is characterized by very low concentrations of all five immunoglobulin classes. IgM may be low in Wiskott–Aldrich syndrome and IgA is commonly low in ataxia telangiectasia. However, abnormal immunoglobulin levels alone do not necessarily distinguish between the different forms of immunodeficiency [8].

Measurements of immunoglobulin concentrations in other body fluids such as saliva, tears, colostrum and cerebrospinal fluid may occasionally be of clinical value. Patients with serum IgA deficiency may have mucosal IgA synthesizing plasma cells as determined by rectal or jejunal biopsy.

Quantitation of serum immunoglobulin is of value in the diagnosis of certain lymphoproliferative disorders which result in secondary immunodeficiencies, e.g. myeloma, Waldenström's macroglobulinaemia and lymphomas. Monoclonal bands usually correlate with increased B cell numbers of the appropriate immunoglobulin class. Bone marrow or lymph node biopsies may reveal plasma cells containing intracellular immunoglobulin of the same class as that in the serum. Quantitation of serum immunoglobulins in these patients may be a useful monitor for therapy and there is some evidence that the immunoglobulin class or subclass may give an indication of the ultimate prognosis and the response to treatment.

Patients with normal or high levels of immunoglobulins or with isolated immunoglobulin deficiencies may fail to respond to one or more antigens. Responses to antigenic stimulation are needed if immunodeficiency is suspected but immunoglobulin concentrations are normal [8].

Method for determination of immunoglobulin concentration

Commercially available kits can be used for determination of immunoglobulins (and C3 and C4) by the conventional Mancini technique [30].

Alternatively 'home made' plates and antisera can easily be produced and if large numbers of assays are to be done then they may be economically justified.

The only modifications that we have found necessary when using commercial kits are to dispense the test serum into the wells using a microsyringe as an alternative to the capillary pipette and to incubate at a standard temperature rather than 'room temperature'.

Assay for IgE

This may be performed using a commercially available radioimmunoassay kit such as 'Phadebas' (Pharmacia, London, UK). It is usually possible to render such kits more 'economical' by adjusting the volumes of reagents used. For example a 1/2 scale method will give twice the number of tests.

More recently an immunodiffusion plate has been produced for measurement of IgE but would appear to be only suitable for those patients with considerably elevated levels.

Salivary immunoglobulins

On occasions it may be necessary to measure the immunoglobulins in other body fluids, e.g. saliva.

The method utilizes conventional commercial plates as for serum immunoglobulins and differs only in the method of sample preparation. Samples

should be processed as soon as possible after collection.

Materials

(1) Sterile plastic Universal containers.
(2) Glass beads of 2–3 mm in diameter.

Procedure

(1) At least 1–2 ml of saliva is produced into a sterile Universal container.
(2) Glass beads of 2–3 mm diameter are added to the specimen and shaken vigorously for 5 min.
(3) The homogenized saliva is transferred to a clean polycarbonate centrifuge tube and spun at 25 000 *g* for 30 min at 4° C.
(4) The supernatant is removed and stored at −80° C until the immunoglobulin levels are assessed, usually on commercial low or ultra low level plates.

Specific antibodies

Measurement of antibody levels is an indication of the capacity of B lymphocytes to produce functional immunoglobulins. Even with normal T cell function, failure to detect antibodies following an appropriate antigenic challenge does not necessarily indicate B cell dysfunction as certain individuals may be non-responders to one antigen whereas the response to other antigens is normal. Antibody responses may be assessed by measuring antibody levels to (a) antigens to which the population is commonly exposed, and (b) antigens used in active immunization.

Live vaccines (smallpox, polio, measles, rubella and mumps) should *never* be given in suspected immunodeficiency disease since severe complications or even death may occur.

Isohaemagglutinins against blood groups A and/or B give an indication of IgM antibody production in those having the appropriate blood group and these are usually detectable by 3–6 months of age. By late childhood they attain a level which is maintained with very little variation throughout life, tending to decrease again in old age. Maternal IgG anti-A or anti-B, if present, can cross the placenta and so give false positive results during the first few months following delivery. Heteroagglutinins (mainly IgM) may occur as 'natural' antibodies (heterophil antibody against sheep red cells) in many individuals and therefore their measurement may provide a simple screening test. Antibodies to the O antigen of commensal *E. coli* are seen in the sera of most normal children and remain detectable throughout adult life. The specificity of the *E. coli* antibody responses depends on the serotypes prevalent in the community and pooled appropriate antigens can be used to identify IgG and IgM antibodies both in adults and children [32]. This test is not satisfactory in the very young. A search for appropriate bacterial or viral antibodies can be undertaken with the knowledge of the appropriate exposure to infections. Therefore measurements of antistreptolysin-O, antistaphylococcal antibodies, antibodies to respiratory syncytial virus, influenza virus, rubella, measles, chickenpox and mumps, etc. may give additional information. A clear history of exposure with failure to produce the appropriate antibody is suggestive of a defect. However, these studies should be interpreted with caution since a virus may be isolated in culture without it necessarily being the pathogenic agent. Viral antibody levels are of limited value because of the variability of responses in normal individuals and the difficulty in defining normal ranges.

Method for determination of isohaemagglutinins

Principle

Antibodies to blood group substances A and B are invariably found in the serum of healthy normal individuals of the appropriate ABO blood group. They are described as isohaemagglutinins because of their ability to sensitize and agglutinate suspensions of human red cells containing the relevant antigen.

Group O individuals have anti-A and anti-B in their serum.

Group A* individuals have anti-B in their serum.

Group B individuals have anti-A in their serum.

Group AB* individuals have neither anti-A nor anti-B in their serum.

(* Subgroups A_2 and A_2B may form anti-A_1 which reacts with the more common A_1 subgroup.)

The principle of the test is that serum containing or suspected to contain isohaemagglutinins is incubated with red cells of known ABO type. The presence of such antibodies results in sensitization and agglutination of the appropriate red cells. A semi-quantitative result is obtained by recording the agglutination end-point of a series of doubling dilutions of serum.

The ABO group of the patient is also required for correct interpretation of the results.

IgM antibodies may be differentiated from IgG and IgA antibodies by preincubation of sera with dithiothreitol (DTT) which dissociates the IgM

antibodies into subunits, resulting in loss of agglutinating ability.

Materials

(1) Clotted whole blood (test sample).

(2) 5 per cent suspension in 0·9 per cent saline of standard group A_1, A_2, 'A mix' ($A_1 + A_2$), B and O red cells.

(3) Complete anti-A standard serum.

(4) Complete anti-B standard serum.

(5) Complete anti-A + B (group O serum).

Any of the above reagents are obtainable from Ortho Diagnostics (Raritan, New Jersey, USA).

Procedure

(1) Centrifuge sample to obtain serum and remove to a clean glass tube.

(2) Prepare a 5 per cent red cell suspension from test sample in 0·9 per cent saline.

(3) Identify ABO group of test cells:

(a) Set up six glass tubes (60 mm × 8 mm).

(b) To the first three are added 1 vol of 5 per cent test red cells.

(c) To the last three are added 2 vols (e.g. 2 drops with Pasteur pipette) of test serum.

(d) Standard reagents are then added as follows:

Tube 1—add 1 vol A mix cells.
2—add 1 vol B cells.
3—add 1 vol O cells.
4—add 1 vol complete anti-A.
5—add 1 vol complete anti-B.
6—add 1 vol complete anti-A + B.

Incubate at room temperature (15–18° C) for 1 hour and read the results macroscopically, observing agglutination of red cells. The results are interpreted as follows:

Sample	A mix cells	B cells	O cells	Anti A	Anti B	Anti A+B	ABO group of sample
(1)	+	+	−	−	−	−	O
(2)	−	+	−	+	−	+	A
(3)	+	−	−	−	+	+	B
(4)	−	−	−	+	+	+	AB

Any other pattern indicates that further tests are required (see Comments).

(4) Isohaemagglutinin titration:

(a) Prepare a master titre on the separated test serum in 0·9 per cent saline, the dilutions being as follows: neat, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512.

(b) Place 1 vol of each dilution into four glass tubes (1 drop with Pasteur pipette is usually sufficiently accurate for most purposes provided that the same pipette is used throughout).

(c) To one set of dilutions add 1 vol of standard group A_1 cells per tube, to the second set of dilutions add 1 vol of group A_2 cells, to the third set group B cells and to the fourth set group O cells. Shake to mix and incubate at room temperature (15–18° C) for 1 hour.

(d) Read for agglutination. The end point is determined microscopically, when an even distribution of clumps of three or more red cells is evidence of agglutination.

(e) The titre of isoagglutinin is expressed as the reciprocal of the greatest dilution causing agglutination.

(5) Identification of IgM/IgG agglutinin [33]:

(a) Prepare 0·01 mol/l dithiothreitol in 0·9 per cent sodium chloride or PBS (see p. 47.16).

(b) Add equal volumes of test serum and DTT to a clean glass tube and incubate at 37° C for 1 hour.

(c) Control consists of equal volumes of test serum plus dilution buffer (without DTT) treated in an identical manner.

(d) Proceed with preparation of master dilutions as above, starting at 1/2 to allow for initial dilution.

(e) Determine isoagglutinin titre as above. Activity due to IgM isoagglutinin will be abolished resulting in complete loss of agglutination, or a marked reduction in titre.

Comments

These techniques are performed on a routine basis by blood banks and transfusion centres and it may be convenient to refer samples directly for testing.

Blood grouping. There may be apparent discrepancies due to subgroups of A or B, and false positive results due to autoantibodies, cold agglutinins, rouleaux formation, and other less common red cell antibodies present in the test sample. The group O cells are added as a screening check for cold agglutinins outwith the ABO system which may give false positive results. If this test is positive, expert advice should be sought for the interpretation of the results.

Isoagglutinin titre. Even in normal individuals the anti-A titres are usually greater than the anti-B titres. Furthermore, group O individuals tend to

have higher values than group A or B for the respective isoagglutinins.

Method for determination of *Escherichia coli* antibody levels (after Webster *et al.*) [32]

Principle

E.coli antigens, prepared by boiling a suspension of bacteria, are coated on to human group O rhesus negative erythrocytes, which are then used to detect specific antibody by direct haemagglutination.

Samples are titrated before and after treatment with dithiothreitol to determine the relative activity of specific anti-*E. coli* IgM and IgG [33] (see p. 47.15).

Materials

(1) *E. coli* antigen—a bacterial suspension is boiled (water bath) for 2 hours, then centrifuged at 5000 *g* for 30 min. The supernatant is removed, adjusted to optimal concentration as determined by optical density measurements, aliquoted and frozen at −20° C.

(2) Fresh human group O rhesus negative blood—up to one week old in anticoagulant (3·8 per cent tri-sodium citrate).

(3) Dithiothreitol—(BDH) (British Drug Houses Ltd., Poole, Dorset, UK) stored as powder at −20° C and prepared immediately before use.

(4) Haemagglutination plates—microtitre, U-bottomed, disposable.

Procedure

Coating of cells

(1) Human O negative erythrocytes are washed three times in saline, and 0·1 ml of packed cells incubated with 5 ml of antigen solution for 30 min at 37° C. The cells are then adjusted to a 1 per cent suspension in saline.

Test procedure

(1) Sera under test are inactivated at 56° C for 30 min and divided into two aliquots. One is heated with dithiothreitol whilst the other is heated with an equal volume of phosphate buffered saline.

(2) Two-fold dilutions of test sera are made in microtitre haemagglutination trays (25 μl volumes).

(3) An equal volume of antigen coated red cells is added to each well and the trays incubated at 37° C for 3 hours before reading, followed by an overnight incubation at 4° C and recheck before discarding.

Comments

Best results are obtained if cells are coated with antigen on the day of testing. Evidence accumulated so far with this technique suggests a definite correlation between serum anti-*E. coli* titre, serum immunoglobulin levels and a diagnosis of immunodeficiency. False positives may occur due to the presence of 'atypical' red cell antibodies (see isoagglutinin methodology p. 47.15).

Immunization

In a previously immunized person antibodies to diphtheria (DT) or tetanus toxoid (TT) may be detectable. A better assessment of the secondary response can be made if a booster injection of DT or DT plus TT is given for subsequent antibody determination. In unimmunized children commercial DT or TT may be given at standard doses (0·5 ml intramuscularly). A complete schedule of three to four injections at 4–6 week intervals should be given before concluding a failure to respond. Antibody levels should be determined 7, 14, and 21 days following the last injection. *Killed* polio virus can also be given intramuscularly (instead of the usual oral route) in three doses 2 weeks apart and the neutralizing antibody assessed. Measurement of the IgM and IgG antibodies following the above immunizations may be useful in defining an inadequate primary or secondary response, but normal ranges and responses are not known.

Bacteriophage $\Phi X174$ has been shown to be a potent, safe, useful and discriminating antigen and techniques have been developed which allow measurement of antigen clearance, and of the primary and secondary immune response [34]. The secondary responses of normal individuals to this antigen have been shown to be remarkably uniform and consistent. The special facilities required for these determinations have probably limited the potential widespread use of such a useful antigen.

Method for determination of tetanus antibody levels (after Nelson [35])

Principle

Tetanus vaccine is coupled to glutaraldehyde-fixed human group O rhesus negative erythrocytes using chromic chloride. These 'antigen carriers' are capable of detecting specific antibody in a direct haemagglutination assay. Sera found or suspected to contain specific anti-tetanus activity are titrated in parallel

with a standard of known unitage, and the units/ml of the test sample calculated.

Materials

(1) Human group O rhesus negative erythrocytes —up to 7 days old.

(2) Glutaraldehyde solution—1 per cent in phosphate buffered saline pH 8·0.

(3) Tetanus vaccine—40 LF units/ml in simple solution (Wellcome).

(4) Chromic chloride solution—freshly made by dissolving 1 g chromic chloride in 100 ml saline, dilute 1/2 in saline just before use to give a final concentration of 0·5 per cent.

(5) Phosphate buffered saline pH 8·0 (PBS):

1 vol Na_2HPO_4, 0·15 mol/l

9 vols NaCl, 0·15 mol/l

5 vols sterile distilled water (SDW)

Adjust pH to 8·0 with HCl

(6) Diluting fluid—10 ml 1/800 tetanus vaccine in saline.

83·4 ml saline.

6·6 ml 30 per cent bovine serum albumin.

(7) 0·9 per cent normal saline—36 g NaCl in 4000 ml SDW.

(8) Positive controls—high titre antibody, either serum or immunoglobulin of known unitage, e.g. Humotet (Wellcome) containing 250 international units per ml of anti-tetanus activity.

(9) Haemagglutination plates—microtitre, U-bottomed, disposable.

Procedure

Glutaraldehyde fixing of cells

(1) Human group O negative erythrocytes are washed three times in saline and the packed cell volume established by centrifugation at 1000 *g* for 5 min.

(2) A 2 per cent suspension of cells in 1 per cent glutaraldehyde is incubated at 4° C for 30 min, with frequent mixing.

(3) The cells are then washed three times in saline and three times in SDW and stored as a 10 per cent suspension in SDW containing 1 mg/ml sodium azide.

Coating of cells

(1) Glutaraldehyde fixed cells are washed three times in saline and the packed cell volume established as above.

(2) 1 ml of tetanus vaccine is mixed with 3·5 ml saline and 1 ml of packed cells added. This is followed by the addition of 0·5 ml of 0·5 per cent chromic chloride, and the mixture incubated at 25° C for 6 min.

(3) The cells are washed three times in saline and stored as a 20 per cent suspension in saline at 4° C for at least one week before use.

Haemagglutination assay

(1) Test sera are initially diluted 1/40 in diluting fluid with subsequent doubling dilutions in a haemagglutination plate. A positive control (serial dilutions of anti-tetanus standard) and a negative control (diluting fluid) are set up in each plate.

(2) One drop (25 ml) of test serum dilution is mixed with 1 drop of a 0·6 per cent suspension of 'coated cells', the plate covered and left at room temperature (25° C) for 1½–2 hours before reading.

(3) The plates are also read after overnight incubation before discarding.

(4) The test sample titre is converted into international units of anti-tetanus activity as follows:

$$\text{IU} = \frac{\text{titre of test} \times \text{known units of standard}}{\text{titre of standard}}$$

Comments

The haemagglutination test described is simple to perform, easy to read, very sensitive and specific (specificity may be checked by inhibition with antigen), and reagents may be stored for at least 6 months at 4° C without affecting sensitivity, provided azide is present to prevent microbial contamination.

INVESTIGATION OF PHAGOCYTE FUNCTION

Quantitation of neutrophils and monocytes

Alterations in the percentage or absolute number of circulating neutrophils or monocytes will often be the earliest indication of immune deficiency. The results must be interpreted with caution. For instance neutropenia in infancy can either be uniformly lethal as in congenital neutropenia (Kostman disease) [36] or compatible with good health as in chronic benign neutropenia of infancy [37]. Rare disorders such as cyclical neutropenia require regular white cell counts as the oscillations in the numbers of circulating cells are sometimes as short as 14 or as long as 35 days. It is important to differentiate the primary neutropenias from the many causes of secondary neutropenias

found in association with infections, the administration of drugs, megaloblastic anaemia, hypersplenism, leuco-erythroblastic anaemia, acute leukaemia, aplastic anaemia, paroxysmal nocturnal haemoglobinuria, ionizing radiation, cytotoxic drugs and a number of miscellaneous conditions such as systemic lupus erythematosus (SLE). Maternal-derived leucocyte-agglutinating antibodies may be found in neonatal neutropenia and these should be sought where appropriate. Neutropenia following transfusions has also been reported in association with anti-neutrophil antibodies. Leucocyte autoantibodies in SLE may also result in low neutrophil counts and these antibodies should be sought in cases of unexplained persistent neutropenia.

Method for detection of leucoagglutinating antibodies [38]

Principle

When leucocytes are mixed with a serum containing leucocyte antibodies, the cells and serum interact to form clumps or agglutinates.

Materials

(1) Dextran 150 in 5 per cent dextrose (Dextraven 150, Fisons Ltd., Loughborough, Leics., UK).

(2) Toluidine blue 0·2 per cent in distilled water.

(3) Glass tissue typing trays (Searle Laboratories, High Wycombe, Bucks., UK).

(4) Mineral oil (Shell Ondina 33, Shell-BP, Grangemouth, Scotland, UK).

(5) Phosphate buffered saline 1·8 per cent.

Procedure

(1) 2 μl of the serum under investigation, previously heat inactivated at 56° C for 30 min, is dispensed using a micro-syringe into the wells of a tissue typing tray containing mineral oil.

A known positive and negative control serum is included on each tray and the plate can be stored at −20° C until required.

(2) 7 ml of defibrinated blood is mixed with 3 ml of Dextran 150 (or other red cell sedimenting agent) and incubated at 37° C for 30–45 min. Usually a panel of 5–10 donors are used to cover a variety of leucocyte antigens.

(3) The supernatant, usually containing between 3000–6000 leucocytes/mm^3 is removed into a clean tube and the cell count standardized to 3000–6000/mm^3.

(4) 1 μl of the cell suspension is added to the test plate with a microsyringe and incubated for 1 hour at 37° C.

(5) After incubation the plates are 'rotated' on an orbital bench rotator at a speed sufficient to 'button' the test mixture.

(6) A volume of 0·2 per cent toluidine blue in distilled water is mixed with an equal volume of 1·8 per cent buffered saline and centrifuged. 1 μl of the stain is then added to each well on the plate.

(7) Examine after a period of 5 min at a magnification of × 100 with bright light. Agglutinates when present are easily observed and should be compared with positive and negative controls for scoring purposes.

Comments

Leucocytes and test sera must be ABO compatible. For convenience group O cell donors may be used.

Morphology and staining methods

Abnormal morphology of neutrophils can often aid the diagnosis of a number of primary and secondary immune deficiencies. Giant lysosomal granules are found in Chediak–Higashi disease. The presence of large numbers of immature granulocytes in the peripheral blood is suggestive of chronic granulocytic leukaemia.

A number of simple staining procedures are of value for screening certain primary immune deficiencies. Leucocytes from patients with chronic granulomatous disease are unable to reduce the dye nitroblue tetrazolium (NBT). Myeloperoxidase deficiency should be considered in individuals susceptible to systemic infections with *Candida albicans*. Myeloperoxidase staining of peripheral blood leucocytes should probably be undertaken in all cases where a defect in phagocytic cells is considered.

Method for nitroblue tetrazolium (NBT) test (after Park *et al.* [39])

Principle

Ingesting phagocytes convert the yellow soluble dye (NBT) to an insoluble dark blue precipitate (formazan) in the phagocytic vacuoles. The process is dependent on an intact nucleotide oxidase within the cell, the NBT substituting for oxygen in the oxidase reaction or being reduced by the superoxide radical.

In patients with systemic bacterial infection there is an increase in NBT reduction in contrast to

patients with chronic granulomatous disease when 90+ per cent are unable to reduce the dye.

Comparisons are normally made between unstimulated NBT reduction and cells that are actively phagocytosing. The estimation of reduction is determined by counting all cells containing a deposit of formazan.

Materials

(1) Nitro blue tetrazolium–Ficoll solution: 2 g of Ficoll are added slowly to 10 ml of phosphate buffered saline pH 7·2 (Oxoid tablets, Oxoid Ltd., London, UK) until all the Ficoll is dissolved. 10 mg of nitro blue tetrazolium (BDH) are then dissolved in this solution. The solution is filtered through a Millex 0·22 μ filter (Millipore S.A., 78530 Buc-France) and used at room temperature.

(2) Stimulating agent: *Escherichia coli* endotoxin (Difco *E. coli* 126B) at a concentration of 0·2 mg per ml in phosphate buffered saline.

(3) Stain: May-Grunwald (50 per cent stock in buffered distilled water pH 6·8). Giemsa (10 per cent stock in buffer distilled water pH 6·8).

Procedure

(1) 2·5 ml of venous blood is taken in to a potassium EDTA tube.

(2) 0·5 ml of blood is aliquoted into two tubes. 0·05 ml of phosphate buffered saline is added to one tube (unstimulated) 0·05 ml of stimulating agent added to the other tube (stimulated). The tubes are left at 20° C for 15 min.

(3) 0·1 ml aliquots are taken into the wells of a Boerner tray (Clay Adams cat. no. 5419—Parsippany, N.J., U.S.A.) with both unstimulated and stimulated mixtures being duplicated.

(4) 0·1 ml of the NBT–Ficoll solution is added to each well, mixed, and the slide incubated in the humidified box for 30 min at 37° C, followed by a further incubation at 20° C for 15 min.

(5) At the end of incubation the wells are gently mixed with a Pasteur pipette and a drop of the reaction mixture placed on a clean glass slide and a smear prepared.

(6) The slide is air dried and fixed in methanol for 2 min.

(7) Stain the slide in May-Grunwald for 3 min.

(8) Transfer directly to Giemsa for a further 3 min.

(9) The excess stain is rinsed with a wash bottle containing distilled water buffered at pH 6·8.

(10) Differentiate for approximately 1 min with distilled water buffered at pH 6·8 checking visually at intervals with a low power objective on the microscope.

(11) Observe wet at a magnification of ×400 or the slide can be air dried and observed with an oil immersion objective at ×1000.

(12) 100 cells and two fields are counted on each slide and the number of formazan containing cells scored.

Comment

EDTA used as an anticoagulant prevents cell clumping. The sensitivity of the test and cell membrane integrity are maintained by adding Ficoll (a sucrose polymer) to the test system.

Method for myeloperoxidase staining (after Kurstak & Kurstak [40])

Principle

The peroxidase reaction depends upon the presence of peroxidases within the cytoplasm of granulocytes and to a lesser extent, of monocytes. The peroxidase of neutrophils is generally termed myeloperoxidase. Peroxidase activity is indicated after appropriate staining by discrete blue granules in the cytoplasm of granulocytes and monocytes.

Materials

(1) Nadi reagent is prepared by dissolving 15 mg α-naphthol and 22 mg p-phenylene diamine in 20 ml 0·07 mol/l PO_4 buffer, pH 7·2 containing 0·17 mol/l NaCl (i.e. in phosphate buffered saline pH 7·2). Prepare fresh reagent several hours before use and filter. Store in dark bottle at 4° C.

(2) Formal–ethanol fixative (10 ml 37 per cent formaldehyde + 90 ml absolute ethanol).

(3) Safranin (0·2 per cent in buffer pH 6·8).

(4) 0·2 per cent methyl green or Giemsa stain.

Procedure

(1) Use fresh smears of blood. Heparin, oxalate and EDTA are not inhibitory as anticoagulants, but EDTA reduces the tendency of leucocytes to clump.

(2) Fix slides for 1 min at room temperature in 10 per cent formal–ethanol.

(3) Wash for 15–30 sec with gently running tap-water. Shake off excess water.

(4) Apply 2–3 drops of Nadi reagent to smears. Leave for 5 min or until stain develops.

(5) Wash briefly (5–10 sec) in running tap-water.

(6) Counterstain with safranin for 1 min.

(7) Recounterstain with (0·2 per cent) methyl green or Giemsa stain if greater nuclear detail is required.

Comments

The peroxidase reaction occurs at once in the presence of H_2O_2 and the sites of peroxidase activity appear in the form of blue coloured spots.

Examine microscopically under ×400 magnification.

Chemotaxis and random migration

It is sometimes convenient to consider increased susceptibility to infection, especially by pyogenic organisms, in terms of the various biological events which result in microbial killing. Thus the cells must have the capacity to respond in chemotaxis and random migration; the individual must generate the appropriate chemoattractants; the particles must be opsonized by antibody and complement; the phagocytic cell must adhere to the particle (immune adherence); there must be a functional phagocytic mechanism and the cell must have the appropriate biochemical pathways to kill the micro-organisms.

The micropore technique of Boyden is a sensitive and reproducible *in vitro* model for studying chemotaxis and random mobility [41]. It has been used for the identification of chemotactic factors and the response of target cells both in health and disease [42]. The Boyden chambers consist essentially of two compartments divided by a micropore filter. A suspension of leucocytes is placed in the upper compartment and the chemotactic agent is introduced into the lower part of the chamber. Following an incubation period the filter is removed, fixed and stained and chemotaxis quantified either by counting the number of cells which have traversed the entire thickness of the membrane or by measuring the distance the 'leading front' of cells has migrated from the origin [43]. Further refinements to the technique include a method in which leucocytes are labelled with ^{51}chromium and chemotaxis is expressed in terms of radioactivity of a second filter placed on the side to which the cells are migrating [44].

A number of patients have been described with intrinsic defects of leucocyte movement although these in general are rare conditions and poorly classified. Two groups of disorders, the lazy leucocyte syndrome and familial chemotactic defects, both show diminished chemotaxis *in vitro* although in the latter random migration was normal [45]. In general these patients have normal phagocytic and bactericidal activities. Chemotactic response is also impaired in the Chediak–Higashi syndrome but this is presumably due to slow migration as a result of large lysosomal granules. A number of patients with chronic mucocutaneous candidiasis have defects of both monocyte and neutrophil chemotaxis, some of which can be corrected by the administration of transfer factor [46, 47]. There are also a number of conditions in which a defect in neutrophil chemotaxis is associated with markedly raised levels of immunoglobulin E, atopic dermatitis and recurrent infection [45]. The reason for these associations is unclear. Defects in chemotaxis are also found in association with a number of disease states such as tumours, diabetes and rheumatoid arthritis [42].

The skin-window (Rebuck) technique may also be of value as an ancillary investigation [48] although in general a diminished skin-window response is always reflected by impairment in chemotaxis *in vitro*. The main value of this test is its simplicity as there are no requirements for special apparatus. It is a satisfactory primary screen when a chemotactic defect is suspected, but difficult to quantitate. The early response (2–4 hours) consists of neutrophils but by 24 hours these are replaced by monocytes. By changing coverslips at appropriate times the chemotactic response of both cell types can be assessed.

Method for measurement of chemotaxis (after Zigmond & Hirsch [43])

Principle

This *in vitro* technique measures the ability of leucocytes to migrate towards a chemotactic stimulant. A leucocyte suspension is separated from a chemotactic agent by a filter through which neutrophils or monocytes can migrate. Either the distance of migration into the filter (leading front) or the number of cells migrating to the underside of the filter are measured.

Materials

(1) Chemotaxis chambers (see Procedure) made from 1 ml plastic syringe barrels; Millipore filters nos. SSWPO 1300 3 μ or SCWPO 1300 8 μ; UHU adhesive.

(2) Tek lab. tubes.

(3) Materials for neutrophil preparation (see p. 47.24).

(4) Medium 199 (Gibco-Biocult, HEPES-buffered).

(5) Ficoll/Triosil solution (specific gravity 1·076) (see Lymphocyte transformation, p. 47.5).

(6) Materials for differential cell count (see NBT test, p. 47.18).

(7) Casein, Hammerstein (BDH).

(8) Ethanol, Harris haematoxylin (Searle), butanol, xylene, DPX.

Procedure

Preparation of chemotaxis chambers

(1) Chemotaxis chambers are prepared from cut-off upper parts of 1 ml syringe barrels retaining the flanges. The length of the chamber is such that there is no difference in hydrostatic pressure between the contents of the chamber and the chemotactic solution in which it is suspended.

(2) The chambers are prepared by gluing Millipore filters to the end of the barrel with UHU adhesive, taking care to ensure a good seal without blocking the end of the chambers. When dry, the excess filter is trimmed away. For neutrophil chemotaxis the 3 μ filter (Millipore SSWPO 1300) is used and for monocytes an 8 μ filter (Millipore SCWPO 1300) is used. Great care should be taken in preparation to avoid damage and contamination of the filters.

Preparation of neutrophils

(1) Neutrophils are prepared as outlined for the phagocytic and Candida killing assays (see p. 47.24).

(2) The cell suspension is made up in HEPES-buffered medium 199 (Gibco-Biocult) containing 100 units per ml of penicillin/streptomycin solution and a cell concentration of 1×10^6/ml.

Preparation of monocytes

(1) 10 ml of heparinized venous blood is diluted with 10 ml of sterile saline and layered on to a mixture of Ficoll/Triosil (specific gravity 1·076) after Böyum [19] (see Lymphocyte transformation, p. 47.5).

(2) Centrifuge at 150 *g* for 25 min then remove the cell band from the interface and wash three times in medium 199. The cells are suspended in 5 ml of medium prior to counting in a haemocytometer.

(3) The suspension is diluted in medium 199 to give 3×10^6 mononuclear cells per ml (monocytes approximately 30 per cent). A differential count may be performed as a check.

Preparation of chemotactic agent

(1) 2 g of casein (Hammerstein, BDH) is dissolved in 180 ml of sterile distilled water adjusted to pH 11 with sodium hydroxide solution.

(2) 20 ml of ten times concentrated 199 solution (Difco) is added and the pH adjusted to 7·2 with phosphoric acid.

(3) The final solution containing 10 mg of casein per ml in medium 199 is dispensed into 5 ml aliquots and stored at −40° C.

Chemotaxis

For neutrophils and monocytes the test systems are identical except that 3 μ or 8 μ filters are used respectively.

(1) Test chemotactic solutions are made up by diluting casein stock solution in medium 199 (Gibco-Biocult) to give: 0 mg, 0·5 mg and 1·0 mg casein per ml. 1 ml of each test solution is placed in Tek lab. tubes, in triplicates, for each cell suspension to be tested.

(2) 0·2 ml of cell suspension is placed into the chemotaxis chambers which are then suspended by the flanges in the Tek lab. tubes containing the test solutions of chemotactic agent. A suspension of cells from a healthy individual, possibly age/sex matched, is included in each assay as a control.

(3) The system is incubated for 75 min at 37° C in a humidified air atmosphere.

(4) The chambers are then removed, the remaining cells ejected, and triplicate chambers are immersed in beakers of ethanol. The ethanol fixes the reaction and dissolves the glue, permitting the filters to detach from the chambers. The following sequence is used for staining and mounting the filters (minimum times):

Rehydration:	distilled water, 2 min
Staining:	Harris haematoxylin, 2 min
Wash:	distilled water, 3 min
Blueing:	alkaline water, until blue (≏ 10 min)
Dehydration:	ethanol 96 per cent, 5 min
	ethanol 96 per cent, 10 min
	ethanol/butanol (80/20), 15 min
	xylene, 15 min

The filters are then mounted origin side uppermost (using the rough texture and convex-upwards shape as a guide) on a glass slide in DPX, care being taken to express air bubbles trapped under the filters.

(5) The filters are examined under ×400 magnification with a microscope equipped with micrometer scale fine adjustment. Note the micrometer reading when the origin is in focus (N.B. top of the filter, not cells) and then measure the distance to

where the furthest migrating front of cells are found by focusing down using the fine adjustment. Five random readings are taken on each filter and the readings averaged for the triplicate. The results are given as the migration distance in microns.

Comments

Sources of error:

(1) False negatives are usually due to torn or poorly sealed filters.

(2) All apparatus should be thoroughly washed and rinsed free of detergents.

(3) Poor triplicates are usually the result of excess glue, inconsistent 'reader' definition of the leading front or reading areas of the filter close to the extremities of the migration area.

Method for determination of *in vivo* leucocyte migration by the 'Rebuck skin window' (after Rebuck & Crowley [48])

Principle

This is an *in vivo* assessment of the complex sequence of events involved in leucocyte migration to a site of induced inflammation. Only the end-stage of these events is measured, i.e. appearance of cells in the inflammatory exudate. The method is simple to perform and allows visualization of cellular events, but does not permit accurate quantitation of the cellular inflammatory response; furthermore it may represent a model of foreign body reaction rather than one depicting the early phase of neutrophil host defence.

The stimulus to cell migration is a skin abrasion which should not be deeper than the dermal–epidermal junction otherwise severed blood vessels may result in direct 'leak' of cells in the exudate. The abrasion is covered with a glass coverslip to which migrating cells adhere and the cell types can be identified by conventional staining techniques. The predominant cell over the first 6–8 hours is the neutrophil and this cell is gradually replaced by monocytes until at 24 hours these predominate.

Materials

(1) Razor.

(2) Sterile gauze swabs.

(3) Isopropyl alcohol.

(4) Adhesive tape (micropore).

(5) Circular glass coverslips (15 mm diameter).

(6) Sterile disposable scalpel (or high speed lapidary drill with sterilized interchangeable cylindrical grinder heads (e.g. Expo (Drills) Ltd., 62 Neal Street, London WC2H 9PA, UK).

(7) Haematological fixatives and stains.

Procedure

The most convenient site for the procedure is the volar aspect of the forearm, but other sites, e.g. anterior aspect of the thigh, can also be used provided they can be protected from accidental blows.

(1) Shave the area to be used, together with the surrounding area of the skin.

(2) Cleanse the skin with isopropyl alcohol and dry with a sterile swab.

(3) The area to be abraded is stretched taut whilst the abrasion is made.

(a) Using a sterile disposable scalpel held vertical to the skin surface the epithelium is gradually scraped away over an area of approximately 0·5 cm^2. The depth of the lesion is critical. The correct level is reached when a 'smarting' discomfort is felt on scraping and fine capillary loop bleeding points are visible. If abrasion is continued, excessive bleeding will be produced giving an unsatisfactory site.

(b) An alternative method of abrasion is to slide a grinding cylinder rotating at high speed over the taut skin, resulting in a rectangular abrasion of appropriate size in a matter of seconds. Operator experience is required to produce a lesion of the correct depth and the risk of going too deep is greater than with the scalpel method. It is, however, much quicker. The grinder heads should be sterilized by autoclaving after each patient to prevent accidental transmission of disease, e.g. serum hepatitis or syphilis.

(4) The abrasion is then immediately covered with a cleansed sterile glass coverslip, followed by a square of sterile gauze and secured with adhesive tape (micropore tape is the most convenient). A circular coverslip is slightly less susceptible to accidental breakage *in situ*, than a square one.

(5) The coverslip is changed at intervals (depending on the requirements), e.g. 2 hours, 6 hours, 12 hours and 24 hours and replaced by a fresh sterile coverslip.

(6) The coverslips are air dried, fixed in methanol and stained with May-Grunwald-Giemsa.

(7) After staining, slips may be mounted on microscope slides giving permanent records.

(8) The wound should finally be covered with a sterile dressing until a dry scab is formed. In some patients with a neutrophil defect infection may be

produced at the site of abrasion and this will require treatment in the usual manner with sterile dressings and appropriate topical antibiotics.

Opsonization

It is generally agreed that the third component of complement is an essential prerequisite for the preparation of micro-organisms for ingestion by phagocytic cells. Cells are coated with C3 following interaction either with specific antibody and the classical complement pathway components C1, C4 and C2 or via the components of the alternate pathway. Other cofactors are probably required for the full expression of opsonic activity but in clinical practice defective opsonization is unusual in the presence of normal levels of immunoglobulins and complement (see below). The sera from patients with hypogammaglobulinaemia and the rare deficiencies in the third component of complement are relatively ineffective in preparing particles for optimal phagocytosis [49].

Phagocytosis and microbial killing (see also Chapter 32)

A defect in the rate of ingestion of particles alone, that is in the absence of other phagocytic defects such as chemotaxis and microbial killing, is rare. A patient has been described with deficient polymerization of G-actin who suffered from recurrent bacterial infections [50]. This individual's neutrophils did not respond in chemotaxis and ingested particles at a very slow rate.

Patients with neutropenia, by and large, have a poor rate of ingestion when compared, on an equal cell to cell ratio, with normal leucocytes. Similarly, patients with hyposplenism have been reported to have a poor phagocytic response.

There is considerable variation between laboratories as to the choice of tests for phagocytic function on a clinical basis. Such procedures usually incorporate both measurements of the rate of phagocytosis and microbial killing capacity. For example, procedures which measure the rate of ingestion and killing of organisms such as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* are commonly employed. The tests involving 'plating-out' procedures are laborious and time-consuming. We find the Candida-killing test to be, in general, the more convenient [51]. Culture techniques are not necessary with the Candida-killing assay as viability can be quantified by exclusion of methylene blue. It should be noted that there have been reports of cases, largely anecdotal, in which selective defects have been reported in terms of an inability to kill only certain micro-organisms. If a patient is susceptible to infections with one particular micro-organism it would seem prudent to assess the capacity of the individual's leucocytes to ingest and kill the same organism *in vitro*. The elegant assay described by Stossel [49] which measures the rate of phagocytosis of opsonized lipopolysaccharide coated oil red O particles (which can be combined with a quantitative NBT test) has been found in our experience to be largely impracticable for routine clinical work. However, this test and others of oxygen consumption and glucose oxidation, such as the generation of the superoxide radical and hexose monophosphate shunt activity, may be required in special circumstances such as the detection of carrier states in chronic granulomatous disease [52, 53]. In these situations as much information as possible is required in relation to phagocytic function for informed genetic counselling.

Method for estimation of Candida killing and phagocytosis (after Lehrer & Cline [51])

Principle

A quantitative method for measuring the phagocytic and Candidacidal activity of human leucocytes based on the uptake of methylene blue by non-viable *Candida albicans*.

Materials

(1) *Candida albicans* suspension.

(2) Sabouraud glucose agar. Sabouraud liquid broth.

(3) 0·01 per cent methylene blue in distilled water.

(4) 6 per cent Dextran 70.

(5) Red cell lysing solution (0·8 per cent NH_4Cl/EDTA/KCO_3 pH 7·4).

(6) Hanks's balanced salt solution (HBSS) pH 7·4.

(7) 2·5 per cent sodium deoxycholate pH 8·7.

(8) Methanol.

(9) May-Grunwald stain.

(10) Giemsa stain.

Procedure

Preparation of Candida suspension

(1) *Candida albicans* is inoculated into Sabouraud

liquid broth from a stock culture maintained on a Sabouraud agar slope and left overnight at 37° C.

(2) The culture is centrifuged at 400 *g* for 10 min and the supernatant discarded prior to resuspension in 2 ml of 0·9 per cent sterile saline and shaken vigorously to give a single cell suspension. A 0·1 ml aliquot is suspended in 5 ml of saline and counted in a haemocytometer and the concentration in millions/ml calculated.

(3) The viability of the Candida is checked by dye exclusion. An equal volume of suspension is mixed with an equal volume of 0·01 per cent methylene blue and left for 5 min at room temperature before checking microscopically for dead cells. The Candida suspension may be left on ice to prevent further growth prior to use.

Neutrophil suspension

(1) 7·5 ml of heparinized (preservative free) blood is mixed with 3·75 ml of 6 per cent Dextran 70 and the erythrocytes sedimented at 37° C for 30–40 min in a container inclined at 45°.

(2) The leucocyte rich plasma is removed and centrifuged at 100 *g* for 10 min.

(3) The cells are washed twice more at 100 *g* for 10 min with Hanks's balanced salt solution and suspended in 5 ml of HBBS prior to counting and adjusting to a concentration of 7×10^6/ml.

Phagocytosis of Candida

(1) The following combinations are prepared in Eppendorf plastic centrifuge tubes using a ratio of 1:4 (neutrophil v. *Candida* at 7×10^6 and 28×10^6/ml respectively).

Volume

0.062 ml AB serum, autologous serum or control serum.
0·062 ml HBSS.
0·062 ml Leucocytes (7×10^6/ml).
0·062 ml Candida (28×10^6/ml).

All reactions are carried out in duplicate and a *blank* is incorporated with no neutrophils as well as comparing all reactions with an age/sex matched control where possible.

(2) The tubes are incubated for 1 hour at 37° C with continuous slow mixing and then slide smears are made of all the suspensions and also of a centrifuged pellet.

(3) The slides are air dried, fixed in methanol and stained with May-Grunwald-Giemsa in the usual way (see NBT, p. 47.18).

(4) One hundred cells are examined microscopically from each culture and the per cent phagocytosis and the phagocytic index calculated.

Candida killing

(1) The following reaction mixtures are prepared in Eppendorf plastic centrifuge tubes.

Volume

0·062 ml AB or autologous serum or control serum.
0·062 ml HBSS.
0·062 ml Leucocyte suspension (7×10^6/ml).
0·062 ml Candida suspension (7×10^6/ml).

All reactions are carried out in duplicate with an appropriate blank and an age/sex matched control where possible.

(2) The tubes are incubated at 37° C for 1 hour with continuous mixing.

(3) A slide smear is made of each culture after 30 min to check that phagocytosis is taking place.

(4) After 60 min incubation 0·062 ml of sodium deoxycholate (pH 8·7) is added to each culture to lyse the neutrophils and then 1 ml of ice-cold 0·01 per cent methylene blue is added to each tube prior to centrifuging at 400 *g* for 5–10 min.

(5) The supernatant is removed and the cells suspended in the remaining volume and mixed thoroughly. The tubes may be left on ice for up to 2 hours, until examination.

(6) The suspension is examined on a haemocytometer and 300 Candida cells counted and the percentage killed (blue) scored.

Method for quantitative phagocytic and NBT assay (after Stossel [49])

Principle

A technique for assaying the rate of ingestion by phagocytes from human peripheral blood. Isolated phagocytic cells ingest *E. coli* lipopolysaccharide-coated paraffin oil droplets containing oil red O. The pre-ingested particles are opsonized with fresh human serum. After incubation, the uningested oil particles are separated from the cells containing ingested particles by centrifugation. Oil red O is extracted from the washed cell pellets with dioxane and measured spectrophotometrically. A simultaneous quantitative determination of nitroblue tetrazolium reduction (NBT) can also be included.

Materials

(1) Oil red O from Gurr Chemicals (Searle Diagnostics Ltd.).

(2) Paraffin oil from BDH Chemicals.

(3) p-Dioxane from BDH Chemicals.

(4) Hanks's buffered salt solution pH 7·4 (HBSS).

(5) *E. coli* lipopolysaccharide (026:B6 Difco) 10 mg/ml in HBSS.

(6) Nitroblue tetrazolium (BDH) 2 mg/ml filtered solution in HBSS. Stored at 4° C in the dark.

(7) n-Ethylmaleimide (Koch Light Laboratories Ltd., Colnbrook, Bucks., UK).

(8) Fresh human serum.

Procedure

Granulocyte isolation

Granulocytes are prepared as outlined under Superoxide radical (see p. 47.26).

Cells are resuspended at a concentration of 10×10^6/ml.

Preparation of oil red O in paraffin oil

Method (1)

2 g oil red O is added to 50 ml heavy paraffin oil (density 0·89) in a mortar and ground carefully. The saturated suspension is centrifuged hard in an MSE centrifuge to remove undissolved dye (re-usable). The dye-containing oil can be stored indefinitely at room temperature.

Method (2)

Using di-iso-decyl phthalate instead of heavy paraffin oil. Prepare as for method (1)—a more viscous solution which does not pack so well.

To permit normalization and comparison of results a factor conversion for optical density (O.D.) of dye to mg paraffin oil is calculated. 10 μl oil red O is added to 10 ml p-dioxane and O.D. read at 525 nm.

K: conversion factor (mg/O.D.)

$$K = \frac{1}{\text{O.D./mg} \times 0{\cdot}89}$$

$$K = \frac{0{\cdot}89 \text{ (density of oil)}}{\text{O.D. actual reading at 525 nm}}$$

Preparation of particles

3 ml of 10 mg/ml *E. coli* lipopolysaccharide solution is dispersed by brief sonication (5 sec). 1 ml of oil red O oil is layered over the aqueous solution and sonicated for 45–90 sec (the probe being just below the oil–aqueous interface).

Preparations can be frozen at −20° C and thawed and sonicated before use.

Standardization of NBT solution (2 mg/ml)

To permit standardization and obtain a conversion factor from O.D. to μg formazan, a known weight of NBT was reduced to formazan (blue precipitate) and the O.D. measured at 580 nm.

Conversion factor from O.D. to μg formazan is 14·4 per 1 cm of light path in p-dioxane at 25° C.

Opsonization of particles before ingestion

An equal volume of fresh serum is added to the prepared emulsion and incubated at 37° C for 25–30 min to give optimal C3 deposition.

Assay

Using siliconized glass tubes for incubation procedure:

(1) The following reaction mixtures are prepared.

	1	2	3	4	
Opsonized particles	0·2	0·2	0·2	0·2	ml
Cells (10×10^6/ml)	0·4	0·4	0·4	0·4	
HBSS pH 7·4	0·4	—	0·4	—	
NBT (2 mg/ml)	—	0·4	—	0.4	

Total reaction volume = 1 ml

Tube 1—measures rate of ingestion
2—measures rate of NBT reduction
3—measures rate of ingestion (control on ice)
4—measures rate of NBT reduction (control on ice)

(2) Start reaction by adding cells to other prewarmed (37° C) premixed reagents.

(3) After 5 min incubation in a shaking water bath at 37° C, stop reaction by adding 6 ml ice-cold 1 mmol/l *N*-ethylmaleimide in 0·15 mol/l NaCl to each tube. Centrifuge at 1500 rpm for 15 min.

(4) Carefully discard the rim of oil red O and uningested cells by shaking tube vertically and pour off supernatant. Resuspend pellet and repeat *N*-ethylmaleimide wash.

(5) Drain pellets and wipe sides of tubes with dioxane soaked tissue to remove any uningested dye.

(6) Add 1·5 ml dioxane to cell pellet to solubilize the oil red O.

(7) To complete dissolution of any precipitated formazan in tube 2 or 4, place tubes in a boiling water bath for 15 min.

(8) Centrifuge to remove cell debris and measure O.D. of the supernatants.
Measure O.D. for tubes 1 and 3 at 525 nm.
Measure O.D. for tubes 2 and 4 at 580 nm.

Calculation of results

Tubes 1 and 3:

$$\text{Initial rate of ingestion} = \frac{\text{O.D. (525 nm)} \times \text{K (conversion factor)}}{\text{time of incubation} \times \text{no. of phagocytic cells} \times 10^7}$$

= mg paraffin oil ingested/10^7 cells/min.

Tubes 2 and 4:

$$\text{• Rate of NBT reduction} = \frac{(\text{O.D.(580 nm) tube 2}) - (\text{O.D.(580 nm) tube 1}) \times 14{\cdot}14}{\text{time of incubation} \times \text{no. phagocytic cells} \times 10^7}$$

= μg formazan/10^7 cells/min.

Comments
Since oil red O itself does absorb at 580 nm, then the O.D. (580 nm) of extracts containing oil red O alone are subtracted from those containing oil red O and NBT (see calculation above •).

Method for determination of superoxide radical production (after Weening *et al.* [52])

Principle
The superoxide radical (O_2^-) is a highly reactive intermediate produced in biological systems by the one-electron reduction of oxygen and is generated by phagocytes during the 'metabolic burst'. It has been shown that O_2^- is an intermediate in the formation of H_2O_2 and may participate in bacterial killing. Detection of O_2^- is based on its ability to reduce cytochrome C when incubated with isolated granulocytes. This reaction is inhibited by superoxide dismutase, an enzyme which destroys O_2^- activity.

Materials
(1) Horse-heart cytochrome C and superoxide dismutase from bovine blood are obtained from Miles Laboratories.
(2) Dow-latex (10 per cent solution) from Serva. Used as a 1 per cent solution.
(3) Washing medium (a phosphate-buffered saline solution at pH 7·4) with 5·5 mmol/l glucose.
(4) Incubation medium (as for washing medium with 0·5 per cent albumin (or 0·5 per cent pooled AB serum) and 5·5 mmol/l glucose).
(5) 6 per cent Dextran 70.
(6) Solution for lysing red cells—0·8 per cent NH_4Cl, 10 mmol/l KCO_3, 0·1 mol/l EDTA, pH 7·4 at 0° C.
(7) Serum treated zymosan (10 mg/ml). Serum is added to zymosan A (Sigma) and homogenized with ice-cold Potter-homogenizer. Incubate mixture for 20 min at 37° C, mixing frequently. Wash twice with 0·9 per cent NaCl. Resuspend to appropriate volume.

Procedure

Preparation of granulocytes
(1) Approximately 15 ml blood is adequate for one assay. Heparinized blood is mixed with half its volume of 6 per cent Dextran 70 in 0·9 per cent NaCl and the erythrocytes allowed to sediment at 37° C for 30–40 min at an angle of 45°.
(2) Remove supernatant plasma containing granulocytes. Centrifuge at 100 *g* for 10 min.
(3) Resuspend cell button in ice-cold lysing solution and leave on ice for 5–10 min with frequent mixing. This lyses any remaining erythrocytes.
(4) Centrifuge at 100 *g* for 10 min. Wash cells twice in washing medium and resuspend cell button in 5 ml. Count granulocytes and resuspend to final concentration of 2×10^6/ml in incubation medium.

Incubation procedure
(1) The following reaction mixtures are prepared:

	1	2	3	4
Cells (2×10^6/ml)	0·85	0·85	0·85	0·85 ml
STZ (10 mg/ml)	—	0·1	—	0·1
or Latex particles				
0·9 per cent NaCl	0·12	0·02	0·1	—
1 mM Cyt. C	0·05	0·05	0·05	0·05
S.D. (43 μM)	—	—	0·02	0·02

Total reaction volume = 1·02 ml

Tube 1—resting value
2—phagocytosis of serum treated zymosan/latex
3—superoxide dismutase inhibition of O_2^- production (resting)
4—superoxide dismutase inhibition of O_2^- production (stimulated)

(2) Incubate for 1 hour in a shaking water bath (180 cycles/min) at 37° C.
(3) Terminate reaction by transferring samples to ice-cold Eppendorf centrifuge tubes and centrifuge for 6 min.

(4) Store supernatants in Eppendorf tubes in liquid N_2 vapour.

Determination of O_2^-

Using Pye Unicam SP 800 Recording Spectrophotometer.

(1) The absorbence of cytochrome C in the samples is measured from 540–560 nm.

(2) To determine the amount of reduced cytochrome C, one grain of potassium ferricyanide was added to the sample in the cuvette and the decrease in absorbence followed at 550 nm (i.e. oxidation of cytochrome C).

(3) Some grains of sodium dithionite are added to show total amount of cytochrome C in the incubation mixtures and the increase followed from 540–560 nm.

Absorbence coefficient of (red-ox.) cytochrome C is 21·1 mmol/l^{-1} cm^{-1}. The results of O_2^- determination are expressed as μ moles cyt.C reduced/10^{10} cells/hour.

Comments

Identical control reaction mixtures can be kept on ice as reference blanks. These samples would have the same amount of reduced cytochrome C as those containing superoxide dismutase.

Method for determination of hexose monophosphate shunt activity (after Pachman *et al.* [53])

Principle

The respiratory burst following phagocytosis is accompanied by an increase in oxygen consumption and glucose oxidation through the hexose monophosphate shunt pathway (HMP). Stimulation of the HMP is determined by measuring the amount of 1-^{14}C-glucose converted to $^{14}CO_2$.

Materials

(1) Dow latex (10 per cent solution) from Serva.

(2) Zymosan A (Sigma). Serum treated (see Superoxide radical, p. 47.26).

(3) D-(1-^{14}C) glucose from Radiochemical Centre, Amersham, Bucks., UK, diluted to concentration of 20 μCi/ml with 0·9 per cent NaCl.

(4) Hyamine hydroxide from Nuclear Enterprises Ltd.

(5) Sodium azide (NaN_3) from BDH Chemicals.

(6) Washing medium (phosphate buffered saline pH 7·4 with 5·5 mmol/l glucose).

(7) Incubation medium (phosphate buffered saline pH 7·4 with 0·5 per cent albumin or 0·5 per cent pooled AB and 5·5 mmol/l glucose).

(8) NE 260 scintillation fluid from Nuclear Enterprises Ltd.

Procedure

Isolation of granulocytes is as for O_2^- determination (see p. 47.26).

The granulocytes are used at a final concentration of 4×10^6/ml in phosphate buffered medium (pH 7·4) with 5·5 mmol/l glucose and 0·5 per cent human pooled AB serum.

Incubation assay

(1) The following reaction mixtures are prepared:

	1	2	3	4	
Cells (4×10^6/ml)	0·85	0·85	0·85	0·85	ml
G 1-^{14}C (0·4 μCi)	0·002	0·002	0·002	0·002	
STZ/latex	—	0·1	—	0·1	
0·9 per cent NaCl	0·128	0·028	0·128	0·028	
100 mM NaN_3	0·02	0·02	0·02	0·02	

Total reaction volume = 1 ml

Tube 1—resting value
2—phagocytosis of serum treated zymosan (STZ) or latex
3—control for resting value } on ice
4—control for phagocytosis }

(2) Tubes 1 and 2 are stoppered and incubated at 37° C for 1 hour in a shaking water bath. The CO_2 is trapped in 0·5 ml hyamine hydroxide in a separate vial attached to the stopper of the incubation tube.

(3) The reaction is stopped by injecting 1 ml 2N HCl through the rubber stopper. Incubation for a further 30 min at 37° C ensures maximum CO_2 absorption.

(4) The hyamine hydroxide is dissolved in 9·5 ml of scintillation fluid and the radioactivity counted in a liquid scintillation counter. Results are expressed as counts per minute.

Comments

Addition of the metabolic inhibitors NaN_3 or KCN (inhibiting catalase or myeloperoxidase activity) ensures there is added stimulation of HMP activity.

INVESTIGATION OF THE COMPLEMENT SYSTEM (see also Chapter 5)

Defects of the complement system in association with primary immune deficiencies are extremely rare.

Genetically determined deficiencies of C3 have been reported [54] as well as an extensively investigated patient with an absence of the naturally occurring C3b inactivator [55]. Certain families have been described with unusual skin conditions and bacterial infections who responded to treatment with infusions of fresh plasma. These patients are said to have a functional abnormality of C5 as assessed by defective opsonic activity and generation of chemotactic factors [56]. However, in these individuals C5 was antigenically and haemolytically intact. Furthermore, an individual with complete absence of C5 has been reported who does not have these defects.

In general measurement of the total haemolytic complement (CH_{50}) and the individual components C3 and C4 is a satisfactory 'complement screen'. Thus normal levels of C3 and C4 with an absent CH_{50} are suggestive of a congenital defect of one of the individual components required to complete the haemolytic sequence. Such deficiencies are, by and large, unassociated with recurrent infections although various connective tissue disease-like states have been described in individuals with absence of C2 or the subunits of C1 [57]. A low C4 suggests activation of the classical components by immune complexes whereas a low C3 with a normal C4 indicates alternate pathway activation such as found in association with certain forms of mesangiocapillary glomerulonephritis and certain types of partial lipodystrophy [58]. Many of these conditions cannot be considered as primary immune defects although the inclusion of a 'complement screen' should be included in the general immune profile especially as this is a rapidly developing field in terms of the association of complement defects with disease. It should be noted that complement components like other plasma proteins can be antigenically intact but functionally inactive. An example is genetic variants of C1 esterase inhibitor deficiency. If complement defects are recognized then an extended complement profile is required in terms of measurements of other individual components of the classical and alternate pathways as well as measurements of terminal complement components.

Estimation of the concentrations of C3 and C4 can be measured by the Mancini technique (see p. 47.13).

Method for determination of the total haemolytic complement (CH_{50}) (after Fischer [59])

Principle

The haemolysis of sensitized sheep cells is followed with a continuous recording spectrophotometer and the time for 50 per cent lysis of the indicator sheep cells (CH_{50}) is a function of total complement activity.

Materials

(1) 0·15 mol/l sodium chloride.

(2) 0·01 mol/l EDTA gelatin Veronal buffer composed of:

5 × Veronal 200 ml
10 per cent gelatin 10 ml
Distilled water to 1 litre

The gelatin Veronal buffer is used to dilute a stock solution of EDTA (e.g. 1 mol/l) to a molarity of 0·01 mol/l.

(3) Dextrose gelatin Veronal buffer made up fresh each day by mixing equal volumes of (i) and (ii).

(i) Composed of:

5 × Veronal 200 ml
10 per cent gelatin, 10 ml
0·03 mol/l calcium chloride, 5 ml
0·1 mol/l magnesium chloride, 5 ml
Distilled water to 1 litre

(ii) Composed of:

Dextrose 50 g
0·03 mol/l calcium chloride, 5 ml
0·1 mol/l magnesium chloride, 5 ml
Distilled water to 1 litre

Solutions (i) and (ii) are made up fresh every day and stored at 4° C.

(4) Sheep cells (E) in Alsever's solution stored at 4° C. Usable for up to one month.

(5) Sheep erythrocyte antibody (A). Prepared in rabbits by repeated injections of autoclaved sheep erythrocyte stroma. The antisera are heat treated at 56° C for 30 min. The optimum concentration of the antibody to use is obtained by titration against sheep cells in a known source of complement.

(6) Disposable 1 cm plastic cuvettes (cat. no. 45/1010, Sarstedt, Leicester, UK).

Procedure

Preparation of sensitized sheep cells (EA)

(1) 50 ml (optimal) of sheep cells in Alsever's are washed twice in 0·15 mol/l NaCl and then once in 0·01 mol/l EDTA in gelatin Veronal buffer. The cells are adjusted to a concentration of 1×10^9 cells/ml using either the spectrophotometer or a haemocytometer.

(2) Antibody is added to the sheep cells at the optimal concentration. For example, 0·1 ml of antibody with a titre 1/300 is added to 30 ml of sheep cells.

(3) The mixture is incubated at 37° C for 30 min in a shaking water bath followed by incubation at 0° C for a further 30 min.

(4) The cells are washed once in EDTA gelatin Veronal buffer and then twice in dextrose gelatin buffer with added magnesium and calcium. They can be stored in the latter for up to 2 weeks at 4° C providing the buffer is changed daily.

Estimation of haemolytic complement (CH_{50})

(1) The sensitized sheep cells (EA) prepared as outlined above are washed three times in dextrose gelatin Veronal buffer with added magnesium and calcium and adjusted to 5×10^8 cells/ml using a spectrophotometer. This suspension is diluted approximately 1:26 to give an optical density of 0·9 in a 1 cm cuvette using transmitted light at 545 mμ. These figures may vary somewhat from instrument to instrument.

(2) For an assay the following is added to each cuvette—0·1 ml of the serum under test, 0·5 ml of dextrose gelatin Veronal buffer with magnesium and calcium and 2·4 ml of EA solution at 37° C. The latter is added fiercely to ensure mixing and then recording started immediately. A continuous recording spectrophotometer with a carrier holding four cuvettes and a chart recorder is used. Controls necessary are a cuvette containing 3·0 ml of the dextrose gelatin Veronal buffer with added calcium and magnesium (blank) and a pooled serum control for comparison with the test serum.

(3) The results are calculated using the mean height of the peak and base. From this a time for 50 per cent haemolysis can be estimated. This figure is then expressed as a percentage of the normal human pool, e.g.

$$\frac{\text{Normal human pool time in seconds} \times 100}{\text{Test serum time in seconds}}$$

SUMMARY

It must be emphasized that the technology and approach to the investigation of immune deficiencies is a rapidly developing field and therefore it is not possible to give a comprehensive list of investigations for use as standard practice. Many of the tests described are laborious, expensive and require considerable technical skills. Furthermore, the number of patients where these laboratory investigations are of value in the diagnosis, management and treatment is relatively small. Most laboratories will inevitably have to make compromises and it is our practice to have a three-tiered screen along the lines indicated in Table 47.1. For instance, extensive tests of lymphocyte function are not usually helpful in the presence of positive delayed-type skin tests to the common antigens. Similarly, if the white cell and differential counts are normal and the patient responds in the skin-window test, reduces NBT and has a positive myeloperoxidase stain it is in general unlikely that further investigations of phagocyte function will yield additional information. However, as has already been emphasized, a careful clinical history will be the main guide as to what emphasis should be placed in terms of the various laboratory investigations.

TABLE 47.1. Laboratory investigations in immunodeficiency

Primary Screen
White cell count and differential
Immunoglobulins (IgG, IgA, IgM and IgE)
Antibodies (isohaemagglutinins, anti-viral antibodies, *E. coli* antibodies)
Complement screen (CH_{50}, C3 and C4)
Delayed-type skin tests (PPD, Candida, SK–SD and mumps)
Skin window (Rebuck) test
NBT test and myeloperoxidase stain
Candida killing
*Secondary Screen**
T and B lymphocyte estimations
Lymphocyte transformation (mitogens, antigens, allogeneic cells)
Phagocytic function (chemotaxis, ingestion and killing of *E. coli*, *S. aureus*, etc.)
Anti-leucocyte antibodies
Extended complement profile
Salivary immunoglobulins (especially IgA)
Response to immunization with tetanus toxoid
*Tertiary Screen**
Lymphokine production
Extended tests of phagocyte function (Stossel assay)
Immunofluorescence of tissue sections (biopsy material)
Antigen transformation to any relevant antigen not included in initial screen

* Done where indicated by results of primary screen

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