STUDIES ON ANTI-LYMPHOCYTIC ANTIBODIES AND OTHER PROTEINS

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TO MY WIFE

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SUMMARY

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

Section A - Describes the development of preparative ultracentrifugal and chromatographic procedures which have been used extensively in characterizing and isolating the proteins described in the subsequent sections.

Section B - This comprises the major part of the work and consists of a detailed investigation of the properties of heterologous antisera to mouse, rat, dog and human lymphoid tissue. These antisera have been fractionated by a variety of physico-chemical procedures including G200 sephadex gel filtration, DEAE cellulose chromatography and Porath column electrophoresis, and the major part of the antibody activity was found to be associated with the IgG fraction of most of the antisera. The whole sera and/or their IgG fractions have been shown to agglutinate, lyse and transform lymphocytes in vitro, delay the rejection of skin allografts in mice and rats and renal allografts in dogs, suppress the graft versus host reaction in mice and the onset of experimentally induced thyroiditis and autologous complex nephritis in rats. Further studies in rats have shown that anti-lymphocytic antibody also suppresses the primary humoral response to alum precipitated bovine serum albumin and sheep erythrocytes and that this effect may be antigen and strain dependent. In contrast cell transfer and other experiments revealed that anti-lymphocytic antibody appears to have little effect on the secondary humoral response of sensitized lymphoid tissues. Finally in an attempt to elucidate the mode of action of anti-lymphocytic antibody, detailed investigations have been performed with the divalent ($F(ab')_2$) and univalent (Fab') fragments obtained by pepsin digestion of the intact IgG molecule, and with the non-

cytotoxic derivative obtained by acid treatment. These studies revealed that the immunosuppressive properties of anti-lymphocytic IgG are dependent upon the antibody molecule possessing an intact Fc region.

Section C - The physico-chemical and immunological changes occurring in normal human IgG on storage have been studied and methods of isolating an abnormal 7S gamma globulin (the P M protein) and an abnormal 19S gamma globulin (rheumatoid factor) are described together with their immunological and physico-chemical properties.

Section D - The immunological properties of human serum α_2-macroglobulin and a low molecular weight ($S_{20}W \simeq 3S$) derivative obtained by pepsin digestion have been compared with the α_2-macroglobulin homologues of other species. The amounts of this protein in normal, pathological and cord sera have been determined immunologically and its effects upon the proteolytic and esterolytic activity of a number of enzymes have been investigated.

Section E - The final section details preliminary immunosuppressive studies in rodents with 'naturally' occurring and synthetically prepared polyribonuclease derivatives. Additional publications outline investigations on synovial cell proteins and on the lymphocyte transforming products of staphylococcal filtrate.

SECTION A - PROTEIN FRACTIONATION PROCEDURES

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Application of Zone Centrifugation to the Study of Normal and Pathological Human Sera

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INTRODUCTION

Since the initial work of Pickels (7) on sedimentation in the angle centrifuge, there has been relatively little application of density-gradient centrifugation to the analysis of serum and other protein mixtures. De Lalla and Gofman (2) have applied flotation procedures to the study of lipoproteins, and Hogeboom and Kuff (6) have used the preparative ultracentrifuge in the determinations of sedimentation coefficients of proteins over a 4 to 2000 S range. The main use of density-gradient centrifugation has been, however, in the fractionation of cellular particles.

Following the success of its application in this field, it seemed worthwhile attempting to develop the technique for the quantitative analysis of protein mixtures on a molecular level. The results obtained by zone electrophoretic methods, such as those developed by Flodin and Porath (4), have already indicated the value of preparative fractionation procedures which afford a measure of the composition of the system under investigation as well as providing fractions for further analysis. Moreover, zone centrifugation of human serum should yield data complementary to that obtained from zone electrophoresis, fractions being separated according to their molecular size rather than charge. The significance of such an approach is well illustrated by the results reported by Wallenius *et al.* (10). These workers studied the distribution of ultracentrifugal components in the various zones obtained by starch-block electrophoresis of human serum and found that nearly all the major electrophoretic fractions were heterogeneous in terms of composition of sedimenting constituents.

An additional advantage of the zone-centrifugation procedure is that it provides a valuable alternative to the use of partition ultracentrifuga-

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tion cells in the characterization of biologically active substances, as was shown recently by Stanworth (9) in a study of the physicochemical properties of horse-dandruff reagin. This report will be confined, however, to a description of the technique employed and its application to the comparative analysis of normal and pathological human sera.

EXPERIMENTAL AND RESULTS

Zone-Centrifugation Technique

In all experiments a *gradient differential* technique has been employed, the tube contents being stabilized against convection and other disturbances by means of buffered sucrose gradients. This is the development of a method used previously by Edelman *et al.* (3) in the purification of rheumatoid factor. Those workers fractionated 1-ml samples of serum (in 5-ml tubes in a swing-out rotor) above layers of 10 and 30% buffered sucrose solutions. A modification of this method was used by Stanworth (9) in the characterization of skin sensitizing antibodies. More recently, however, the technique has been refined with a view to achieving greater resolution of the complex mixture of proteins comprising human serum.

Ideally, of course, maximal resolution would be expected by starting with an infinitely narrow band of an infinitely dilute protein solution. Obviously, for practical reasons, it was necessary to compromise, and so by starting with 0.5 ml serum (in a 5-mm deep band), fractions of sufficiently high concentration for subsequent analysis and biological testing were obtainable. Moreover, by narrowing down the band as compared with the width (10 mm) of the starting sample used in the initial studies, it was possible to extend the effective length of separation.

The arrangement found to give optimum resolution of human serum is that shown in Fig. 1. A combination of 10 and 30% buffered sucrose solutions proved to be the most effective of several tested. Such solutions were dense enough to uphold the starting band of serum and to stabilize the separated protein bands, both during centrifugation (at a temperature of 12°C) and afterward at room temperature. In this medium only the high density lipoproteins (of density greater than 1.063) would sediment. This means that the zone-centrifugation procedure frees the serum of the low density lipoprotein constituents, which remain in the starting layer well above any non-lipid-containing protein zone. In this respect, it is interesting to note that one serum showed three discrete lipoprotein bands near the top of the tube after zone centrifugation.

A small volume (0.3 ml) of carbon tetrachloride at the bottom of the tube is useful in avoiding pellet formation by the high molecular weight

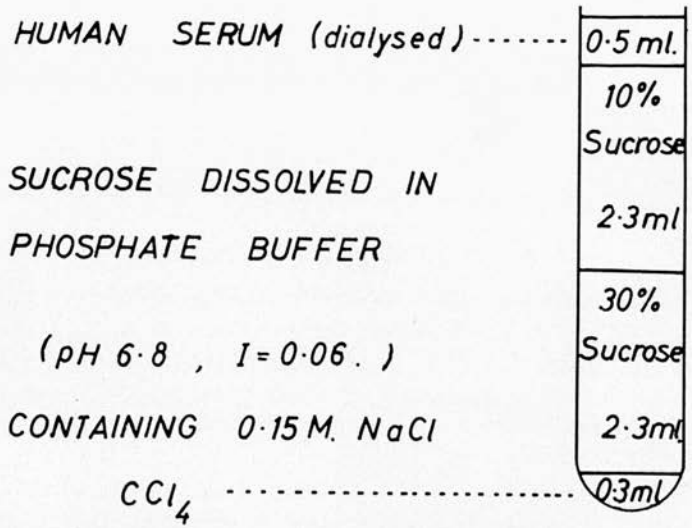

FIG. 1. Zone-centrifugation procedure, showing initial arrangement and positions of slicing of tube.

serum protein constituents—a process which can lead to insolubility and loss of biological activity. With such an arrangement it is most important to preclarify the serum by centrifugation for 30 min at 10,000 rpm and 5°C. Otherwise particulate matter will carry down adsorbed serum proteins nonspecifically, and these become layered above the carbon tetrachloride.

Lusteroid tubes, carefully selected for uniformity in shape and bore, are set up in this way, using solvents precooled to 4°C. They are then centrifuged in precooled buckets in a Spinco SW 39 swing-out rotor (refrigerator setting of −1°C). This cools the tube contents to 12.5°C during the run. Afterward the tubes are immediately sliced in a mechanical slicer of similar design to that described by Randolph and Ryan (8). By slicing systematically with a highly sharpened blade it is possible to obtain as many as 13 sections (each 3 mm deep) from a single tube.

If the tube and blade are well lubricated with silicone grease, 80% recoveries of liquid are achieved indicating an average loss of about 0.08 ml at each slicing. The final fraction is usually sucked off from above the carbon tetrachloride with a pipet. No other form of sampling the contents of the tube after centrifugation has been tried. The successive slicing of tubes containing colored protein solutions indicated that this form of sampling causes only slight disturbance of separated protein zones.

Use of Dye-Labeled Reference Proteins

Selection of suitable positions of slicing of the tubes after zone centrifugation of human sera has been guided by the use of reference proteins labeled with various colored dyes. A purified protein from each of the main sedimenting classes of serum proteins, namely 4.5, 7, and 19 *S*, has been labeled with a different colored dye and used in this way. Thus albumin has been labeled with Evans Blue (and more recently bromophenol blue). γ-Globulin has been labeled with Brilliant Procion Yellow (and Red) and 19 *S* α_2-glycoprotein[3] with Brilliant Procion Red.

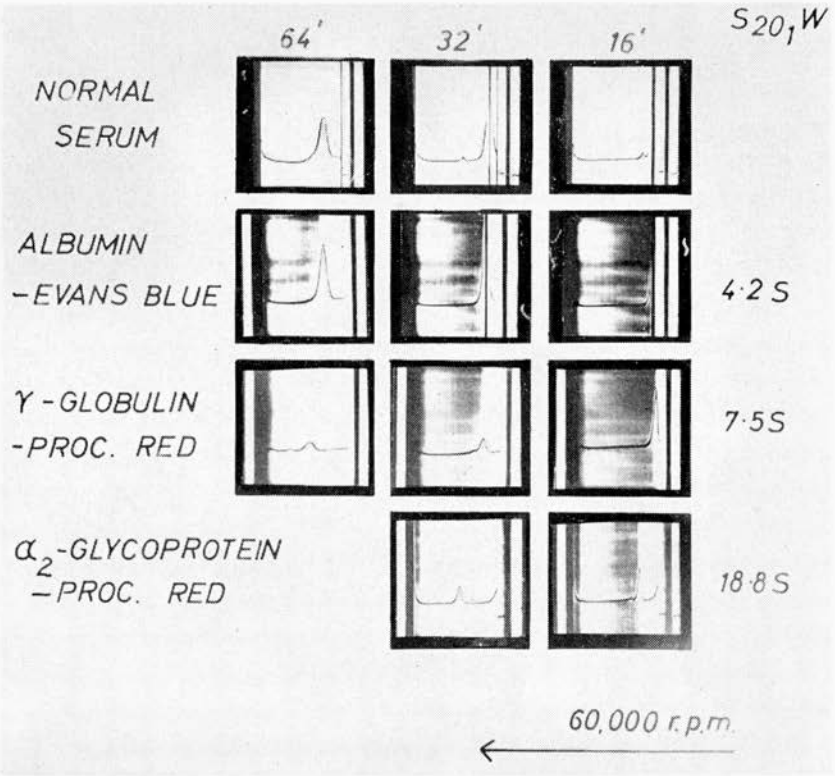

FIG. 2. Analytical ultracentrifugal patterns of dye-labeled albumin, γ-globulin, and α_2-glycoprotein. The sedimentation coefficients ($S_{20,w}$) of the labeled proteins are included.

[3] A purified preparation of this high molecular weight glycoprotein constituent of normal human serum was obtained by a method (D. R. Stanworth, 1960; unpublished work) involving zone centrifugation of a fraction (comprising only 4.5 and 19 *S* components) eluted from a diethylaminoethylcellulose (DEAE-cellulose) chromatography column with 0.05 *M* NaH_2PO_4 solution.

The procion dyes are cellulose-reactive dyes possessing a triazine chloride radical via which they become linked to proteins. They have been used by Hess and Pearse (5) for labeling antigens. It is easy to irreversibly precipitate the protein during the labeling procedure, which is accomplished merely by mixing with dye in buffered (pH 7.4) saline at room temperature for 30–60 min and then dialyzing away the unbound dye. The coupling of γ-globulin with Procion Yellow proved particularly difficult in this respect, whereas it was much easier to couple Procion Red on to this and other proteins.

As is shown in Fig. 2, ultracentrifugal analysis of the labeled proteins in a Spinco model E machine indicated that the dye-binding had not affected the sedimentation coefficients of the proteins. Here the ultracentrifugal patterns of the labeled proteins are compared with the pattern of normal human serum.

Comparative zone centrifugation of the three labeled proteins in separate tubes was carried out at varying times in order to establish optimum conditions for achieving complete resolution of a mixture in the same tube. Such a separation was ultimately accomplished by centrifuging for 16 hr at 39,000 rpm, the carbon tetrachloride layer preventing the pelleting of the labeled 19 *S* α_2-glycoprotein (Fig. 3). Primary colors

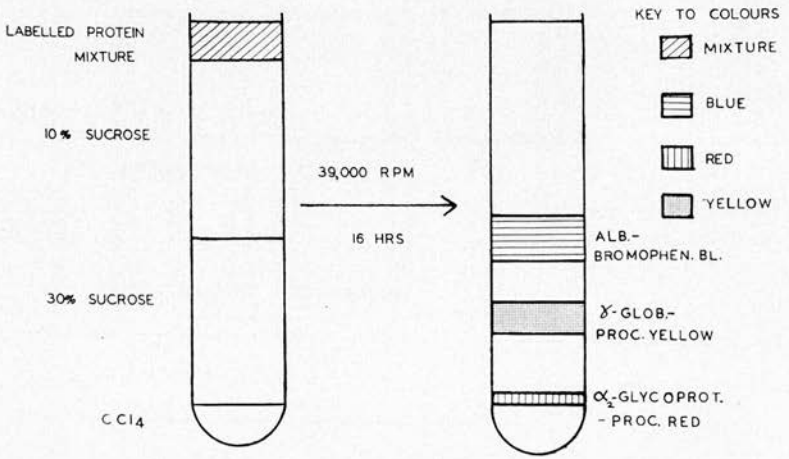

FIG. 3. Result obtained by zone centrifugation of a mixture of dye-labeled albumin (4.5 *S*), γ-globulin (7 *S*), and α_2-glycoprotein (19 *S*).

were chosen deliberately as dye labels so that any overlapping would be revealed as secondary color formation. There was no evidence of any such effect in the separation shown in Fig. 3.

The tubes containing the labeled proteins were afterward sliced according to the separation of the colored zones. Gel-diffusion precipitin analysis of the fractions thus obtained (employing rabbit antihuman serum) revealed only slight overlapping of the labeled proteins. Moreover, ultracentrifugal analysis indicated that the labeled proteins were still intact after zone centrifugation.

It has been the custom to include a tube containing a mixture of the different labeled proteins in each zone centrifugation analysis of human serum. By placing such a reference tube, after the run, directly alongside the serum tubes in a plastic holder, it is possible to select the best positions of slicing in order to obtain maximal separation of 4.5 from 7 *S* serum component and of 7 from 19 *S* component.

Application of the Zone-Centrifugation Technique to the Analysis of Pathological Human Sera

A problem which sometimes confronts the clinical biochemist is illustrated in Fig. 4, which shows the paper electrophoretic patterns of a normal and two different pathological sera (each containing about 12 gm protein/100 ml). It will be noted that both pathological sera contain an abnormally intense protein band in the γ-globulin region. Serum WA is from a patient showing typical myeloma symptoms, whereas serum WI is from a patient showing no such symptoms. The important question left unanswered by the paper electrophoresis is whether serum WI contains abnormal macroglobulins.

Zone-centrifugation patterns of the two pathological sera and the normal serum obtained by the technique just described are shown in Fig. 5. These patterns were obtained by plotting the total protein in each fraction (as obtained by optical density measurements at 280 mμ on diluted aliquots) against the mean distance from the meniscus. The demarcation positions between labeled reference proteins centrifuged simultaneously are illustrated by the vertical dotted lines. The values shown are the percentages of the total serum protein falling in each of the three main zones. It will be seen at once that serum WI can be differentiated from serum WA by means of its marked content of 19 *S* component. On the other hand, serum WA contains considerably more 7 *S* component than is found in serum WI.

These findings are confirmed by examination of the analytical ultracentrifugal patterns of these sera (shown in Fig. 6). Macroglobulin components comprise 53% of the total protein in serum WI, while 7 *S* component comprises 52% of the total protein in serum WA.

Hence, without having carried out an analytical ultracentrifugal

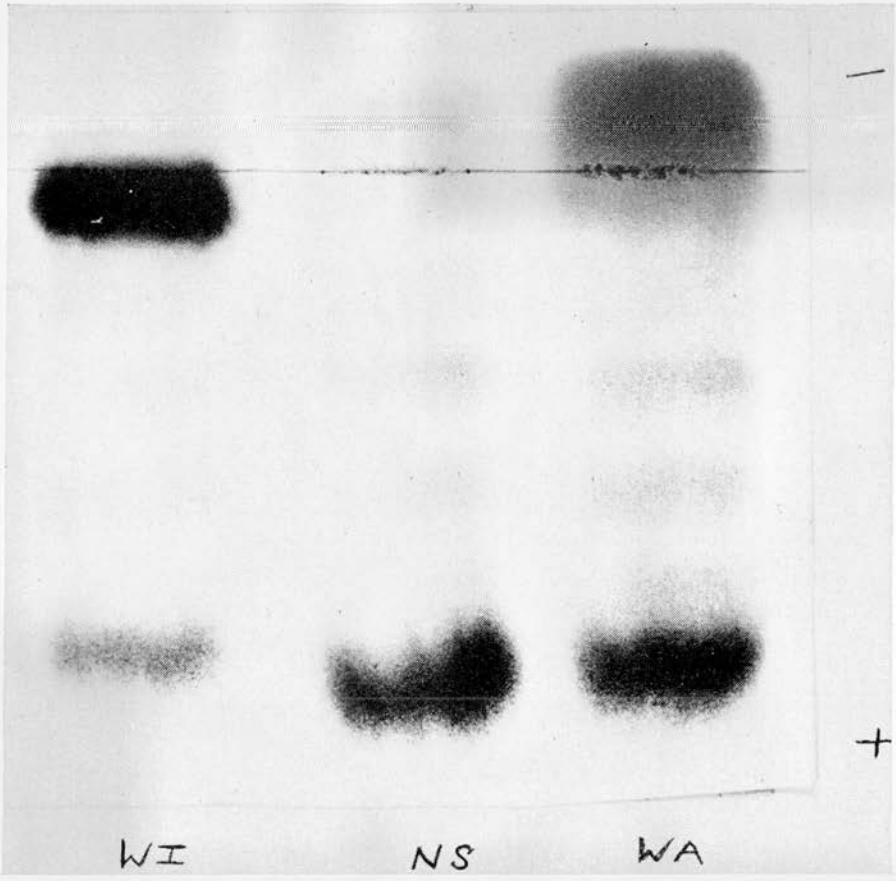

FIG. 4. Patterns obtained by paper electrophoretic analysis (barbitone buffer, pH 8.6, $I = 0.05$) of normal human serum (NS), myeloma serum (WA), and macroglobulinemic serum (WI). Each serum was diluted 1:4 before analysis.

examination of the two pathological sera, it has been possible, by means of a *single* zone-centrifugation analysis, to demonstrate that the serum from patient WI (who failed to show myeloma symptoms) contained an abnormal amount of macroglobulin responsible for the "M" band revealed by paper electrophoresis. Moreover, the pathological serum fractions obtained from the zone-centrifugation procedure were available for further study.

DISCUSSION

Normally, it would be necessary to establish the presence of abnormal macroglobulin in a serum by use of an analytical ultracentrifuge (from

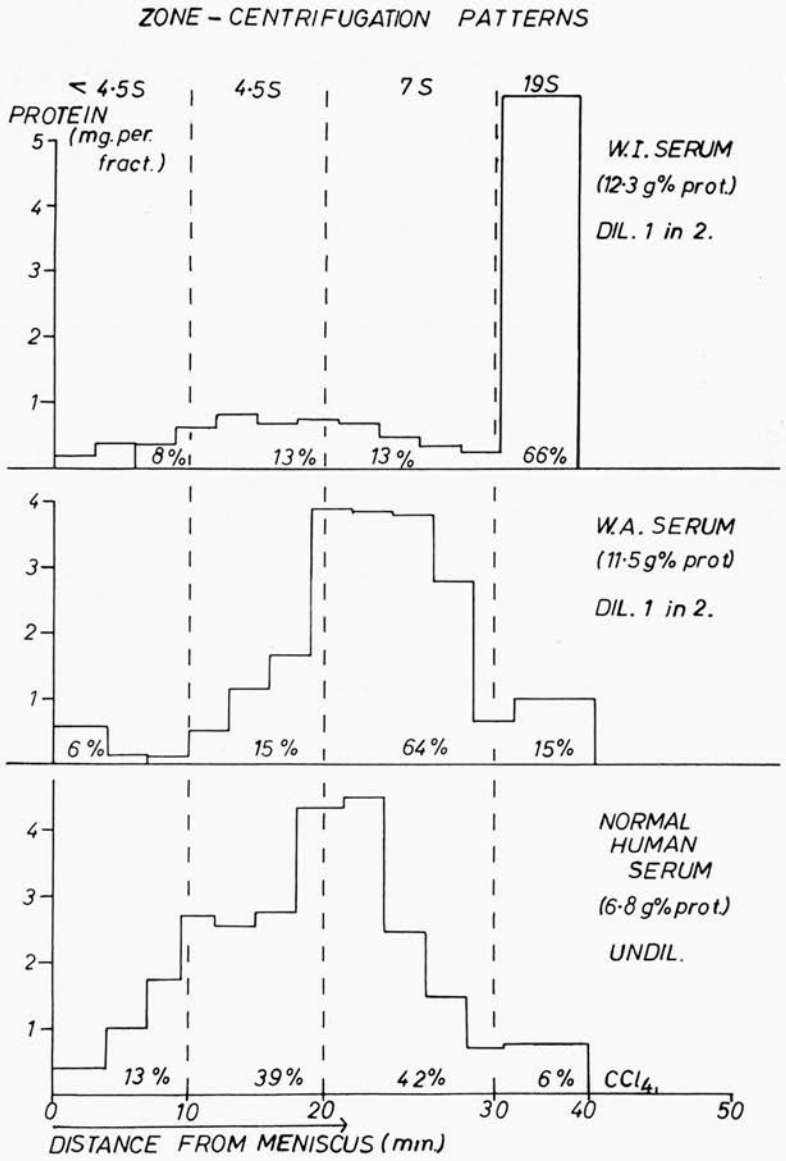

FIG. 5. Zone-centrifugation patterns of normal human serum, myeloma serum (WA), and macroglobulinemic serum (WI). The vertical dotted lines indicate the demarcation levels of the various ultracentrifugal classes of serum proteins as revealed by comparative sedimentation of dye-labeled components. Percentages of total protein falling in the different zones are given.

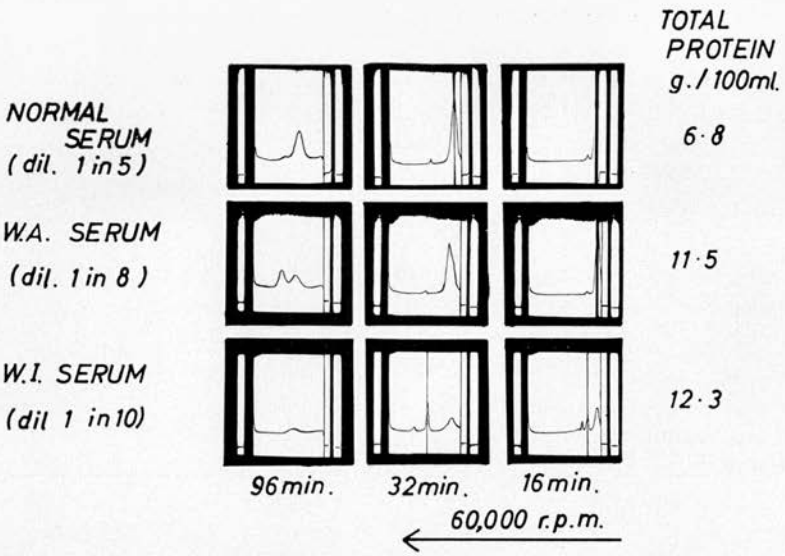

FIG. 6. Analytical ultracentrifugation patterns of normal human serum, myeloma serum (WA), and macroglobulinemic serum (WI). Analyses were carried out at 20°C on diluted sera which had been dialyzed against barbitone buffer (pH 8.6, $I = 0.05$) containing 0.2 M NaCl.

which the separated protein is not recoverable). Providing the concentration of macroglobulin is sufficiently high, however (as in typical cases of Waldenström macroglobulinemia), it can be detected and isolated simultaneously by the zone-centrifugation procedure described here. The inclusion in each run of a tube containing reference proteins labeled with different colored dyes facilitates selection of the positions of slicing of the tubes. Such a practice leads to the isolation of more clear-cut fractions besides serving as an independent index of sedimentation rate.

As might be expected, complete resolution of the relatively low molecular components (3, 4, 5, and 7 S) of whole serum was not achieved. This was particularly noticeable in the zone centrifugation of normal human serum, where comparative paper electrophoresis and gel-diffusion precipitin analyses on the fractions revealed considerable overlapping of the 4.5 S albumin component with a globulin component (probably 5 S siderophilin). In addition, there was some overlapping of the albumin with 7 S γ-globulin.

The results of preliminary zone-centrifugal analyses, involving the use of narrower (2-mm) bands of starting serum, suggest that some of this overlapping could be reduced (at the expense of a reduction in the

yields of the fractions recovered). In this connection the zone-centrifugation pattern of an analbuminemic serum is of interest. A protein deficiency was observed in the 4.5 *S* region, but there was no marked trough as might have been expected in the pattern of a serum containing only 400 mg albumin/100 ml (according to electrophoretic analysis).

There is good reason to assume that the resolution achieved in the zone-centrifugal analysis of serum could be improved even further by adoption of technical refinements such as the use of longer Lusteroid tubes and better methods of sampling. Mixing of the tube contents could be minimized by stricter stabilization of the swing-out rotor during acceleration and decceleration (as employed, for example, by DeDuve *et al.* (1) in the zone centrifugation of cellular particles).

At the moment, it seems necessary to recycle fractions in order to purify serum components other than the 19 *S* macroglobulin. Alternatively, zone centrifugation has been applied to the purification of crude serum protein preparations isolated initially by other complementary fractionation procedures (such as DEAE cellulose chromatography).

SUMMARY

The development of a zone-centrifugation technique suitable for the fractionation of soluble protein mixtures such as human sera is described. This involves gradient differential sedimentation in buffered sucrose at 39,000 rpm in a swing-out rotor.

Selection of optimum positions of slicing of the tubes (Lusteroid) after centrifugation is indicated by the use of reference proteins of various molecular sizes (4.5, 7, and 19 *S*) labeled with different colored dyes. Pelleting of the high molecular weight (19 *S*) components of serum is avoided by placing a layer of carbon tetrachloride on the bottom of the tube.

Characteristic histograms, representative of serum ultracentrifugal composition, are obtained by plotting the optical densities (at 280 mμ) of fractions (zones) against their distance from the meniscus. It is, for example, possible to distinguish between myeloma and macroglobulinemic sera.

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STUDIES ON THE CHROMATOGRAPHY OF HUMAN SERUM PROTEINS ON DIETHYLAMINOETHYL(DEAE)-CELLULOSE

I. THE EFFECT OF THE CHEMICAL AND PHYSICAL NATURE OF THE EXCHANGER

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(Received December 2nd, 1963)

INTRODUCTION

Although ion-exchange chromatography on substituted cellulose derivatives[1], particularly diethylaminoethyl-cellulose, has found wide application in the fractionation of serum proteins[2,3], surprisingly few detailed investigations have been undertaken to establish the factors influencing the separation of proteins accomplished by this procedure. Recently the need for such investigations have been emphasized (PORTER[4] and PETERSON AND CHIAZZE[5]).

The work to be described here was undertaken with the object of improving the effectiveness of the DEAE-cellulose chromatographic procedure as a means of fractionating serum proteins. In addition to column chromatography, a rapid batch procedure (STANWORTH[6]) has been used to study a number of the factors influencing the ion-exchange process.

Results of studies of the effect of the degree of substitution and physical form of the exchangers, and also the temperature of elution, are reported in this paper (Part 1). The standard procedure thus evolved has been employed in further studies (reported in Part 2), involving the detailed investigation of the chromatographic properties of individual purified serum proteins and artificial mixtures of these.

METHODS

Preparation of samples

Three different samples of human serum were used. Sample 1 (serum separated from a normal human donor) was used in all the column experiments with laboratory prepared exchanger.

Sample 2, used in the column chromatographic experiments on the commercial exchangers, was prepared by the defibrination of pooled acid citrate dextrose (ACD) plasma which had been stored at 4°.

Sample 3 comprised serum separated from a single donation of blood from a rheumatoid arthritic patient and was used in all the batch chromatographic experiments, providing, in addition, data on the chromatographic behaviour of rheumatoid factor.

Prior to chromatography, all the samples were dialysed against 200 volumes of the starting buffer (pH 7.6, 0.01 *M* phosphate) for 20–24 h. The samples were then centrifuged at 3,000 r.p.m. for 10 min, before application to the chromatographic columns.

Adsorbents

The DEAE-cellulose used was obtained from two sources. One sample ("W") was prepared in the laboratory from wood cellulose, according to the method of PETERSON AND SOBER[1]. This exchanger was powder-like in form and had a degree of substitution of 0.87 mequiv./g, as revealed by titration (with *N*/100 hydrochloric acid) of a suspension of the exchanger in 0.15 *M* saline. The other samples were obtained from commercial sources. These exchangers were floc-like in form, having been prepared from fibrous cotton linters. Their exchange capacities ranged between 0.05 and 1.2 mequiv./g. In this series of experiments the exchangers are referred to as C 5, C 25, C 65 and C 120, where the letter "C" indicates their cotton source and the number their degree of substitution in mequiv./g × 100.

CHROMATOGRAPHIC PROCEDURE

The exchanger "W" (2 g) was washed successively with two 50 ml volumes of 0.05 *M* NaH_2PO_4 solution and phosphate buffer (pH 7.6, 0.01 *M*) before being suspended in 30 ml of the latter buffer and poured into a column (diameter 1 cm). The column was then washed overnight with at least 100 ml of phosphate buffer (pH 7.6, 0.01 *M*), the final length of the column being about 16 cm.

Due to the floc-like nature of the "C" type exchangers, even packing of the columns by means of pouring a slurry of the exchanger into the column and applying positive pressure proved difficult. In addition, the low capacity of these exchangers necessitated the use of larger weights of material (see Discussion). The dry exchanger (3 g) was, therefore, packed a little at a time into the column (diameter 1 cm). After packing, the column was equilibrated by washing with 300–500 ml of the pH 7.6, 0.01 *M* phosphate buffer, giving a column of length between 13 and 15 cm.

The batch chromatographic experiments to be described were performed using 0.5 g quantities of exchanger "W", and 1.0 g samples of the "C" type exchangers. These samples were washed initially with 25 ml volumes of 0.05 *M* NaH_2PO_4 solution and finally equilibrated with phosphate buffer (pH 7.6, 0.01 *M*).

Throughout all the investigations, fresh exchanger was used in each experiment. On no occasion was regenerated material used. In addition all equilibrations were accomplished at room temperature, whilst chromatography was performed at 4° (except where otherwise stated).

ELUTION PROCEDURE

Column experiments

The dialyzed protein samples (volume 5 ml) were carefully applied to the top of the column by means of a Pasteur pipette and then allowed to run in under gravity. Elution was performed by a stepwise procedure using the following series of solvents:

1. Phosphate buffer: pH 7.6, 0.01 *M*.
2. Phosphate buffer: pH 6.3, 0.02 *M*.
3. 0.05 *M* NaH_2PO_4 solution.

The flow-rates for the columns prepared from the "W" and "C" type exchangers were 3-5 ml and 60 ml per hour, respectively.

The use of an additional solvent, 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl, was found to result in the elution of only a small amount of protein of very heterogeneous composition and so was not used in these column chromatographic studies. The effluent was collected in 3 ml fractions using an automatic fraction collector, incorporating a weight-balance syphon.

Examination of the effluent

The protein distribution in the effluent was determined by measuring the $E^{1\,cm}_{280\,m\mu}$ value in silica cells, in a Unicam SP 500 spectrophotometer. Effluent pH was determined by means of a micro electrode and direct reading pH meter, whilst the electrical conductivity was measured with a Mullard conductivity bridge incorporating a cathode ray (magic eye) indicator.

A standard system of pooling effluent fractions was adopted which was based upon the distribution of the main protein peaks in the chromatographic pattern of the whole serum (see Fig. 2).

Batch experiments

After equilibration, as described above for the column procedure, the exchanger ("W") was filtered through a sintered glass disc and the resultant damp exchanger was then intimately mixed with the equilibrated serum sample 3 (volume 1 ml). Following equilibration for a further 30 min, 20 ml of phosphate buffer (pH 7.6, 0.01 *M*) were added and the contents stirred to give a suspension. The sample was then allowed to stand for 30 min at 4° with frequent stirring. Exchanger and supernatant were then separated either by centrifugation, as in the case of the exchanger "W" or by filtration (see above), as in the case of the "C" type derivatives. This process was repeated with 20 ml volumes of the solvents described previously for the column procedure, but with the addition of a fourth solvent, namely 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl.

In experiments investigating the effect of temperature, equilibration was performed in a thermostatically controlled centrifuge (for temperatures 0°, 10° and 22°) or in a thermostatically controlled water bath (for 37° and 50°). The equilibrated exchanger and solvent were then separated by centrifugation with minimum temperature change.

Concentration of fractions

Concentration of samples was performed at 4° by ultra-filtration through visking tubing ($^8/_{32}$ in. in diameter) using a negative pressure of 50–60 cm of Hg. After ultra-filtration adherent protein was carefully massaged from the sides of the sac and the concentrated fractions stored at —20° in polythene containers.

Analysis of fractions

Protein determinations were carried out on the concentrated fractions by the LOWRY modification[7] of the FOLIN phenol procedure, and their compositions were determined by the immunoelectrophoretic technique using a rabbit antiserum raised against human serum.

RESULTS

Column separations

Four column chromatographic fractionations of defibrinated plasma (serum sample 2) were performed on each of the "C" type exchangers. An analysis of variance indicated that there was no significant difference between the total product recovered from these exchangers at varying degrees of substitution (ranging from 0.05–1.20 mequiv./g).

For comparison, results obtained from the analysis of normal human serum (sample 1) on exchanger "W" are also included in Table I. A more direct comparison

TABLE I

THE EFFECT OF THE DEGREE OF SUBSTITUTION OF DEAE-CELLULOSE EXCHANGERS ON PROTEIN RECOVERY IN THE COLUMN CHROMATOGRAPHIC PROCEDURE

The values are given as means of four sets of results together with their standard deviations ($6\bar{x}$)

Sample	*Exchanger*	*Degree of substitution (mequiv./g)*	*Amount of protein applied (mg)*	*Protein recovery (percentage of total applied)*			
				Solvent 1	*Solvent 2*	*Solvent 3*	*Total*
				Phosphate buffer		*0.05 M NaH_2PO_4*	
				pH 7.6, 0.01 M	*pH 6.3, 0.02 M*		
Defibrinated	C5	0.05	240.6 ± 17.6	80.4 ± 6.4	3.0 ± 0.6	9.0 ± 2.1	92.8 ± 4.4
Human	C25	0.25	210.0 ± 15.4	56.7 ± 5.5	2.8 ± 1.1	11.1 ± 2.3	70.5 ± 6.5
A.C.D.	C65	0.65	265 ± 15.8	39.5 ± 4.7	9.7 ± 0.9	32.5 ± 3.8	81.6 ± 7.6
Plasma	C120	1.2	227.6 ± 6.8	13.6 ± 3.9	7.6 ± 1.5	48.6 ± 7.5	68.9 ± 2.0
Normal human serum	Laboratory prepared W	0.87	365.0	8.2 ± 2.3	7.5 ± 1.1	57.4 ± 6.8	73.0 ± 8.8

of the properties of the two types of exchangers was made, however, by employing the batch procedure (see later). In general, the total recoveries of protein from the "C" type exchanger were not significantly different from those observed with exchanger "W", although significantly greater recoveries were obtained with exchanger C5 (*i.e.* 0.05 mequiv./g).

The elution patterns varied markedly with the chemical nature of the exchanger (see Fig. 1). For instance, the amount of protein eluted with the initial solvent (phosphate buffer; pH 7.6, 0.01 *M*) was inversely related to the degree of substitution of the exchanger. Conversely, there was a direct relationship between the amount of protein recovered with the final solvent (0.05 *M* NaH_2PO_4 solution) and the degree of substitution of the exchanger.

As shown in Fig. 2 a characteristic serum elution pattern was obtained using exchanger "W", pure 7S-γ-globulin being eluted in the initial fractions. On the other hand, comparative immunoelectrophoretic analyses revealed the breakthrough of other proteins in the corresponding fractions from the "C" type exchangers. For instance, proteins such as the β- and α-globulins and the albumin, which are normally firmly bound to the exchanger at pH 7.6 (0.01 *M* phosphate), were eluted along with the 7S-γ-globulin. Consequently, the amount of protein eluted with further solvents was much reduced. Nevertheless, the protein fractions eluted with solvent 3 from these exchangers showed the heterogeneity characteristic of this part of the chromatogram (compare with Fig. 2), indicating that even ex-

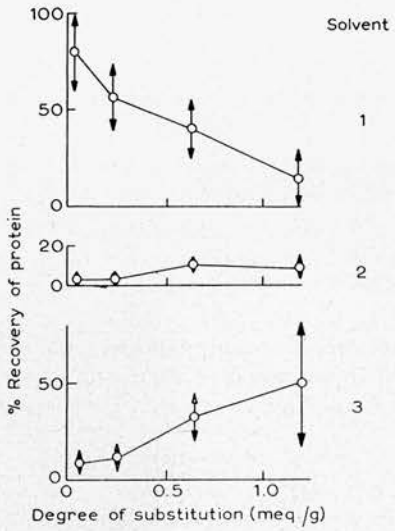


Fig. 1. The effect of the degree of substitution of "C" type exchangers on protein recoveries. The chromatographic technique employed is described in the text, whilst the amounts of protein (serum sample 2) fractionated are recorded in Table I. Each point on the graph represents the mean of four observations. The deviations illustrated by the arrows are derived by multiplying the standard deviations of the mean by the value of t at the 5% significance (0.05 probability) level for the respective number of degrees of freedom.

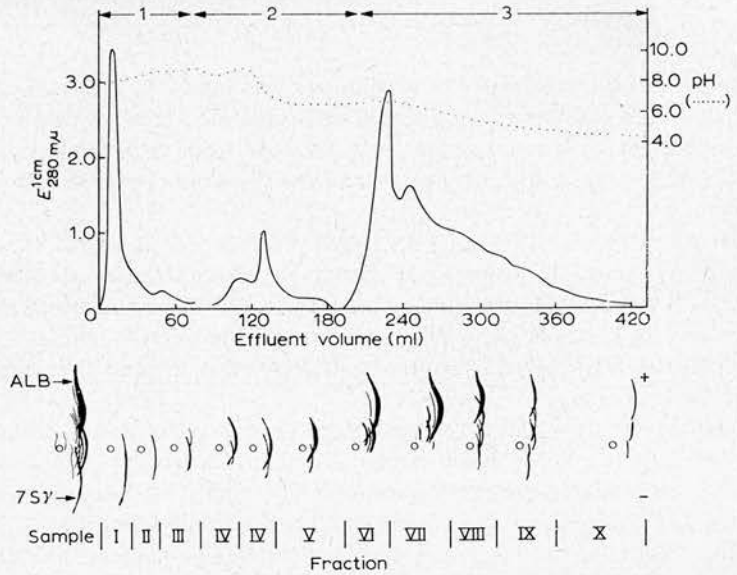


Fig. 2. A typical human serum chromatographic pattern based on the results of four different column separations of 5 ml of serum, sample 1, on type "W" exchanger. The dotted line indicates the pH of the effluent whilst the heavy line represents the protein concentration as measured by absorbancy at 280 mμ. Tracings of immunoelectrophoretic patterns obtained by testing the fractions with rabbit anti-whole human serum are also included. For chromatographic conditions see text.

TABLE II

THE EFFECT OF THE DEGREE OF SUBSTITUTION OF DEAE-CELLULOSE EXCHANGERS ON PROTEIN RECOVERY BY THE BATCH PROCEDURE

The results given are those obtained in experiments using 1 ml samples of serum 3, 1 g amounts of the "C" type exchangers and 0.5 g amounts of exchanger "W"

Exchanger	*Degree of substitution (mequiv./g)*	*Protein recovery (percentage of total applied)*				
		Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*	*Total*
		Phosphate buffer		*0.05 M NaH_2PO_4*	*0.05 M NaH_2PO_4 containing 0.25 M NaCl*	
		pH 7.6, 0.01 M	*pH 6.3, 0.02 M*			
C5	0.05	57.5	13.2	9.9	15.8	96.4
C25	0.25	39.3	12.9	11.9	22.5	86.6
C65	0.65	22.0	8.8	15.0	36.2	82.0
C120	1.2	28.3	6.3	13.5	46.9	95.0
W	0.87	13.0	6.0	9.3	54.5	82.8

changers of a very low degree of substitution (*i.e.* 0.05 mequiv./g) have a retentive capacity for a certain number of serum proteins.

Batch separations

In all the batch experiments performed (see Table II), there were no significant differences in the recovery of total serum protein (sample 3) from the various exchangers ("C" and "W").

These recoveries were greater than those achieved by the corresponding column procedures described above. However, the relative amounts of protein eluted with the various solvents were similar to those observed with the column technique (see Fig. 3).

As in the case of the column separations, breakthrough of proteins other than 7S-γ-globulin occurred only in the fractions eluted from "C" type exchangers with solvent 1.

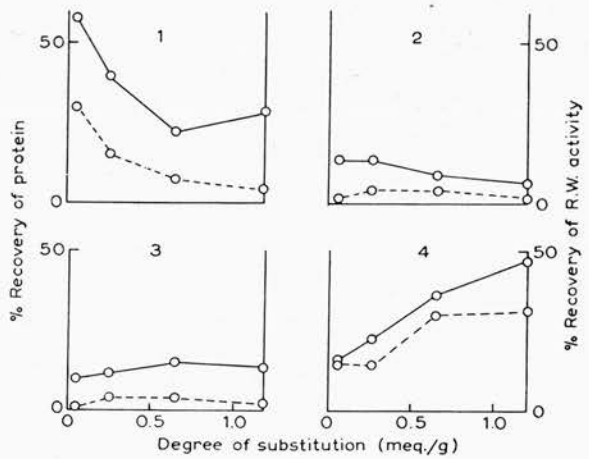


Fig. 3. Effect of the degree of substitution of "C" type exchangers on mean protein recovery (———) and recovery of ROSE-WAALER activity (– – –), during batch experiments using serum sample 3. For chromatographic conditions see text.

As the degree of substitution of the exchangers increased, the α_2-macroglobulin and albumin content in the initial effluent decreased. The major components of fractions eluted from all the different exchangers by the final solvent, however, were the α-globulins (haptoglobin and ceruloplasmin), 19S-γ-globulin and albumin, suggesting that at least these proteins were retained.

By employing rheumatoid serum (sample 3), it was also possible to study the chromatographic behaviour of a human 19S-γ-globulin, the "rheumatoid factor". This protein was detected in the effluent by means of its capacity to agglutinate sensitised sheep erythrocytes, (*i.e.* by the ROSE-WAALER technique, as modified by BALL[8]). It was hoped that the strong affinity shown by this protein for DEAE-cellulose exchangers of about 1 mequiv./g substitution, a factor which complicates its isolation on these derivatives, would be overcome by employing less highly substituted materials. In this way, it might have been possible to first selectively adsorb the rheumatoid factor and subsequently to recover it readily. Unfortunately, however, the widespread distribution of serological activity amongst the various chromatographic fractions (see Fig. 3) indicated no preferential adsorption on "C" type exchangers of relatively low degrees of substitution, thus limiting their use in the isolation of rheumatoid factor.

The effect of temperature on the chromatographic separation of human serum proteins on DEAE-cellulose was also investigated. Changes of temperature between 0–50° had a negligible effect on the specific adsorption capacity of the laboratory prepared exchangers for human serum proteins (see Fig. 4).

The amounts of protein remaining adsorbed at pH 7.6 (0.01 *M* phosphate), at

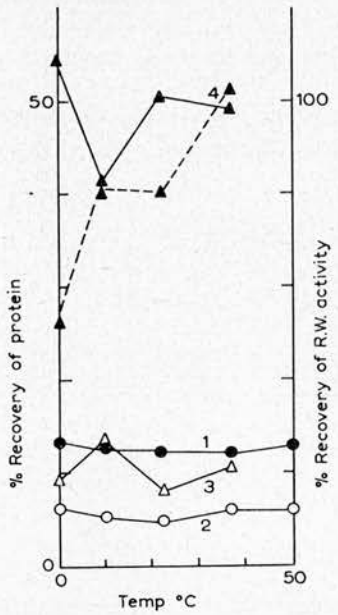


Fig. 4. Effect of temperature on the mean protein recovery (———) and recovery of ROSE-WAALER activity (– – –) during the batch chromatography of serum sample 3 on exchanger "W". Recoveries of activity with solvents 1, 2 and 3 were negligible and so have been omitted from the diagram. For chromatographic conditions see text.

the various temperatures tested, were almost identical and the effluent compositions were very similar. The quantitative and qualitative composition of the protein eluted with the other solvents were comparable, although there was some variation in the recovery of rheumatoid factor; this could, however, be partially attributed to the limitations of a doubling dilution technique of estimation of rheumatoid factor activity.

From the mean protein recoveries obtained in the above experiments, the specific adsorption capacities of the various exchangers in both the column and batch procedures have been determined and these are plotted in Fig. 5. In these experiments the "specific adsorption capacity" is defined as the total amount (mg) of serum protein adsorbed per 100 mg of exchanger, after both have been equilibrated with phosphate buffer (pH 7.6, 0.01 *M*). This permits a comparison of values obtained in these studies with the results reported by PETERSON AND SOBER[1]. As will be seen, the amount of protein adsorbed was related to the degree of substitution of the exchanger. The adsorption curves reached a plateau, above which the substitution of further reactive groupings had little effect on protein adsorption.

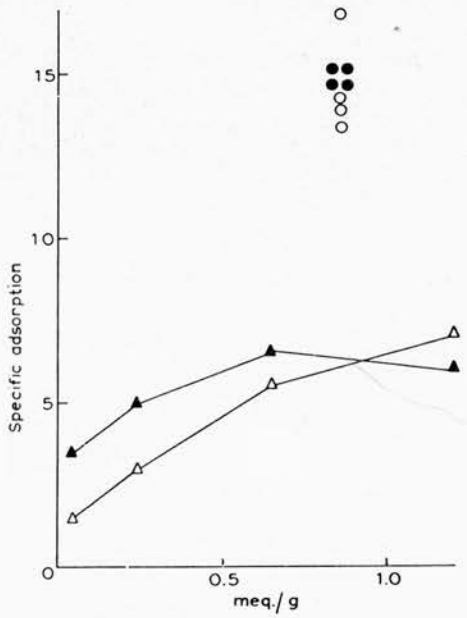


Fig. 5. A comparison of the specific adsorption capacities of "C", (–△–△–, –▲–▲–) and "W" (○, ●) type exchangers for total human serum proteins, by both column (–△–△–, ○) and batch (–▲–▲–, ●) techniques. The batch experiments on "W" type exchangers were performed between the temperatures of 0 and 50°.

DISCUSSION

Although the present study has not led to the ready development of an improved procedure for the chromatographic fractionation of human serum proteins on DEAE-cellulose, the data presented offer an indication of how various factors influence the ion-exchange process.

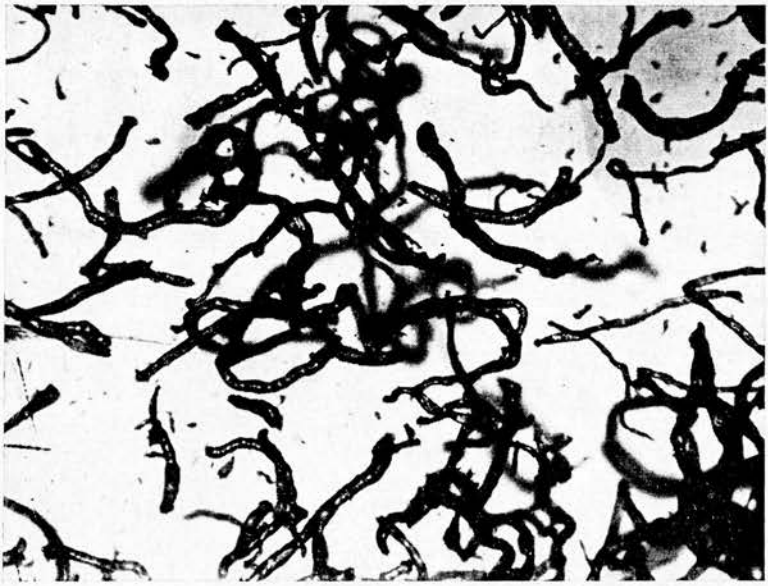
By employing exchangers of varying degrees of substitution it was hoped to

achieve the selective adsorption of certain proteins. Furthermore, by using exchangers of low degrees of substitution it should have been possible theoretically to overcome the irreversible binding which seemed to accompany the chromatography of some of the serum proteins, notably the 19S-γ-globulins. It was found, however, that there was no selective retention of the 19S-γ-globulin (rheumatoid factor) molecule on exchangers of low degrees of substitution (see Fig. 3). On the other hand, smaller molecules such as ceruloplasmin and haptoglobin were preferentially adsorbed under similar conditions in both the column and batch procedures. Hence it would appear that some proteins compete for the available sites on the surface of the exchanger, molecules with a relatively high affinity for the exchanger displacing those with a smaller affinity. It is possible that this property could be applied to the partial purification of specific serum proteins. For example α_2-macroglobulin can be separated from the bulk of the other serum proteins (including 19S-γ-globulin and the other α-globulins) by the batch chromatography of serum on ion exchangers of 0.25 mequiv./g substitution or less, which had been equilibrated with phosphate buffer (pH 7.6, 0.01 *M*). Theoretically, it should then be possible to adsorb selectively the α_2-macroglobulin on an exchanger of high substitution (0.7–1.0 mequiv./g). In practice, however, it was found that recoveries by such a two-stage process were small.

Another useful fraction rich in ceruloplasmin and other α-globulins could also be obtained by a selective adsorption procedure, *i.e.* by equilibrating a mixture of exchanger and serum with 0.05 *M* NaH_2PO_4 solution and then eluting the exchanger with a solvent of high ionic strength (such as 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl). It should be mentioned, in this respect, that such selective adsorption techniques have already been applied by other investigators (*e.g.* CONNEL AND SHAW[9], STEINBUCH AND QUENTIN[10] and STEINBUCH AND LOEB[11]) to the isolation of serum α-globulins. In order to obtain further information about the relative selectivity of exchangers of differing degrees of substitution it will be necessary to study the adsorption characteristics of individual isolated serum proteins.

It is significant that the specific adsorption capacities obtained in the present series of experiments are approximately one tenth of those observed by PETERSON AND SOBER[1]. Although those investigators employed bovine serum albumin, in elutions with pH 7.0, 0.01 *M* phosphate buffers, it is difficult to explain the observed differences. Nevertheless, the two sets of results showed the same trend, the amount of protein adsorbed being proportional to the degree of substitution of the exchanger. In contrast, however, the adsorption curves shown in Fig. 5 reached a plateau above which the substitution of further reactive groupings had little effect upon protein adsorption. This is not apparent from the results of PETERSON AND SOBER, where marked increases in adsorption capacity were observed between the ranges 0.45 to 1.34 mequiv./g substitution. The higher adsorption capacity observed during the batch (as opposed to the column chromatography) procedure with "C" type exchangers of less than 0.65 mequiv./g substitution can probably be attributed to the fact that the elution with the first solvent is less efficient than in the column procedure.

As previously mentioned, some protein is adsorbed by exchangers of low substitution. For example, the exchanger C 5 adsorbs 20 % of the total amount of protein adsorbed by exchanger C 120, although its degree of substitution is only 4 % of that of the latter exchanger. This would suggest that either C 5 exhibits considerable non-ionic adsorption, or else a high proportion of the ionizing groups in the exchanger of



(a)

(b)

Fig. 6. Photomicrographs of "C" and "W" type exchangers. A. Exchanger C120, magnification × 100. B. Exchanger W, magnification × 250.

high substitution are not available for combination with the protein. It is assumed here, that the washing procedure has been efficient, and that no protein is trapped within the exchanger.

The differences in the chromatographic properties of the "W" type and "C" type exchangers (of similar substitution) can readily be explained in terms of differences in their physical form, as will be seen from the photomicrographs shown in Fig. 6. The "C" type exchangers are of a coarse physical form being prepared from fibrous cotton linters, whereas the "W" type product was crystalline-like. The effect of the physical form of DEAE-cellulose on its chromatographic behaviour has previously been discussed by PETERSON AND SOBER[1], who showed that gelatinous adsorbents (of fine physical form) possess higher capacities than non-gelatinous adsorbents. As well as affecting the capacity of the exchanger, the physical form also influences the resolution which was not so pronounced on the "C" type exchangers as in the laboratory product. A consideration of the factors which affect chromatographic phenomena on columns could explain these discrepancies (GLUECKAUF[12]). Disturbances due to non-equilibrium between protein solution and exchanger are liable to be more serious in exchangers of coarse form, for there is a greater tendency for longitudinal diffusion and channelling effects as well as less time for equilibration. All these factors tend to reduce the efficiency of the chromatographic process, although considerably higher rates of elution are obtained.

The results of the investigations of the effect of temperature are consistent with the observations of HJERTÉN[13], using calcium phosphate adsorbents. This factor was found to be of limited importance in the DEAE-cellulose chromatography of serum proteins. However, the effect of temperature may prove to be more marked in column procedures employing floc-like exchangers, where rises in temperature will increase diffusion and channelling of protein and solvent thus affecting resolution. The limitations of chromatography at temperatures above 4° are not inherent in the technique itself. Nevertheless, the susceptibility of proteins to denaturation and the risk of increased bacterial activity at these elevated temperatures necessitate fractionation at low temperatures.

The findings discussed here suggest that DEAE-cellulose exchangers prepared from cotton cellulose are inferior to those prepared from wood cellulose, at least as far as the separation of serum proteins is concerned. Ideally, the ion exchangers should be substituted to a degree of about 1 mequiv./g and should be of a physical form which permits ready packing into columns without showing a marked resistance to flow. Nevertheless, exchangers prepared from cotton cellulose have application in batch procedures where packing is not a problem, in spite of the necessity of using relatively greater amounts of such exchangers (owing to their low adsorption capacities).

With the solvent systems employed, the batch procedure was found to effect a resolution of serum proteins comparable to that achieved by the column technique. This means that when a large number of chromatographic fractionations are to be undertaken, the more rapid batch procedure provides a satisfactory alternative.

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SUMMARY

Studies have been undertaken to establish the influence of various factors on the fractionation of human serum proteins by DEAE-cellulose chromatography. The following points emerged:

1. In both column and batch procedures, the specific adsorption capacities of the various exchangers tested were related to their degree of substitution.

2. Exchangers of low degrees of substitution selectively adsorbed a number of serum proteins, especially α-globulins such as ceruloplasmin and haptoglobin, although they failed to retain preferentially rheumatoid factor.

3. The adsorption capacity of exchanger prepared from wood cellulose (of fine physical form) was twice the capacity of exchangers of similar substitution prepared from cotton cellulose.

4. Variation in the temperature of elution over a range from 0–50° had no effect on the properties of DEAE-cellulose exchanger (prepared from wood cellulose) as revealed by batch chromatographic experiments.

5. The batch technique would appear to provide a rapid alternative method to the column procedure for the chromatographic separation of serum proteins.

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STUDIES ON THE CHROMATOGRAPHY OF HUMAN SERUM PROTEINS ON DIETHYLAMINOETHYL(DEAE)-CELLULOSE

II. THE CHROMATOGRAPHIC CHARACTERISTICS OF PURIFIED HUMAN SERUM PROTEINS

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INTRODUCTION

Although, as already mentioned[1], DEAE-cellulose chromatography has been used extensively in the fractionation of serum proteins, there have been no previous reports of the investigations of well characterized individual proteins on this exchangers.

In the work to be described the chromatographic properties of several purified serum proteins have been studied. The group of proteins studied comprised macromolecules with a wide range of molecular size and charge density.

The effect of protein–protein interaction has also been determined by the chromatography of artificial protein mixtures, some of which included isotopically labelled proteins.

Studies of this type are essential if the resolution of serum protein mixtures affected by DEAE-cellulose chromatography is to be improved and if new selective isolation procedures are to be developed.

METHODS

Purified proteins

The human serum proteins used in these investigations are listed in Table I, together with their mode of preparation. The 7 S-γ-globulin, β-lipoprotein and α_2-macroglobulin preparations were shown to be free of other serum proteins by ultracentrifugal analysis and immunoelectrophoretic analysis employing a rabbit antiserum to whole human serum. By similar techniques the siderophilin preparations were found to contain small amounts of contaminating 7 S-γ-globulin (less than 5 %) and the albumin, a β-globulin impurity (2–5 %). In the case of the Lister albumin preparation the β-globulin contaminant was siderophilin.

Isotopic labelling of proteins

Various protein preparations were labelled with ^{131}I by the iodine monochloride technique of McFARLANE[6], as modified by DAVIES *et al.*[7], whilst siderophilin was labelled with ^{59}Fe by an exchange procedure (VEAL AND VETTER[8]). The activity of

TABLE I

METHODS OF PREPARATION OF THE PROTEINS INVESTIGATED

Protein	*Sample numbers*	*Method of separation*
7S-γ-Globulin	1	DEAE-cellulose column chromatographic separation from normal human serum
	2	DEAE-cellulose batch chromatographic separation from outdated acid citrate dextrose plasma (STANWORTH[2])
Siderophilin	1 and 3	DEAE-cellulose column chromatographic separation of F IV–4 obtained by the low temperature ethanol procedure of COHN *et al.*[3]
	2	DEAE-cellulose chromatographic separation of outdated acid citrate dextrose plasma followed by further DEAE-cellulose chromatography of the siderophilin-rich fraction
β-Lipoprotein	1	Subfractionation of Fractions II and III, obtained by COHN method 6[3], by method 9 (ONCLEY *et al.*[4]) to give fraction III-O; zone centrifugation of this fraction in saline density gradient
α_2-Macroglobulin	1	Zone centrifugation in sucrose density gradient of the pellet obtained in the above centrifugation procedure
Albumin	1	Fraction V obtained by COHN method 6[3]
	2	Commercial preparation (LISTER) obtained by the ether fractionation procedure of KEKWICK AND MACKAY[5]

labelled fractions was determined by counting measured aliquots in a thallium activated, well shaped, sodium iodide crystal.

Column chromatographic procedure

With one exception (discussed later) all chromatographic separations were carried out in columns containing 2 g of DEAE-cellulose exchanger prepared in the laboratory from wood cellulose (Solka Floc Grade BW 100) according to the method of PETERSON AND SOBER[9] (column dimensions 16.0 × 1 cm). This material had a degree of substitution of 0.87 mequiv./g. Elution was effected by a stepwise procedure using the following series of solvents:

1. Phosphate buffer: pH 7.6, 0.01 *M*.
2. Phosphate buffer: pH 6.3, 0.02 *M*.
3. 0.05 *M* NaH_2PO_4 solution.

Other practical details are discussed in the previous paper[1].

RESULTS

(a) Studies of single proteins

The amounts of protein eluted with the various solvents are recorded in Table II in which the mean recoveries and the standard deviation of the mean are given. Statistical analysis revealed that the total recoveries of 7S-γ-globulin and albumin were significantly greater than those observed with total serum protein, whilst the recovery of siderophilin was comparable. On the other hand, the recoveries of β-lipoprotein and α_2-macroglobulin were significantly less than that shown by total serum protein.

TABLE II

THE AMOUNTS OF THE VARIOUS SERUM PROTEINS CHROMATOGRAPHED AND THEIR RECOVERIES

Mean recoveries expressed together with their standard deviations ($6\bar{x}$).

Sample	*Number of experiments*	*Amount protein applied (mg)*	*Protein recovery (percentage of total applied)*			
			Phosphate buffer		*0.05 M NaH_2PO_4*	*Total*
			pH 7.6, 0.01 M	*pH 6.3, 0.02 M*		
Normal human serum	4	365	8.2 ± 2.3	7.5 ± 1.1	57.4 ± 6.8	73.0 ± 8.8
7S-γ-globulin	4	20.5 ± 4.2	72.4 ± 4.6	11.9 ± 4.4	6.1 ± 2.6	90.5 ± 3.9
Siderophilin	5	119.4 ± 25.6	52.7 ± 8.0	10.1 ± 3.7	2.9 ± 0.8	65.7 ± 7.3
β-Lipoprotein	2	13.5	0.65 ± 0.65	7.2 ± 3.2	15.4 ± 3.7	23.1 ± 6.4
α_2-Macroglobulin	4	70.1 ± 4.6	2.7 ± 0.9	0.9 ± 0.2	29.4 ± 3.0	33.4 ± 3.1
Albumin	6	157.0 ± 47.8	0.8 ± 0.2	1.1 ± 0.2	82.1 ± 1.3	84.1 ± 1.2
7S-Globulin and siderophilin	3	51.7 ± 15.3	61.0 ± 6.7	13.1 ± 3.9	1.3 ± 0.4	75.2 ± 5.0
7S-Globulin and albumin	2	125 ± 15.0	32.8 ± 4.6	2.4 ± 0.2	52.9 ± 4.9	88.2 ± 0.3
α_2-Macroglobulin and albumin	1	114	0.3	0.8	37.0	38.1
7S-Globulin, siderophilin, α_2-macroglobulin and albumin	1	168	18.9	15.7	46.9	81.5

As was to be expected most of the 7S-γ-globulin failed to bind to the exchanger and so was recovered in the initial solvent front. Nevertheless, this protein exhibited the "trailing" effect also observed by other investigators[10,11].

The elution characteristics of isolated siderophilin were found to differ markedly from those exhibited by this protein during the DEAE-cellulose chromatography of whole serum[12]. For instance, the major part of the isolated siderophilin was eluted by the first solvent (0.01 *M* phosphate, pH 7.6) whereas in separation of whole serum it was completely eluted by the second solvent (0.02 *M* phosphate, pH 6.3). This effect does not appear to have been observed by TOMBS *et al.*[13], in their studies on the chromatographic behaviour of electrophoretically prepared β-globulin fraction.

Recoveries of β-lipoprotein were extremely low (23.1 % ± 6.4), the eluted material being dispersed throughout the greater part of the chromatogram. Similarly the recovery of α_2-macroglobulin was disappointingly low (33.4 % ± 3.1). In contrast to the β-lipoprotein, however, almost all of this protein was recovered with one solvent (the 0.05 *M* NaH_2PO_4 solution). It was eluted ahead of the normal 19S-γ-globulin position[11], and also prior to the albumin (see Fig. 1).

As mentioned earlier, the total recoveries of albumin were constantly high (84.1 % ± 1.2). Again the major portion of this protein (82.1 % ± 1.3) was recovered with the final solvent (0.05 *M* NaH_2PO_4 solution). The chromatographic patterns obtained by duplicate analysis of the same albumin sample showed considerable variability, however. Compare for instance patterns A and B in Fig. 2 which were obtained by parallel analyses on the same albumin preparation (a COHN Fraction V). Comparison with patterns (E and F) given by larger amounts of a different albumin preparation (LISTER) revealed even greater variation. It should be mentioned, however, that the leading minor peaks in these patterns can be attributed to contaminating siderophilin.

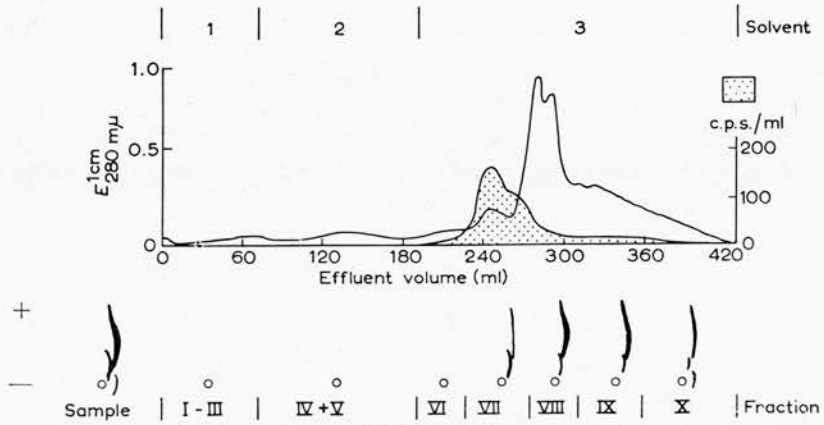

Fig. 1. Chromatography of a mixture of ^{131}I labelled α_2-macroglobulin (12.6 mg) and COHN F V albumin (101.4 mg of preparation 1) on DEAE-cellulose exchanger W. For chromatographic details see text.

In general, the recoveries of the various proteins studied were found to be independent of the amount of material applied to the DEAE-cellulose column. For instance, although the amounts of albumin chromatographed varied between 84–320 mg, the total recovery was remarkably constant, *e.g.* 84.1 % ± 1.2. This is supported by the results of statistical analysis.

(b) Studies of protein mixtures

The mixtures of serum proteins investigated are tabulated in Table II where it can be seen, that with the exception of the ^{131}I labelled α_2-macroglobulin–albumin mixture, the overall protein recoveries were comparable with those shown by the individual

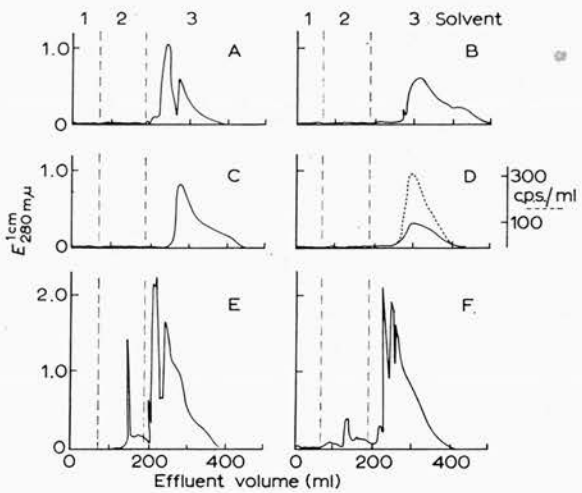

Fig. 2. The chromatographic distribution of albumin on DEAE-cellulose exchanger W. A, B and C are 82.0, 82.0 and 106.0 mg respectively of COHN F V albumin (preparation 1); D is 43.0 mg of ^{131}I labelled COHN F V (preparation 1); and E and F are 320.0 and 293.0 mg of LISTER albumin (preparation 2). For chromatographic details see text.

proteins. Moreover, the positions of elution of various components in the protein mixtures were similar to those exhibited by these proteins in isolated form. Recoveries of labelled siderophilin, and α_2-macroglobulin, which had been added to whole serum were comparable with those shown by the isolated proteins. On the other hand, the recoveries of ^{131}I labelled 7 S-γ-globulin, β-lipoprotein and albumin from whole serum were lower than the corresponding recoveries observed with isolated proteins and simple protein mixtures.

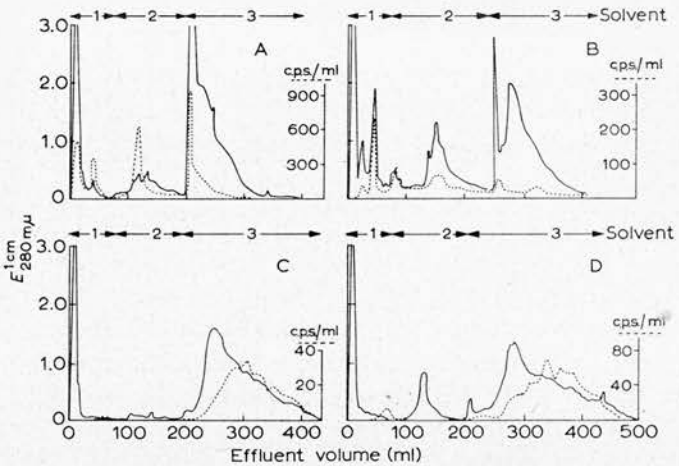

Fig. 3. The chromatography of mixtures of isotopically labelled serum proteins and whole serum on DEAE-cellulose exchanger W. The following isotopically labelled serum proteins were added to 5 ml of normal human serum: A = 6.3 mg of ^{131}I labelled 7 S-γ-globulin preparation 2; B = 141.8 mg of ^{59}Fe labelled siderophilin preparation 3; C = 12.6 mg of ^{131}I labelled α_2-macroglobulin; D = 45.7 mg of ^{131}I labelled albumin preparation 1. Distribution of radioactivity indicated thus: · · ·. For further chromatographic details see text.

In these experiments, involving the addition of labelled proteins to whole serum, the distribution of protein-bound isotope was often found to differ from that observed during the chromatography of simple mixtures containing the labelled protein. This was particularly noticeable in experiments employing ^{131}I labelled 7 S-γ-globulin (see Fig. 3), where the major portion of the labelled protein was eluted with solvents other than the 0.01 *M* phosphate buffer (pH 7.6). It should also be noted that the total recoveries of protein from isotopically labelled protein–serum mixtures were less than those obtained with serum alone.

DISCUSSION

On the whole, the chromatographic behaviours of isolated serum proteins proved similar to those exhibited during the fractionation of total serum.

As suggested from earlier studies on whole serum, the position of elution of an individual component depends largely on its charge density at the pH employed. Other factors, however, appear to influence the chromatographic separation of certain serum proteins. For example, although both α_2-macroglobulin and ceruloplasmin fall into the α_2-globulin electrophoretic class these proteins exhibit quite

distinct chromatographic properties. Similarly, there are marked differences in the chromatographic behaviour of siderophilin (a β-globulin) and 19S-γ-globulin, in spite of their relatively small differences in electrophoretic mobility.

The results of the studies on artificial protein mixtures failed to demonstrate that protein–protein interaction was responsible for the differing behaviour of these pairs of proteins. It is more likely, however, that molecular size plays a critical role in the ion-exchange chromatography of proteins on substituted cellulose as suggested by PETERSON AND SOBER[14], resulting in the phenomenon known as "size compensation". This could explain the difference in chromatographic behaviour of 7S-γ- and 19S-γ-globulin. Another complication results from variations in the distribution, availability and degree of ionization of the charged groups within a protein molecule. This renders difficult the precise duplication of chromatographic separations of serum proteins. In addition, it is also possible that certain charged groups are not revealed until the protein molecule becomes unfolded on binding to the ion exchanger. Such groupings might be expected to play an active part in irreversible binding, which is assumed to be responsible for the low recoveries of proteins such as α_2-macroglobulin and β-lipoprotein.

The weak affinity of 7S-γ-globulin for exchanger, probably due to its relatively high isoelectric point, is reflected by both its position of elution and its good recovery. Nevertheless, as already mentioned, a certain degree of trailing of the 7S-γ-globulin was observed, successive fractions showing progressively greater electrophoretic mobility (see refs. 10 and 11). The rather abnormal distribution of ^{131}I labelled 7S-γ-globulin (see Fig. 3A) was probably due to configurational changes produced in this molecule due to excessive iodination. Immunoelectrophoresis failed to reveal any such changes, however.

The alterations in the chromatographic behaviour of siderophilin following its isolation were puzzling. This could have been due to irreversible changes in its molecular configuration resulting from the disruption of its combination with other kinds of protein molecules. Such an irreversible change is suggested by the results of experiments involving the addition of ^{59}Fe labelled siderophilin to whole serum (see Fig. 3B), which showed that a large proportion of the added protein was still eluted prematurely. However, this effect could have been due to the use of a large excess of added siderophilin. Alternatively, structural changes not attributable directly to any protein–protein interaction effect could have played a part in the observed anomalous behaviour. The elution of the siderophilin preparations in a number of distinct peaks could have been due to the presence in the mixture of a range of siderophilin molecules saturated to varying extents with iron, or due to the existence of several distinct siderophilins[15].

The poor recoveries and "trailing" of both the β-lipoprotein and α_2-macroglobulin can be attributed to the molecular size of these proteins. Having a large partial specific volume they are susceptible to entrapment between the cellulose particles. In addition, it seems reasonable to assume that the simultaneous disruption of all the groups involved in the adsorption processes is never realised. In the case of the β-lipoprotein there is the additional complication of the high lipid content, which may render the molecule surface active, thereby causing increased affinity between exchanger and protein leading to denaturation. Moreover, this protein is rather insoluble in the aqueous eluting solvents. The elution of the α_2-macroglobulin

prior to albumin would appear to be a reversal of the size compensation phenomenon observed with 7S-γ- and 19S-γ-globulin. The most plausible explanation of this effect is a difference in the availability of the ionizable groups of the two proteins, for their total dicarboxylic and sialic acid contents are similar.

Because of the high dicarboxylic acid content (and hence low isoelectric point), the albumin molecule is adsorbed strongly onto DEAE-cellulose at alkaline pHs. However, this adsorption process must be readily reversible because the albumin is efficiently recovered. This could be due to the marked configurational adaptability of the albumin molecule. The appearance of albumin in a number of peaks and over a wide area of the chromatogram (see Fig. 2) has been attributed to a number of factors including the binding of small ions, especially fatty acids[16], dimer formation[17,18] facilitated by the presence of fatty acid[19,20] and to the concentration dependence of the adsorption isotherms[21]. It has also been suggested that this phenomenon might result from successive stepwise decreases in the capacity of the column under the influence of the eluting buffer[22,23]. Experiments involving the chromatography of COHN F V on DEAE-cellulose prepared from cotton linters (illustrated in Fig. 4),

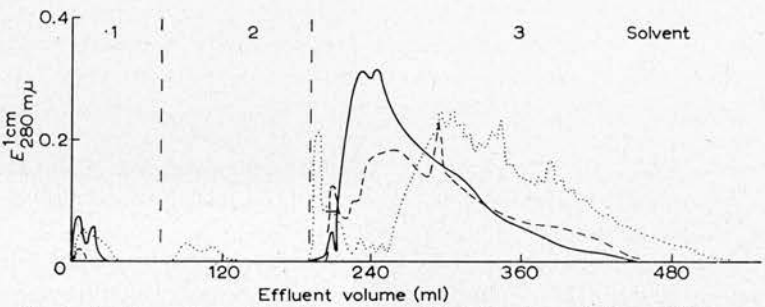

Fig. 4. The effect of oleic acid on the chromatographic behaviour of COHN F V serum albumin (preparation 1) on DEAE-cellulose (commercial exchanger 1.2 mequiv./g substitution). Chromatographic distribution represented as follows: albumin ———, ether extracted albumin – – – and oleic acid saturated albumin · · ·. For additional chromatographic details see text.

indicate that oleic acid saturated protein is more firmly bound to the column and less readily recovered than the other proteins. These results support the observations of previous workers[14,16]. Hence the binding of small, immunologically undetectable molecules, is most probably a factor of paramount importance in the observed chromatographic anomalies of human serum albumin.

As the investigation of isolated serum proteins has confirmed a wide variation in affinity for DEAE-cellulose, and has also failed to demonstrate the occurrence of any obvious protein–protein interactions, it seems probable that further studies of a similar nature could lead to the development of selective adsorption techniques for the isolation of specific serum components. Preliminary studies have demonstrated that batch chromatographic procedures, involving selective adsorption under strictly controlled conditions, could be used for such purposes.

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SUMMARY

1. The chromatographic behaviour of several purified human serum proteins on columns of diethylaminoethyl-cellulose has been established. The group of proteins investigated included 7 S-γ-globulin, siderophilin, β-lipoprotein, α_2-macroglobulin and albumin, *i.e.* molecules with a wide range of physico-chemical properties.

2. Individual proteins were found to differ widely in their recoveries. For example, 7 S-γ-globulin and albumin were recovered in high yield in spite of their widely different affinities for DEAE-cellulose. On the other hand, a major portion of the β-lipoprotein and α_2-macroglobulin investigated (proteins of large molecular size) could not be recovered from the exchange cellulose.

3. The chromatographic characteristics of the isolated proteins closely paralleled those shown by these proteins during the fractionation of simple mixtures, though such a close relationship was not observed in whole serum. Purified siderophilin, however, proved an exception in showing a much reduced affinity for exchanger whether in isolated form or even when added to more complex mixtures.

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SECTION B - ANTI-LYMPHOCYTIC ANTIBODIES

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Oberarzt an der Medizinischen Universitätsklinik Bonn

Mit einem Geleitwort von

Prof. Dr. E. LETTERER
Präsident der Deutschen Gesellschaft für Allergie- und Immunitätsforschung

In Zusammenarbeit mit der Chirurgischen Universitätsklinik Bonn
(Direktor: Prof. Dr. A. GÜTGEMANN)

Mit 151 Abbildungen, davon 3 mehrfarbig, und 94 Tabellen

19 69

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The Preparation and Fractionation of Anti-lymphocytic Sera

K. James

Introduction

A large number of potent species specific anti-lymphocytic antibody preparations have been described in the literature [see reviews (11, 12)]. These have been prepared by a variety of conventional immunization and protein fractionation procedures. However at least as far as human anti-lymphocytic antibody preparations are concerned, there is still the urgent need for the production of less toxic and more potent products. Therefore in the present paper, in addition to summarizing basic procedures, an attempt will be made to outline some of the possible methods of improving the production and properties of antibody to human lymphocytes. For further details on these and other aspects of anti-lymphocytic antibody the reader is advised to consult a recent extensive review (12).

The nature of the antigen

The anti-lymphocytic sera described to date have been produced using a variety of antigens including relatively homogenous populations of cells such as those obtained by thoracic duct cannulation and the more heterogeneous mixtures obtained from peripheral blood and lymphoid organs such as spleen (Table 1) (11, 12).

Table 1. Antigens used in the production of human anti-lymphocyte serum.

Antigen	Some principal references
Thoracic duct lymphocytes	Traeger et al. (1968); Pichlmayr et al. (1968)
Peripheral blood lymphocytes	Balner et al. (1968)
Thymocytes	Shorter et al. (1967)
Lymph node cells	Monaco et al. (1967)
Spleen cells	Iwasaki et al. (1967); James (1968)

N. B. On some occasions cells from a number of lymphoid organs have been used e.g., spleen and lymph node.

As the properties of the resulting antiserum are determined by composition of the immunizing antigen, the choice of this material is of critical importance. In this

connection recent reports suggest that less toxic and perhaps most effective antisera to human lymphocytes may be produced by using thymocytes, thoracic duct lymphocytes and preparations obtained from the buffy coat of fresh or outdated blood (2, 4, 28). These preliminary observations are of great practical importance and are currently being subject to critical investigation.

Although effective antisera have been obtained against sub-cellular fractions of mouse and rat lymphoid tissue (9, 19, 21) insufficient attention has been paid to the possible advantages of using similar products, or »purified« histocompatibility antigen preparations, to produce antisera to human lymphocytes. In addition to possibly overcoming some of the problems associated with production of antibody against shared antigenic determinants, the use of stable freeze dried preparations of lymphocyte antigens should enable the implementation of the desired standardized immunization schedules. For this reason, antisera are now being produced using thoracic duct lymphocyte and spleen cell suspensions which have been stored at — 180° C in dimethylsulphoxide (4, 33). Subsequent *in vivo* and *in vitro* investigations have revealed that effective antisera can be produced using materials stored under these conditions.

Immunization schedule

The most effective methods of antiserum production have still to be ascertained. Aspects of the immunization procedure requiring detailed investigation include the choice of animal for antiserum production, the route of administration and dose of antigen, the length of the immunization course and so on (Table 2). However for practical reasons it is difficult to obtain a conclusive answer on all of these variables.

Table 2. Immunization procedure — factors to be investigated.

1. The most suitable species in which to produce the antisera
2. The most satisfactory antigen
3. The amount of antigen per injection, the number of injections and the route of administration
4. The value of adjuvants
5. The possible suppression of undesirable antibodies by passive transfer inhibition

N. B. In addition to governing the titre of specific anti-lymphocyte antibodies, these and other factors, will influence the levels of undesirable antibodies e.g., anti-platelet and anti-erythrocyte.

Ideally the animal in which the antiserum is produced should be one which exhibits the maximum specific response to the minimum amount of lymphoid antigen. At the same time this animal should not exhibit a marked response to contaminating antigen or shared antigenic determinants (anti-platelet) and its proteins (especially IgG) should

be weakly immunogenic in the animal which is to receive the anti-lymphocytic antibody. While this is a theoretical ideal it is not always an advisable one for BALNER and DERSJANT (1) demonstrated that antisera produced in cymolgus monkeys to rhesus monkey lymphoid tissue were less effective immunosuppressants than those produced in rabbits even though they were less immunogenic.

Considerable debate has centred around the most efficient immunization schedule. The advantages of the short two pulse procedure advocated by LEVEY and MEDAWAR (19) are obvious. Antisera produced by this procedure require less absorption with homologous erythrocytes and presumably contain less antibody against minor irrelevant, although perhaps undesirable contaminants of the injected antigen. Preliminary data obtained in humans indicates that antisera prepared by this procedure are less toxic (i.e., less painful) than antisera prepared by more chronic immunization procedures but the immunosuppressive properties of such material have still to be determined (28). Antisera produced against the lymphoid tissue of animals by the 2 pulse procedure have not always proved effective immunosuppressants, nevertheless this aspect of antiserum production is still worthy of further consideration.

Because of the difficulties frequently experienced in obtaining large amounts of suitable antigenic material, the minimum amount of antigen required to stimulate the production of effective titres of anti-lymphocytic antibody should be critically investigated, at least in small animals, recognizing of course the inherent difficulties in extrapolating this data to other species. These studies should incorporate examination of other variables such as the route of administration, the use of adjuvants and the possible inhibition of undesirable antibodies by the passive transfer of antibody (25). Furthermore where small amounts of »purified« antigenic material are available the obvious potential of intra-lymph node immunization procedures should be exploited.

The data obtained from the above procedures should enable the production of antisera in which a greater proportion of the molecules are capable of inactivating lymphocytes. Alternatively the specific anti-lymphocyte content of the antisera may be increased by producing the antibody in animals whose plasma IgG levels are naturally low, or has been reduced by selective plasmaphoresis prior to immunization (35).

Characterization of anti-lymphocytic antibody

The advantages of using »purified« anti-lymphocytic antibody preparations, especially in humans, have been stressed on previous occasions [JAMES (1967, 1968)]. However as the nature of antibodies produced is dependent upon a variety of factors, it is desirable, wherever possible, to ascertain the precise distribution of antibody activity in the anti-lymphocytic sera prior to large scale fractionation. This may be achieved by a number of standard procedures which result in the separation of the serum into a small number of well characterized fractions whose antibody activity can be assessed (Table 3). Gel filtration on G200 sephadex (7) and gradient ultracentrifugation (30) procedures which separate proteins on the basis of molecular weight (and size and shape) enable the resolution of serum into the classical 19S, 7S, 4.5S fractions. The 19S fraction

Table 3. Some basic methods for determining the molecular characteristics of antibodies.

Basic property	Technique	Application to characterization of anti-lymphocytic antibody
Size	Gel-filtration on G200 Sephadex	James and Medawar (1967); James and Anderson (1967); Woodruff et al. (1967)
	Mercaptoethanol lability	James and Medawar (1967); Monaco et al. (1967)
	Zonal-ultracentrifugation	
Charge	Preparative electrophoresis	
	DEAE cellulose column chromatography	Iwasaki et al. (1967); Pichlmayr (1967); Pichmayr et al. (1968)

N. B. The above procedures permit a general characterization of the antibodies involved but for more specific characterization, immunological and physicochemical analysis of »purified« antibody preparations is essential.

contains the IgM class of antibodies (mol wt. approx. 1,000,000) and the 7S the so called IgG proteins (mol wt. 160,000). The other major immunoglobulin (the IgA protein) is located between the 19S and 7S protein fractions. A partial resolution of the main immunoglobulin classes may also be achieved by DEAE cellulose chromatography which separates the proteins on the basis of their surface charge (size also plays a less prominent role). The precise location and nature of the various immunoglobulins can be determined by the use of specific antisera against the proteins in question and by using suitable purified marker proteins, labelled isotopically or with protein reactive dyes (30).

The susceptibility of IgM type antibodies to mercaptoethanol treatment has also been used to characterize antibodies and while this is a simple procedure it should be born in mind that all 7S antibodies, including anti-lymphocytic, are not mercaptoethanol resistant (15). Having determined the distribution of the antibody activity procedures can be adopted which permit the maximum recovery of antibody activity with the minimum amount of contamination. Fortunately in many of the antisera examined a major part of the antibody activity has been associated with the most stable and readily isolated immunoglobulin fraction of serum namely the IgG globulin. A number of well established procedures are available for isolating this component and these are outlined in Table 4. Almost all the human anti-lymphocytic globulin has been prepared by salt precipitation using ammonium or sodium sulphate (4, 10). Further purification of this crude material has sometimes been achieved by chromatography on DEAE cellulose

(12)[1]), 20) or DEAE sephadex (33). The latter step removes contaminating albumin and globulins (including IgA and IgM) and has recently been claimed to render the anti-lymphocytic globulin less antigenic (31).

Table 4. Some basic methods for isolating anti-lymphocytic antibody.

	Technique	Some key references
1. Precipitation	Neutral salts — sodium or ammonium sulphate	Iwasaki et al (1967); Carraz et al. (1968)
	Organic solvents — ethanol or ether	Watt (1968)
	Organic cations — rivanol	
2. Chromatography	DEAE cellulose	Guttman et al. (1967); Naysmith and James (1968); Denman and Frenkel (1968)
	DEAE Sephadex	Perper et al. (1967)
3. Electrophoresis	Column or slab	
	Forced flow	Watt (1968)
4. Combined procedures	Salt precipitation followed by chromatography	James and Anderson (1967); James (1968); Monaco et al. (1967); Woiwod and Phillips (1967); Denman et al. (1967); Starzl (1968)

Methods of anti-lymphocytic globulin production currently under investigation in Edinburgh include the popular Cohn fractionation procedure involving alcohol precipitation of IgG globulin under controlled conditions of pH and temperature and the technique of selective plasmaphoresis (32) originally described by Bier (3). The former technique permits the fractionation of extremely large volumes of serum and is widely used in the production of normal human IgG for therapeutic use. The selective plasmaphoresis procedure involves the application of forced flow electrophoresis to the fractionation of whole blood maintained in extracorporeal circulation. By this technique relatively pure gamma globulin can be separated from large volumes of blood, the

[1]) In the author's department this step is usually carried out by modification of the batch procedure of Stanworth 1961 using Whatman DE11 or DE52 exchanger supplied through H. Reeve Angel and Co. Ltd., 14 New Bridge Street, London, E.C.4.)

remainder (cells and protein) being returned to the donor animal. Recent experiments have indicated that using the most up to date equipment it should be possible to process the complete blood volume of an immunized horse in 4 hours. It should be possible to repeat this process at frequent intervals (32).

The absorption and sterilization of anti-lymphocytic antibody

Most anti-lymphocytic sera are not specific for lymphocytes but cross react with other cells including erythrocytes and platelets. This cross reactivity is due in part to the heterogeneous cell mixtures used in producing the antisera and also to the presence of shared histocompatibility antigens on the various cells. In addition the antisera may also cross react with serum proteins suggesting, as might be expected, that the cell mixtures used were »contaminated« with serum proteins. As these cross-reacting antibodies are extremely toxic they have to be removed by absorption. In our own laboratories the antibodies to erythrocytes are now routinely removed using stroma prepared from outdated AB blood and studies are under way to determine the minimum number of platelets required to absorb the anti-platelet activity. If high levels of antiserum protein antibodies are detected they may be absorbed with normal human serum (10). However if such a step is necessary it is advisable to ensure that the resultant soluble antigen antibody complexes are removed during the subsequent fractionation procedure, or by high speed centrifugation (30,000 G for 1 h), for they could prove troublesome, especially if this material is administered intravenously. This centrifugation may be conveniently performed prior to sterilization for it also removes material which blocks the Seitz or Millipore filters (0.2 mu) and is preferable to pre-filtration which frequently accentuates this problem. The final product should be stored at —20° C to prevent undesirable structural changes in the antibody molecules which could result in the loss of immunosuppressive activity (14).

Throughout fractionation and absorption, especially where open systems are used, the emphasis should be on speed and wherever possible the fractionations should be performed in the cold (4° C). These precautions will reduce the risks of denaturation and bacterial contamination which in turn will minimize the release of pharmacologically active peptides such as bradykinin and the production of pyrogens which may have resulted in some of the side effects observed to date. However it should perhaps be stressed that a number of these side reactions reported on administration of heterologous anti-lymphocytic IgG are also observed on the intravenous injection of human IgG and have been attributed to structural changes in this molecule. These adverse reactions include tachycardia, respiratory distress, chills, fever and in more severe cases nausea, vomiting and circulatory collapse may occur (16).

Future developments in the fractionation of anti-lymphocytic globulin

Recent data from our own laboratory using ^{131}I-labelled antilymphocytic IgG has indicated, as might be expected, that less than 5% of the IgG in our preparations is

Table 5. Some properties of equine anti-hapten antibody.

Antibody class	IgG_a	IgG_b	IgG_c	»10S« IgG	IgG(T)
Binds antigen	+		+	+	+
Precipitates antigen	+		±		—
Fixes complement	+		—		—
Electrophoretic mobility	γ_2		γ_1	γ_1	γ_1

This table is based on data published by KLINMAN, KARUSH and ROCKEY (see text) and is ncluded to illustrate the heterogeneity of horse antibody to a relatively simple hapten (p-azo-henyl-β lactoside group). Experimentel data from our own laboratories suggests that horse ntiserum to human lymphoid tissue is equally complex.

nti-lymphocytic i.e., binds to lymphocytes (35). Based on the detailed reports on horse nti-hapten antibodies it is highly probable that this small percentage is representative f several distinct types of IgG globulin with widely different properties (17, 18, 26). t would appear that there are at least 4 different proteins namely the IgGa, IgGb, gGc and a »10S« IgG globulin. Although all these proteins may exhibit a marked ffinity for the antigen, the IgGc globulin (like IgG(T) — the Tequine globulin) fails o bind complement or precipitate the antigen and it is highly probable that molecules f this type could block the immunosuppressive effect of the cytotoxic IgGa, b mole-ules. Immunoelectrophoretic analysis of anti-lymphocytic antibody preparations btained in our own department by a variety of procedures confirms the heterogeneity f the horse immunoglobulins. It is possible therefore that the specific activity of anti-ymphocytic antibody preparations and hence the therapeutic effect, could be greatly ncreased by the removal of this non cytotoxic »blocking« antibody. In order to do this will be necessary to resort to electrophoretic and chromatographic procedures which hould permit the partial resolution of the IgGa, b and the electrophoretically faster gGc component. However with the presently available equipment, the amount of rotein that could be fractionated by this procedure is limited, and therefore the sub-ractionation of the IgG protein is a question of academic rather than practical mportance.

Summary

The present brief report outlines some of the more important techniques currently mployed in the production and fractionation of anti-lymphocytic antibody.

Various aspects of the immunization procedure are considered including the choice f antigen, the selection of the recipient and the course of immunization. The advantages f using fractionated and stored antigenic materials and adjuvants are discussed, and

the potential value of the passive inhibition of undesirable antibody formation stressed.

The desirability of characterizing anti-lymphocytic antibodies prior to large sca fractionation is emphasized and procedures permitting such an analysis are outline The techniques presently used to fractionate antilymphocytic antibody are present together with details on absorbing and sterilizing the antibody preparations.

In the final section the heterogeneity of anti-lymphocytic antibody preparations cu rently available is stressed and the possible advantages, and practical difficulties, further fractionation are discussed.

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Characterization of Antilymphocytic Antibody

ANTILYMPHOCYTIC serum (ALS) is now well known to have the power of prolonging the life of homografts on the animals into which it is injected[1–3]. All who have studied ALS agree that a high proportion of its immunosuppressive power resides in the 7*S* globulin fraction[2]. The experiments described here represent a formal analysis of the power of different fractions of ALS raised in rabbits and horses to prolong the life of A-strain tail skin homografts on CBA mice. Rabbit antisera against mouse thymocytes were prepared by the method of Levey and Medawar[3]. The horse antiserum, made by a scaled-up variant of the same technique, was kindly supplied to us by the Wellcome Foundation. The serum fractions were normally reconstituted to the concentrations at which they were originally present in whole serum, and assayed by the subcutaneous injection of 0·5 ml. on the second and again on the fifth days after skin grafting[3].

All sera were inactivated at 56° C for 30 min before further treatment. Separation into the 19*S*, 7*S*, 4·5*S* size classes (and an intermediate class, 10*S*) was accomplished by filtration on 'Sephadex' gel at 4° C as described by Flodin and Killander[4], using 17–20 ml. serum volumes on 'Sephadex *G*-200' columns (90 × 5·7 cm) equilibrated with 0·15 molar sodium chloride containing 0·06 molar phosphate buffer *p*H 7·2. The pooled fractions were concentrated to the original serum volume by ultrafiltration at 4° C through dialysis tubing.

Reduction was carried out at 20° C for 2 h using 0·1 molar 2-mercaptoethanol. The reduced material was alkylated by dialysis against 100 volumes of 0·06 molar phosphate buffer (see above) containing 0·02 molar sodium iodoacetamide[5]. The reduced and alkylated preparation was finally dialysed against phosphate buffered saline to remove the excess iodoacetamide.

The IgG fractions were made by two procedures. Rabbit antisera were fractionated by diethylaminoethyl (DEAE) cellulose batch chromatography[6], and the horse antiserum by first precipitating with sodium sulphate (final concentration 14 per cent w/v) and then batching on DEAE cellulose. Whatman DEAE cellulose (*DE*11) with an exchange capacity of 1·0 molar equiv./g was used, and equilibration and elution were performed with 0·025 molar sodium chloride containing 0·01 molar phosphate buffer *p*H 7·2.

The composition of the various fractions was assessed both by immunoelectrophoresis[7], using either sheep

Table 1. GRAFT MORTALITY DISTRIBUTIONS ASSOCIATED WITH DIFFERENT FRACTIONS OF RABBIT AND HORSE ANTI-MOUSE ALS

	Days after transplantation															
	10	11	12	13	14	15	16	17	18	19	20	21–25	26–30	31–40	41–50	>50
Rabbit ALS XVIII:																
Whole serum (5·8%)											1	3		1		
19*S* (0·75%)		4	1	1	2	1	1	1								
"10*S*" (0·28%)		4	2	3	1		1			1						
7*S* (1·3%)												4	1	1	1	
4·5*S* (2·9%)		1	1	1	2											
Rabbit ALS XX:																
Whole serum (6·25%)						1		1			2	2	2			
19*S* (0·88%)		2	2	1	1	2	2									
"10*S*" (0·53%)*		2		2	3	3	2									
7*S* (1·63%)									1	3		2	1	1		
7*S*-γ (IgG) (0·85%)						1		1	1	1		2	2			
4·5*S* (2·36%)	1	2	2	3	2	1	1									
2-ME treated (6·25%)									1			3	1		4	1
Horse ALS 4896A:																
Whole serum (6·6%)						1		1			5	4	1	1	2	
19*S* (0·39%)				3	1	3	1									
"10*S*" (0·28%)			2		1	2	2	1								
7*S* (3·25%)									1	1	4			1		
7*S*-γ (IgG) (1·1%)								1			2	2	1	2		
4·5*S* (1·5%)		1	1	1	1	3	1									
2-ME treated (6·6%)		3	1	9	2					1						

Each entry in the table shows the number of skin graft breakdowns registered as complete on the day indicated. All grafts A→CBA. All sera and serum fractions injected subcutaneously on the second and again on the fifth day after grafting. Figures in brackets represent protein concentrations (grams per cent).

* Twice serum concentration.

anti-rabbit serum or rabbit anti-horse serum, and by electrophoresis on polyacrylamide gel[8]. Protein content was estimated by the Folin-phenol procedure[9]. Analysis showed that the rabbit IgG preparation was free from contaminating proteins. The horse preparation, on the other hand, contained small amounts of another globulin, presumably the IgA(T) globulin[10].

The survival time of A-strain tail skin grafts on normal untreated CBA males is $11{\cdot}6 \pm 1{\cdot}3$ days[3]. Table 1 records the daily incidence of graft breakdowns in mice treated with rabbit or horse antisera and their various fractions. With rabbit serum it is clear that all the activity is accountable for in the IgG (7*S*-γ) fraction and that the serum fully retained its potency after reduction with 2-mercaptoethanol. The activity of horse antiserum resides almost wholly in the 7*S* size fraction, but it was destroyed by reduction and alkylation under conditions that did not affect the potency of rabbit antiserum, and we must leave open the possibility that fractions other

than IgG, possibly the IgA(T) fraction, may contribute to its immunosuppressive power.

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IN VITRO STUDIES ON THE HETEROGENEITY OF EQUINE ANTIBODIES TO HUMAN LYMPHOID TISSUE

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Abstract—The development of lymphocyte agglutinating, lysing and transforming activities in the serum of a horse undergoing prolonged immunization with human spleen have been investigated. The antibody activities of successive bleeds were assessed by standard *in vitro* immunological procedures and their molecular characteristics were determined by G200 sephadex gel filtration. Anti-lymphocytic globulin was prepared from a selected serum sample and the distribution of the various activities in this sample, and the original serum, was extensively studied by a variety of physicochemical procedures. These included G200 sephadex gel filtration, gradient ultracentrifugation, DEAE cellulose chromatography and Porath column electrophoresis. The results indicate that both these samples contain a complex mixture of proteins with widely differing physicochemical properties and *in vitro* activities. Finally a preliminary assessment has also been made of the various methods currently used in preparing anti-lymphocytic antibody for therapeutic use.

INTRODUCTION

During recent years the immunosuppressive properties of anti-lymphocytic sera have been well documented and in the limited number of studies performed to date the major part of this activity has been found to be associated with the IgG fraction[1]. However absorption studies using ^{131}I-labelled IgG globulin prepared from hyperimmune equine antisera to human spleen have indicated, as might be expected, that only a small proportion of the molecules in these preparations were anti-lymphocytic i.e. bind specifically to lymphocytes[2]. Furthermore based on previous observations on equine antibodies to a variety of much simpler antigens, it is highly probable that many of the anti-lymphocytic IgG (and globulin) preparations used are extremely complex[3–8]. For example studies on equine anti-hapten antibodies have indicated that there are at least four different proteins in the IgG fraction alone, namely IgGa, IgGb, IgGc and a 10*S* IgG globulin, as well as the IgG(T) and IgM components[3, 4]. Although all of these antibodies exhibit affinity for the antigen (p-azophenyl B lactoside), they vary in their ability to precipitate the protein bound antigen and to fix complement. Experiments have shown that the non-cytotoxic non-precipitating antibody molecules are able to block the activity of those molecules capable of precipitating the antigen and fixing complement[4]. In addition sequential

studies on antibody formation have revealed that the concentrations and binding affinities of the various antibody molecules may vary appreciably during the course of immunization [4].

On the basis of the above observations on the complexity of equine antibodies we have re-investigated the distribution of the *in vitro* anti-lymphocytic activity in an equine antiserum to human spleen and in its IgG fraction using a variety of physicochemical procedures. In addition to these extensive characterization studies on a single antiserum sample we have studied the distribution of antibodies throughout the period of immunization and also made a preliminary assessment of various methods of isolating anti-lymphocytic antibody. It is believed that these studies might explain to some extent the difficulty experienced in correlating the *in vitro* properties of anti-lymphocytic antibody with its immunosuppressive activity and might also explain the loss of activity which has been observed on prolonged immunization [9, 10] and fractionation [11]. Furthermore in the long term it is anticipated that investigations of this nature will lead to the development of less toxic and more active products for therapeutic use.

MATERIALS AND METHODS

The preparation of anti-lymphocytic serum and anti-lymphocytic globulin

The anti-lymphocytic serum was produced using fresh human spleen as antigen. Following perfusion of the isolated spleen with saline or other physiological buffers the spleen was chopped up and finely disrupted by passage through stainless steel screens [1]. The final cell suspension contained, in addition to lymphocytes, a large number of other leukocytes and erythrocytes although the exact number of these was not determined. This inoculum was injected subcutaneously, the course of immunization and bleeding being outlined in Table 1 and Fig. 1. Following bleeding the samples, which were collected in a standard acid citrate dextrose mixture, were incubated at 56°C for 30 min to precipitate fibrinogen and to inactivate complement.

The sequential development and molecular characteristics of the antibodies elicited was investigated by performing G200 sephadex gel separations of successive bleeds. However, a more precise characterisation of the antibodies produced was restricted to a single bleed (obtained 5/3/68) and an IgG globulin sample derived therefrom. This IgG globulin preparation was obtained by a combined salt precipitation and chromatographic technique as follows. The initial step involved adjusting the pH of the serum to 8 with 0·1 *N* sodium hydroxide and precipitating a crude globulin fraction by the slow addition of 28 per cent (w/v) sodium sulphate, the final concentration being 16 per cent (w/v). The resultant precipitate was then dissolved in, and dialyzed against, 0·02 *M* phosphate buffer, pH 6·5, and finally batched on diethylaminoethyl cellulose equilibrated to the same pH and ionic strength. Approximately 120 g dry weight of Whatman DE11 exchanger with an ion exchange capacity of 1·0 m Eq/g was used for each original litre of inactivated plasma. The final product was concentrated by lyophilization, and reconstituted in phosphate buffered saline (pH 7·2, 0·06 *M* containing 0·15 *M* sodium chloride). Immunoelectrophoretic analysis (see later) revealed that the produce contained several IgG components.

Table 1. A summary of the injection and bleeding schedule used in producing equine antiserum to human spleen

Date	Spleen cell injections*		Bleed† volume (l.)	Date	Spleen cell injections		Bleed volume (l.)
	No.	Per cent viable			No.	Per cent viable	
13/12/66	Not determined		—	5/3/68	—	—	10
10/2/67	28×10^9	82	—	22/3/68	$63{\cdot}2 \times 10^9$	52	—
1/3/67	—	—	10	1/4/68	—	—	8
7/3/67	—	—	10	18/4/68	—	—	10
14/3/67	—	—	10	30/4/68	—	—	8
28/9/67	20×10^9	80	—	20/4/68	Not determined		—
3/10/67	$11{\cdot}8 \times 10^9$	77	—	17/5/68	—	—	8
25/10/67	—	—	8	24/5/68	—	—	6
2/11/67	—	—	10	27/5/68	$22{\cdot}9 \times 10^9$	61	—
9/11/67	—	—	10	26/7/68	Not determined		—
18/11/67	Not determined		—	11/9/68	Not determined		—
23/2/68	$43{\cdot}6 \times 10^9$	13	—	18/9/68	—	—	9

*On several occasions cell counts were not determined (see Table) but each injection contained the cells from one spleen.

†The blood was collected in A.C.D. and after separation of the plasma the erythrocytes were retransfused into the donor. A number of minor bleeds (less than 500 ml) were performed in addition to the above bleeds.

Gel filtration

This procedure was performed at room temperature (20°C) on columns of G200 sephadex (particle size 40–120 μ) equilibrated in phosphate buffered saline (pH 7·2, 0·06 *M* containing 0·15 *M* sodium chloride). Small aliquots (2 ml) of the various bleeds were fractionated on columns* 32 × 2·5 cm, the rate of elution being maintained by peristaltic pump at 16 ml per hour. The larger serum samples (40–60 ml) and IgG globulin fractions (containing 1–2 g of protein) were separated on columns 100 × 5·7 cm, the rate of elution being 80 ml per hr. The protein concentration (percentage transmission) of the effluent was monitored continuously at 254 mμ in a Uvicord 1 absorptiometer (See Figs. 2 and 7). The void volumes of the columns were determined using Blue Dextran 2000 (M_W^-, 2,000,000).

Gradient ultracentrifugation

Preparative ultracentrifugation was performed in an MSE Superspeed 50 centrifuge under the conditions illustrated in Fig. 3 [ref. 12]. On the completion of the run the base of the tube was pierced and 0·5 ml fractions collected by a drop wise procedure, the flow being controlled by a syringe attached to the top of the tube. The relative protein concentration of the fractions was determined at 280 mμ in a Unicam spectrophotometer, an $E^{1\,cm}_{280\,m\mu}$ of 1·0 being equivalent to one unit. The protein composition of the fraction was assessed immunoelectrophoretically.

DEAE cellulose column chromatography

This chromatographic procedure was performed at room temperature on Whatman DE 52 cellulose exchanger with an ion exchange capacity of 1 m Eq/g dry wt. The column (bed height 58 × 3 cm) contained the equivalent of 50 g dry exchanger. Prior to fractionation the exchanger and the protein samples (15 ml of serum, 1·0 g of IgG) were equilibrated with 0·01 *M* phosphate buffer, pH 8·0 by washing and dialysis respectively. The column was eluted initially with the equilibrating buffer (400 ml) after which gradient elution was commenced using the cone-sphere system described by Fahey *et al.* [13] the final buffer being 0·3 *M* sodium dihydrogen phosphate. The protein distribution in the effluent was measured in a 1 cm cell in a Unicam Sp 500 spectrophotometer at 280 mμ (See Figs. 4 and 9) and the pH and resistance of the fractions were also determined.

Column electrophoresis

This preparative procedure was performed on a polyvinyl chloride powder supporting medium (supplied by British Geon Ltd.) in a large scale Porath electrophoresis column (LKB Model 5800 A). This equipment enables high resolution of the slowly moving components of protein mixtures, since the faster zones are extracted by counter current elution during the electrophoresis. The supporting medium and the protein samples (150 ml of serum or 7 g of IgG) were equilibrated against the electrophoresis buffer (0·05 *M* Tris-HCl buffer, pH 8·2) by washing and dialysis respectively. The protein samples were applied to the column (bed height 60 cm) by means of a bent pipette and electrophoresis was performed for 24–32 hr using an LKB 5806 A power supply.

*Chromatographic columns supplied by Wright Scientific Ltd., Kenley, Surrey.

Electrophoresis was then continued for a further period of 20–53 hr during which counter current elution of the anodic end of the column was performed using a buffer flow rate of 100–200 ml per hr. At the end of this period the electrophoresis and counter current elution were terminated and the remainder of the protein displaced from the column by elution with electrophoresis buffer. Throughout the counter current and normal elution period the column effluent was monitored at 254 mμ in a Uvicord I absorptiometer and was also checked at 280 mμ in a Unicam SP 500 spectrophotometer. The fractions obtained were pooled to give fifty samples of 200 ml volume which were concentrated ten fold by ultrafiltration. The protein content (g per cent) of the concentrated fractions is plotted in Figs. 5 and 11. For further details of the equipment and the conditions of electrophoresis the reader should consult Figs. 5 and 11 and previous references [14, 15].

Additional fractionation procedures

Preliminary experiments were also performed to determine the most efficient procedures for purifying anti-lymphocytic antibody. These involved the repeated precipitation (1–3 times) of the anti-lymphocytic serum with sodium sulphate (final conc 14 per cent w/v) or ammonium sulphate (final conc 40 per cent with respect to saturated ammonium sulphate) followed in some cases by batch chromatography on Whatman DEAE 52 cellulose exchanger under the conditions outlined on page ii. In the case of the sodium sulphate precipitates, 20 g wet weight of DE52 was used for each 100 ml initial volume of serum whilst 50 g was used for the ammonium sulphate precipitates obtained from 100 ml of serum. The pH of the serum was adjusted to 8 with 0·1 *N* sodium hydroxide prior to the initial precipitation and the procedures were carried out at room temperature. Further details of these experiments are recorded in Table 3.

Protein concentration

Large volumes of protein were concentrated by lyophilization whilst the smaller samples were concentrated at 4°C by ultrafiltration through 8/32 Visking tubing. The samples were then dissolved in and/or dialyzed against phosphate buffered saline (pH 7·2, 0·06 *M* containing 0·15 *M* NaCl) and all the samples from any particular fractionation were made up to the same volume prior to *in vitro* assay.

Protein determinations

These were performed by the Folin Phenol procedure as described by Lowry *et al.* [16] using a crystalline bovine serum albumin standard.

Analytical ultracentrifugation

Analyses were carried out in 10 mm cells in an MSE analytical ultracentrifuge operating at 55,000 rev/min and 20°C. The protein solutions (concentration $\simeq$ 1 g per cent) were dissolved in and dialyzed against phosphate buffered saline (pH 7·2, 0·06 *M* containing 0·15 *M* sodium chloride) and centrifuged at 5000 rev/min for 10 min prior to analysis. The relative compositions of the protein solutions were determined by measuring the areas under the peaks,

after fitting the appropriate solvent base line, while the $S_{20,w}$ values were calculated by the traditional method.

Immunoelectrophoresis

A discontinuous buffer system was used in these analyses. Oxoid agar No. 3 (1 per cent w/v) was dissolved in barbitone buffer ($I = 0{\cdot}05$, pH 8·2) whilst a borate buffer was used in the electrode vessels (0·3 *M* boric acid, 0·06 *N* sodium hydroxide, pH 8·2). The protein samples were applied at a 2 g per cent concentration and electrophoresed for 3½ hr at 6–8 V per cm. The antisera used were rabbit anti-equine serum and a goat anti-equine *H* chain*. The latter antiserum distinguishes between the IgGa, IgGb and IgGc proteins and also reacts with common heavy chain determinants of the IgG(T) heavy chain.

The preparation of peripheral blood lymphocytes

Lymphocytes were obtained from 400 ml fresh heparinized human blood by filtration at 37°C through a Fenwal Leuko-Pak which removes the polymorphs, followed by sedimentation of the red cells by addition of 3 per cent (w/v) plasma gel (3 ml per 100 ml of filtrate). After standing for 2 hr at 37°C the lymphocyte rich supernatant was removed asceptically. These lymphocyte preparations were generally of high viability (>95 per cent) and were contaminated to varying degrees with erythrocytes.

In vitro *assay of anti-lymphocytic activity*

The lymphocyte agglutination titres were determined in $8 \times 1{\cdot}2$ cm test tubes by the addition of 2×10^6 peripheral blood lymphocytes (in 0·1 ml) to doubling dilutions of the test sample (final vol. 0·2 ml) in phosphate buffered saline (pH 7·2, 0·6 *M* containing 0·15 *M* sodium chloride). The samples were then incubated for 18 hr at room temperature (20°C) and examined microscopically. Cytotoxic titres were performed by the addition of 4×10^6 lymphocytes (volume 0·2 ml, viability >96 per cent) to doubling dilutions of the test sample in Hanks solution (prepared as previously described[17]). Following the addition of 0·1 ml of rabbit complement, the samples were incubated for 1½ hr at 37° after which 1·6 ml of 0·067 per cent (w/v) trypan blue in Hanks solution was added. Five minutes later the cells were fixed by means of 0·1 ml of 40 per cent formalin, and the number of cells failing to exclude trypan blue determined. The cytotoxic titre of the initial dilution of each test sample was also determined in the absence of complement and the end point was taken as that dilution in which the number of trypan blue stained cells was 10 per cent higher than in the appropriate complement free control.

The treatment of lymphocytes with anti-lymphocytic antibody in a complement deficient tissue culture medium has been shown to transform the cells into blast forms and this process is accompanied by a marked increase in RNA and DNA synthesis within the cell[18]. We have therefore determined the capacity of the various fractions to stimulate the incorporation of tritiated uridine into lymphocyte RNA and throughout this paper we refer to this as a measure of transformation. The cultures were set up in $10 \times 1{\cdot}3$ cm stoppered test tubes and incubated at 37°C in an atmosphere of 95 per cent air 5 per cent

*Kindly provided by Dr. J. H. Rockey, Department of Microbiology, University of Pennsylvania, Philadelphia.

carbon dioxide. Each culture initially contained 2×10^6 viable lymphocytes in 2 ml of medium 199 (Glaxo Laboratories Ltd., Greenford, England) plus 0·5 ml of autologous heat inactivated plasma (56°C for 30 min) and 0·1 ml of the sample under test. Tritiated uridine* was added when the cultures were set up, in all cases the final concentration being 0·18 μc/ml (i.e. 0·5 μc/culture). Eighteen hours later the cells were spun down and washed three times in 5 ml volumes of phosphate buffered saline and then successively in similar volumes of 5 per cent trichloroacetic acid, phosphate buffered saline, and absolute methanol (twice). After a final spin the precipitate was dissolved in 0·3 ml of hyamine hydrochloride and incubated for 10 min at 60°C. Following cooling, 5 ml of scintillant (NE, 213, Nuclear Enterprises, Edinburgh) was added and the samples counted in a Packard 'Tricarb' scintillometer for 10–20 min or a minimum count of 20,000. The cultures were set up in triplicate and all the samples from any particular fractionation were examined simultaneously using the same batch of cells. Control tubes were also set up containing cells, autologous plasma, tritiated uridine and medium 199. The activity of the various samples has with one exception (see Fig. 3) been expressed as a ratio of the mean counts of the antibody treated culture to that of the control culture (see above) set up at the same time with cells from the same donor. In these studies no correction was made for possible quenching though previous results from this laboratory using similar materials have shown that there was no significant variation in the degree of quenching from culture to culture[19]. External standard counts indicated that the efficiency of counting was the same for each batch of samples i.e. for each experiment.

Measurement of erythrocyte agglutinins

The erythrocyte agglutinating activity was determined in $8 \times 1{\cdot}2$ cm test tubes by adding 0·1 ml of 2·5 per cent (w/v) AB erythrocytes to 0·25 ml volumes of doubling dilutions of the test sample. All the dilutions were performed in the phosphate buffered saline originally described. After incubation for 18 hr at room temperature (20°C) the contents of the tube were agitated and examined macroscopically and microscopically for agglutination.

In the figures the antibody levels have been expressed as the reciprocal $\log_2$ of the titre.

RESULTS

THE SEQUENTIAL DEVELOPMENT OF ANTI-LYMPHOCYTIC ANTIBODIES

Due to the difficulty in obtaining suitable antigenic material the course of immunization was somewhat irregular and the antigen extremely complex, nevertheless several points of interest emerged. Following the first four spleen cell injections the anti-lymphocyte and anti-erythrocyte titres remained at fairly steady levels, apart from some indication of a slight decline in the lymphocytotoxic titres of the fractions towards the end of the immunization course (see Fig. 1). Although the peak antibody titres occurred in the 7*S* fraction, considerable activity was also observed in the 19*S* and 10*S* fractions, the activity of the 19*S* fraction closely paralleling that of the 7 fraction. After the initial

*Code No. TRA. 27. Specific activity 2·73 c/mmole. Supplied by Radiochemical Centre, Amersham, England.

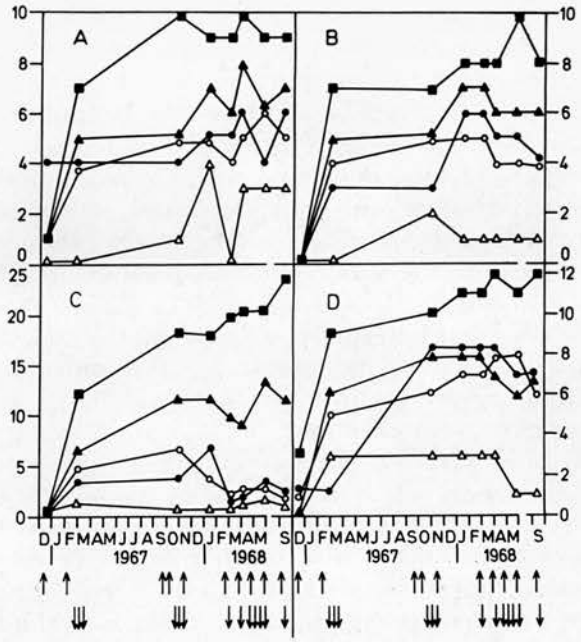


Fig. 1. The development and characteristics of anti-lymphocytic antibodies in a horse receiving a prolonged course of immunization with human spleen.

The horse received subcutaneous injections with human spleen cell suspensions and was bled repeatedly over a 2 yr period as indicated in Table 1. Selected samples were fractionated on G200 sephadex as described in the text and the lymphocyte agglutinating (A), lytic (B) and transforming activities (C) and the erythrocyte agglutinating activities (D) of the various fractions obtained were assessed. The lymphocyte transforming activities in all the figures are expressed as a multiple of the control sample. All other activities are recorded as $\log_2$ of the observed titre. Note—Following the first ~~three~~ four spleen cell injections most of the activities remain fairly constant. In addition although peak activities are associated with the 7*S* fraction, considerable activity is also observed in the 19*S* and 10*S* fractions throughout the period of immunization. Key: ↑ injection; ↓ bleed; -■-■-serum; -●-●-19*S*; -○-○-10*S*; -▲-▲-7*S*; -△-△-4·5*S*.

immunization period the transforming activity of the whole sera and the 7*S* fraction also remained fairly constant. There was however a significant decline in the transforming activity of the 19*S* and 10*S* material.

THE DISTRIBUTION OF ANTI-LYMPHOCYTIC ACTIVITY IN WHOLE ANTISERUM

As previously indicated all the following studies were performed on a single sample of antiserum and on a globulin sample derived therefrom.

The large scale G200 sephadex fractionation procedure in which the major peaks were subdivided, indicated that a higher proportion of the lympho-agglutinating, lymphocytotoxic and erythrocyte agglutinating activity was located in the 7*S* region than was revealed by the small scale procedures, a reflection perhaps of the finer subdivision and possible greater resolution of the larger column (see Fig. 2). There was however a certain amount of 'weak non specific' lymphoagglutinating and erythrocyte lytic activity in the 4·5*S* fractions.

In contrast to the somewhat selective concentration of the above activities in the 7*S* region, the lymphocyte transforming activity was present in high concentrations in all fractions, including the 4·5*S* fractions. The above observations were made on two separate large scale fractionations.

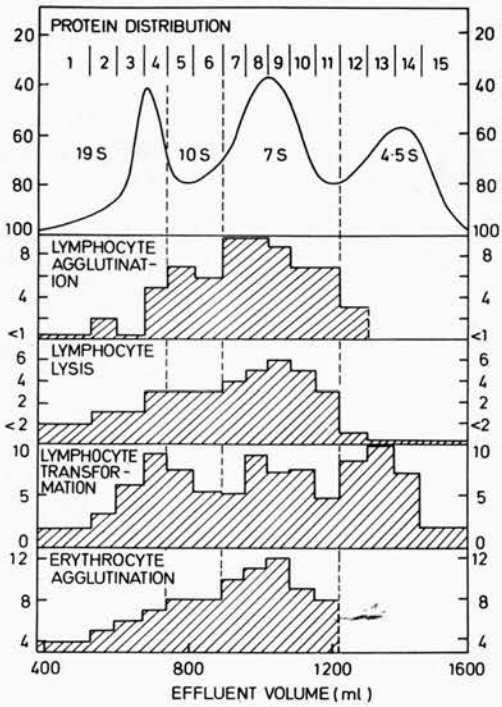


Fig. 2. The distribution of *in vitro* activities in fractions obtained by G200 sephadex gel filtration of anti-lymphocytic serum.

Forty millilitres of equine antiserum to human spleen was fractionated on a G200 sephadex column (dimensions 100 × 5·7 cm). The effluent was pooled as shown in the diagram and concentrated to give 15 fractions of 2 ml volume. Note that the bulk of the lymphoagglutinating, lymphocytotoxic and erythrocyte agglutinating activities were associated with the 7*S* fraction, whilst the transforming activity was fairly evenly distributed throughout the various molecular weight classes.

Ultracentrifugation in a discontinuous sucrose density gradient confirmed that the bulk of the lymphoagglutinating was located in the 7*S* region of this serum (see Fig. 3). However, the distribution of lymphocyte transforming activity was less widespread than observed in the previous G200 runs.

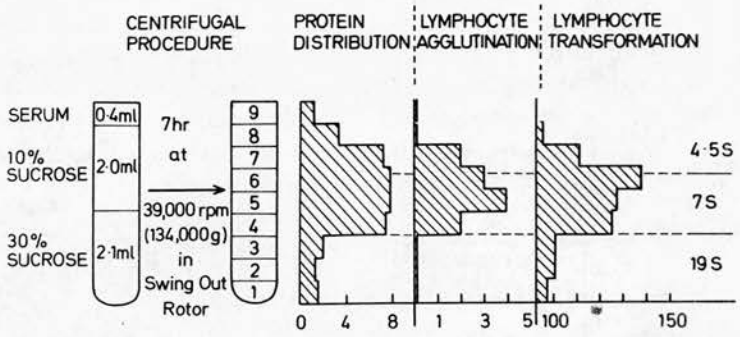


Fig. 3. The distribution of *in vitro* activities in fractions obtained by gradient ultra-centrifugation of anti-lymphocytic serum. The equine antiserum to human spleen was separated at 5°C as outlined in the figure. The lymphocyte transforming activity has been expressed as a percentage of that observed in the control culture. Note that the major part of the anti-lymphocytic activity associated with the 7*S* region. Compare with Fig. 2.

Column chromatography on DEAE cellulose indicated that the major part of the lymphocyte agglutination, lymphocyte lysis and erythrocyte agglutinating activity was associated with the slow IgG fraction of serum thus confirming the G200 sephadex results indicating that these activities were predominantly associated with the 7*S* fraction of serum (see Fig. 4). The distribution of these activities once more closely paralleled each other. In contrast however, the lymphocyte transforming activity did not closely follow the distribution of the other anti-lymphocytic activities. Thus, although transforming activity was associated with fractions containing slow IgG (F1–F3) and fast IgG (F6), considerable activity also resided in fractions possessing relatively small amounts of agglutinating and lytic activities (see F2, 3 and 6). Furthermore fractions 8–15 appeared to inhibit the incorporation uridine into lymphocyte RNA.

According to expectations, Porath column electrophoresis of whole serum revealed that the various agglutinating and cytotoxic antibodies were located in the cathodic (gamma) region (see Fig. 5). Immunoelectrophoresis using a polyvalent antiserum confirmed that these activities were associated with proteins of gamma 1 (fast) and gamma 2 (slow) electrophoretic mobility (see Fig. 6). The major part of the transforming activity was also confined to the gamma region though some activity was observed in fractions of faster electrophoretic mobility (see fractions 5, 19 and 23).

THE DISTRIBUTION OF ANTI-LYMPHOCYTIC ACTIVITY IN ANTI-LYMPHOCYTIC GLOBULIN

Two protein peaks were obtained following G200 sephadex gel filtration of the anti-lymphocytic globulin preparation, a major 7*S* peak preceded by a

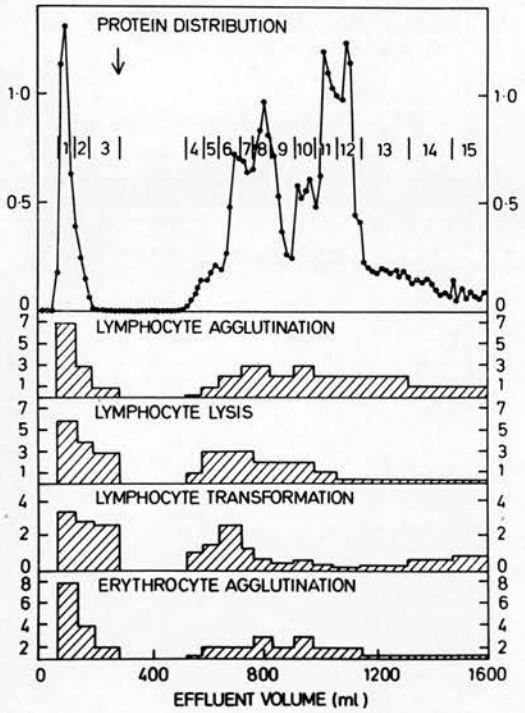


Fig. 4. The distribution of *in vitro* activities in fractions obtained by DEAE cellulose column chromatography of anti-lymphocytic serum. Fifteen millilitres of equine antiserum to human spleen was fractionated as outlined in the text on a column of Whatman DE52 cellulose ion exchanger containing 50 g dry weight exchanger with an ion exchange capacity of 1 mEq/g. Gradient elution was commenced at the point indicated by the arrow and the effluent was pooled as shown in the diagram to give 15 fractions of 5 ml volume. Note that Fractions 2, 3 and 6 exhibit transforming activity comparable to that shown by the more strongly agglutinating and cytotoxic Fraction 1. Observe also the broad distribution of the various activities indicating a wide spectrum of antibodies.

minor peak (see Fig. 7). The bulk of the lymphocyte agglutinating and lytic activity and the anti-erythrocyte activity were associated with the 7*S* peak. On the other hand the lymphocyte transforming activity was more evenly distributed throughout the chromatogram, considerable activity being observed in the high molecular weight fraction (F1–F2) which exhibited extremely low lymphocytotoxic and lymphoagglutinating titres. Subsequent G200 sephadex studies with a mixture of anti-lymphocytic IgG preparation and Blue Dextran

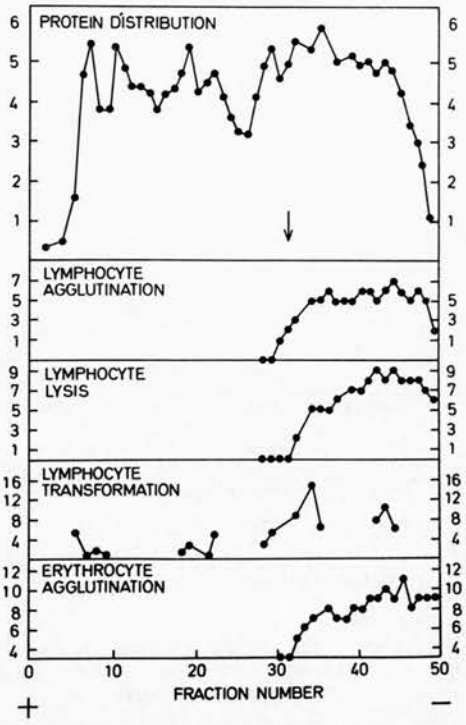


Fig. 5. The distribution of *in vitro* activities in fractions obtained by the Porath column electrophoresis of anti-lymphocytic serum.

One hundred and fifty millilitres of equine antiserum to human spleen was electrophoresed on an LKB Model 5800A electrophoresis column as outlined in the text. During the initial stages electrophoresis was performed at 450 V and 0·2 A for 24 hr after which it was performed at 500 V and 0·25 A for a further 53 hr and counter current elution was commenced from the anodic end of the column. At the point indicated by the arrow, electrophoresis and counter current elution were terminated and elution was commenced from the cathodic end (the top) of the column. Note. The location of the various activities in the cathodic region, that is in fractions containing immunoglobulins of gamma 1 and gamma 2 mobility (see Fig. 6). However transforming activity was also observed in fractions of faster mobility.

2000, indicated that the protein in F1 was excluded in the void volume, and that the transforming activity of this fraction was enhanced by the Blue Dextran. Analytical ultracentrifugation revealed that F1, which was turbid, contained appreciable amounts of 10*S* protein and other higher molecular weight components (see Table 2) a pattern similar to that frequently observed in IgA rich solutions. Immunoelectrophoretic analysis using both polyvalent antiserum and

Table 2. A summary of the properties of anti-lymphocytic globulin fractions

Fractionation procedure	Fraction No.	Protein conc. (g%)	Anti-lymphocytic activity			Relative ultracentrifugal composition					Immuno-electrophoret. composition
			Agg.	Cytotoxic	Transform.	19S > 19S* > 19S < 10S		10S	7S	4·5S	
Original serum		5·0	256	256		6·2	—	2·6	28·9	62·3	
Original IgG		10·0	640	640	16·5	—	—	6·2	93·8	—	See Figs. 10 and 12
G200 Sephadex	*1	1·2	80	40	5·9	24·4		13·3	64·6	—	See Fig. 8
	4	15·0	1280	2560	5·2	1·8		5·6	92·6	—	
DEAE Cellulose Column Chromatography	1	2·2	160	640	3·8						
	2	3·7	160	640	3·2	4·3	5·3	10·5	79·9	—	
	3	0·83	80	160	7·7						
	*4	0·52	20	80	8·5	8·3	34·0	31·4	25·8	—	See Fig. 10
	10	0·78	40	80	6·4						
	11	1·2	20	80	11·2 1·2	4·2	—	10·7	85·1	—	
	14	1·04	80	<20	2·5 0·9						
Porath Column Electrophoresis	15	0·36	40	40	120 1·2	—	—	5·5	76·5	18·0	
	22	6·0	2560	320	1510 1·5	3·1	3·5	7·8	85·6	—	
	24	7·24	1280	640	1375 1·4						See Fig. 12
	27	6·52	5120	1280	1220 1·2	5·1	—	4·5	90·4	—	
	*32	1·3	160	160	1475 1·5	3·8	24·9	30·1	42·3		
	36	0·44	40	<40	1280 1·3						

*These fractions exhibited a marked capacity for lymphocyte transformation even though they possessed low levels of other anti-lymphocytic activities. Analytical ultracentrifugation revealed that they were rich in proteins with $S_{20,w}$ values of 10 or greater and were polydisperse (suggesting aggregation). It is possible that the complexity of these protein samples may be due in part to their susceptibility to physicochemical changes during isolation and storage that this results in their high transforming activity.

antiserum to the *H* chain of equine IgG showed that it contained at least 2 antigenically distinct components, namely a gamma 2 globulin and another protein with immunoelectrophoretic properties similar to the 10*S* IgG previously described [3]. By comparison Fraction 4 contained IgG proteins of both gamma 1 and gamma 2 mobility (see Figs. 7 and 8).

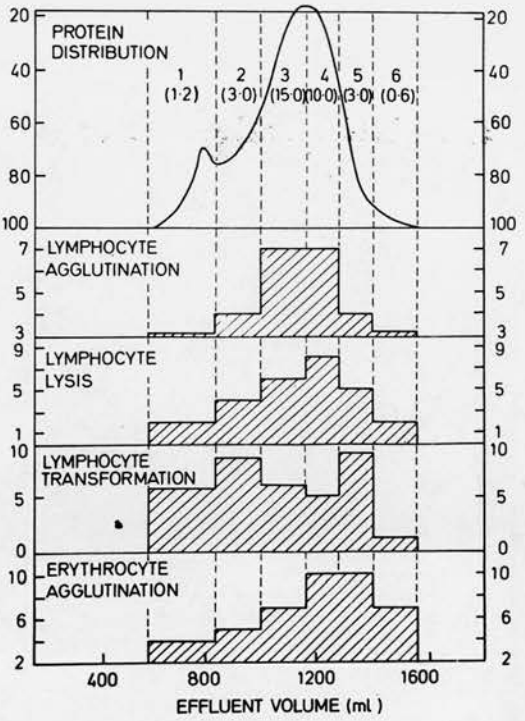
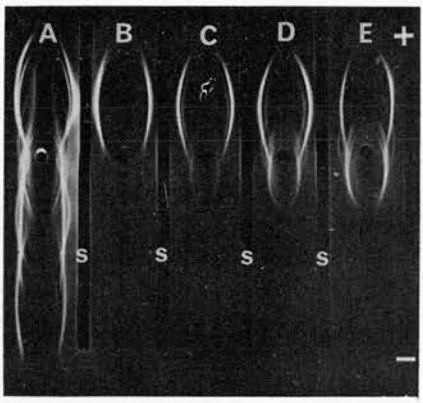
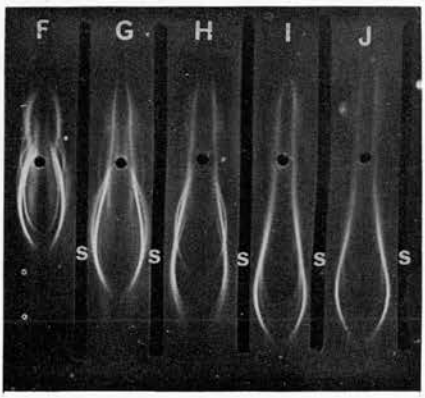


Fig. 7. The distribution of *in vitro* activities in fractions obtained by the G200 sephadex filtration of anti-lymphocytic globulin.
Two grammes of anti-lymphocytic IgG prepared as described in the text was fractionated on a G200 sephadex column (dimensions $100 \times 5{\cdot}7$ cm). Note that the bulk of the lymphoagglutinating, lymphocytotoxic and erythrocyte agglutinating activities were located in the 7*S* peak (Fractions 3 and 4). In contrast the lymphocyte transforming activity was more evenly distributed, considerable activity being observed in fractions excluded in the void volume (F1 and 2) which were relatively deficient in other anti-lymphocytic activities.

The distributions of the various biological activities in the DEAE cellulose chromatographic fractions of anti-lymphocytic IgG were in general similar to those observed on the fractionation of whole serum (see Fig. 9, compare Fig. 4). There did, however, appear to be a greater proportion of lymphoagglutinating activity eluted with the higher ionic strength buffer (see Fractions 12–15). Once



6A



6B

Fig. 6. The immunoelectrophoretic analysis of fractions obtained by the Porath column electrophoresis of anti-lymphocytic serum.

A. Initial serum; B. Fraction 5; C. Fraction 10; D. Fraction 15; E. Fraction 20; F. Fraction 25; G. Fraction 30; H. Fraction 35; I. Fraction 40; J. Fraction 45; K. Fraction 50. S = rabbit anti-equine serum. Note the heterogeneity of the protein fractions in the gamma 1 region (H-fraction 35) and the gamma 2 region (J-fraction 45) containing anti-lymphocytic activity. For further details of the samples and the immunoelectrophoresis see the text.

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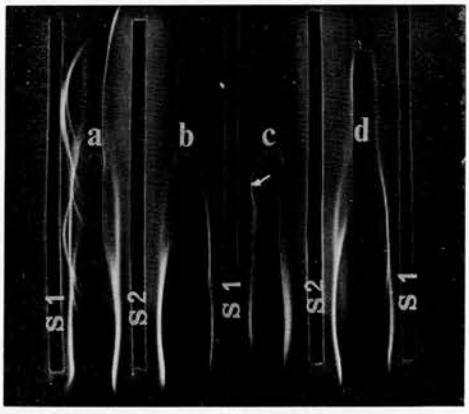


Fig. 8. The immunoelectrophoretic analysis of fractions obtained by the G200 sephadex gel filtration of anti-lymphocytic globulin.
A. Initial serum; B. Anti-lymphocyte IgG; C. G200 Fraction 1; D. G200 Fraction 4; S1. Rabbit anti-equine serum; S2. Goat anti-equine IgG heavy chain. Note the major components in fraction 1 are gamma 2 protein and a fast moving component possibly 10*S* IgG (see arrow) while fraction 4 is rich in proteins of gamma 1 and gamma 2 mobility.

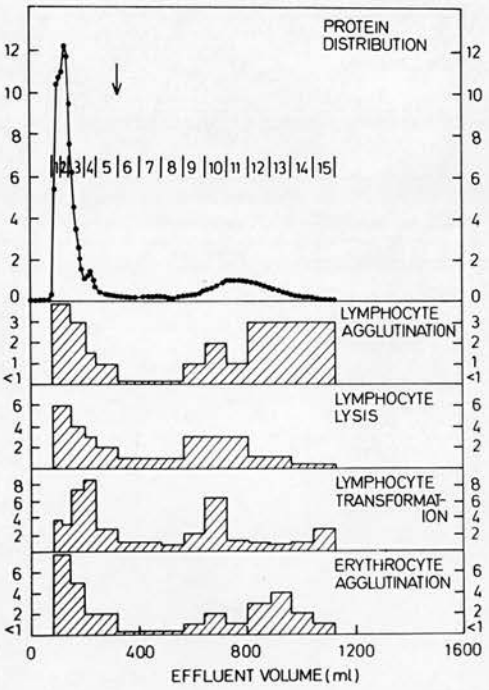


Fig. 9. The distribution of *in vitro* activities in fractions obtained by the DEAE cellulose column chromatography of anti-lymphocytic globulin.
One gramme of anti-lymphocytic IgG was fractionated on DE52 cellulose as outlined in Fig. 4 and the text. Note that as in Fig. 4 some fractions with relatively low lymphoagglutinating and lymphocytotoxic titres exhibited marked transforming activity (see Fractions 3, 4 and 10) compared with fractions high in these activities (Fractions 1 and 2). Observe also the distribution of the various activities in proteins with widely varying affinities for the DEAE cellulose (See also Fig. 4.).

again peak levels of lymphocyte transforming activity did not coincide with the peak levels of other anti-lymphocytic activities (see Fractions 3, 4 and 10). Immunoelectrophoretic analysis revealed that the protein eluted with the initial buffer (0·1 *M* phosphate, pH 8·0) was relatively homogeneous, successive fractions exhibiting progressively increasing electrophoretic mobility (see Fig. 10). In contrast the protein eluted on the commencement of the gradient was immunoelectrophoretically complex containing a number of proteins of gamma 1 and beta mobility. Analytical ultracentrifugation indicated that the fractions possessing high transforming activities were rich in 10*S* and other higher molecular weight components (see Table 2).

Porath column electrophoresis confirmed the heterogeneity of the anti-lymphocytic globulin (see Fig. 11). The lymphoagglutinating activity occurred in 2 major peaks, one associated with proteins of gamma 1 mobility and the

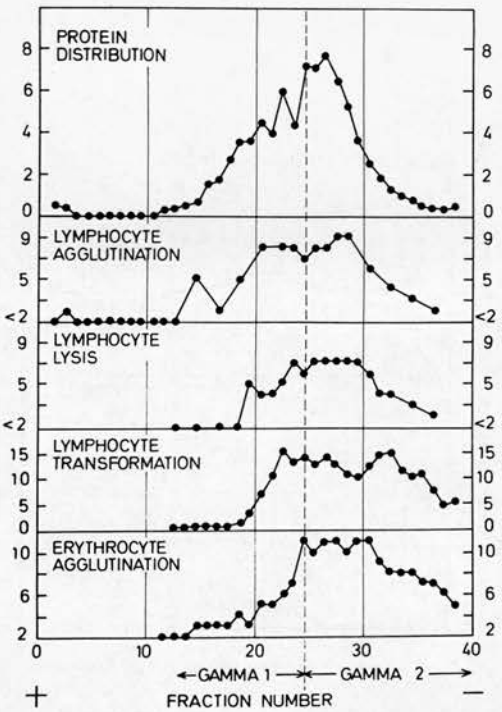
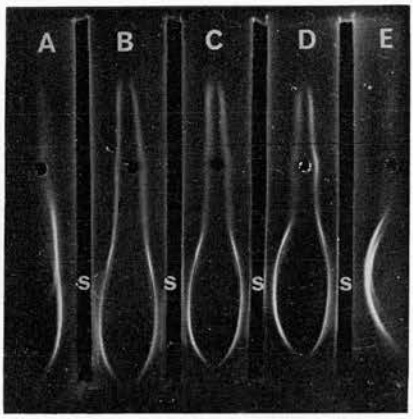


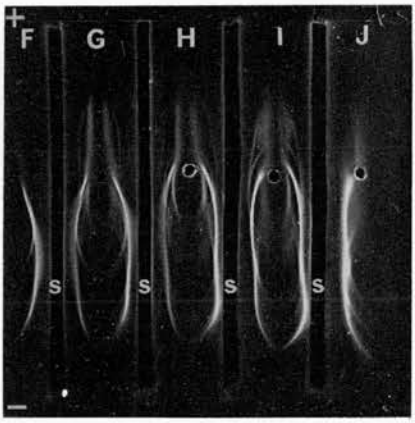
Fig. 11. The distribution of *in vitro* activities in fractions obtained by the Porath column electrophoresis of anti-lymphocytic globulin.

Seven grammes of anti-lymphocytic IgG was electrophoresed on an LKB Model 5800A electrophoresis column as outlined in the text. Electrophoresis was performed at 550 V and 0·275 A for a total of 52 hr, counter current elution from the anodic end (base) of the column being commenced after 32 hr. At the point indicated by the arrow electrophoresis and counter current elution were terminated and elution commenced from the cathodic end (top) of the column. Note. The wide distribution of the various activities in fractions of both gamma 1 and gamma 2 mobility. Nevertheless apart from the lymphoagglutinating activity, there is a displacement towards the gamma 2 region. The fractions exhibit a number of peaks of lymphocyte transforming activity, two of these occurring in regions where the other anti-lymphocytic activities are sharply declining (see F32 and 35). In contrast relatively low lymphocyte transforming activity was observed in fractions with peak lymphocyte agglutinating and lymphocytotoxic activity (see F27–30). For the immunoelectrophoretic composition of selected fractions see Fig. 12.

other with proteins of gamma 2 mobility (see Fig. 12). The lymphocyte and erythrocyte agglutinin activity were less evenly distributed, a greater proportion of the activity being associated with proteins of gamma 2 mobility. The lymphocyte transforming activity was again eluted in a number of peaks confirming the marked heterogeneity of proteins possessing this activity. One of the peaks coincided with the peak lymphoagglutinating activity in the gamma 1 region (see Fraction 23). In contrast, however, the fractions in the gamma 2 region possessing maximum lymphoagglutinating and lymphocytotoxic activity were



(A)



(B)

Fig. 10. The immunoelectrophoretic analysis of fractions obtained on the DEAE cellulose chromatography of anti-lymphocytic globulin.
A. Anti-lymphocytic IgG; B. Fraction 1; C. Fraction 2; D. Fraction 3; E. Fraction 5; F. Fraction 11; G. Fraction 12; H. Fraction 13; I. Fraction 14; J. Fraction 15. S = rabbit anti-equine serum. Referring to Fig. 9 it will be noted that the various biological activities are associated with proteins of a wide range of electrophoretic mobilities and immunological characteristics. Observe the heterogeneity of the protein eluted with the high ionic strength buffer due in part to the presence of proteins other than immuno globulins.

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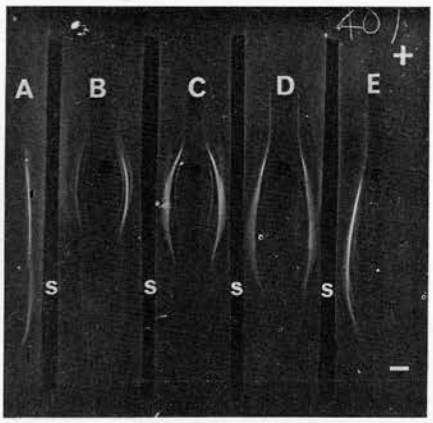
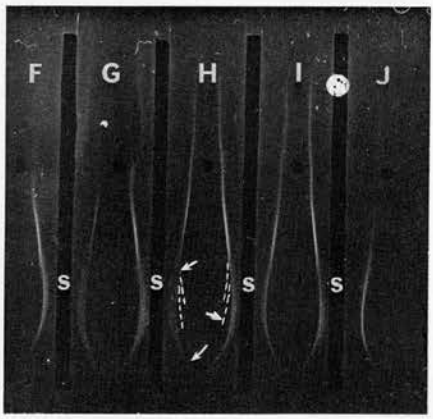
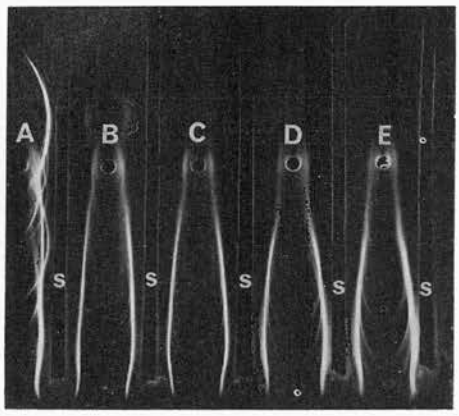


(A)

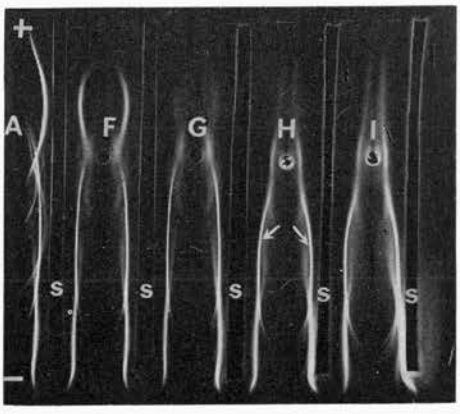


(B)

Fig. 12. The immunoelectrophoretic analysis of fractions obtained by the Porath column electrophoresis of anti-lymphocytic globulin. A. Anti-lymphocytic IgG; B. Fraction 12; C. Fraction 15; D. Fraction 18; E. Fraction 21; F. Fraction 24; G. Fraction 27; H. Fraction 30; I. Fraction 33; J. Fraction 36. S = Rabbit anti-equine serum. Note the heterogeneity of the protein eluted in both the gamma 1 and gamma 2 regions, many fractions in the latter region containing at least 3 proteins presumably IgGa, IgGb and IgGc (see arrows). For further details of the samples see Fig. 11.



(A)



(B)

Fig. 13. The immunoelectrophoretic analysis of anti-lymphocytic globulin prepared by various procedures.
A. Initial serum. B. Sodium sulphate precipitation (X1) C. Sodium sulphate precipitation (X3) D. Sodium sulphate precipitation (X1) followed by DEAE cellulose batch chromatography. E. Sodium sulphate precipitation (X3) followed by DEAE cellulose batch chromatography. F. Ammonium sulphate precipitation (X1). G. Ammonium sulphate precipitation (X3). H. Ammonium sulphate precipitation (X1) followed by DEAE cellulose batch chromatography. I. Ammonium sulphate precipitation (X3) followed by DEAE cellulose batch chromatography. S = Rabbit anti-equine serum. Note the heterogeneity of all the products including those prepared by 3 successive salt precipitations and batch chromatography. The sodium sulphate precipitated materials were extremely rich in gamma 2 globulins especially IgGa, IgGb and IgGc. In contrast ammonium sulphate derived products were very rich in the IgG(T) globulin of gamma 1 mobility (see arrow).

associated with a trough in the lymphocyte transforming activity (see Fraction 28). Furthermore, other peaks of transforming activity also occurred in fractions where these anti-lymphocytic activities were relatively low (Fractions 32 and 35). Immunoelectrophoretic analysis again revealed that the fractions in the gamma 1 region (Fractions 12–24) were immunoelectrophoretically complex (see Fig. 12). The proteins in the gamma 2 region were probably less complex but still contained a number of proteins including IgGa, IgGb and IgGc subcomponents. Analytical ultracentrifugation of Fraction 32 possessing high transforming activity revealed that this sample was rich in proteins with $S_{20,w}$ values of 10 or greater (see Table 2).

A COMPARISON OF METHODS FOR PREPARING ANTI-LYMPHOCYTIC IgG

From the results of these preliminary experiments it will be observed that there were marked differences in both the quantity and the quality of the protein recovered depending on the salt used for precipitation (see Table 3 and Fig. 13). In general 2·5–3 times as much protein was recovered in procedures utilizing ammonium sulphate and the products obtained contained large amounts of protein of gamma 1 mobility, presumably IgG(T)[3]. The sodium sulphate products on the other hand contained much higher concentrations of gamma 2 protein (IgG subcomponents) (compare samples D and H Fig. 13).

On a weight basis the lymphocyte lysing and agglutinating properties and the erythrocyte agglutinating activities of the various products were remarkably similar. In contrast however, the transforming activity of the sodium sulphate products appeared to be potentiated by batch chromatography whilst that of the ammonium sulphate precipitates were reduced. Nevertheless the overall recoveries of *in vitro* anti-lymphocytic activity were much greater when ammonium sulphate was used for the initial precipitation.

Throughout all these studies the anti-lymphocytic and anti-erythrocyte activity of normal horse serum and normal horse IgG were frequently assessed and shown to be negligible (for example see pre-immunization samples Fig. 1).

DISCUSSION

The antigen used in these studies was extremely complex and most probably elicited antibodies against cells other than lymphocytes, such as macrophages and plasma cells. Nevertheless these studies were performed because this antigen (spleen) has been widely used in producing anti-lymphocytic antibody for therapeutic use[1]. In spite of this limitation the results obtained indicate that the antibodies in our preparation which reacted with lymphocytes were physicochemically and functionally complex, characteristics previously observed with equine antibodies to other purer antigens[3–8].

Sequential studies on the development of lymphocyte agglutinins and cytotoxins in horses undergoing prolonged courses of immunization with human lymphoid tissue have previously been reported[20, 21]. However, in the present study additional data has been obtained on the molecular characteristics of the antibodies and on the development of lymphocyte transforming activity. Although following the initial period of immunization no really significant quantitative and qualitative antibody changes were observed, important

Table 3. A comparison of the properties of anti-lymphocytic globulin prepared by different procedures

Property	Whole serum	Na_2SO_4 Precipitation						$(NH_4)_2SO_4$ Precipitation					
		Alone			+DEAE batching			Alone			+DEAE batching		
		X1	X2	X3	X1	X2	X3	X1	X2	X3	X1	X2	X3
Mg protein recovered per ml serum	57·5	10·0	8·7	7·0	4·4	4·3	3·8	25·0	25·0	20·5	13·0	12·5	13·0
Lymphocyte agglutination	1024	512	512	512	256	512	512	512	512	256	512	512	512
Lymphocyte lysis	160	80	80	80	160	160	160	160	160	160	160	160	160
Lymphocyte transformation	9·0	8·1	4·9	5·5	8·0	9·7	8·3	7·8	7·8	5·6	4·2	6·1	5·5
Erythrocyte agglutination	2048	512	256	256	512	512	512	512	512	512	1024	1024	512

A crude globulin sample was precipitated from the serum by the addition of either sodium sulphate or ammonium sulphate and the resultant precipitate was redissolved and either reprecipitated with salt or batched on DEAE cellulose. The number of precipitations performed varied between one and three. In contrast to previous studies all the *in vitro* activities were determined on 2 g per cent solutions and the transformation was determined in 40 hr cultures.

variations in the avidity, concentration, complement fixing ability, and electrophoretic properties may have occurred in the earlier stages of immunization, a phenomenon observed following the immunization of horses with other antigens [4, 6].

Apart from the sequential experiments discussed above the rest of the investigations were restricted to antiserum obtained from a single bleed. Whilst the limitations of this approach are appreciated, it was adopted because it made a detailed study of the distribution of anti-lymphocytic activity a practical exercise. Furthermore the IgG component of this sample was investigated in preference to the other immunoglobulins, because of its known heterogeneity and its widespread experimental and clinical use, the latter being attributable in part to its ease of isolation [1].

These investigations have clearly demonstrated the complex distribution of the various 'anti-lymphocytic' activities in a single antiserum sample from a horse hyperimmunized with human spleen a property previously noted by other investigators [21, 22]. In the present studies this heterogeneity has been observed in the whole antiserum and in its IgG fraction. For example the present chromatographic and electrophoretic results on the anti-lymphocytic IgG preparation indicated that its lymphoagglutinating activity is almost evenly distributed between its gamma 1 and gamma 2 components [see Figs. 7 and 11]. In contrast a greater proportion of the lymphocytotoxic activity was associated with the gamma 2 fraction which has been shown to contain the complement fixing antibodies of a number of species. Furthermore the 10*S* containing fraction obtained on the G200 sephadex chromatography of the so called anti-lymphocytic IgG was relatively deficient in lymphocyte agglutinating or lytic activity. It should be stressed however that the observed electrophoretic distribution of these activities may not be representative of that occurring in whole serum, for the process of preparation of the IgG results in the selective concentration of the gamma 2 component (see Fig. 13).

In addition to the heterogeneities discussed above, the present studies also revealed the marked complexity of proteins capable of lymphocyte transformation, a property not previously observed in preliminary observations from our laboratory [19]. Of particular interest was the observation that high levels of transforming activity were often observed in fractions possessing low lymphoagglutinating and lymphocytotoxic activity, and these were located in the gamma 2 region. Such proteins were relatively rich in 10*S* protein and higher molecular weight components (presumably aggregates). Equine immunoglobulins with similar sedimentation properties have previously been observed in other antisera [3, 23], however, further studies will be necessary to determine if this protein is a naturally occurring 10*S* IgG globulin [3] or IgA like protein, or has resulted from denaturation and complex formation during fractionation.

At the moment we possess no satisfactory explanation of the superior transforming activity of the above fractions. It is possible that the development and distribution of the lymphocyte transforming properties is completely independant of the other anti-lymphocytic activities. Alternatively the true lymphocyte transforming activity of the more concentrated fractions may not have been manifested for a variety of reasons including saturation of the system, non-

specific destruction and various forms of inhibition. Preliminary investigations would suggest that some form of inhibition (feedback or otherwise) may be operative for dilution of Fraction 2 (see Fig. 9) results in a marked increase in its transforming activity. Indeed 0·5 mg of this material in culture is more active than 0·5 mg of Fraction 4. On the other hand viability studies and transformation studies with erythrocyte absorbed fractions indicated that the observed differences could not be attributed to non specific cell destruction or the presence of erythrocyte agglutinins.

The picture is further complicated by the fact that anti-lymphocytic sera frequently contain antibodies to serum proteins[1, 21] and these may cause lymphocyte transformation. For example xenogeneic antisera to rabbit whole serum, rabbit IgG and IgG subunits and allogeneic anti-allotype sera will directly transform rabbit lymphocytes in tissue cultures[24–26]. Furthermore recent studies have indicated that transformation may be 'indirectly' affected through antigen antibody complexes[27] which could be produced by the interaction of the samples under test with the autologous plasma in the culture medium. However, determination by the tanned cell procedure of the anti-human serum protein titres of the samples under test suggested that transformation of this nature was insignificant in the present studies. In the first instance the antiserum protein activity of all the samples tested was extremely low and in addition the most potent transforming fractions of IgG (see F4, Fig. 9) possessed lower titres than those of the less active fractions (see F1 and F2, Fig. 9).

The marked transforming activity of the 4·5*S* fractions obtained following the large scale G200 sephadex fractionation of anti-lymphocytic serum was unexpected, especially as such activity was not observed in equivalent fractions obtained in small scale separations or on preparative ultracentrifugation. While it is feasible that such differences could be due to higher levels of growth promoting substances or low molecular antibodies[3] in more concentrated fractions obtained following the large scale separation, recent data obtained in our laboratory suggests that this activity may be the result of contamination with small amounts of sephadex. This could also explain the weak agglutination and lysis observed in these fractions.

The preliminary comparative studies on the various methods of preparing anti-lymphocytic globulin are of great importance in relationship to the large scale preparation of these materials for therapeutic use. It is quite apparent that greater recoveries of *in vitro* anti-lymphocytic activity can be achieved using the ammonium sulphate procedures, presumably due to the precipitation of large amounts of the IgG(T) globulin. It should be stressed that greater protein recoveries can be achieved with sodium sulphate by increasing the concentration to 16–18 per cent (w/v). Whether or not however this material is a more effective immunosuppressant than the sodium sulphate derived material has still to be determined. In this respect it is feasible that qualitative differences between the products may significantly influence their *in vivo* activities.

Although in the present report there has been no attempt to relate the immunosuppressive capacity of the various antibody preparations with their physicochemical properties and *in vitro* biological activity, the data obtained

is of value in our further understanding of the known properties of anti-lymphocytic antibody and in relation to its future clinical use. As previously stated the anti-lymphocytic antibody preparations described to date have been physico-chemically and functionally complex, even those preparations obtained by immunoabsorption procedures[2]. The observed *in vitro* and *in vivo* activity of such preparations will be the net effect of a variety of activities, a number of which are acting in competition. For example we may have direct competition between non-cytotoxic and cytotoxic antibodies for sites on the lymphocyte surface resulting in the inhibition of complement fixation and hence cell lysis, a situation similar to that observed in other equine antibody systems[4, 7]. A variation in the relative concentration of 'competitive' activities of this type which can occur during immunization or as a result of fractionation might explain the loss of immunosuppressive activity which has occasionally been noted following hyperimmunization or fractionation[9–11]. Furthermore the marked lymphocyte transforming capacity of relatively non-cytotoxic fractions may help to explain previous observations that lymphocyte transforming activity is not a reliable measure of immunosuppressive activity[28–30].

The present results emphasize the need for further studies on the development, fractionation and immunosuppressive properties of anti-lymphocytic antibodies. Experiments are already in progress to ascertain the precise development of various anti-lymphocytic activities in horses undergoing immunization with human peripheral blood and thoracic duct lymphocytes, and a study of the immunosuppressive properties of equivalent preparations from equine antisera to rat lymphoid tissue is planned. In order to correlate precisely the physicochemical and immunological properties of anti-lymphocytic antibodies and develop reliable immunoassay procedures it may be necessary to isolate the various anti-lymphocytic antibodies in purer form and this will most probably involve combined physicochemical and immunoabsorption procedures. Finally, it is assumed that studies of this nature will contribute greatly to our understanding of the properties of anti-lymphocytic antibodies and to the development of improved preparations for therapeutic use.

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Quantitative Studies with Antilymphocytic Antibody

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Radioactive labelling has been used in measuring the amount of IgG taken up from antilymphocytic serum by lymphocytes, and the proportion of antilymphocytic IgG in various preparations of this immunoglobulin.

LYMPHOCYTES exposed *in vitro* to specific antilymphocytic serum (ALS) become coated with antibody[1,2]. In the absence of complement, ALS agglutinates lymphocytes[3–5], and in appropriate conditions of culture stimulates blast transformation[6] and uptake of uridine and thymidine[7,8]; in the presence of complement it causes lysis[3,4].

In experiments with horse-anti-human ALS based on measurements of uptake of tritiated uridine and thymidine by lymphocytes *in vitro*, it was found that most of the stimulating activity resides in the IgG component of the serum[8]. It was further shown that when lymphocytes were exposed to ALS for short periods of time and then washed twice before being put up in culture, exposure for 15 min resulted in only very slight stimulation, whereas exposure for 60 min resulted in nearly maximal stimulation, as judged by subsequent uptake of tritiated uridine. We set out to extend this work in two ways.

In the first place, we have used radioactive labelling to determine the mean number of IgG molecules taken up when lymphocytes are incubated *in vitro* with IgG prepared from ALS (referred to hereafter as ALS IgG) and from normal serum.

Second, by combining radioactive studies with experiments in which the stimulating activity of various preparations of IgG is measured before and after absorption with standard numbers of lymphocytes, we have attempted to estimate the proportion (R) of antilymphocytic IgG in each preparation, that is, the proportion of IgG molecules which are antilymphocytic in the sense that they stimulate lymphocyte transformation.

Horse-anti-human ALS and IgG were prepared as described earlier[9]. Samples of ALS IgG and normal IgG were labelled with iodine-131 by the method of Hunter and Greenwood[10]. Suspensions of lymphocytes were prepared and cultures were set up as described before[8] except that, unless otherwise stated, the number of lymphocytes in a culture was 3×10^6 instead of 10^7 and the dose of tritiated uridine in a culture was 0·5 μc. The total volume of each culture was 3 ml. and the medium consisted of medium 199 with 25 per cent inactivated autologous plasma.

As a preliminary, dose response curves were established showing the uptake of tritiated uridine in cultures containing either iodine-131-labelled or unlabelled antilymphocytic IgG in amounts ranging from 0·25 to 2·0 mg per culture. Cultures containing tritiated uridine but no iodine-131 were treated as previously described. The cells were washed successively in phosphate buffered saline (three washes), 5 per cent trichloroacetic acid, phosphate buffered saline, and absolute methanol, and digested with the minimum possible quantity of hyamine hydrochloric acid. Scintillant was then added and counting was carried out with a Packard 'Tricarb' scintillometer. The ratio of the c.p.m. of cultures containing IgG preparations to the mean c.p.m. of control cultures without added IgG was calculated and is referred to in what follows as the relative count. Cells and supernatants from suspensions containing labelled IgG were counted for iodine-131 in a well-type scintillation spectrometer incorporating a 2 in. sodium iodide crystal (Nuclear Enterprises 'Gammamatic'). Cells from cultures which contained both iodine-131-IgG and tritiated uridine were prepared for liquid scintillation counting as described and counted on two channels, one adjusted for tritium and the other for iodine-131. By counting known amounts of isotope it was found that for values of the external standard within the range accepted in the experiment, the efficiency of the tritium channel was $12{\cdot}5 \pm 1{\cdot}6$ per cent for tritium and $2{\cdot}90 \pm 0{\cdot}17$ per cent for iodine-131, and the efficiency of the iodine channel for iodine-131 was $74{\cdot}7 \pm 2{\cdot}9$ per cent. (This calibration was kindly undertaken by Dr John Simpson of the Department of Medical Physics, whose help we gratefully acknowledge.) In analysing the experimental results allowance was made for the influence of iodine-131 on the count in the tritium channel. As Fig. 1 shows, the labelled IgG was slightly less stimulating than the unlabelled material, but even

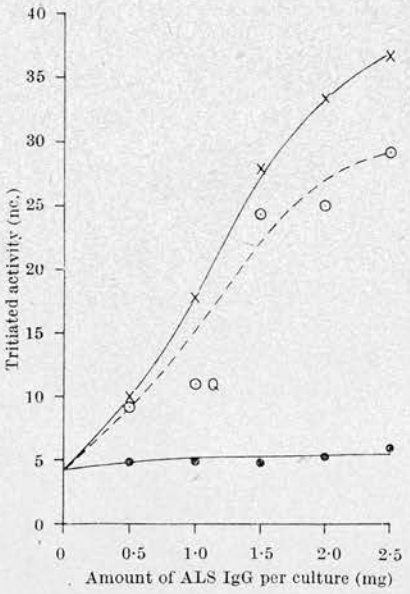

Fig. 1. Graphs showing the effect of ^{131}I-labelled (○) and unlabelled (×) ALS IgG, and of ^{131}I-labelled normal horse IgG (●), on the uptake of tritiated uridine by human blood lymphocytes *in vitro*. The point *Q* is an underestimate caused by quenching.

in the largest dose the difference was less than 20 per cent and it was considered that this could be neglected for the purposes of the investigation.

To determine the number of molecules of IgG taken up by each cell, lymphocytes suspended in culture medium were exposed to labelled IgG prepared from ALS. The cells were then spun down, separated from the supernatant, washed twice and counted, and an aliquot of supernatant was counted separately. Control tubes were set up with labelled normal IgG in place of IgG prepared from ALS. Assuming that all IgG molecules were labelled equally with iodine-131 and that the suspension contained no cells other than lymphocytes, the mean amount of IgG taken up by a cell (M) in each tube is then given by the equation $M = \frac{p\,G}{n}$, and the mean number of molecules

$$\text{taken up per cell} = \frac{M\,N}{m} = \frac{p\,G\,N}{n\,m}$$

$$\text{where } p = \frac{\text{Count of cells}}{\text{Count of cells + count of total supernatant}}$$

N = Avogadro's number ($\simeq 6{\cdot}10^{23}$ molecules/mole), G = amount of IgG per tube, n = number of lymphocytes per tube, m = molecular weight of IgG ($\simeq$160,000). It clearly does not matter which units are used for G and M provided they are the same for both.

In practice, allowance must be made for the possible uptake of IgG by erythrocytes, which constituted 60–75 per cent of the total cells in the suspension. The proportion of erythrocytes could have been further reduced, but only at the cost of either greatly reducing the total yield of lymphocytes or by using procedures, such as agglutination of erythrocytes by an appropriate antiserum or lysis in a hypotonic solution, which we thought might modify the subsequent behaviour of the lymphocytes. The relatively high proportion of erythrocytes was therefore accepted and appropriate controls were set up to determine the uptake of IgG in the conditions of the experiment by erythrocytes alone. The amount of IgG taken up by the lymphocytes was then calculated on the assumption that the erythrocytes in the mixed population adsorbed as much IgG as the same number of erythrocytes in the absence of lymphocytes.

The protocol of one experiment is set out in Table 1, and the results of this and three other similar experiments are summarized in Table 2. The results obtained with cells from different donors differed appreciably, but the uptake of ALS IgG was always much greater than that of normal IgG in similar conditions of culture, the maximum observed values being $5{\cdot}34 \times 10^6$ and $0{\cdot}94 \times 10^6$ molecules per lymphocyte, respectively. The relationship of the mean uptake of ALS IgG and normal IgG to the amount present in the culture is shown in Fig. 2. The similarity of the curves relating to ALS IgG in this figure and Fig. 1 suggests that uridine uptake is linearly related to the number of IgG molecules taken up by a cell, and Fig. 3 shows that this is approximately true.

The ratio (R) of antilymphocytic to total IgG molecules in the preparation has been estimated by combining the results of studies of the uptake of ^{131}I-IgG with absorption experiments based on stimulating activity as indicated by the uptake of tritiated uridine by lymphocytes in culture. In these experiments, a dose-response curve was first established in which the uptake of tritiated uridine by a fixed number of lymphocytes from a particular donor was plotted against the total amount of IgG added to the culture medium. The stimulating activity remaining after standard amounts of the same preparation had been absorbed for 1 h with a known number of lymphocytes from the same donor was then determined by using the absorbed material as a culture medium for fresh lymphocytes. Tritiated uridine was added to these cultures and the degree of stimulation was determined as usual. The amount (E) of unabsorbed IgG per culture which would

Table 1. UPTAKE OF ^{131}I-IgG BY LYMPHOCYTES AND ERYTHROCYTES

Source of IgG	Amount of IgG/tube (mg)	Exposure for 1 h at 37° C No. of cells/tube (millions) Erythrocytes	Lymphocytes	Mean c.p.s. (duplicate tubes) Cells	Supernatant	Total	Cell donor II Uptake of IgG ($g \times 10^{-8}$) By erythrocytes	By lymphocytes
ALS	**1**	9	Nil	28·0	23,636	1·18	1·18	Nil
	2	9	Nil	45·2	48,274	1·87	1·87	Nil
	3	9	Nil	64·1	66,632	2·98	2·98	Nil
	2	90	Nil	47·0	47,400	1·98	1·98	Nil
NS	1	9	Nil	1·5	6,217	0·24	0·24	Nil
	2	9	Nil	3·6	12,392	0·58	0·58	Nil
	3	9	Nil	4·3	18,290	0·71	0·71	Nil
	2	90	Nil	4·2	12,121	0·69	0·69	Nil
ALS	1	9	3	60·3	24,627	2·43	1·18*	1·25
	2	9	3	74·5	49,032	3·04	1·87*	1·17
	3	9	3	149·9	73,337	6·13	2·98*	3·15
	2	18	6	117·4	47,551	4·94	1·88*	3·06
	2	36	12	179·9	46,782	7·68	1·90*	5·78
	2	54	18	268·9	48,364	11·0	1·93*	9·07
	2	72	24	330·2	46,800	14·0	1·96*	12·04
	2	90	30	483·8	49,450	19·4	1·98*	17·42
NS	1	9	3	2·9	6,351	0·45	0·24*	0·23
	2	9	3	5·3	12,410	0·85	0·58*	0·27
	3	9	3	6·9	18,470	1·12	0·71*	0·41
	2	90	30	10·0	12,230	1·63	0·69*	0·94

This table shows the protocol of the first experiment.

* Calculated on the assumption that the presence of lymphocytes has not affected the amount of IgG taken up by the erythrocytes.

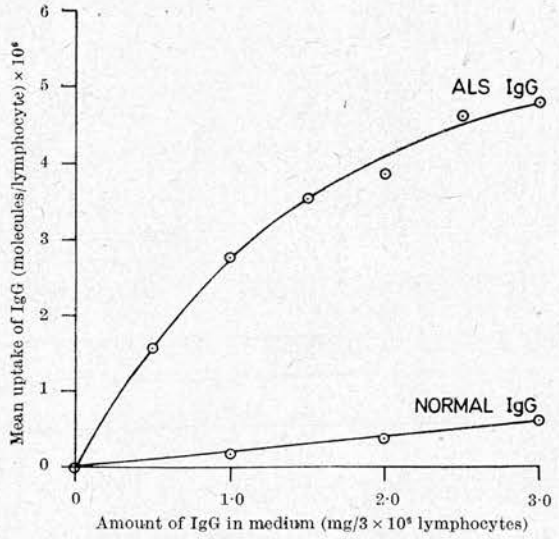

Fig. 2. Graphs showing the mean uptake of ^{131}I-labelled ALS IgG and normal horse IgG by human lymphocytes against the amount of IgG added to each culture. The means have been calculated from the data shown in Table 2 after completing the table for the cells of each donor at each dose level by graphical extrapolation.

produce the same degree of stimulation was read from the dose-response curve. To allow for the possible effect of erythrocytes in the cell suspension, control tubes were set up to determine how much, if any, antilymphocytic activity was absorbed out in the conditions of the experiment by erythrocytes alone. The absorption due to lymphocytes alone was then calculated on the assumption that the erythrocytes in the mixed population absorbed out as much antilymphocytic activity as the same number of erythrocytes in the absence of lymphocytes.

To calculate R it is necessary to make some assumptions about the types of IgG molecule present in the preparation and their properties. The simplest set of assumptions is as follows. (*a*) The preparation contains only two classes of IgG molecule: antilymphocytic molecules which bind to lymphocytes and stimulate them to an equal extent (as measured by increase in uptake of tritiated uridine) and non-antilymphocytic molecules which neither bind nor stimulate. (*b*) The degree of stimulation produced in tests with a standard number of lymphocytes is a function of the number of antilymphocytic molecules in the preparation but independent of the number of non-antilymphocytic molecules. On this basis, using the symbols already defined, the medium contained $\frac{G\,N}{m}$ molecules of IgG before absorption, of which $\frac{G\,N\,R}{m}$ were antilymphocytic and $\frac{G\,N\,(1-R)}{m}$ were non-antilymphocytic. After absorption the medium must have contained $\frac{E\,N\,R}{m}$ antilymphocytic molecules to cause the observed uptake of tritiated uridine, so that the mean number of molecules taken up by a cell was $\frac{(G-E)\,N\,R}{n\,m}$ (because, *ex hypothesi*, only antilymphocytic molecules are taken up). Thus

$$\frac{M\,N}{m} = \frac{(G-E)\,N\,R}{n\,m} = \frac{p\,N\,G}{n\,m} \quad \therefore R = \frac{M\,n}{(G-E)} = \frac{p\,G}{G-E}$$

Table 2. UPTAKE OF ^{131}I-IgG BY LYMPHOCYTES

Source of IgG	Amount of IgG per tube (mg)	No. of lympho-cytes per tube ($\times 10^6$)	Cell donor	Amount of IgG taken up per lymphocyte g 10^{-13}	Amount of IgG taken up per lymphocyte Molecules ($\times 10^6$)
ALS	0·5	3	I	6·35	2·38
	1·0	3	I	9·28	3·48
			II	4·18	1·57
			III	8·29	3·11
	1·5	3	I	10·08	3·78
	2·0	3	I	11·95	4·48
			II	3·89	1·46
			III	14·24	5·34
			IV	7·40	2·78
	2·5	3	I	13·33	5·00
	3·0	3	II	10·50	3·94
			III	14·24	5·34
	2·0	6	II	5·09	1·91
		12	II	4·80	1·80
		18	II	5·01	1·88
		24	II	5·01	1·88
		30	II	5·81	2·18
			IV	4·59	1·72
NS	0·5	3	I	0·11	0·04
	1·0	3	I	0	0
			II	0·77	0·29
			III	1·09	0·41
			IV	0·10	0·04
	1·5	3	I	0·90	0·34
	2·0	3	I	0·56	0·21
			II	0·90	0·34
			III	1·40	0·52
			IV	0·87	0·33
	2·5	3	I	1·07	0·40
	3·0	3	II	1·37	0·49
			III	2·50	0·94
			IV	1·50	0·56
	2·0	30	II	0·31	0·11
			IV	0·63	0·25

This is a summary of the results of four experiments employing cells from different donors.

Table 3. STIMULATING ACTIVITY OF ALS IgG BEFORE AND AFTER ABSORPTION WITH LYMPHOCYTES

Purpose of observations	Initial amount of IgG per tube (g) G	Stimulating activity before absorption expressed as mean relative count in standard uridine uptake test	No. of cells used for absorption ($\times 10^6$) Lympho-cytes n	Erythro-cytes	Stimulating activity after absorption expressed as mean relative count in uridine uptake test using absorbed IgG and fresh lymphocytes	Amount of IgG per tube which would have the same stimulating activity as the absorbed material (read from dose-response curve) (g) E	$G-E$
To establish dose-response curve	$0{\cdot}5.10^{-3}$	1·53					
	$1{\cdot}0.10^{-3}$	1·95					
	$1{\cdot}5.10^{-3}$	2·98					
	$2{\cdot}0.10^{-3}$	4·72					
	$2{\cdot}5.10^{-3}$	6·39					
Absorption experiment	$2{\cdot}0.10^{-3}$	4·72	30	80	1·45	$0{\cdot}4.10^{-3}$	$1{\cdot}6.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	24	64	1·53	$0{\cdot}5.10^{-3}$	$1{\cdot}5.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	18	48	1·51	$0{\cdot}5.10^{-3}$	$1{\cdot}5.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	12	32	1·63	$0{\cdot}65.10^{-3}$	$1{\cdot}35.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	6	16	1·80	$0{\cdot}8.10^{-3}$	$1{\cdot}2.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	3	8	1·74	$0{\cdot}8.10^{-3}$	$1{\cdot}2.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	0	80	4·71	$1{\cdot}90.10^{-3}$	$0{\cdot}1.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	0	8	4·74	$1{\cdot}93.10^{-3}$	$0{\cdot}07.10^{-3}$
	$2{\cdot}0.10^{-3}$	4·72	30	0		$0{\cdot}5.10^{-3}$*	$1{\cdot}5.10^{-3}$*
	$2{\cdot}0.10^{-3}$	4·72	24	0		$0{\cdot}6.10^{-3}$*	$1{\cdot}4.10^{-3}$*
	$2{\cdot}0.10^{-3}$	4·72	18	0		$0{\cdot}6.10^{-3}$*	$1{\cdot}4.10^{-3}$*
	$2{\cdot}0.10^{-3}$	4·72	12	0		$0{\cdot}7.10^{-3}$*	$1{\cdot}3.10^{-3}$*
	$2{\cdot}0.10^{-3}$	4·72	6	0		$0{\cdot}9.10^{-3}$*	$1{\cdot}1.10^{-3}$*
	$2{\cdot}0.10^{-3}$	4·72	3	0		$0{\cdot}9.10^{-3}$*	$1{\cdot}1.10^{-3}$*

This table shows the protocol of experiments with cells of donor V.

* Calculated on the assumption that the presence of lymphocytes has not affected the amount of stimulating activity absorbed by the erythrocytes in a mixed population.

Table 4. CALCULATION OF R FROM RESULTS OF TWO ABSORPTION EXPERIMENTS COMBINED WITH DATA ON UPTAKE OF ^{131}I-ALS IgG FROM TABLE 2

No. of lymphocytes ($\times 10^6$) n	Amount of ALS IgG per G	No. of molecules taken up/cell (from Table 2) ($\times 10^6$) MN^*/m				Amount of IgG/tube which would produce the same degree of stimulation as G grams IgG absorbed with n lymphocytes E		100 R Calculated from formula $R = \frac{Mn}{(G-E)}$							
		Lymphocytes of donor I	Lymphocytes of donor II	Lymphocytes of donor III	Lymphocytes of donor IV	Lymphocytes of donor IV (from Table 3)	Lymphocytes of donor V	Donors I, V	Donors I, VI	Donors II, V	Donors II, VI	Donors III, V	Donors III, VI	Donors IV, V	Donors IV, VI
3	$1{\cdot}0.10^{-3}$	3·48	1·57	3·11			$0{\cdot}85.10^{-3}$		1·86		0·84		1·66		
3	$2{\cdot}0.10^{-3}$	4·48	1·46	5·34	2·78	$0{\cdot}9.10^{-3}$	$1{\cdot}6.10^{-3}$	0·34	0·90	0·11	0·37	0·31	1·07	0·21	0·56
3	$3{\cdot}0.01^{-3}$		3·94	5·34			$1{\cdot}9.10^{-3}$				0·29		0·39		
6	$2{\cdot}0.10^{-3}$		1·91			$0{\cdot}9.10^{-3}$				0·28					
12	$2{\cdot}0.10^{-3}$		1·80			$0{\cdot}7.10^{-3}$				0·44					
18	$2{\cdot}0.10^{-3}$		1·88			$0{\cdot}6.10^{-3}$				0·65					
24	$2{\cdot}0.10^{-3}$		1·88			$0{\cdot}6.10^{-3}$				0·86					
30	$2{\cdot}0.10^{-3}$		2·18		1·72	$0{\cdot}5.10^{-3}$				1·16				0·91	

*m, Molecular weight of IgG. N, Avogadro's number. M, Mean amount (g) of IgG taken up/cell.

Other possible assumptions on which the calculation of R might be based will be considered later in the light of the experimental results.

The protocol of a typical absorption experiment is set out in Table 3, and Table 4 summarizes the results of this and another similar experiment and shows the values obtained for R when these are combined with the measurements of uptake of ALS IgG labelled with iodine-131 contained in Table 2. As will be seen, these values, expressed as percentages, varied from 0·11 to 1·86.

Rather more precise values might have been obtained if it had been possible to use lymphocytes from the same donors in the absorption experiments and in the experiments on the uptake of labelled IgG. It is also possible that the allowance made for the presence of erythrocytes in the cell suspensions may have resulted in slightly too low a value of R because, contrary to the assumption which was made, the amount of IgG bound to erythrocytes may have been less when lymphocytes were present. An upper limit can be determined, however, by making no allowance at all for the presence of erythrocytes, and if this procedure is followed with the pair of experiments yielding the highest value of R the result is only 2·3 per cent.

A more cogent criticism concerns the validity of the assumptions on which the calculation of R has been based. If these were true the values of R should be independent of the amount of IgG (G) or the number of lymphocytes (n) used in the experiment. As Table 4 and Fig. 3 show, however, in experiments with cells from the same pair of donors, the values obtained for R are inversely proportional to G/n. The most likely explanation would seem to be that the ALS IgG used in the experiments, far from containing just two types of molecule as postulated, was much more heterogeneous. Such heterogeneity might take various forms, but the simplest hypothesis is that the antilymphocytic molecules are heterogeneous only in respect of their avidity, and that all IgG molecules which become attached to a lymphocyte exert an equal stimulating effect. Heterogeneity of this type may be defined in an operational way as implying simply that when ALS IgG is absorbed with lymphocytes the stimulating capacity of the absorbed solution is less than the stimulating capacity of a dilution of the unabsorbed solution containing the same total number of antilymphocytic IgG molecules, because the more avid molecules are preferentially absorbed whereas during dilution the population distribution remains the same.

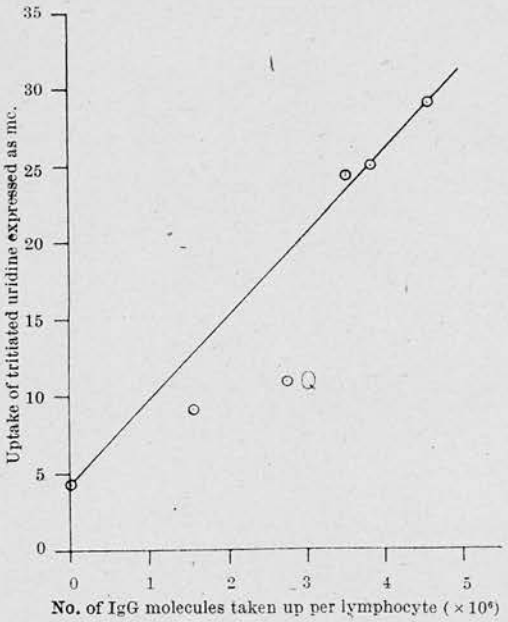

Fig. 3. Graph showing the approximately linear relationship between uptake of tritiated uridine and the number of IgG molecules taken up by a lymphocyte. The uptake of tritiated uridine denoted by the point Q is erroneously low because of marked quenching in the tubes on which this result is based.

It is easy to see that if such heterogeneity exists the experimental methods used would yield a value of R which was less than the true value*. It would seem to be premature to go further and attempt to develop a quantitative theory of the reaction until more experimental data are available with which the predictions of such a theory can be compared. It is worth noting, however, that the observed linear increase of R' with n/G, within the range of values which obtained in the experiment, suggests that even the highest observed value of R' may be a gross underestimate of R. Indeed, once the postulate of heterogeneity in respect of avidity is accepted it raises the possibility that R attains or comes very close to the theoretical limit of 1, because much, if not all, of what we have classed as non-antilymphocytic IgG might be antilymphocytic IgG of extremely low avidity. In this event

* Suppose a solution containing G grams of IgG, of which GR grams is made up of antilymphocytic molecules of varying degrees of avidity and $G(1-R)$ grams is not antilymphocytic, is absorbed with n lymphocytes. Let Mn denote the amount of IgG absorbed, E the total IgG in a dilution of the original solution with the same stimulating effect in a standard test as the absorbed material, and $R' = \frac{Mn}{G-E}$ the experimental value obtained for R. Suppose further that in standard tests performed with dilutions of the original solution the degree of stimulation is $f(x)$, where x is the total amount of IgG in a tube, and $f(x)$ is a function which increases with x. After absorption the solution contains $G-Mn$ grams of IgG, of which $GR-Mn$ grams are antilymphocytic, and its stimulating effect is $f(E)$. A dilution of the original solution containing the same number of antilymphocytic molecules as the absorbed material would contain a total of $G - \frac{Mn}{R}$ grams of IgG and its stimulating capacity would be $f\left(G - \frac{Mn}{R}\right)$. Thus, *ex hypothesi*, $f\left(G - \frac{Mn}{R}\right) > f(E)$ and therefore because $f(x)$ is an increasing function of x, $G - \frac{Mn}{R} > E$. Replacing Mn by $R'(G-E)$, we have $G - \frac{R'}{R}(G-E) > E \therefore G-E > \frac{R'}{R}(G-E) \therefore R' < R$.

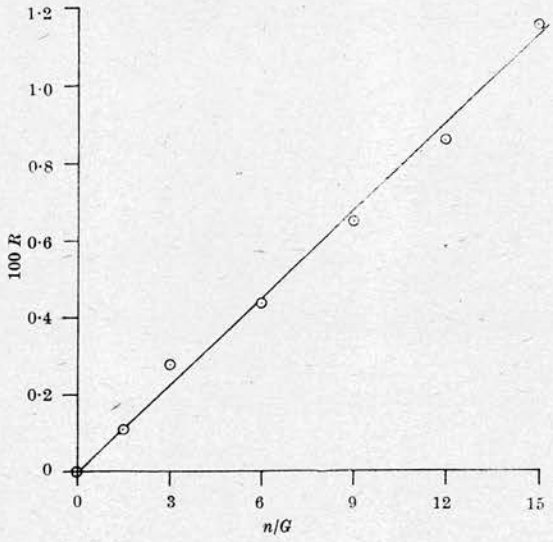

Fig. 4. Values obtained for R using the formula $R = \frac{M\,n}{(G-E)}$ in experiments with cells of donors II and V plotted against n/G. Symbols are explained in the text and Table 4.

R, as defined, would obviously not be a useful parameter for comparing different preparations of ALS IgG. The ideal would be to determine the distribution of avidity in the population of molecules making up each preparation, but useful comparisons could be made by determining R' for each for one or two values of G/n. This might be of some practical importance because it seems likely from the results of these experiments that a great deal of the IgG in the preparation tested has at any rate very little stimulating effect on lymphocytes *in vitro*. This is scarcely surprising, for the horse in which the ALS was raised cannot be expected to have lacked previous immunological experience. It is not yet known whether there is a positive correlation between the immunosuppressive activity of ALS *in vivo* and its lymphocyte-stimulating activity *in vitro*; if there is, however, it would follow that even a highly purified preparation of ALS IgG, such as the one used in these experiments, contains a great deal of protein which is of no value as far as immunosuppression is concerned. It is conceivable that preparations containing a higher proportion of "useful" IgG might be obtained by raising sera in animals whose plasma concentration of IgG before immunization was either naturally low or had been reduced by plasmaphoresis. Alternatively, the same end might perhaps be achieved by modifying the schedule of immunization.

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Effect of Antilymphocytic Antibody and Antibody Fragments on Human Lymphocytes *in vitro*

The mode of action of horse anti-lymphocyte serum (IgG) and its pepsin fragments has been investigated by lymphocyte agglutination tests, cytotoxic tests and the uptake of uridine and thymidine. It is found that its divalent pepsin fragment ($F(ab')_2$) agglutinates lymphocytes and stimulates the uptake of uridine and thymidine whereas the univalent fragment (Fab') is inactive in all these tests.

by

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THERE have been numerous reports from this[1] and other laboratories describing the immunosuppressive properties of antilymphocyte serum* (ALS) and its effects on lymphocytes *in vitro*; its mode of action is, however, still obscure, although various theories have been proposed. The present experiments, which are concerned with the effects of horse-anti-human ALS, serum fractions and antibody fragments on human blood lymphocytes *in vitro*, form part of a systematic study designed to elucidate this question.

ALS in the form of filtered inactivated plasma was prepared from the last two collections of blood obtained from a horse which had received nine injections of intact cells from human infant thymus or adult spleen over a period of about 10 months (see Table 1). Previous investigations[2] showed that IgG and 7*S* 'Sephadex' fractions

* Serum denotes not only serum separated in the usual way from clotted blood but also plasma from which the fibrinogen has been removed by heating (56° C for 30 min) and filtration. ALS denotes any serum having demonstrable effects on lymphocytes *in vivo* or *in vitro* whether raised with injections of intact lymphoid cells (as in the present experiments), intact cells of other types, or cellular fractions.

Table 1. SCHEDULE OF IMMUNIZATION OF HORSE

Date	Cells used for immunization			Route of
Day-mth-yr	Source*	Total No.	Per cent viable	injection
7– 3–1966	Infant thymus	$5{\cdot}4 \times 10^9$	20	Intravenous
18– 3–1966	,, ,,	$4{\cdot}1 \times 10^9$	90	,,
29– 3–1966	,, ,,	$5{\cdot}4 \times 10^9$	76	,,
4– 5–1966	,, ,,	$16{\cdot}6 \times 10^9$	Nil	,,
24– 6–1966	,, ,,	$10{\cdot}0 \times 10^9$	,,	,,
18–10–1966	Adult spleen	$8{\cdot}5 \times 10^9$	80	,,
19–10–1966	,, ,,	$16{\cdot}5 \times 10^9$	82	Subcutaneous
27–10–1966	,, ,,	$62{\cdot}2 \times 10^9$	84	,,
13–12–1966	,, ,,	$25{\cdot}3 \times 10^9$	62	,,

Bleeding, 10 l. on 23–12–1966; 10 l. on 30–12–1966.

* The thymuses were obtained at routine autopsy (by courtesy of Dr A. D. Bain). One spleen was obtained within 30 min of death and the others were removed in the course of radical gastrectomy for carcinoma of the stomach.

from earlier batches of serum from the same animal contained most of the material responsible for lymphocyte agglutination and cytotoxicity, and for the stimulation of uridine and thymidine uptake by lymphocytes in culture, whereas much of the erythrocyte agglutinin was in the 19*S* 'Sephadex' fractions and could be absorbed out of whole serum without significantly reducing its lymphocyte agglutinin and cytotoxic titres or its stimulating effect on lymphocytes in culture. In the present experiments, therefore, all preparations in which the erythrocyte agglutinin reciprocal titre exceeded four were absorbed with human erythrocytes to bring the titre below this level before they were tested against lymphocytes. Normal horse and antilymphocytic IgG globulins were prepared by diethylaminoethyl (DEAE) cellulose batch chromatography on Whatman '*DE*11' exchanger with an ion exchange capacity of 1·0 m.equiv/g. The products were concentrated by lyophilization and reconstituted to 1 g per cent (w/v) in 0·06 molar phosphate buffer (*p*H 7·2) containing 0·15 molar sodium chloride. Their purity was checked by immunoelectrophoresis using a rabbit-anti-horse serum and polyacrylamide gel electrophoresis. The antibody preparation contained only small amounts of contaminating protein, probably IgA (T). The $F(ab')_2$ portion of the IgG molecule, which contains both antigen combining sites, was prepared by digestion in pepsin[3]. The digestion was performed at 37° C for 48 h in 0·1 molar acetate buffer *p*H 4·0 (using one part enzyme to fifty parts protein by weight). The products were finally dialysed against the above phosphate buffered saline. Degradation of the IgG preparations to the $F(ab')_2$ fragment was shown to be complete by immuno-diffusion and, in the case of the antilymphocytic preparation, by subsequent cytotoxic analysis. The univalent Fab′ fragment was prepared by reduction of the divalent $F(ab')_2$ fragment with 0·1 molar cysteine in 0·06 molar phosphate buffer (*p*H 7·2, containing 0·15 molar sodium chloride) and then alkylated. This was achieved by dialysing the reduced preparations

Table 2. EFFECT OF ANTILYMPHOCYTIC AND NORMAL HORSE SERUM AND DERIVATIVES, AND PHYTOHAEMAGGLUTININ, ON THE UPTAKE OF TRITIATED URIDINE AND TRITIATED THYMIDINE BY HUMAN LYMPHOCYTES IN CULTURE

Preparation tested*	Cell donor	Uridine test C.p.m.† (thousands)	Uridine test Relative counts Observed†	Uridine test Relative counts Mean	Thymidine test C.p.m.† (thousands)	Thymidine test Relative counts Observed†	Thymidine test Relative counts Mean
ALS and derivatives							
ALS	B	1,000, 834, 811	12·3, 10·2, 9·9	10·8	652, 882, 600	14·7, 19·9, 13·5	16·0
	C	200, 236	11·5, 13·4	12·5	608, 656	84·6, 91·3	88·0
	D	310, 297, 294	4·59, 4·41, 4·36	4·45			
	G	629, 429	11·0, 7.50	9·27			
	N	141, 158, 119	4·27, 4·78, 3·64	4·23	150, 294, 195	18·2, 32·0, 23·6	24·6
	O	253, 266	2·71, 2·86	2·79			
IgG	C	118, 138	6·69, 7·95	7·32	415, 266	57·8, 37·0	47·4
	J	146, 143, 107	3·24, 3·18, 2·38	2·94			
	K	407, 379, 435	6·72, 6·24, 7·17	6·71			
	N	146, 160, 155	4·33, 4·87, 4·71	4·64	130, 211	20·4, 31·4	25·9
	O	196, 206, 169	2·10, 2·21, 1·81	2·04	129, 169	3·13, 4·09	3·61
$F(ab')_2$	J	177, 181, 155	3·94, 4·02, 3·43	3·78			
	N	289, 156, 146	8·79, 4·75, 4·34	5·96	169, 138, 206	26·6, 21·7, 32·4	26·9
	O	526, 476, 385	5·64, 5·10, 4·12	4·95	217, 216, 236	5·25, 5·23, 5·72	5·40
Fab'	G	68·4, 57·5	1·20, 1·01	1·10			
PHA	G	629, 508	11·0, 8·90	9·95			
	L	447, 271	11·3, 6·85	9·08			
	M	89·7, 74·3	6·76, 5·59	6·18			
	O	476, 541	5·10, 5·79	5·45			
NHS and derivatives							
NHS	A	43·0, 42·7	1·14, 1·13	1·14	56·5, 37·9, 67·2	2·62, 1·76, 3·12	2·50
	B	62·0, 82·4, 78·9	0·76, 1·01, 0·97	0·91	85·1, 29·8, 35·3	1·91, 0·80, 0·67	1·12
	J	51·6, 54·0	1·13, 1·20	1·16			
	L	46·9, 19·1	1·31, 0·54	0·93			
Fab'	G	65·3, 41·1	1·15, 0·72	0·93			

* The dose per culture of serum and serum derivatives contained 2·0 mg IgG or fraction. The dose per culture of PHA was 0·1 ml. reconstituted dried phytohaemagglutinin (Wellcome).

† Figures on the same horizontal line relate to replicate cultures set up at the same time with cells from the same donor.

against large volumes of 0·02 molar iodoacetate in the same buffer. The excess iodoacetamide was removed by dialysis for 24 h against three changes of large volumes of phosphate buffer (100 volumes) through which nitrogen was continuously bubbled. This treatment was found to be essential because trace amounts of iodoacetamide (less than 0·0002 molar) were extremely toxic to lymphocytes in culture. Extensive dialysis procedures (greater than 2 days) in the absence of nitrogen resulted in a significant amount of recombination of the Fab′ units to the $F(ab')_2$ fragment. All these preparations were sterilized by 'Millipore' filtration and stored at $-20°$ C.

The IgG globulin content of the sera was determined by quantitative cellulose acetate electrophoresis and by the antibody-agar radial diffusion procedure[4] using a rabbit-anti-horse IgG serum and horse IgG standards. The protein content of the samples was determined by the Folin phenol procedure[5] and in the case of the purified preparations and fragments it was also determined by measuring the extinction coefficient at 280 mμ in a 1 cm cell.

Agglutination and cytotoxic tests were performed as described by Abaza and Woodruff[6] with the following modifications. (*a*) Lymphocytes were obtained from 400 ml. fresh heparinized human blood by filtering it through a Fenwal 'Leuko-Pak', to remove the polymorphs, and then sedimenting the red cells by adding 120 ml. of 3 per cent gelatine and allowing it to stand for 45 min at 37° C and then for 30 min at room temperature. (*b*) In both tests the number of viable lymphocytes in each tube or well was four million. (*c*) Rabbit complement was used instead of guinea-pig complement in the cytotoxic test. With rabbit complement a higher value is obtained for the titre and, what is more important, the end point is sharper. (*d*) The final serum dilutions were adjusted according to the concentration of IgG or antibody fragment in the preparation so that the first tube or well contained approximately 1 mg IgG or fragment in a total volume of 0·4 ml. This is referred to as a dilution of 1 : 4 because our standard preparation of IgG contains 1 mg/0·1 ml. On this basis the reciprocal lymphocyte agglutinin titres of antilymphocytic serum, IgG and $F(ab')_2$ were essentially the same and ranged from 1024 to 4096 in tests performed with lymphocytes from various donors. The Fab′, on the other hand, had a negligible titre ($\text{titre}^{-1} \leqslant 4$). The cytotoxic titre^{-1} (with rabbit complement) was 1024 for antilymphocytic serum and IgG, and negligible for both fragments ($\leqslant 4$). The agglutinin and cytotoxic titres of normal horse serum, and of IgG and fragments derived from it, were negligible ($\text{titre}^{-1} < 4$).

Lymphocyte cultures were set up in Bijoux bottles and incubated at 37° C. Each culture initially contained

about 10 million viable lymphocytes and 10–20 million erythrocytes in 2 ml. medium 199 plus 0·5 ml. autologous plasma. Usually the plasma was inactivated by heating (56° C for 30 min), but in experiments designed to study the effect of complement unheated plasma was used. The various preparations to be tested for stimulating activity were added as described here. Some cultures were set aside for morphological study, but the majority were used to study the incorporation of tritiated uridine or thymidine. Tritiated uridine (specific activity 2·73 c./mmole) was added when the cultures were set up, and tritiated thymidine (specific activity 5 c./mmole) was added to cultures which had been incubated for 72 h. In each case the final concentration of isotope was 1·67 μc./ml. Eighteen hours after the addition of tritiated uridine, or 24 h after the addition of tritiated thymidine, the cells were spun down and washed three times in phosphate buffered saline and then successively in 5 per cent trichloroacetic acid, phosphate buffered saline, and absolute methanol. After the final spin the supernatant was discarded and the precipitate was taken up in the minimum possible quantity of hyamine hydrogen chloride and heated for a few minutes in a water bath at 60° C. After cooling, 10 ml. of scintillant (*NE* 213, Nuclear Enterprises, Edinburgh) was added and counting was performed with a Packard 'Tricarb' scintillometer. Cultures were set up usually in triplicate (occasionally duplicate or quadruplicate). The counts per minute in control cultures which contained only cells, medium 199 and autologous plasma showed not more than 25 per cent variation from the mean value (and often much less than this) when each contained cells from the same donor, but the mean c.p.m. in control cultures of cells from different donors ranged from 17,520 to 81,600 (fourteen donors; mean $46{,}250 \pm 21{,}800$) in cultures incorporating tritiated uridine, and from 6,400 to 44,430 (four donors; mean $19{,}930 \pm 12{,}790$) in cultures incorporating tritiated thymidine. It has seemed desirable therefore to calculate for each treated culture the relative count, that is, the ratio of c.p.m. in that culture to the mean c.p.m. of the control cultures set up at the same time with cells from the same donor.

The results of tests with a standard dose of each of the various preparations are summarized in Table 2, from which it will be seen that antilymphocytic serum, IgG and $F(ab')_2$ were all highly active in stimulating uptake of both uridine and thymidine, and when given in comparable dosage to the same donor all produced approximately the same degree of stimulation. Initial experiments with Fab′ were invalid because the preparation contained toxic amounts of iodoacetamide, but the preparation referred to in Table 2, which had no stimulating effect, was non-toxic as shown by the trypan blue

Table 3. EFFECT OF TIME OF EXPOSURE TO STIMULATING AGENT AND PRE-TREATMENT WITH Fab′ ON UPTAKE OF ^{3}H URIDINE BY HUMAN LYMPHOCYTE CULTURES

Stimulating agent		Pre-treatment			Relative count	
Agent and dosage	Duration of exposure	Agent and dosage	Duration of exposure	Cell donor	Observed	Mean
ALS (0·2 mg IgG).	18 h	Nil		G	11·0, 7·50	9·27
		Fab′ from ALS	15 min	G	4·65, 7·59, 3·04	5·09
		Fab′ from NHS	15 min	G	4·62, 5·85, 10·2	6·89
	1 h	Nil		H	4·91, 4·96, 5·65	5·17
		Fab′ from ALS	15 min	H	10·8, 11·2, 17·4	13·1
		Fab′ from NHS	15 min	H	14·4, 13·3, 14·9	14·2
	15 min	Nil		H	1·94, 1·91, 1·75	1·87
		Fab′ from ALS	15 min	H	2·70, 2·89, 2·29	2·62
		Fab′ from NHS	15 min	H	2·52, 2·27	2·40
PHA (0·1 ml.)	18 h	Nil		G	11·0, 8·90	9·95
		Fab′ from ALS	15 min	G	8·86, 8·76	8·80
		Fab′ from NHS	15 min	G	17·0, 10·5	13·7
	15 min	Nil		H	13·0, 14·7, 13·5	13·7
		Fab′ from ALS	15 min	H	8·06, 5·25, 5·92	6·41
		Fab′ from NHS	15 min	H		11·9

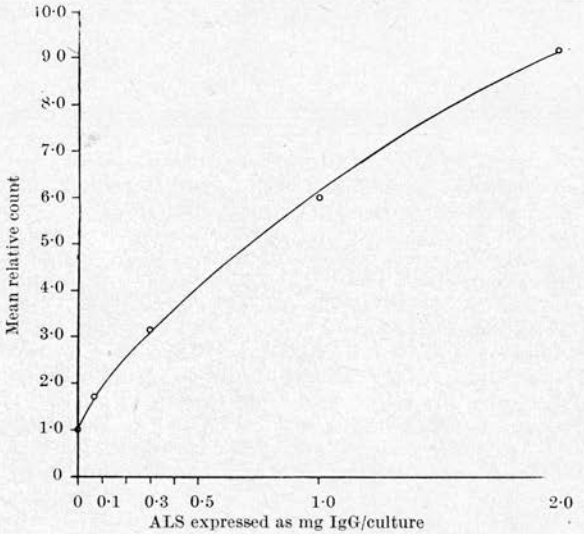


Fig. 1. Curve showing relationship between the degree of stimulation of uridine uptake (expressed as mean relative count) and the amount of ALS added to the culture. Based on experiments using the same serum but lymphocytes from six different donors.

test and by the fact that a preparation made in the same way from normal horse serum did not depress the uptake of uridine.

In the absence of complement, stimulation of the uptake of uridine is, within the limits of the experiment, an increasing function of the amount of antilymphocytic antibody added to the culture. The dose response curve is shown in Fig. 1. The effect of complement is shown in Fig. 2. When the lymphocytes were suspended in medium 199, and ALS and complement (in the form of raw autologous plasma) were added later, there was extensive cytolysis, and stimulation of uridine uptake was completely inhibited (Fig. 2*a*). When, on the other hand, the cells were suspended in a mixture of medium 199 and autologous plasma, and ALS was added 30 min or so after the suspension had been prepared, the degree of inhibition was very much less (Fig. 2*b*). These results confirm in a slightly different system some recent observations of Holt, Ling and Stanworth[7]. As would be expected, complement caused little, if any inhibition of the stimulating effect of $F(ab')_2$. Thus the ratio

$$\frac{\text{Mean relative count in cultures with complement}}{\text{Mean relative count in cultures without complement}}$$

for cultures containing 0·2 μg $F(ab')_2$ was 0·80 and 0·92 in two separate experiments, and these figures do not

differ significantly from the ratios observed in untreated control cultures which, for some unknown reason, consistently showed a somewhat lower count when raw autologous plasma was used instead of inactivated plasma (Fig. 2).

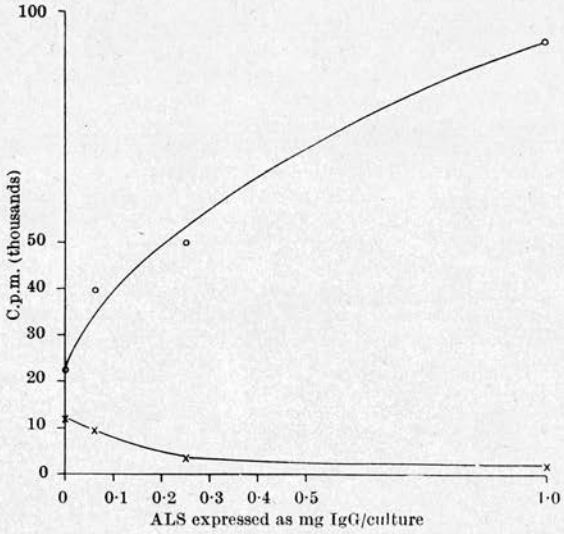
Experiments in which lymphocytes were exposed to stimulating agents for short periods of time and then twice washed before being put up in culture (Table 3) showed that exposure to ALS for 15 min results in only very slight stimulation, and exposure for 60 min to less than maximal stimulation, whereas exposure to phytohaemagglutinin (PHA) for 15 min is as effective as exposure throughout the 18 h of culture.

Pre-treatment of lymphocytes by exposing them for 15 min to Fab′ prepared from ALS, followed by two washes, did not significantly block the stimulation of the uptake of uridine by subsequent exposure to ALS (Table 3); indeed when the exposure to ALS was limited to 60 min, uptake appeared to be increased by pre-treatment with Fab′ from either ALS or normal serum. On the other hand, there is a suggestion that the stimulating effect of PHA is to some extent inhibited by pre-treatment with antilymphocytic Fab′ though not with Fab′ from normal serum. The significance of these observations is difficult to assess because we do not know for how long Fab′ remains attached to the surface of the lymphocyte. It should be possible to answer this question by using either the Coombs test or immunofluorescence, and experiments of this kind are being undertaken.

As we have seen, antilymphocytic serum and IgG stimulate the uptake of uridine and thymidine by lymphocytes in the absence of complement, but when complement is present they fail to do so, and at high concentration cause cell lysis. This suggests that the action of ALS *in vivo* may depend on such factors as the local concentration of antibody and complement, and the degree of anti-complementary activity, in lymphoid tissue and also in the blood stream.

It is noteworthy that $F(ab')_2$ is as effective as IgG not only in respect of lymphocyte agglutination but also in respect of the stimulation of uptake of uridine and thymidine.

Clearly it will be of great interest to determine the effect of $F(ab')_2$ on the blood lymphocyte count and also on immunological responsiveness *in vivo*. This cannot readily be determined in man, but experiments with $F(ab')_2$ from horse-anti-rat ALS are in progress. The most likely explanation of the failure of Fab′ to block subsequent stimulation by ALS or IgG would seem to be that the Fab′ does not become firmly attached to combining sites on the surface of the lymphocyte. Alternatively, it is possible that a "homoreactant" phenomenon[8] is operative. If this is so, intact antilymphocytic IgG or normal IgG



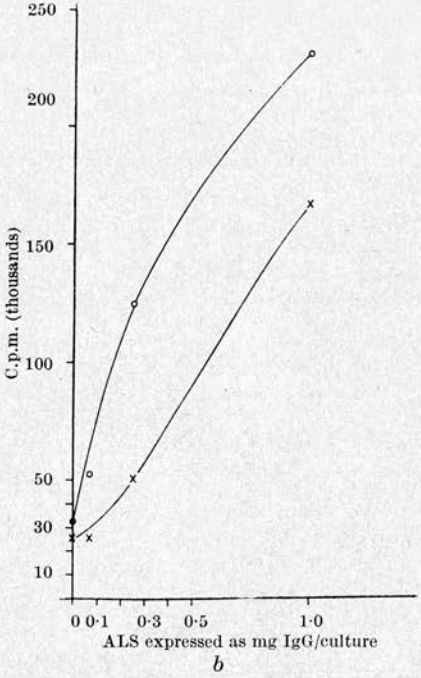


Fig. 2. Effect of complement on uridine uptake at different ALS concentrations. ○. Without complement; ×, with human complement. (*a*) 2·0 ml. of a suspension of lymphocytes (donor E) in medium 199 was put in each culture bottle. ALS and either raw or inactivated autologous plasma was added later, followed by tritiated uridine. Counting was done after 18 h. (*b*) 2·5 ml. of a suspension of lymphocytes (donor I) in a mixture of medium 199 (four parts) and raw or inactivated autologous plasma (one part) was placed in each culture bottle. About 30 min after the suspension had been prepared ALS and tritiated uridine were added. Counting was done after 18 h.

would be capable of agglutinating Fab′ coated cells and thus possibly causing transformation. The question of whether ALS and PHA attach to the same receptors, which we had hoped to be able to answer, remains open.

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EFFECT OF ANTILYMPHOCYTIC ANTIBODY AND ANTIBODY FRAGMENTS ON SKIN-HOMOGRAFT SURVIVAL AND THE BLOOD-LYMPHOCYTE COUNT IN RATS

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Summary Antisera have been raised in a horse and in rabbits to thoracic-duct lymphocytes from hooded rats. These antisera and IgG globulin prepared from them prolonged skin-homograft survival in rats and produced a sustained lymphopenia. On the other hand $F(ab')_2$ and Fab′ fragments of the horse IgG, given in a dosage equal to the dose of IgG which increased homograft survival by a factor of 2·5, had no effect on homograft survival and produced only a transient lymphopenia. Normal horse and rabbit serum had no striking effect.

Introduction

SERUM prepared by immunising an animal with xenogeneic lymphoid tissue causes a fall in blood-lymphocyte count when injected into a member of the species which provided the lymphoid tissue (Chew and Lawrence 1937). Woodruff and Anderson (1963, 1964) showed that the survival of homografts may be greatly prolonged in animals given injections of serum prepared

in this way; Woodruff (1967) has reviewed the reports which confirm this finding. (By *serum* we mean not only serum separated in the usual way from clotted blood but also plasma from which the fibrinogen has been removed by heating at 56°C for 30 minutes followed by filtration.)

We have determined the extent to which these two properties are exhibited by IgG prepared from anti-lymphocyte serum (A.L.S.), and by divalent and univalent antibody fragments of the IgG molecule.

Materials and Methods

Rabbit-anti-rat A.L.S. was prepared as described by Woodruff and Anderson (1964) by immunising rabbits by repeated intraperitoneal injections of rat thoracic-duct lymphocytes. The first three injections were given at weekly intervals; each contained 2×10^8 lymphocytes and the rabbits were bled 10 days after the last injection. The rabbits were bled again on several occasions 10 days after they had received a booster injection of 1 to 2×10^8 lymphocytes.

Horse-anti-rat A.L.S. was prepared by immunising a horse with three intravenous injections of $1{\cdot}07 \times 10^9$ to $1{\cdot}30 \times 10^9$ thoracic-duct lymphocytes (>95% viability) at weekly intervals, followed by a fourth injection 2 weeks later, and bleeding 11 days after the last injection.

IgG was prepared from horse-anti-rat A.L.S. by repeated (twice) sodium-sulphate precipitation (final concentration 14% w/v) followed by diethylaminoethyl cellulose batch chromatography on Whatman 'DE11' exchanger. The final product was concentrated by lyophilisation and reconstituted to yield a preparation containing 1 g. protein per 100 ml. This product was shown by immunoelectrophoresis (using a rabbit-anti-horse serum) and polyacrylamide-gel electrophoresis to contain only small amounts of contaminating protein, presumably IgA(T)—a protein present in large amounts in hyperimmunised horses and is the main source of commercial antitoxins (see Weir and Porter [1966]).

The $F(ab')_2$ portion of the antibody molecule was prepared by digestion with pepsin in 0·1 *M* acetate buffer (pH 4·0) at a ratio of 1 part enzyme to 50 parts IgG w/w. Digestion was shown to be complete, by immunodiffusion analysis and by the fact that while the product retained its capacity to agglutinate rat lymphocytes it did not lyse them in the presence of complement. The univalent Fab′ fragment was obtained by reduction of the $F(ab')_2$ in phosphate buffer (pH 7·2) containing 0·1 *M* cysteine hydrochloride and subsequent alkylation by dialysis against large volumes of iodoacetamide (0·02 *M*) in the same buffer. Completeness of reduction was shown by the fact that the product did not agglutinate lymphocytes in vitro (inverse titre, <8). The preparations of both antibody fragments was

TABLE I—EFFECT OF ANTILYMPHOCYTIC ANTIBODY AND ANTIBODY FRAGMENTS ON SURVIVAL OF SKIN HOMOGRAFTS IN RATS

Preparation	Dose schedule*	Recipient strain	Skin-homograft survival (days)	
			Observed values	Mean
Nil (no treatment)	..	Hooded	8, 8, 8, 8, 8, 8, 8, 8, 8, 8, 9	8·1
		White	8, 8, 8, 8, 9, 9, 9, 10	8·6
Normal horse serum (N.H.S.)	1	Hooded	8, 8, 8, 8, 9	8·2
Horse-anti-rat A.L.S.	1	Hooded	18, 19, 19, 20, 21, 28	20·8
IgG from horse-anti-rat A.L.S.	1	Hooded	14, 16, 17, 17, 19, 21, 21, 25, 25, 26, 30, 31	21·8
$F(ab')_2$ horse-anti-rat	1	Hooded	8, 8, 9, 9, 9, 9	8·7
Fab′ horse-anti-rat	1	Hooded	8, 8, 8, 9, 9, 10	8·7
Normal rabbit serum (N.R.S.)	1	Hooded	8, 8, 8, 8, 8, 8, 8, 8, 8, 8	8·0
Rabbit-anti-rat A.L.S.	1	Hooded	18, 18, 22, 27, 29, 30, 30, 32, 32, 46	28·4
		White	31, 33, 33, 35, 37	33·8
	2	Hooded	30, 30, 35, 47	35·5
IgG rabbit-anti-rat	2	Hooded	20, 23, 24, 24, 28, 40, 43	30·3

* Dose schedules: (1) 2 ml. on days −7 to −1 and 1 ml. on days +1 to +14; and (2) 2 ml. on days −2 to 0 and 1 ml. on days +1 to +10. Day of grafting = day 0. Sera were inactivated and absorbed, if necessary, with rat erythrocytes to reduce the erythrocyte-agglutination titre^{-1} to $\leqslant 8$. The horse-anti-rat and rabbit-anti-rat A.L.S. both contained approximately 1·0 g. IgG per 100 ml. The IgG, $F(ab')_2$, and Fab′ preparations contained 1·0 g. protein per 100 ml.

concentrated by ultrafiltration to yield a protein content of 1 g. per 100 ml.

Crude A.L.S. contained erythrocyte agglutinins in fairly high titre. Before use it was inactivated by heating (56°C for 30 minutes) and absorbed with rat erythrocytes (1 volume erythrocytes for horse-anti-rat and $^1/_2$ volume for rabbit-anti-rat A.L.S.). IgG and antibody fragments did not require absorption.

Further details of these procedures, and the lymphocyte agglutinin and cytotoxin titres of the various preparations, are given elsewhere (James and Anderson 1967, Woodruff, James, Anderson, and Reid 1967).

Two types of experiment were performed. In the first, rats were given a course of daily intraperitoneal injections of A.L.S., normal horse serum (N.H.S.), normal rabbit serum (N.R.S.), or one of the preparations derived therefrom, and during the course each animal was given a homograft of skin. Total and differential blood-leucocyte counts were performed at least once per week. The injections were given at the same time each day and if a blood-sample was required it was taken just before the injection. In the second type of experiment total and differential blood-leucocyte counts were determined in rats before, 4 hours after, and 24 hours after a single intraperitoneal injection of A.L.S., N.H.S., N.R.S., or one of the preparations derived therefrom.

Blood-samples were obtained by amputating the tip of the tail under ether anæsthesia. Skin grafting was performed by the method of Woodruff and Simpson (1955).

Results

The survival of skin homografts in treated and control animals is shown in table I. Whole A.L.S., and IgG derived from it, produced the same degree of prolongation of skin-homograft survival. On the other hand, neither divalent nor univalent antibody fragment given in the same dosage (i.e., same weight of fragment and of IgG) produced any significant increase in homograft-survival time. N.H.S. and N.R.S. also had no effect.

The fall in the absolute blood-lymphocyte count 4 hours and 24 hours after a single intraperitoneal injection of N.H.S., horse-anti-rabbit A.L.S., and preparations derived therefrom, expressed as a percentage of the pre-injection count, is shown in table II. Once again A.L.S. and IgG in comparable dosage produced a similar effect, namely a sharp fall in the blood-lymphocyte count which had not returned to normal after 24 hours. Both divalent and univalent antibody fragment produced a smaller but quite definite fall in the lymphocyte-count 4 hours after injection, but by 24 hours this had returned to or exceeded the pre-injection level.

TABLE II—EFFECT ON BLOOD-LYMPHOCYTE COUNT OF A SINGLE INTRAPERITONEAL INJECTION OF ANTILYMPHOCYTIC ANTIBODY AND ANTIBODY FRAGMENTS

Material injected	Change in lymphocyte-count (%)					
	$\frac{\text{Count at 4 hours} - \text{initial count}}{\text{Initial count}} \times 100$			$\frac{\text{Count at 24 hours} - \text{initial count}}{\text{Initial count}} \times 100$		
	Observed values	Mean	P*	Observed values	Mean	P*
Horse-anti-rat A.L.S. (2 ml. containing 20 mg. IgG)†	−56·7, −59·7, −49·2, −65·0	−57·9	<0·001	−44·9, −33·7, −25·7, −36·3	−35·2	<0·005
IgG from horse-anti-rat A.L.S. (20 mg.)	−81·2, −72·6, −76·5, −86·2	−79·1	<0·001	−53·1, −41·6, −42·2, −34·7	−42·9	<0·002
$F(ab')_2$ from horse-anti-rat A.L.S. (20 mg.)	−73·9, −50·7, −44·0, −59·4	−57·0	<0·005	+16·9, −18·5, +3·0, −28·6	−6·8	0·5
Fab′ from horse-anti-rat A.L.S. (20 mg.)	−45·2, −48·1, −47·9, −50·7	−48·0	<0·001	+16·1, −1·9, +11·9, +117·0	+35·8	..
Normal horse serum (2 ml.)	+46·7, −17·0, +0·9, −20·2	+2·6	..	+47·4, +17·2, +44·8, +35·1	+36·1	..

* P denotes probability that observed fall in lymphocyte-count is not significant by *t*-test.

† IgG content of antiserum estimated by quantitative immunodiffusion and quantitative cellulose acetate electrophoresis.

The effect of repeated injections is shown in table III. Horse-anti-rat A.L.S. and IgG behaved indistinguishably and produced a sustained fall in blood-lymphocyte count; at 7 days, however, this was significantly less than that produced by the rabbit-anti-rat IgG. Rats treated with horse-anti-rat Fab′ showed only a slight fall in lymphocyte-count at 7 days and none at 14 days; those which received $F(ab')_2$ showed no fall even at 7 days.

TABLE III—EFFECT ON BLOOD-LYMPHOCYTE COUNT OF REPEATED DAILY INTRAPERITONEAL INJECTIONS OF ANTILYMPHOCYTIC ANTIBODY AND ANTIBODY FRAGMENTS

Material injected *	No. of rats treated	Change in lymphocyte-count (mean ± S.D.) (%)	
		7 days after start of treatment	14 days after start of treatment
Horse-anti-rat A.L.S.† ..	6	−56·3 (±12·5)	−30·7 (±28·1)
IgG from horse-anti-rat A.L.S.	12	−55·2 (±22·2)	−33·5 (±27·7)
$F(ab')_2$ from horse-anti-rat A.L.S	4	−3·2 (±13·8)	+26·3 (±25·7)
Fab′ from horse-anti-rat A.L.S.	6	−24·9 (±16·7)	+6·4 (±41·1)
IgG from rabbit-anti-rat A.L.S.	6	−91·7 (±4·8)	Not measured

* See table I, dose schedule (1), for dosage and strength of each preparation.
† IgG content of antiserum estimated by quantitative immunodiffusion and quantitative cellulose acetate electrophoresis.

Discussion

IgG has a similar effect to A.L.S. on homograft survival and on blood-lymphocyte count.

The failure of $F(ab')_2$ and Fab′ prepared from A.L.S. to cause prolongation of skin-homograft survival when given in a dosage equal to the dose of antilymphocytic IgG which increased homograft survival by a factor of 2·5, like their failure to inhibit humoral antibody production (James and Anderson 1967), would seem to be related to the lack of toxicity of these preparations for lymphocytes. This conclusion is consistent with the observation reported by Woodruff, Reid, and James (1967) that $F(ab')_2$ from horse-anti-human A.L.S. is as effective as an equal dose of IgG in stimulating uptake of tritiated uridine and tritiated thymidine by human lymphocytes in culture. If, as seems likely, this holds good also in vivo, and in species other than man, it would suggest that the immuno-suppressive effect of A.L.S. and IgG is not due simply to sterile activation of lymphocytes, though, as Levey and Medawar (1966) have suggested, this may play some part. One way to obtain further light on the question would be

to examine the effect on immunological responsiveness of considerably larger doses of $F(ab')_2$ than those we have used so far, and this we plan to do.

The cytotoxic effect of A.L.S. and IgG may be manifested in vivo by a fall in the blood-lymphocyte count. There is evidence, however, that A.L.S. *may* cause a high degree of immunosuppression, including suppression of homograft rejection, in the absence of striking lymphocytopenia (Woodruff 1967). It seems possible that in this event a subpopulation of sensitised lymphocytes may have been selectively destroyed or inactivated.

When there is a striking and persistent fall in the blood-lymphocyte count it seems likely that many lymphocytes are destroyed. Sequestration of lymphocytes may also play a part, however, and may well account for the rather transient lymphocytopenia which follows injection of $F(ab')_2$ and Fab', since these agents do not bind complement. It would scarcely be surprising if antibody-coated cells were to become trapped in various parts of the capillary bed or even removed from the circulation. Direct evidence of this is lacking, however, and we are therefore embarking on an autoradiographic study of the distribution of IgG-coated lymphocytes when injected into untreated isogeneic animals, and also of labelled but non-coated lymphocytes injected into isogeneic animals which are subsequently treated with antilymphocytic IgG.

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PROLONGATION OF CANINE RENAL ALLOGRAFT SURVIVAL WITH ANTILYMPHOCYTIC SERUM

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SUMMARY

Twenty-nine dogs received renal allografts with simultaneous bilateral nephrectomy. Five recipients were untreated, five were given daily s.c. injections of normal horse serum, 14 received daily injections of horse anti-dog antilymphocytic serum (ALS) at varying intervals before and after transplantation, and five were treated with daily injections of immune globulin prepared from the ALS by sodium sulphate precipitation and diethylaminoethanol cellulose chromatography.

The mean survival times of the untreated control animals and those receiving normal horse serum were 9.6 days and 10 days respectively. No animal in these groups survived for more than 12 days.

In the ALS-treated dogs, some prolongation of survival was achieved when the serum was started as late as 5 days postoperatively, but the best results were obtained with 5 days' pretreatment and continued postoperative administration, the dogs in this group showing a mean survival of 68.4 days even with a limitation of survival credit to 100 days.

Immune globulin admistered on this schedule produced a mean survival of 28 days, so that some immunosuppressive activity was lost in the processing of the serum.

Previous studies in this laboratory (*1*) demonstrated the value of the i.v. injection of a horse anti-dog antilymphocytic serum (ALS) in the prolongation of canine renal allograft survival. Further experiments have been carried out to determine the effects of s.c. administration of the ALS, the importance of timing of injection in relation to transplantation, and the effect of an immune globulin (IgG) fraction prepared from the ALS.

MATERIALS AND METHODS

Preparation of ALS. Horse-anti-dog antilymphocytic serum was prepared in an 11-year-old gelding. Immunization was begun in 1963 and consisted initially in four i.v. injections of dog thoracic duct lymphocytes at weekly intervals followed by monthly booster injections for 6 months. The serum thus

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produced was used in the study of renal allografts reported previously by Abaza et al. (*1*), and details of the initial immunization schedule and the lymphocyte agglutinin and cytotoxic titres have already been reported (*1, 2*). In October and again in November, 1966, the horse was given a s.c. injection of dog spleen cells (37×10^9 nucleated cells 95 % viable, and 15×10^9 cells, 70 % viable, respectively), and 1 month later it was exsanguinated. All the antilymphocytic serum and IgG used in the present experiments was from this last bleeding. The blood was left overnight at room temperature, and the serum separated and centrifuged to eliminate contaminating red cells. The serum was inactivated by heating to 56 C for ½ hr and absorbed with washed dog red cells or stroma until the erythrocyte agglutinin titre, which was initially $\frac{1}{4096}$, was reduced to ¼; it was then sterilized by filtration through HA Millipore filter and stored in 10- or 20-ml ampoules at −20 C. The serum had lymphocyte agglutinin and cytotoxin titres of $\frac{1}{512}$ which were not affected by the absorption with red cells. The methods of assay were those described by Abaza and Woodruff (*2*).

Normal horse serum (NHS) was obtained from this animal before immunization, and had a haemagglutinin titre of $\frac{1}{16}$ and a lymphagglutinin titre of ¼.

Preparation of IgG. IgG was prepared by sodium sulphate precipitation of the ALS followed by diethylaminoethanol (DEAE) cellulose batch chromatography (*3*). The product was concentrated by lyophilization and reconstituted in phosphate-buffered saline. Immunoelectrophoretic analysis, using rabbit anti-horse serum and polyacrylamide gel electrophoresis, indicated that the preparation contained only small amounts of contaminating protein (probably IgT). The product contained 3 g of protein/100 ml, as compared with 1 g/100 ml of IgG in the original ALS, and had a haemagglutinin titre of $\frac{1}{1024}$ which was reduced to ½ by absorption. The lymphagglutinin titre was $\frac{1}{1024}$ and was unaffected by absorption with red cells.

Protocol. The 29 dogs were divided into seven groups, and all were given renal allotransplants. The details of treatment are summarized in Table 1.

Transplantation. The kidney donors and recipients were adult male beagles 1.5–4 years of age weighing between 8 and 12 kg. All dogs were immunized against distemper and hepatitis with a single injection of a commercial vaccine (Epivax-T.C.-plus (Burroughs Wellcome)). Transplants were carried out simultaneously in pairs of recipients, the donors being bled out to provide blood for absorption. Details of the operative technique have been described previously (*1*). Ischaemic periods were between 18 and 32 min. Bilateral recipient nephrectomy was carried out after completion of the anastomoses.

Investigations. Blood samples were obtained before the injection of serum, before operation, and at 3-day intervals thereafter. Total and differential white cell count, haemoglobin, platelet count, and blood urea, were estimated on these samples.

No attempt was made to collect urine specimens, but bedding was observed for evidence of passage of urine.

Autopsies were carried out in animals which died and histological sections

TABLE 1. Survival of renal allograft recipients

Group	No. of dogs	Treatment[a]	Survival times (days)	
			Observed values	Mean
1	5	None	12, 12, 7, 6, 11	9.6
2	5	Normal horse serum 3 ml/kg/day from day −5	10, 9, 12, 11, 8	10.0
3	3	ALS[b] 3 ml/kg/day from day 5	28, 15, 14	19.0
4	3	ALS 3 ml/kg/day from day 3	24, 16, 24	21.3
5	3	ALS 3 ml/kg/day from day 0	17, 21, 24	20.6
6	5	ALS 3 ml/kg/day from day −5 to day 45, then 3 ml/kg twice weekly to day 75	74, >100, >100, 50, 18	68.4
7	5	IgG 1 ml/kg/day from day −5 to day 45	40, 28, 17, 22, 33	28.0

[a] All serum and IgG was given by s.c. injection.
[b] ALS, Antilymphocyte serum.

prepared from kidney, lymph glands, thymus, spleen, liver, and lung. Renal biopsy with light and electron microscopy was carried out in one long-surviving animal.

RESULTS

Behaviour of transplants. Data showing survival times are summarized in Table 1.

None of the untreated controls or those dogs receiving NHS survived for longer than 12 days, and the two groups showed a mean survival of 9.8 ± 1.5 (SD) days. The transplanted kidneys in these groups showed the typical features of allograft rejection, with marked swelling, interstitial haemorrhage, round cell infiltration, and tubular necrosis. No difference was noted in the two groups.

In the ALS-treated animals, prolongation of survival was obtained when the serum was started as late as 5 days postoperatively in one dog, but in the other two animals in this group, only slight prolongation was achieved, and the appearance of the kidneys was similar to that of the controls. With ALS started on day 3 or day 0, some prolongation was produced in all six animals, and the histological pattern of rejection was less acute than in the control group showing only focal tubular necrosis, with little interstitial haemorrhage but marked mononuclear infiltration. The over-all mean survival of dogs receiving ALS postoperatively was 20.3 ± 5.0 days ($P < 0.01$).

A mean survival of 68.4 ± 35 days was obtained in the five animals receiving serum pre- and postoperatively, even when credit for survival was limited to 100 days to allow data comparison, and one animal is still alive at 350 days after transplantation. The kidneys of three of the animals which died

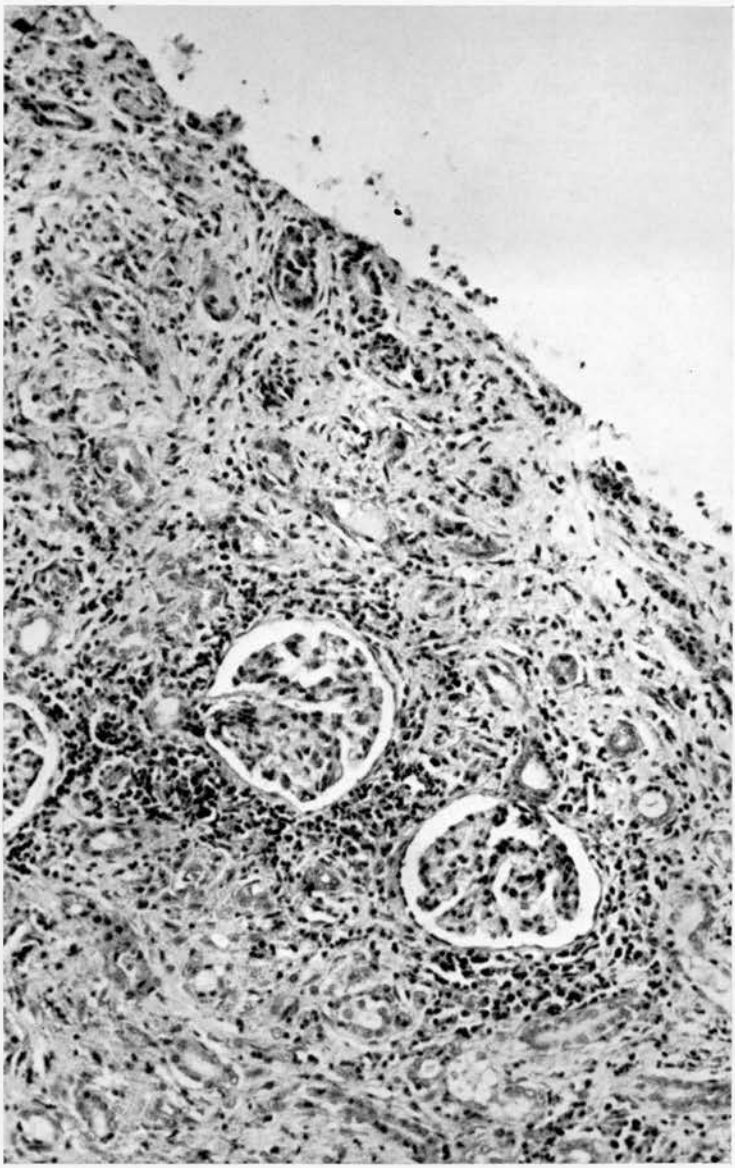

FIGURE 1. Postmortem section of kidney allotransplant from a dog in group 6 at 74 days, showing moderate round cell infiltration. H & E; ×150.

were large and pale, and showed only moderate round cell infiltration with some glomerular capillary basement membrane thickening (Fig. 1). The dog which died at 18 days showed moderate interstitial haemorrhage with heavy mononuclear infiltration.

There is a highly significant difference ($P < 0.001$) in the survival times of the animals receiving ALS postoperatively and those receiving it preoperatively.

The IgG-treated group showed a mean survival of 28 ± 9.2 days. The microscopic features of the transplants at autopsy were similar to those in the pretreated ALS group in the longer surviving animals, and were comparable to the controls in the other two.

Effect on peripheral lymphocyte count and lymphoid tissues. The degree of lymphopenia produced was variable, but all animals which received ALS or antilymphocytic IgG showed a fall in peripheral lymphocyte count in spite of a marked initial granulocytosis. This lymphopenia was more consistent and sustained than after i.v. injection of ALS (*1*).

In the pretreated ALS or IgG groups, the initial degree of lymphopenia did not appear to be related to eventual survival of the transplant, but all animals showed a definite rise in count at the time of rejection (Figs. 2 and 3). In the two long-surviving animals, the lymphocyte count rose to normal levels within 3 weeks of cessation of treatment. One of these dogs died from rejection 34 days after ALS was stopped, but the other is still alive at 350 days.

Most of the animals receiving ALS or IgG showed marked small lymphocyte depletion in spleen and lymph nodes. This depletion was fairly uniform, and there was no evidence of the large central hyperplastic pyronin positive cells noted by Iwasaki et al. (*4*).

Thymuses were within normal limits for the age of the animals, and no germinal centres were seen.

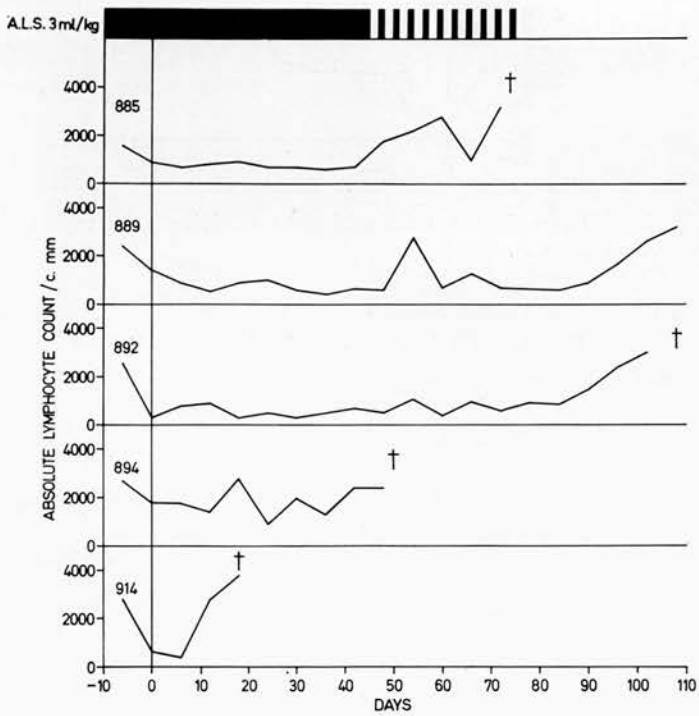

FIGURE 2. Peripheral lymphocyte counts of dogs receiving antilymphocytic serum pre- and postoperatively (group 6).

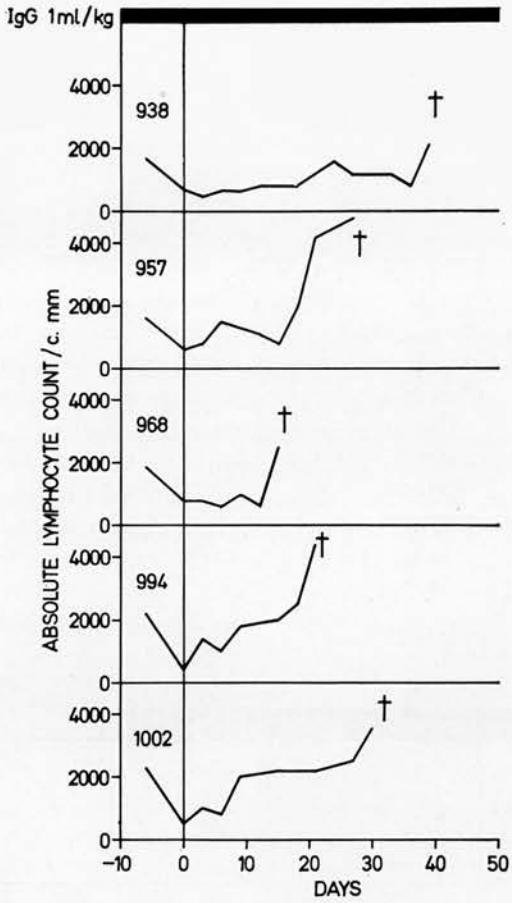

FIGURE 3. Peripheral lymphocyte counts of dogs receiving antilymphocytic IgG pre- and postoperatively (group 7).

Evidence of toxicity. The animals showed no signs of general upset following s.c. administration of the whole serum. The injections were of large volume, and caused slight local swelling which persisted for 24–36 hr. A few dogs appeared to find the injection painful, but this became less obvious as the treatment was continued. No s.c. sepsis was seen in these animals.

IgG was administered s.c. in a third of the volume when compared with normal serum, and appeared to cause no pain and very slight local swelling which had gone within 24 hr.

The general condition of the animals seemed satisfactory while renal function was good, and after slight initial weight loss, the longer surviving dogs ate well and gained weight well.

All dogs showed an initial postoperative anaemia apparently due to blood loss, but the haematocrit returned to normal levels within 4 weeks if renal function remained satisfactory.

Renal biopsy was carried out in the one long-term survivor at 154 days, 79

days after the last dose of ALS, and multiple small dense subepithelial glomerular capillary basement membrane deposits were seen (Fig. 4). There was no evidence of these deposits in a further biopsy at 319 days, so that these changes appear to be reversible (Fig. 5).

Wound infection did not occur in any animals in this series, but two dogs developed urinary tract infections. The pneumonia seen in several animals was terminal in type and probably secondary to uraemia. No evidence of hepatitis or distemper was seen.

DISCUSSION

These experiments provide further evidence of the value of heterologous antilymphocytic serum in the suppression of allograft rejection. The degree of immunosuppression obtained in the group of dogs receiving ALS both before and after operation, with a mean survival of 68 days, is comparable to that achieved in this laboratory with a combination of azathioprine and prednisone (mean survival 45 days) and there was a striking freedom from the usual infection, marrow depression, gastrointestinal haemorrhage, general malaise, and weight loss seen in the drug-treated animals.

Using a mouse skin graft system, Levey and Medawar (*5*) found that one or two i.p. injections given 2 or 3 days postoperatively produced a greater

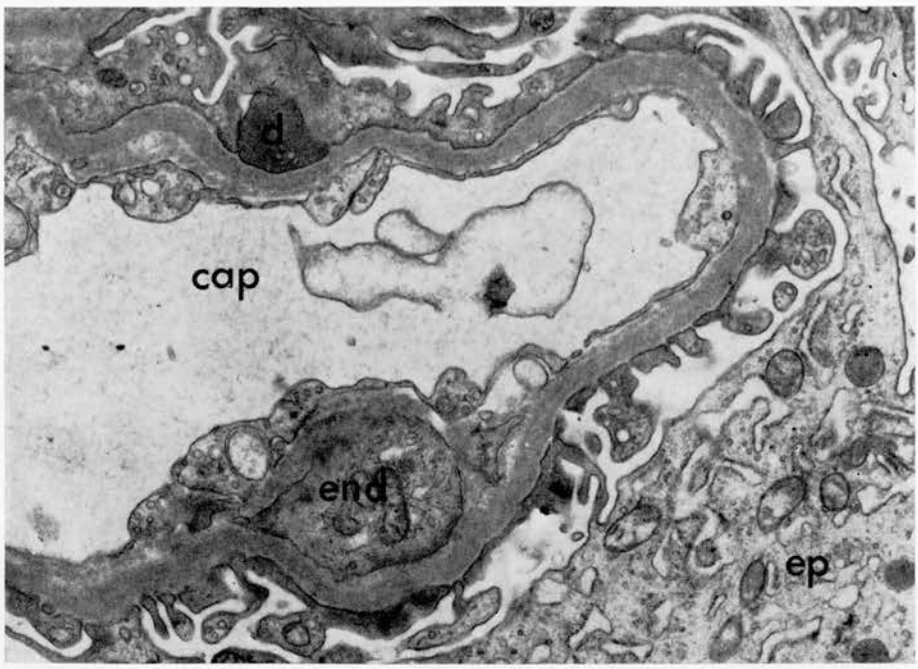

FIGURE 4. Renal biopsy at 154 days. Glomerular capillary with a subepithelial deposit (d) of electron-dense material. cap, Capillary lumen; end, endothelial cell; ep, epithelial cell with foot processes. Electron micrograph. Lead hydroxide stain; ×10,750. (By courtesy of Professor K. A. Porter.)

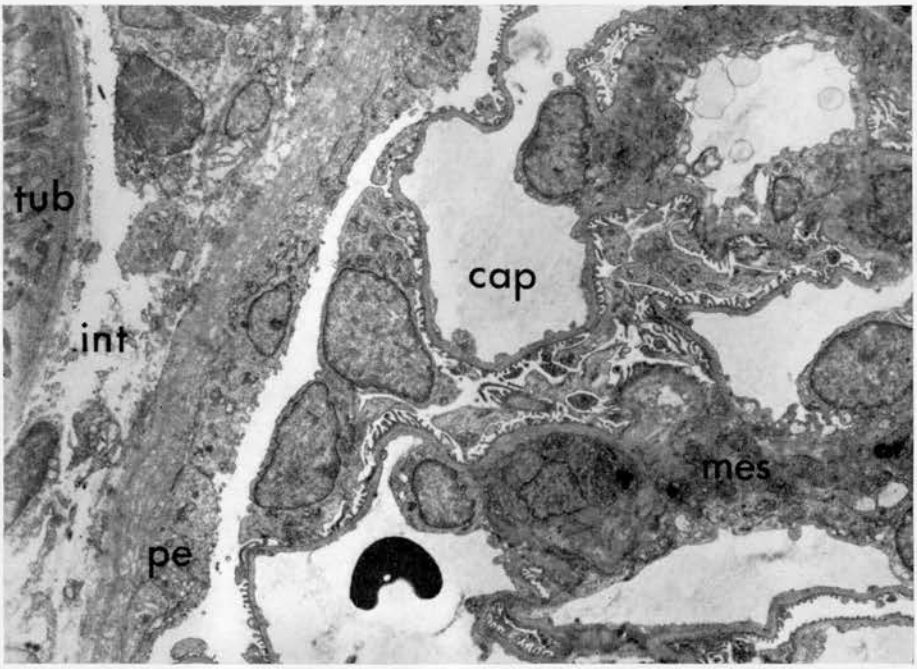

FIGURE 5. Renal biopsy at 319 days. Part of glomerulus. There is some increase in the amount of mesangial matrix (mes), but there are now no deposits on the capillary basement membranes. tub, Proximal convoluted tubule; int, interstitium; pe, parietal epithelium lining Bowman's capsule; cap, capillary lumen. Electron micrograph. Lead hydroxide stain; ×1,400. (By courtesy of Professor K. A. Porter.)

effect than the same dose given preoperatively, but when repeated doses of ALS were administered both pre- and postoperatively in a similar mouse system by Monaco and his colleagues (*6*) pretreatment was shown to give a significantly longer graft survival than postoperative administration alone. This finding has been confirmed in the canine renal allograft situation by the present experiments and by Starzl and his associates (*7*). It seems, therefore, that pretreatment with continued postoperative administration of serum is the method of choice and that ALS might be expected to produce its maximal effect clinically in the living donor situation as opposed to the cadaver, where pretreatment for any length of time would normally be impossible. The slight prolongation of survival seen in the animals not receiving pretreatment suggests that ALS might also be of value in the treatment of established rejection crisis, as suggested by Levey and Medawar (*5*).

The s.c. injection of the ALS and its IgG fraction was well tolerated. Any local reaction had subsided within 24–36 hr and seemed to be due more to the volume of the injected fluid rather than to any specific reaction. There was no evidence of the s.c. sepsis noted by Starzl and his associates (*7*) or of the systemic reactions seen after i.v. injection of a similar serum (*1*).

In an attempt to reduce the volume of fluid injected and the amount of

extraneous horse protein, an IgG preparation which retained most of the in vitro lymphagglutinating activity of the whole serum, but which contained 3 times the weight of immune globulin, was prepared. Volume of injection was therefore reduced by one-third, but some immunosuppressive activity was lost in the processing, the animals treated with this preparation showing a mean survival of 28 days in contrast with the mean 68-day survival in animals given the same amount of IgG in the form of the whole serum. Rabbit anti-rat IgG prepared by fractionation by DEAE cellulose batch chromatography and horse anti-rat IgG prepared by sodium sulphate precipitation and DEAE cellulose chromatography, did not show significant loss of activity in vivo (*3*) and it is not clear why horse anti-dog IgG prepared in the same way should be weakened by the processing. Further definition of the nature of the antibody, and other methods of fractionation of the serum, are under investigation.

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Effect of F(ab′)$_2$ from Rabbit Anti-mouse Lymphocyte IgG on the Graft versus Host Reaction in F_1 Hybrid Mice

HETEROLOGOUS antisera raised against lymphoid cells (ALS) have been found to suppress a large variety of immune responses (see review by James[1]). In particular, lymphoid cells from mice which have been treated with ALS exhibit a diminished ability to produce graft versus host (GVH) reactions[2–5]. Such responses result from immunological interaction between lymphoid graft and the histocompatibility antigens of the host. One such reaction occurs when parental spleen cells are injected into adult F_1 hybrid mice, and its occurrence and severity can be assayed by measurement of the resulting increase in size of spleen and liver[6]. As anti-lymphocytic antibody fragments seem incapable of suppressing humoral antibody formation[7–10] and inhibiting allograft rejection[11], we have investigated the effect of such preparations on the following GVH system.

We have compared the ability of spleen cells from *C57Bl* mice treated with intact IgG and fragments (antibody and normal) to produce a GVH-reaction on subsequent injection into normal $(C57Bl \times CBA)F_1$ hybrid recipients. The mice used were derived from inbred strains maintained in this department; the *C57Bl* donors were males and females weighing 25–30 g and the recipient F_1 hybrids were males between 30 and 35 g.

ALS was raised by intravenous injection of New Zealand white rabbits with about 10^9 *CBA* mouse thymus cells on each of three occasions during a 4 week period. The rabbits were bled 1 week after the last injection. The ALS obtained was inactivated (56° C for 30 min) and absorbed with mouse erythrocytes to remove agglutinins.

Small amounts of this antiserum prolonged the survival (three times) of A strain skin grafts on *CBA* male mice and produced a marked lymphopenia in *C57Bl* mice. The normal rabbit serum (NRS) IgG and rabbit anti-mouse lymphocyte IgG preparations used in these studies were prepared by batch chromatography[12] on Whatman *DE52* ion exchange cellulose (degree of substitution = 1·0 m.equiv./g). Immunoelectrophoresis using a sheep anti-whole rabbit serum indicated that the products contained only trace amounts of contaminating proteins. The non-cytotoxic F(ab′)$_2$ portion of the antibody molecule (which contains both antibody combining sites) was obtained by pepsin digestion[13] at 37° C for 24 h in *p*H 4·0, 0·1 molar acetate buffer. For each 100 mg of protein 2 mg of enzyme

was used. Degradation of the IgG to the $F(ab')_2$ product was shown to be complete by immunodiffusion and cytotoxic analyses. The products were concentrated to 1 g per cent by ultrafiltration and dialysed against 0·15 molar sodium chloride before use.

C57Bl donor animals were given a total of 1 ml. of the appropriate 1 g per cent preparation intraperitoneally in doses of 0·25 ml. on days 4, 3, 2 and 1 prior to cell transfer.

Spleen cell suspensions were prepared from the pooled spleens of four or five treated animals by gently breaking them up in a ground glass homogenizer containing balanced salt solution plus 0·1 per cent normal mouse serum. The suspensions were passed through a fine meshed stainless steel sieve, allowed to stand for 10 min at 4° C, then the cells in the supernatant were washed twice and re-suspended to give a concentration of about 200×10^6/ml. The viability of the cells was assessed by their ability to exclude 0·05 per cent trypan blue, and was never less than 86 per cent. The volume necessary to give a dose of 100×10^6 viable cells was calculated, and this volume of suspension was injected into the tail vein of the appropriate animal.

The $(C57Bl \times CBA)F_1$ animals were arranged in six groups, as shown in Table 1. Nine days after cell transfer the animals were weighed, killed by cervical dislocation, and the weights of their spleens and livers determined.

As Table 1 shows, ALS IgG alone inhibited the production of splenomegaly and hepatomegaly while ALS $F(ab')_2$ and the normal IgG preparations were ineffective. This result is in agreement with other findings that antilymphocytic fragments seem incapable of suppressing humoral antibody formation[7–10] or inhibiting homograft rejection[11], and suggest that the immunosuppressive properties of ALS are dependent on the possession of an intact Fc (complement binding) portion. It is possible, however, that other properties associated with this region

Table 1. MEAN RELATIVE SPLEEN AND LIVER WEIGHTS OF $(C57Bl \times CBA)F_1$ HYBRID MICE, 9 DAYS AFTER INTRAVENOUS INJECTION OF 100×10^6 SPLEEN CELLS FROM *C57Bl* MICE OR SALINE

Group	No.	Relative spleen weights* mean (±1 *S.D.*)	Relative liver weights* mean (±1 *S.D.*)
I Cells from donors treated with ALS IgG	6	42·5 (±7·7)	568·7 (±47·1)
II Cells from donors treated with ALS $F(ab')_2$	5	128·5 (±23·9)	752·4 (±55·4)
III Cells from donors treated with NRS IgG	5	108·0 (±8·3)	698·8 (±54·6)
IV Cells from donors treated with NRS $F(ab')_2$	5	120·3 (±8·1)	637·6 (±49·9)
V Cells from donors treated with saline	5	120·9 (±7·3)	746·1 (±57·3)
VI Saline only	7	43·1 (±19·2)	585·7 (±31·9)

* Relative organ wt. $= \frac{\text{Organ weight (mg)} \times 10}{\text{Body weight (g)}}$. Group I against group V in respect of spleen weight $P < 0{\cdot}01$. Group I against group V in respect of liver weight $P < 0{\cdot}01$. Group II against group V in respect of spleen weight or liver weight, not significant.

of the molecule are involved (that is, membrane attachment, placental transfer, immunogenicity).

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Effect of Anti-rat Lymphocyte Antibody on Humoral Antibody Formation

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Injections of rabbit and horse anti-lymphocytic sera into hooded rats inhibited the primary humoral antibody response to sheep erythrocytes, but did not have a marked effect on the secondary response. The major part of the immunosuppressive activity was associated with the IgG fraction. Antibody fragments were not effective in suppressing the immune reaction.

INTEREST in he immunosuppressive properties of anti-lymphocyticserum has been stimulated by the observations of Woodruff and Anderson[1,2] that the survival of skin homografts in rats can be greatly prolonged if the recipients are treated with rabbit anti-rat lymphocyte serum (ALS). More recent investigations have revealed that administration of anti-lymphocytic serum can also prolong the survival of skin homografts in mice[3,4] and aenal homotransplants in dogs[5,6], and it appears that rnti-human lymphocyte sera may be of therapeutic value in the transplantation of human organs[7].

Because of the potential practical importance of these observations we have investigated the effect of anti-lymphocytic antibody, and of fragments thereof, on the humoral antibody response of rats to sheep erythrocytes. These investigations were carried out to assess the potency of fractionated anti-lymphocytic serum and in order to obtain further data on the mode ot action of this material. Similar investigations have recently been reported in mice using unfractionated antiserum[3].

The anti-lymphocytic sera used were produced in a horse and in rabbits by the injection of thoracic duct lymphocytes obtained by cannulation of hooded strain male rats. The horse received intravenous injections weekly for 3 weeks and was given a fourth injection 2 weeks later. Each injection contained $1{\cdot}07–1{\cdot}3 \times 10^9$ lymphocytes (> 95 per cent viable). The horse was exsanguinated 10 days after the last injection. The rabbits received a total of three intraperitoneal injections, each containing 2×10^8 lymphocytes at weekly intervals, and were bled 10 days after the final injection. Further bleedings were performed on the rabbits 10 days after re-injection intraperitoneally with from 1 to 2×10^8 lymphocytes.

'Sephadex *G*200' gel filtration and reduction with mercaptoethanol (0·2 moles/l.), followed by alkylation, revealed that the antibody produced in horses was distributed in both the 19*S* and 7*S* fractions of serum, while the rabbit antibody was predominantly 7*S* protein. Because of the difficulty of preparing horse antiserum free from rat erythrocyte agglutinin activity, most of this work was performed with a preparation of IgG globulin which was almost devoid of this activity (see Table 1). The limited supply of rabbit antisera prevented extensive work with fractionated material.

The IgG preparation from the horse anti-lymphocytic serum was obtained by repeated (twice) sodium sulphate precipitation (the final concentration of the sodium sulphate was 14 per cent w/v) followed by diethylaminoethyl (DEAE) cellulose batch chromatography on Whatman *DE*11 exchanger with a capacity of 1·0 m.equiv./g (ref. 8). The product was concentrated by lyophilization and reconstituted in phosphate buffered saline (*p*H 7·2, 0·06 molar phosphate containing 0·15 molar sodium chloride). Immunoelectrophoretic analysis, using a rabbit anti-horse serum and polyacrylamide gel electrophoresis, indicated that the preparation contained only small amounts of contaminating protein (probably IgT).

The $F(ab')_2$ portion of the antibody molecule, which contains both antibody combining sites, was obtained by digestion with pepsin[9] at 37° C for 48 h in 0·1 molar acetate buffer, *p*H 4·0, using 2 mg enzyme for each 100 mg of protein. Degradation of the IgG to the $F(ab')_2$ product

Table 1. PROPERTIES OF ANTISERA AND ANTIBODY PREPARATIONS

Sample	Protein conc. (g per cent)	Reciprocal titres: Lymphocyte agglutination	Reciprocal titres: Lympho-cytotoxic	Reciprocal titres: Erythrocyte agglutination	Effect on primary response
(1) Horse ALS inactivated and absorbed with one volume rat erythrocytes	5·0 (1 g per cent IgG)	512	512	128	Suppression
(2) IgG globulin from horse ALS	1·0	64	256	< 2	Suppression
(3) $F(ab')_2$—pepsin digest from 2	1·0	32	8	4	No effect
(4) Fab′—reduced and alkylated sample from 3	1·0	4	8	< 2	No effect
(5) IgG globulin from normal horse serum	1·0	< 2	8	< 2	No effect
(6) Rabbit ALS inactivated and absorbed with a half volume of rat erythrocytes	5·3	512	512	2	Suppression
(7) Normal rabbit serum inactivated	4·9	< 8	< 8	4	Partial suppression

Note: These results are from duplicate assays.

was shown to be complete by immunodiffusion and subsequent cytotoxic analyses (see Table 1). A sample of this material was reduced with 0·1 molar cysteine hydrochloride and alkylated by dialysis against 100 volumes of the phosphate buffered saline containing iodoacetamide (0·02 moles/l.). The univalent antibody fragment obtained (Fab′) did not agglutinate lymphocytes *in vitro*, indicating complete reduction (Table 1). Both these preparations were finally equilibrated against phosphate buffered saline as already described.

Erythrocyte agglutinin activity was determined in 3 × ½ in. test-tubes by adding 0·1 ml. of 2·5 per cent (v/v) erythrocytes to 0·25 ml. volumes of doubling dilutions of the test sample. All dilutions were performed in the phosphate buffered saline used previously. After incubation for 18 h at room temperature (20° C) the contents of the tubes were agitated and then examined macroscopically and microscopically.

Test sera failing to agglutinate sheep erythrocytes were examined for antibodies by the Coombs (anti-globulin technique) using a rabbit anti-rat serum. The non-agglutinating rat sera were incubated with sheep erythrocytes, as in the standard test, after which they were washed (three times) with 3 ml. volumes of phosphate buffered saline. After this the sheep erythrocytes were resuspended in 0·5 ml. of the phosphate buffer and divided into two equal portions. To one sample was added one drop of the rabbit anti-rat serum and to the other one drop of normal rabbit serum. Both sera had been inactivated (56° C for 30 min) and absorbed with equal volumes of sheep erythrocytes. Controls were also performed using normal sheep erythrocytes. The tubes were examined macroscopically after 3 h at room temperature.

formation was achieved only with whole antisera and intact IgG antibody. It should be noted that these samples alone exhibited lymphocytotoxic activity as assayed by *in vivo* and *in vitro* procedures. The preparation of IgG, however, demonstrated only weak cytotoxic activity *in vivo*.

The lymphopenia produced by rabbit antibody was more marked and prolonged than that observed with the horse antiserum. Furthermore, the Coombs anti-globulin test indicated that a greater agglutination was achieved with the rabbit antibody. All the sheep erythrocytes treated with the non-agglutinating sera from rats receiving horse anti-lymphocytic antibody were strongly agglutinated by the rabbit anti-rat serum. In contrast, of the sheep erythrocytes treated with the eleven negative sera from rats receiving the rabbit anti-lymphocytic antibody, only two were agglutinated by the rabbit anti-rat serum. Thus it would seem that rats treated with the horse antibody possessed appreciable amounts of non-agglutinating erythrocyte antibody while this was only rarely detected in rats receiving the more effective rabbit antibody.

The divalent antibody fragment ($F(ab')_2$) agglutinated lymphocytes *in vitro* but failed to suppress humoral antibody formation. Thus degradation of the Fc (the complement and cell surface binding) portion of the molecule, with the expected loss of cytotoxic activity, also destroys the immunosuppressive properties of this antibody. The univalent moiety (Fab′) did not agglutinate or lyse the lymphocytes *in vitro*, nor did it inhibit antibody formation. Nevertheless, this material was shown by the Coombs technique, using a rabbit anti-horse IgG, to bind to lymphocytes *in vitro*.

Table 2. EFFECT OF ANTI-LYMPHOCYTE ANTIBODY AND ANTIBODY FRAGMENTS ON THE PRIMARY RESPONSE OF RATS TO SHEEP ERYTHROCYTES

Nos. of rats	Treatment	Lymphocyte count day*		Reciprocal erythrocyte agglutination titre											Day of test	Effect on primary response
		−3	0	<2	2	4	8	16	32	64	128	256	512	1,024		
12	No pretreatment			—	—	—	—	—	3	1	4	1	2	1	5	No effect
				—	—	—	—	1	5	4	1	—	—	1	10	
8	Horse ALS	5,910	2,180	6	2	—	—	—	—	—	—	—	—	—	5	Suppression
		(100)	(37)	6	1	1	—	—	—	—	—	—	—	—	10	
14	1 g% IgG from horse ALS	5,100	3,810	7	—	4	1	2	—	—	—	—	—	—	5	Suppression
		(100)	(75)	10	2	1	—	1	—	—	—	—	—	—	10	
8	1 g% $F(ab')_2$ from above	5,240	5,320	—	—	—	—	—	—	2	6	—	—	—	5	No effect
		(100)	(101·5)	—	—	—	—	—	1	6	1	—	—	—	10	
10	1 g% Fab′ from above		7,190	—	—	—	—	1	3	2	4	—	—	—	5	No effect
8	1 g% normal horse IgG	5,600	4,950	—	—	—	—	—	—	2	3	—	3	—	5	No effect
		(100)	(88·4)	—	—	1	2	—	2	2	1	—	—	—	10	
24	1 g% IgG from horse ALS to Wistar rats (line bred)	13,560 (100)	8,860 (65·3)	—	—	—	—	—	—	3	5	15	1	—	5	No effect
4	1 g% IgG from horse ALS to Wistar rats (inbred)	10,510 (100)	9,430 (89·7)	—	—	—	1	2	1	1	—	—	—	—	5 10	No effect
12	Rabbit ALS	5,910	780	11	—	—	1	—	—	—	—	—	—	—	5	Suppression
		(100)	(13·2)	12	—	—	—	—	—	—	—	—	—	—	10	
6	Normal rabbit serum	5,380	4,920	—	—	—	1	3	1	1	—	—	—	—	5	Partial suppression
		(100)	(91·5)	—	—	1	1	2	2	—	—	—	—	—	10	

Each animal received 2 ml. of test sample intraperitoneally on days −3, −2 and −1 and was injected (intravenously) with 1×10^9 erythrocytes in 1 ml. on day 0.

* The figures given in parentheses are per cent values based on day −3 count = 100 per cent.

Note: All experiments were performed with hooded rats except where otherwise indicated.

Lymphocyte agglutinins and lymphocytotoxins were determined by procedures described previously[10]. The *in vivo* cytotoxicity of the preparations was assayed by determining their effect on the peripheral blood lymphocyte count.

In most of the experiments, equal numbers of hooded male (210–275 g) and female rats (155–215 g) were used. The quantities of materials administered are indicated in Tables 1–3. A few results obtained with two strains of Wistar rats (one line bred, the other inbred) have also been included in Table 2.

Detailed preliminary investigations revealed that the peak day of primary response in hooded rats to sheep erythrocytes was day 5, and so, in all subsequent analysis of effect on the primary responses, the sera were usually examined on day 5 and day 10. The rat sera were examined 5 days after secondary challenge.

It can be seen from Tables 1 and 2 that effective suppression of humoral antibody (erythrocyte agglutinin)

The preparation of horse antibody IgG to hooded rat lymphocytes failed to inhibit the production of erythrocyte agglutinin in the two other strains of rats tested even though it produced a lymphopenia similar to that observed in the hooded rat. This could be due to a number of factors including the larger size of these animals (260–355 g), their higher initial lymphocyte count and a degree of strain specificity.

The effect of rabbit and horse anti-lymphocytic antibody on the secondary response of hooded rats to sheep erythrocytes can be seen in Table 3. Although only small numbers of rats were used in these experiments, it would appear that the secondary response was suppressed to a slight extent in all the groups receiving anti-lymphocytic antibody (compare rats *A* with rats *B*). This suppression was observed irrespective of the treatment before primary stimulation. As has been observed earlier[3], the inhibition of the secondary response is not so sensitive to anti-lymphocytic antibody as the primary phase. It is possible

that more effective suppression of the secondary response may be achieved by administration of larger amounts of these preparations, or by using stronger (more avid) antisera, such as the rabbit anti-rat lymphocyte material. Monaco *et al.*[3] have already indicated that the degree of inhibition of the secondary response is proportional to the amount of anti-lymphocytic antibody administered. Prolonged pretreatment of this kind might also have prevented the marked primary response observed in the Wistar strain rats.

antibodies (quantitatively and qualitatively), and this may in part explain the reduced humoral antibody production after secondary stimulation.

These results also suggest a number of points of possible practical importance in the production of anti-lymphocytic antibody and its use in transplantation. The preparation of purified IgG possessing anti-lymphocytic activity may in some cases obviate the costly process of absorbing the preparation to remove erythrocyte agglutinins. Furthermore, the use of such preparations makes

Table 3. EFFECT OF ANTI-LYMPHOCYTIC ANTIBODY ON THE SECONDARY RESPONSE OF HOODED RATS TO SHEEP ERYTHROCYTES

Treatment before		Lymphocyte count		Reciprocal agglutination titre											Days after primary challenge
Primary	Secondary	3 Days before secondary	Before injection of antigen	<2	2	4	8	16	32	64	128	256	512	1,024	2,048	
A Horse antibody IgG	Horse antibody IgG	3,250	2,760	—	—	—	1	1	1	1	1	—	—	—	—	14
		(100)	(85)	1	—	—	—	—	—	1	1	1	1	—	—	19
B Horse antibody IgG	Normal horse IgG	2,850	2,750	—	—	1	—	3	2	—	—	—	—	—	—	14
		(100)	(97)	—	—	—	—	—	—	—	1	3	1	—	—	19
A Normal horse IgG	Horse antibody IgG	4,080	3,500	—	—	—	—	1	2	1	—	2	—	—	—	14
		(100)	(86)	—	—	—	—	—	—	2	1	1	2	—	—	19
B Normal horse IgG	Normal horse IgG	4,140	4,500	—	—	—	—	2	1	2	—	—	—	—	—	14
		(100)	(109)	—	—	—	—	—	—	1	1	1	1	—	1	19
A Horse ALS	Horse antibody IgG	6,790	4,300	4	—	—	—	—	—	—	—	—	—	—	—	28
		(100)	(63)	—	2	—	2	—	—	—	—	—	—	—	—	33
B Horse ALS	Normal horse IgG	5,370	6,430	4	—	—	—	—	—	—	—	—	—	—	—	28
		(100)	(120)	—	—	—	—	—	2	2	—	—	—	—	—	33
A Normal horse IgG	Horse antibody IgG	3,930	2,900	—	1	1	1	1	—	—	—	—	—	—	—	21
		(100)	(74)	—	—	—	—	1	2	1	—	—	—	—	—	26
B Normal horse IgG	Normal horse IgG	4,930	3,300	—	—	—	—	1	—	2	—	—	—	—	—	21
		(100)	(67)	—	—	—	—	—	—	—	—	2	1	—	—	26
A Rabbit ALS	Rabbit ALS	4,600	1,430	3	—	—	—	—	—	—	—	—	—	—	—	14
		(100)	(31)	3	—	—	—	—	—	—	—	—	—	—	—	19
B Rabbit ALS	Normal rabbit serum	3,870	2,000	3	—	—	—	—	—	—	—	—	—	—	—	14
		(100)	(52)	2	—	—	—	1	—	—	—	—	—	—	—	19
A Normal rabbit serum	Rabbit ALS	3,700 (100)	1,610 (44)	—	—	1	—	2	—	—	—	—	—	—	—	14
				—	—	1	1	1	—	—	—	—	—	—	—	19
B Normal rabbit serum	Normal rabbit serum			—	—	1	1	—	1	—	—	—	—	—	—	14
				—	—	—	—	—	3	—	—	—	—	—	—	19
A No pretreatment	Rabbit ALS	2,230	1,140	—	1	1	—	—	1	—	—	—	—	—	—	14
		(100)	(51)	—	1	1	—	—	—	—	1	—	—	—	—	19
B No pretreatment	Normal rabbit serum			—	—	1	1	—	—	—	—	—	—	—	—	14
				—	—	—	—	—	1	—	—	1	—	—	—	19

All the rats received daily intraperitoneal injections of 2 ml. of whole serum (antibody or normal) or IgG globulin (antibody or normal) on the 3 days immediately before primary or secondary stimulation with 1×10^9 sheep erythrocytes. The humoral antibody response was assessed 5 days following secondary stimulation.

These results do not, of course, reveal what mechanisms are responsible for the inhibition of antibody formation with anti-lymphocytic serum. Nevertheless, the observation that antibody IgG suppresses the formation of humoral antibody after primary antigenic stimulation without producing a significant lymphopenia merits further comment. It is possible that the preparation of antibody IgG, which we have found also produces prolonged skin homograft survival, preferentially inactivates a small proportion of cells with a pronounced capacity for producing antibody because of a particular responsiveness to antigenic stimulation. The possibility that there are two sets of immunologically competent cells with differential sensitivity to anti-lymphocytic serum has previously been put forward[3]. The surviving lymphocytes may have been present from the start or may have been produced as the result of lymphoid hyperplasia of a small number of cells. These cells may be intrinsically inactive or else rendered so by coating (blindfolding) with antibody. The failure of antibody fragments which bind *in vitro* to inhibit the primary immune response would, however, suggest that a "blindfolding" mechanism may not be involved, although this should be more clear when fluorescent antibody studies have been performed to determine whether these materials do indeed coat lymphocytes *in vivo*. Moreover, on the basis of the results obtained with the rabbit antiserum it is possible that $F(ab')_2$ and Fab′ preparations from more avid antibody may be capable of inhibiting the primary immune response and experiments are at present being performed to test this hypothesis. As well as the possible effect of ALS on the recognition of antigen, this material might also influence the ability of immunologically competent cells to produce unnecessary the administration of large amounts of non-antibody protein, part of which possesses anti-enzyme activity which could disturb normal metabolic processes and perhaps influence subsequent graft survival. The few results so far available with rabbit antisera indicate, at least for rats, that this material may be preferable to horse preparations. The erythrocyte agglutinins were readily absorbed from the rabbit antisera and the resultant extremely cytotoxic preparations caused almost complete suppression of humoral antibody formation after primary stimulation. Finally, the anti-lymphocytic activity of the rabbit antisera was located in the 7*S* region and therefore is readily amenable to isolation.

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EFFECT OF ANTI-RAT LYMPHOCYTE ANTIBODY ON HUMORAL ANTIBODY FORMATION

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Effect of Anti-rat Lymphocyte Antibody on Humoral Antibody Formation

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Horse anti-rat lymphocyte IgG globulin suppresses and/or delays the primary response to alum-precipitated bovine serum albumin in hooded rats. This material does not have a marked effect on the secondary response of sensitized animals.

RECENT work has shown that anti-lymphocytic sera (ALS) are effective immunosuppressive agents prolonging the survival of skin homografts in rats[1,2,15] and mice[3–5] and renal homotransplants in dogs[6–8] and humans[8]. In addition, anti-lymphocytic antibody has been shown to suppress significantly the primary humoral antibody response of both rats[9] and mice[3] to sheep erythrocytes and possibly to exert a slight effect on the secondary response[3,9]. The present report is a detailed study of the effect of anti-lymphocytic IgG on the production of humoral antibody in hooded rats after primary and secondary stimulation with a further commonly used test antigen, namely alum precipitated bovine serum albumin (BSA). Throughout the investigations the humoral antibody response has been assessed by measuring the antigen binding capacity (ABC) of the rat sera by means of the sensitive ammonium sulphate method of Farr[10] which measures the primary interaction of antigen and antibody.

Details of the production of the horse antiserum to thoracic duct lymphocytes, and the isolation and assay of the IgG preparations used, have already been reported[9]. The reciprocal lymphocyte agglutination and lymphocytotoxic titres of the anti-lymphocytic IgG were both 256, while those of the normal IgG were less than 8.

The alum precipitated bovine serum albumin for injection (Armour Pharmaceutical Company, Lot Nos. *C*4101) was prepared as described by Pinckard, Weir and McBride[11]. Soluble BSA iodinated with iodine-131, for use in the Farr procedure, was prepared by the chloramine-T method described by Hunter and Greenwood[12] using carrier-free iodine-131. The nitrogen content of the labelled BSA was determined by the micro-Kjeldahl procedure[13]. The labelled BSA preparations were used only if more than 96 per cent of the radioactivity was precipitated with 10 per cent trichloroacetic acid (TCA). The antibody content of the rat sera obtained following antigenic stimulation was assessed by the Farr procedure[10]. This technique was performed exactly as described by Pinckard, Weir and McBride[11], using appropriate normal serum controls. The antigen binding capacities recorded in Table 1 and plotted in Figs. 1 and 2 were obtained with bovine serum albumin samples containing 0·2 μg nitrogen and are expressed in terms of the number of micrograms of nitrogen bound by 1 ml. of undiluted serum. In order to measure the relative binding affinity (a measure of the quality of the antibody produced) the antigen binding capacity was also determined using samples containing 0·02 μg of bovine serum albumin nitrogen. The latter ABC value was divided by the value obtained using 0·2 μg nitrogen and expressed as a percentage value. As the affinity of the antibody increases, the value obtained approaches 100 per cent. Throughout the investigations all samples were counted in a well-type scintillation spectrometer with a 2 in. sodium iodide crystal.

The effect of anti-lymphocytic IgG on the immune response to alum precipitated BSA was investigated in

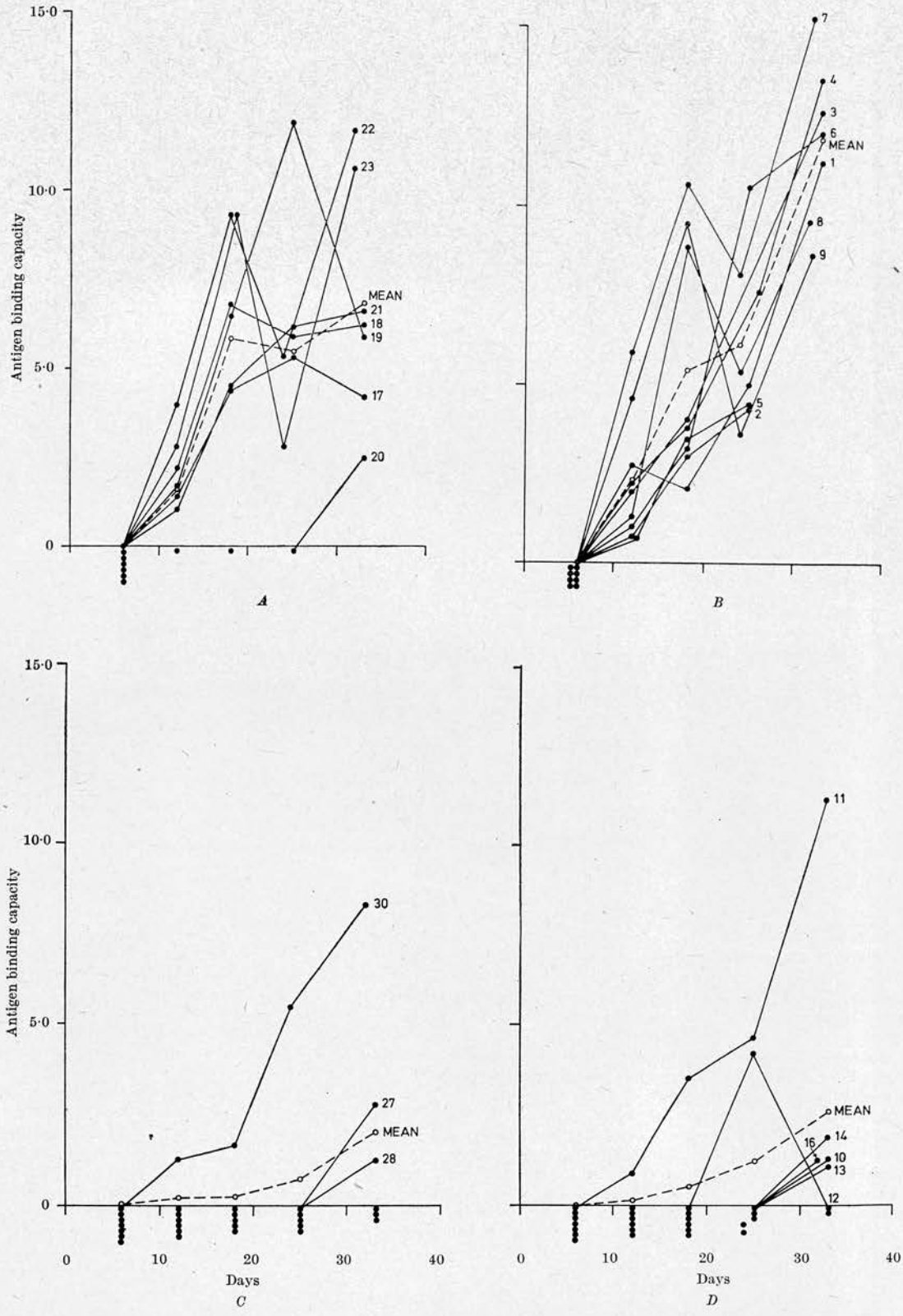

Fig. 1. Effect of normal IgG and anti-lymphocytic IgG on the primary immune response of hooded rats to alum precipitated bovine serum albumin. *A*, Experimental group 1*A*, male rats receiving normal IgG; *B*, experimental group 1*B*, female rats receiving normal IgG; *C*, experimental group 1*C*, male rats receiving anti-lymphocytic IgG; *D*, experimental group 1*D*, female rats receiving anti-lymphocytic IgG. The experimental groups were divided into male and female for ease of presentation of results. The circles under the base-line represent animals in which the immune response was so weak that ABC values could not be obtained using a bovine serum albumin test sample containing 0·2 μg nitrogen. In some groups the number of samples varies slightly from bleed to bleed and this is due to deaths and the occasional inability to obtain sufficient sera for analysis.

hooded strain male and female rats. The animals received intraperitoneal injections of sterile normal horse IgG (experimental groups 1*A* and 1*B*—see Table 1) or anti-lymphocytic IgG (experimental groups 1*C* and 1*D*) on days −3, −2 and −1 and were injected intraperitoneally on day 0 with 5 mg of alum precipitated BSA. The animals were then bled at intervals of 6 days. After 30 days the rats in groups 1*A* and 1*B* were subdivided, half the rats in each group receiving a further course of normal IgG (experimental group 2*A*) and the other half anti-lymphocytic IgG (experimental group 2*B*). Similarly half the rats in group 1*C* and 1*D* received normal IgG (experimental group 2*C*) and the other half anti-lymphocytic IgG (experimental group 2*D*). These injections were given on days 30, 31 and 32 and the animals were rechallenged intraperitoneally with 5 mg of alum precipitated BSA on day 33. The animals were then bled at 7 day intervals. All the serum samples obtained were stored at −20° C until analysed. Further details on the animals used and the course of treatment are given in Table 1.

The effect of the normal and anti-lymphocytic IgG preparations on the primary response of individual hooded male and female rats is shown in Figs. 1*A* to 1*D* and summarized in Table 1. It is seen that anti-lymphocytic antibody causes a marked suppression and delay in humoral antibody formation in most of the rats studied (Figs. 1*C* and 1*D*). This suppression was also associated with a significant reduction in the peripheral blood lymphocyte count (Table 1). Anti-lymphocytic IgG did not markedly inhibit humoral antibody formation in all the rats studied, however, for two out of fourteen (that is, rat No. 30, see Fig. 1*C*, and rat No. 11, see Fig. 1*D*) showed a response of similar magnitude to that found in rats pretreated with normal IgG. It should also be noted that on day 33 all the sera from rats treated with anti-

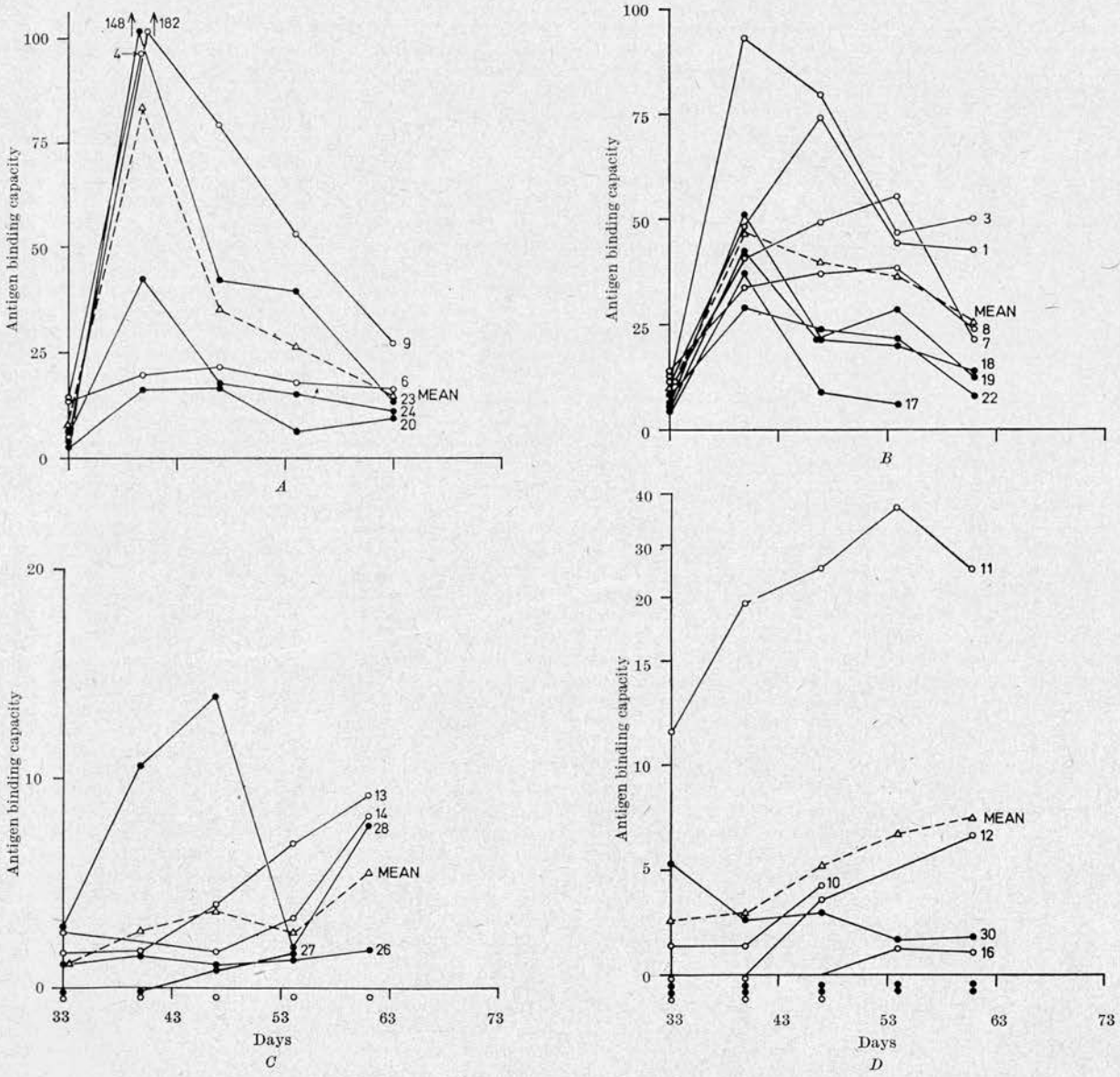

Fig. 2. Effect of normal IgG and anti-lymphocytic IgG on the secondary response of hooded rats (from groups 1*A* to 1*D*) to alum precipitated bovine serum albumin. ●, Male; ○, female. *A*, Experimental group 2*A*, rats receiving normal IgG before primary and secondary antigenic challenge with alum precipitated bovine serum albumin; *B*, experimental group 2*B*, rats receiving normal IgG before the primary and anti-lymphocytic IgG before the secondary antigenic challenge; *C*, experimental group 2*C*, rats receiving anti-lymphocytic IgG before the primary and normal IgG before the secondary antigenic challenge; *D*, experimental group 2*D*, rats receiving anti-lymphocytic IgG before the primary and secondary antigenic challenge. For further details see Fig. 1, Table 1 and text.

Table 1. PRETREATMENT OF HOODED RATS AND ITS EFFECT ON HUMORAL ANTIBODY PRODUCTION

Response	Experimental group		Rats: Sex	Nos.	Mean wt. (g)	Wt. range	IgG treatment		Peripheral blood lymphocyte count (total and %): −3 Day or 30	0 Day or 33	Antigen binding capacity (0·2 μg nitrogen): Initial	Maximum observed*
Primary	1A		Male	7	213	180–275	3 ml.	1 g% normal	3,720 (100)	3,550 (95)	0	7·9 ± 1·3
	1B		Female	9	161	130–195	2 ml.	1 g% normal	4,310 (100)	4,000 (93)	0	12·0 ± 0·7
	1C		Male	7	221	185–330	3 ml.	1 g% ALS	3,900 (100)	2,200 (55)	0	2·1 ± 1·3
	1D		Female	7	162	135–220	2 ml.	1 g% ALS	4,450 (100)	2,370 (53)	0	3·1 ± 1·5
Secondary	2A	Rats from groups 1A and 1B	Male and female	6			3 ml.	1 g% normal	4,630 (100)	4,450 (96)	7·5 ± 2·4 (100 ± 32)	84·2 ± 22·1 (1,122 ± 294)
	2B			8			3 ml.	1 g% ALS	6,140 (100)	3,410 (56)	9·3 ± 1·4 (100 ± 15)	50·1 ± 5·6 (538 ± 60)
	2C	Rats from groups 1C and 1D		6			3 ml.	1 g% normal	3,075 (100)	3,135 (102)	1·2 ± 0·4 (100 ± 30)	5·8 ± 2·4 (580 ± 200)
	2D			7			3 ml.	1 g% ALS	3,850 (100)	2,960 (76)	2·6 ± 1·9 (100 ± 53)	7·5 ± 5·0 (288 ± 192)

* The antigen binding capacities are expressed as mean values together with the standard deviation of the mean. In primary response studies these values were calculated only from rats which had survived the entire experimental period. In the secondary response all rats were considered because very few died and these had usually reached a maximum. The secondary values are also expressed as a percentage of the starting antigen binding capacity of the appropriate experimental group.

lymphocytic IgG contained detectable amounts of anti-BSA antibodies although the levels were not always high enough to allow the determination of antigen binding capacities (that is, a 1/10 dilution of the serum failed to bind 33 per cent of the test antigen[10,11]). In those cases in groups 1*C* and 1*D* where antigen binding capacities were measurable on day 33 the relative binding affinities were similar to those observed on day 12 in animals receiving normal IgG (experimental groups 1*A* and 1*B*). Thus although there was usually a decrease and delay in antibody production, the antibodies eventually produced appeared to be qualitatively comparable with those initially observed in "non-suppressed" animals.

As previously noted in experiments studying the response of rats and mice to sheep erythrocytes[2,9], the effect of anti-lymphocytic IgG on the secondary response was not very marked. Indeed, it is quite apparent from Table 1 and Fig. 2*B* (experimental group 2*B*) that animals which had received normal IgG before the initial injection of alum BSA responded readily to a further injection of alum BSA whether or not this was preceded by injection of anti-lymphocytic IgG. Analysis of the results suggests, on the other hand, that anti-lymphocytic IgG may be influencing quantitative aspects of the secondary response in a number of animals. In support of this there was also a slight indication that the relative response to a secondary challenge in rats which had initially received anti-lymphocytic antibody was also reduced when they received a further course of anti-lymphocytic IgG before the secondary stimulus (experimental group 2*D*). In order to establish whether anti-lymphocytic IgG does influence the magnitude of the secondary response, experiments will have to be carried out using more animals.

The anti-lymphocytic IgG had no obvious effect on the relative binding affinities of the antibodies produced after secondary stimulation in animals which had received normal IgG before primary stimulation. In experimental groups 2*A* and 2*B* the relative binding affinities usually showed marked increases after secondary stimulation. On the other hand, the relative binding affinities in groups 2*C* and 2*D* (those that had received anti-lymphocytic IgG before primary challenge) rarely showed the high values observed in groups 2*A* and 2*B*, thus indicating qualitative as well as quantitative differences in antibody production.

It is clear from these results that anti-lymphocytic IgG suppresses and/or delays the primary response of hooded rats to alum precipitated BSA, in addition to inhibiting erythrocyte agglutinin formation in rats and mice[3,9]. Thus this material affects the onset of both humoral and cellular types of immunity (see homograft experiments with this preparation[14,15]).

It has still to be determined whether anti-lymphocytic antibody interferes with the sensitizing ("triggering") phase of antibody production and/or the proliferative phase. A number of theories have been postulated to explain how this material could affect either, or both, of the processes. These include lymphocytolysis, the "sticky antigen" theory, blindfolding of lymphocytes[4] and sterile inactivation[5]. Whatever mechanisms are operating, it is clear that the present course of treatment, although it caused suppression, did not produce complete tolerance; all the animals receiving anti-lymphocytic antibody eventually produced detectable amounts of anti-BSA antibodies and also responded to a secondary challenge with BSA. Thus either the animals recovered their capacity to recognize and process antigen (the sensitization phase) and/or their ability to produce antibody after antigenic recognition (proliferative phase). This could result from the original cells recovering their immunological capacity or the maturation of additional immunologically competent cells or a combination of these and other phenomena. Cytological investigation[16] of those cells which do survive anti-lymphocytic treatment suggests that "deformation" may have occurred. This could render them incapable of producing normal levels of antibody or developing into cells with this potential. Whatever the explanation, it appears from measurements of relative binding affinity that the antibodies eventually produced after primary stimulation are qualitatively similar to those initially observed in animals receiving normal horse IgG. On the other hand, the affinities of the antibodies produced after secondary stimulation in group 2*C* and 2*D* were appreciably lower than those observed in animals which had received normal IgG before primary stimulation (group 2*A* and 2*B*).

The failure effectively to suppress the secondary response in "sensitized" animals (that is, those from group 1*A* and 1*B*) indicates that the course of treatment used did not "abrogate the pre-existing state of immunity to alum precipitated BSA", a phenomenon observed in relation to the survival of second set homografts[4,5]. Thus in these studies the anti-lymphocytic IgG failed to destroy immunological memory by inactivating memory cells. Nevertheless the results suggest that this form of treatment may be affecting the quantity, though not the quality, of the antibody produced after secondary stimulation in "sensitized" animals.

These investigations also indicate that gross lymphocyte depletion is not a prerequisite for effective immunosuppression[4,9]. Nevertheless, as previously suggested, the possibility that anti-lymphocytic antibody functions by destroying or inactivating a select proportion of vital cells cannot be precluded[3,4,9]. Indeed, it is also possible that this material may interfere with the activity of the macrophage, thus affecting sensitization.

In conclusion, it is apparent that anti-lymphocytic antibody is capable of suppressing or delaying primary

humoral antibody formation against alum precipitated BSA as well as cellular aspects of immunity, but the mechanisms involved in these important phenomena will require further investigation.

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Further studies on the effect of anti-lymphocytic antibody on humoral antibody formation

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FURTHER STUDIES ON THE EFFECT OF ANTI-LYMPHOCYTIC ANTIBODY ON HUMORAL ANTIBODY FORMATION

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SUMMARY

The effect of 3-, 6- and 12-day courses of anti-lymphocytic IgG treatment (1 ml of 2 g/100 ml) on the primary humoral response of random bred Wistar rats has been investigated. The primary response to alum precipitated bovine serum albumin was suppressed by all these courses. In contrast however the primary response to sheep erythrocytes was suppressed by the 6-day treatment alone or by a 3-day course augmented with azathioprine treatment. Additional experiments performed in hooded rats confirm that prolonged courses of anti-lymphocytic globulin failed to exert an antidotal effect.

INTRODUCTION

The ability of heterospecific anti-lymphocytic sera to suppress the primary humoral response of mice, rats and dogs to a number of cellular and soluble antigens is well documented (see reviews: James, 1967, 1968a, b). Nevertheless in certain situations anti-lymphocytic sera fail to suppress primary humoral responses. For example quantities of antisera (produced against thoracic duct lymphocytes from hooded strain rats) which readily suppress the primary humoral response of this hooded strain of rats are not very effective in certain Wistar strains (James & Anderson, 1967; Woodruff *et al.*, 1967). In addition a number of reports suggest that anti-lymphocytic antibody is not very efficient at suppressing antibody formation against itself, a factor of extreme importance in relation to the therapeutic use of this material (Currey & Ziff, 1966; Clark, James & Woodruff, 1967; Lance & Dresser, 1967; James & Anderson, 1968; Jasin *et al.*, 1968).

We have re-investigated therefore the effect of anti-lymphocytic antibody on both these aspects of the humoral response. Experiments have been performed to determine if the primary humoral response of random bred Wistar rats could be suppressed by prolonged courses of treatment with anti-lymphocytic antibody or by the simultaneous administration of other immunosuppressants, such as azathioprine. In addition investigations have been

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undertaken in hooded strain rats to determine the effect of anti-lymphocytic antibody on the formation of circulating antibodies against itself. The levels of circulating antibody in the latter experiments have been assessed by tanned cell and immune elimination procedures.

MATERIALS AND METHODS

The preparation of anti-lymphocytic serum

The anti-lymphocytic serum used was produced in a horse by the intravenous injection of thoracic duct lymphocytes obtained following cannulation of hooded strain male rats. The horse was injected on days 0, 7, 14 and 28 with between 1·07 and $1{\cdot}3 \times 10^9$ lymphocytes (95% viable) and was bled out on day 38 and the serum inactivated by incubating at 56°C for 30 min.

The preparation of IgG globulin

Immunoglobulin G was obtained from the above antiserum, and from normal horse serum, by a combined salt precipitation and chromatographic technique. The initial step involved the precipitation of a 'crude' globulin preparation by the slow addition of 1 volume of 28% (w/v) sodium sulphate. The precipitate was redissolved in 0·15 M-sodium chloride and re-precipitated as above. This material was then dissolved in, and dialysed against, 0·02 M-phosphate buffer, pH 6·5, and finally batched on diethylamino ethyl cellulose (Whatman DE11 exchanger with an ion-exchange capacity of 1·0 mEq/g). The final product was concentrated by lyophilization, reconstituted in phosphate buffered saline (pH 7·2, 0·06 M containing 0·15 M-sodium chloride) and sterilized by filtration through an 0·22 mμ Millipore filter. Immunoelectrophoretic analysis, using a rabbit anti-horse serum, indicated that the product contained several IgG components. The reciprocal lymphoagglutination and lymphocytotoxic titres of a 1 g/100 ml solution of the anti-rat lymphocyte IgG were both 256. The normal horse IgG possessed negligible amounts of anti-lymphocytic activity.

The estimation of humoral antibody formation

Antibodies to bovine serum albumin (crystalline bovine albumin, Armour Pharmaceutical) were determined by the Farr technique (Farr, 1958) using ^{131}I-labelled bovine serum albumin prepared by the chloramine T procedure (Hunter & Greenwood, 1962). As many of the sera under test bound less than 33% of the test antigen (containing 0·2 μg protein nitrogen) the results have been expressed as the percentage of the antigen bound by 0·5 ml of a 1:10 dilution of the serum rather than as antigen binding capacities. All the estimations were performed using normal serum and standard antiserum controls and further details of the procedure can be found elsewhere (Pinckard, Weir & McBride, 1967; James & Jubb, 1967).

The production of agglutinating antibodies to sheep erythrocytes was assessed as previously described from this laboratory (James & Anderson, 1967). The results have been expressed as the reciprocal $\log_2$ of the titre.

The antibodies to horse IgG were estimated using tanned pyruvic aldehyde preserved sheep erythrocytes (Ling, 1961) sensitized with 0·1 g/100 ml solution of normal horse IgG. The serial dilutions (three-fold) were performed using the Takatsky microtitrater. Each analysis included normal serum and standard antiserum controls as well as unsensitized sheep erythrocyte controls. The plates were examined after standing at 20°C for 3 hr and the observed results have been expressed as reciprocal $\log_3$ of the titre.

The presence of circulating antibodies to horse IgG has also been assessed by the immune elimination procedure. This involved injecting the animals with 10 mg of normal horse IgG labelled with 10 μC of iodine-131 by the chloramine T method described by Hunter & Greenwood (1962). Immediately prior to injection the labelled IgG was centrifuged at 30,000 rev/min (58,000 *g* average) for 1 hr to remove aggregated globulin which would complicate the immune elimination picture. Whole body counting of the animals was then performed at regular intervals using a ring counter consisting of twelve J26Pb Geiger–Müller tubes shielded with 1·25 in. of lead shot. Previous investigations had shown that the results obtained by this procedure were similar to those obtained by counting samples of peripheral blood (Clark *et al.*, 1967).

Chi-square analysis was performed to determine the significance of the effect of treatment on the primary response of the rats to alum precipitated bovine serum albumin. Animals whose sera bound more than 20% of the test antigen at any time during the test period were regarded as responders and those binding less than 20% as non-responders. The haemagglutinin and tanned cell titres on the other hand were analysed by means of the Student's *t*-test using the Bessel correction for small numbers and values of P less than 0·1 were taken as significant.

RESULTS

The effect of anti-lymphocytic antibody on the primary humoral response in random bred Wistar rats

These investigations were performed in male rats (weight 267–416 g) maintained by the small animal breeding station of the University of Edinburgh. The animals (six per group initially) were treated with anti-lymphocytic IgG (for 3–12 days) and azathioprine as indicated in Table 1. On the day after the last IgG injection (designated day 0) the animals were injected intraperitoneally with 5 mg of alum-precipitated bovine serum albumin and intravenously with 1×10^9 sheep erythrocytes. The animals were bled by cardiac puncture on days 7, 14, 22 and 28, and the circulating antibody assessed by the above procedures. From Fig. 1 and Table 1 it will be observed that all of the courses of anti-lymphocytic IgG treatment used significantly suppressed the primary response of random bred Wistar rats to alum precipitated bovine serum albumin. Indeed, in all but one case (exception 1C) the levels of circulating antibody in individual animals were much lower than those observed in all the animals in the control group with the exception of animal 5A. In contrast a significant suppression of the primary response to sheep erythrocytes was only observed in two of the anti-lymphocytic IgG treated groups (Groups C and E see Fig. 2 and Table 1). These animals received a 3-day course of anti-lymphocytic IgG treatment in conjunction with azathioprine and a 6-day course of anti-lymphocytic antibody treatment, respectively. Short term treatment (3 days) with anti-lymphocytic antibody alone and the more prolonged course of treatment (12 days), both failed to significantly inhibit erythrocyte agglutinin formation.

While the azathioprine dose used appeared to potentiate the effect of anti-lymphocytic IgG on the primary response to sheep erythrocytes, by itself it failed to suppress the primary response to this antigen and to bovine serum albumin.

In all cases anti-lymphocyte IgG treatment resulted in lymphopenia, the effect being most marked in those animals receiving prolonged treatment (groups E and F).

TABLE 1. The effect of anti-lymphocytic IgG and azathioprine on the primary humoral response of random bred Wistar rats

Group	Days treated with*		Effect on peripheral lymphocyte count (cells/mm³)		Response to alum precipitated bovine serum albumin‡			Response to sheep erythrocytes			
								log₂ reciprocal titre			
	Anti-lymphocytic IgG (1ml of 2 g/100 ml)	Azathioprine (50 mg/kg)	Initial	Final†	No. responding	No. not responding	Significance (χ^2 analysis)	Mean	SD	Difference between means	Significance of difference between means§
A	No treatment	No treatment		12,850	4	1		8·32	1·23		
B	No treatment	0, +2, +4, +6	16,890 (100)	15,440 (91)	4	1	Insignificant	8·32	1·27	Nil	Insignificant
C	−3 to −1	0, +2, +4, +6	15,245 (100)	9,750 (64)	0	3	$\chi^2 = 4·8$ $P<0·05>0·01$ Significant	6·15	0·76	2·17	$n = 9, t = 3·24$ $P<0·1>0·01$ Significant
D	−3 to −1	No treatment	15,210 (100)	7,660 (50)	0	4	$\chi^2 = 5·76$ $P<0·05>0·01$ Significant	8·07	1·71	0·25	$n = 7, t = 0·225$ $P>0·8$ Insignificant
E	−6 to −1	No treatment	20,250 (100)	6,580 (32)	0	5	$\chi^2 = 6·67$ $P = 0·01$ Significant	3·93	1·09	4·39	$n = 9, t = 3·055$ $P<0·1>0·01$ Significant
F	−12 to −1	No treatment	18,620 (100)	6,150 (33)	0	6	$\chi^2 = 7·54$ $P<0·01$ Significant	5·93	3·36	2·39	$n = 9, t = 1·358$ $P>0·02$ Insignificant

* Intraperitoneal injections. Antigens injected day 0.

† In groups A, D, E and F counts performed day 0. In groups B and C on day +7. Values in brackets expressed as per cent initial value.

‡ Animals binding more than 20% of test antigen at peak of response regarded as responding.

§ Significance of the difference between the peak mean values observed in groups B to F compared with those observed in control group A.

The immune response of hooded strain rats to anti-lymphocytic globulin

These studies were undertaken in female rats (weight 185–297 g) of the hooded strain. The animals (ten per group) received intraperitoneal injections of 1 ml of a 2 g/100 ml

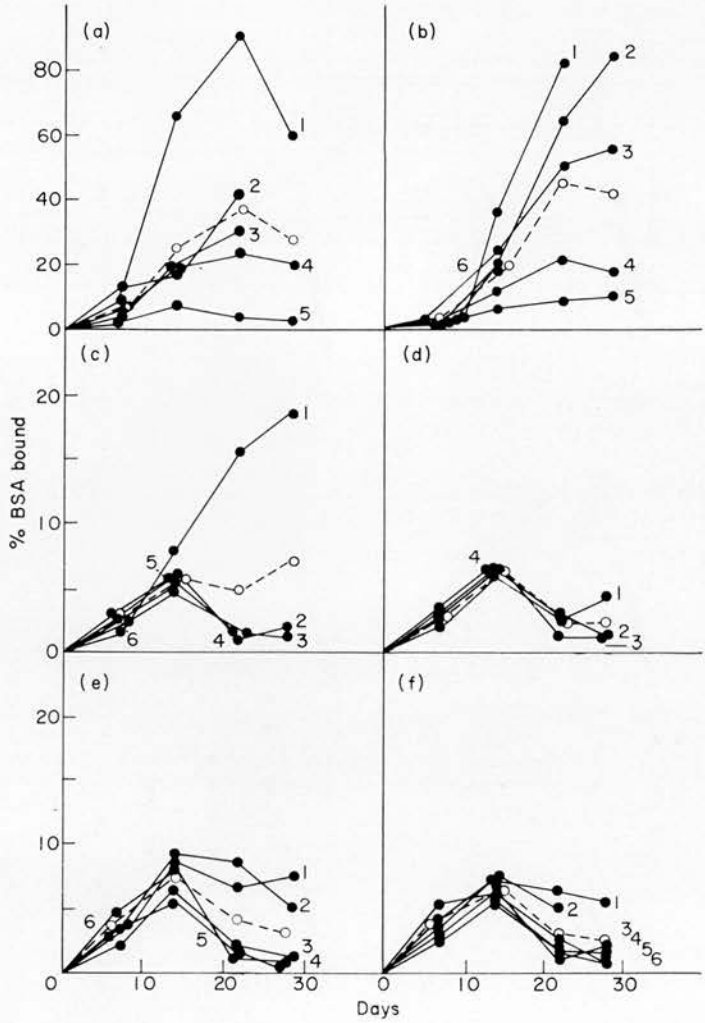

FIG. 1. The effect of various courses of anti-lymphocytic IgG and/or azathioprine treatment on the primary response of Wistar rats to alum precipitated bovine serum albumin. Course of treatment: (a) no treatment, (b) azathioprine alone, (c) anti-lymphocytic IgG for 3 days plus azathioprine, (d), (e) and (f) anti-lymphocytic IgG for 3, 6 and 12 days, respectively. For further details see Table 1. The broken line indicates the mean value.

solution of one of the IgG preparations daily for 7 days (commencing day 0) followed by 0·5 ml daily for a further 15 days. The animals were bled by cardiac puncture on days 0, 6, 12, 19, 30 and 37, and the antibodies to horse IgG were estimated by the previously described tanned cell procedure. Four days after the last IgG injection (day 25) the rate of

elimination of ^{131}I-labelled normal horse IgG was assessed in the surviving animals as outlined above. As will be observed from Fig. 3 the anti-lymphocytic IgG did not exert an antidotal effect for animals treated with this material developed high titres of circulating antibody against normal horse IgG. Indeed there was some suggestion that anti-lymphocytic

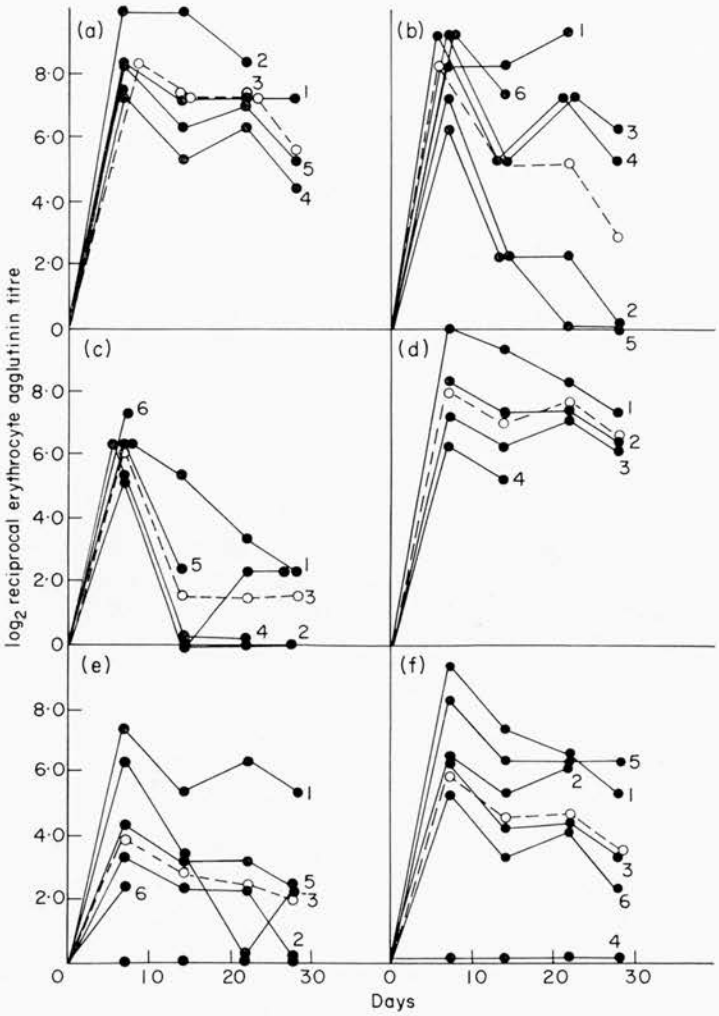

FIG. 2. The effect of various courses of anti-lymphocytic IgG and/or azathioprine treatment on the primary response of Wistar rats to sheep erythrocytes. Courses of treatment: (a) no treatment, (b) azathioprine alone, (c) anti-lymphocytic IgG for 3 days plus azathioprine, (d), (e) and (f) anti-lymphocytic IgG for 3, 6 and 12 days, respectively. For further details see Table 1. The broken line indicates the mean value.

IgG was more immunogenic than normal horse IgG, an observation previously noted by other investigators (Currey & Ziff, 1966; Lance & Dresser, 1967; Jasin *et al.*, 1968). However, a statistical appraisal of the limited data available indicated that anti-horse IgG titres

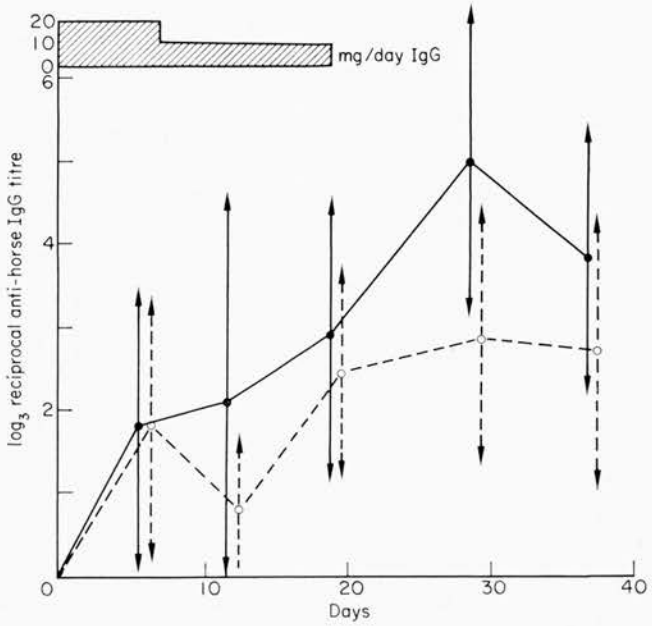

FIG. 3. The measurement of antibodies to horse IgG in animals receiving prolonged courses of treatment with anti-lymphocytic (●) or normal (○) horse IgG. The amount of globulin injected per day is indicated at the top of the figure. The mean agglutinating titres are expressed together with their standard deviations.

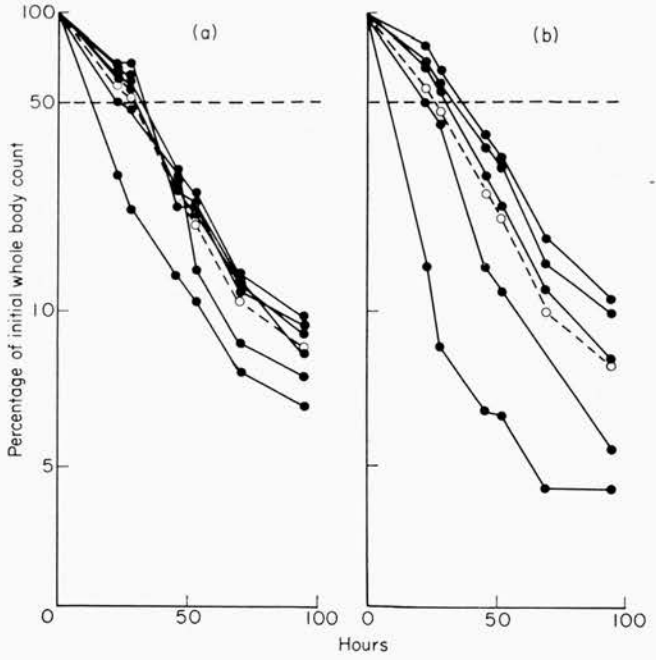

FIG. 4. The elimination of ^{131}I-labelled normal horse IgG by animals receiving prolonged courses of treatment with anti-lymphocytic (a) or normal horse IgG (b). For further details see Fig. 3 and text. The broken line indicates the mean value. (a) $T_{\frac{1}{2}}$ = 27·3 hr, (b) $T_{\frac{1}{2}}$ = 26·7 hr.

were only significantly elevated over the normal horse IgG controls on days 12 and 30, and even then they were just significant at the 10% level.

The immune elimination data supported the above observations that anti-lymphocytic IgG does not suppress humoral antibody formation against itself (see Fig. 4) the mean half life of the injected antigen being 27·3 and 26·7 hr in the normal horse and anti-lymphocytic IgG treated groups, respectively. This value was higher than that previously observed in similar experiments performed in this department (Clark *et al.*, 1967) and could have been due in part to the use of ultracentrifuged labelled globulin.

DISCUSSION

Although only small numbers of animals were used in these investigations the results indicate that the anti-lymphocytic IgG exerted a greater effect on the primary response of these random bred Wistar rats to alum precipitated bovine serum albumin, than was previously observed in other Wistar strain rats (Woodruff *et al.*, 1967). This could possibly be explained in part by antigenic competition, for even in the control group (1A) the mean response was considerably lower (approximately half) than originally detected in animals receiving bovine serum albumin alone. Alternatively it could be due in part to small genetic differences between the random bred and line bred Wistar rats used in these investigations. A statistical comparison within groups receiving anti-lymphocytic IgG alone (groups D–F) indicated that immunosuppressive effect in these experiments was not significantly increased by prolonged anti-lymphocytic globulin treatment.

As in previous experiments in line bred Wistar rats, a short course of pre-treatment with anti-lymphocytic IgG failed to suppress heteroagglutinin formation (James & Anderson, 1967). Nevertheless, doses of azathioprine, which in themselves were ineffective, were able to potentiate the humoral inhibitory capacity of short courses of anti-lymphocytic antibody. A similar potentiation of the immunosuppressive effect of anti-lymphocytic antibody by azathioprine and other immunosuppressive drugs has previously been observed by other investigators in cell mediated immune systems (Levey & Medawar, 1966; Hoehn & Simmons, 1967; Weil & Simmons, 1968).

Whilst the ability of the 6-day course of anti-lymphocytic antibody treatment to suppress erythrocyte agglutinin formation is probably explainable on the basis of a more complete inactivation of antigen reactive small lymphocytes (Martin & Miller, 1967; Denman, Denman & Embling, 1968) the failure of more prolonged treatment to exert a significant effect is more difficult to understand. The observation that the peripheral lymphocyte count may show recovery during or shortly after prolonged courses of anti-lymphocytic antibody treatment may be a possible explanation of the observed effect (Woodruff & Anderson, 1964; Nagaya & Sieker, 1965; Clunie *et al.*, 1968). In this connection Denman *et al.* (1968) have recently demonstrated that the intense proliferation which may follow anti-lymphocytic globulin treatment, usually results in the increased production of short lived small lymphocytes, and these cells are capable of initiating primary immune response (Miller, 1967).

The failure of the anti-lymphocytic IgG to suppress humoral antibody formation against itself is in agreement with previous results from this laboratory and elsewhere (Clark *et al.*, 1967; Currey *et al.*, 1966; Guttman *et al.*, 1967; Lance & Dresser, 1967). Indeed as previously observed there is some suggestion from the tanned cell data (Fig. 3) that anti-lymphocytic IgG may be more immunogenic than its normal IgG counterpart (Currey

et al., 1966; Guttman *et al.*, 1967; Lance & Dresser, 1967). It is theoretically possible that the combination of anti-lymphocytic antibody with lymphocytes or other cells could greatly enhance its antigenicity, for such cells will presumably be aggregated, lysed, or opsonized and so prone to phagocytosis by macrophages thus sensitizing the recipient to the bound protein (Lance & Dresser, 1967; James, 1968a; Jasin *et al.*, 1968). These observations are of course in contrast to those previously observed (Monaco *et al.*, 1966; Iwasaki *et al.*, 1967). However Russell & Monaco (1967) have suggested that the antidotal or immunogenic effect of anti-lymphocytic antibody is dose dependent. In animals receiving large amounts of antibody, all the cells which could produce humoral antibody may be inactivated or destroyed, but with smaller amounts the heterologous protein may persist in the recipient beyond the period of immunosuppression and so antibody formation can occur.

Nevertheless the formation of circulating antibodies against heterologous anti-lymphocytic globulin still constitutes a serious therapeutic problem (Kashiwagi *et al.*, 1968; James, 1968a) and a number of methods of reducing this hazard have been proposed. These include inducing tolerance in the recipient to heterologous antigen and this also appears to have the added advantage of increasing the lymphopenic and immunosuppressive properties of the antibody (Lance, 1968; Denman & Frenkel, 1968) presumably by circumventing the rapid immune elimination of the antibody. However the detailed studies of Denman & Frenkel (1968) also revealed that the continuous administration of anti-lymphocytic IgG to tolerant animals resulted in a wasting disease associated with haemolytic anaemia.

It would appear, therefore, that the most practical approach in humans at the moment is to avoid prolonged therapy with anti-lymphocytic globulin and to concentrate on short intensive courses of treatment which are terminated prior to the formation of high levels of circulating antibodies (Monaco, Wood & Russell, 1967; Iwasaki *et al.*, 1967; James, 1968a, b). In addition this problem may be partially alleviated by the simultaneous use of other immunosuppressants which will permit short courses and also inhibit the formation of antibodies against the administered heterologous protein (Hoehn & Simmons, 1968; see also Starzl *et al.*, 1967).

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Some factors influencing the ability of anti-lymphocytic antibody to suppress humoral antibody formation

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SOME FACTORS INFLUENCING THE ABILITY OF ANTI-LYMPHOCYTIC ANTIBODY TO SUPPRESS HUMORAL ANTIBODY FORMATION

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SUMMARY

The pre-treatment of hooded female rats with horse anti-rat lymphocyte IgG suppresses and/or delays their primary humoral antibody response to alum precipitated bovine serum albumin. Treatment with anti-lymphocytic antibody commencing 4 hr after the administration of antigen proved ineffective.

The non-cytotoxic $F(ab')_2$ fragments prepared from horse and rabbit anti-rat lymphocyte IgG both failed to inhibit the primary response to alum precipitated bovine serum albumin when administered prior to antigen.

The significance of these results in relationship to the immunosuppressive properties of anti-lymphocytic antibody is discussed.

INTRODUCTION

Recent reports have indicated that anti-lymphocytic antibody is capable of suppressing humoral antibody formation (Monaco *et al.*, 1966; James & Anderson, 1967; James & Jubb, 1967). As studies on factors influencing the immunosuppressive activity of anti-lymphocytic antibody are essential in order that we might understand its mode of action, the present paper describes experiments designed to determine the effect of two such factors.

Levey & Medawar (1966a, b) have previously shown that anti-lymphocytic antibody suppresses cell mediated immune processes prolonging homograft survival, even when administered several days after grafting. Experiments have, therefore, been performed to determine if anti-lymphocytic antibody was capable of suppressing humoral antibody formation in animals which had received antigenic stimulation shortly before the commencement of anti-lymphocytic antibody treatment.

Furthermore, studies utilizing antibody fragments* prepared from horse anti-rat lympho-

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* The divalent $F(ab')_2$ fragment obtained on pepsin digestion of IgG, and the univalent Fab′ fragment obtained on the reduction and alkylation of this product.

TABLE 1. The effect of anti-lymphocytic antibody and antibody fragments on the primary response of hooded rats to alum precipitated bovine serum albumin

Experiment group	No. of rats	Weight (g) and mean	Treatment*		*In vitro* properties of materials injected		Effect on peripheral lymphocyte count		Effect on the primary response
			Material injected	When injected	Reciprocal titres†		Initial	Final	
					Lymphoagg.	Lymphocytotox.			
A	9	130–195 (161)	Normal horse IgG	Days −3, −2 and −1	<2	<8	4310 (100)	4000 (93)	No suppression
B	7	135–220 (162)	Horse anti-rat lymphocyte IgG	Days −3, −2 and −1	256	256	4450 (100)	2370 (53)	Suppression
C	6	185–200 (189)	Normal horse IgG	+4, +28, +52 hr	<2	<8	3000 (100)	3830 (128)	No suppression
D	6	175–205 (191)	Horse anti-rat lymphocyte IgG	+4, +28, +52 hr	256	256	4090 (100)	2900 (72)	No suppression
E	6	160–175 (171)	$F(ab')_2$ from horse anti-rat lymphocyte IgG	Days −3, −2 and −1	64	<8	4560 (100)	4440 (97)	No suppression
F	4	165–175 (163)	$F(ab')_2$ from rabbit anti-rat lymphocyte IgG	Days −3, −2, and −1	256	<8	4625 (100)	3780 (82)	No suppression

* Each animal was injected intraperitoneally with 2 ml of a 1 g/100 ml solution at the times shown. The times expressed are in relation to the intraperitoneal injection of 5 mg of alum precipitated bovine serum albumin.

† The lymphocytotoxic titres were determined using fresh guinea-pig serum as a source of complement.

cyte IgG, have shown that those materials are incapable of suppressing the primary response of hooded rats to sheep erythrocytes (James & Anderson, 1967) or prolonging homograft survival in these rats (Anderson, James & Woodruff, 1967). In an attempt to confirm and extend these observations the effect of $F(ab')_2$ preparations from both horse and rabbit anti-rat lymphocyte IgG on the response of hooded rats to alum precipitated bovine serum albumin have been studied.

Throughout these experiments humoral antibody formation has been assessed by the sensitive ammonium sulphate procedure of Farr (1958) utilizing ^{131}I-labelled antigen.

MATERIALS AND METHODS

Details on the production and properties of antisera and the isolation of their IgG components have been reported elsewhere (James & Anderson, 1967). The $F(ab')_2$ portions of the antibody molecules (which contain both antibody combining sites but lack the complement binding and skin attaching Fc portion) were obtained by digestion with pepsin at 37°C for 48 hr in 0·1 M acetate buffer, pH 4·0, using 2 mg enzyme for each 100 mg of protein (Nisonoff *et al.*, 1960). The lymphoagglutinating and lymphocytotoxicity of the preparations were assessed as previously described (Abaza & Woodruff, 1966).

Six groups of animals were investigated and details of the treatment and the properties of the material administered are recorded in Table 1. The animals were challenged by an intraperitoneal injection of 5 mg of alum precipitated bovine serum albumin after (Groups A, B, E and F) or prior to (Groups C and D) treatment with the various IgG and IgG fragment preparations. The animals were bled at intervals of 6 days and the sera stored at −20°C until tested. The antigen binding capacity of the sera was measured by the Farr procedure (Farr, 1958) using a ^{131}I-labelled bovine serum albumin preparation containing 0·2 μg nitrogen per test. Further details on the preparation of alum precipitate bovine serum albumin for injection, the iodination of the bovine serum albumin, and the Farr procedure have been described elsewhere (Hunter & Greenwood, 1962; Pinckard, Weir & McBride, 1967).

RESULTS

The effect of the various treatments on the primary response of the hooded female rats is illustrated in Fig. 1(a–f) and summarized in Table 1.

It will be observed that pretreatment of the rats with anti-lymphocytic IgG suppresses and/or delays the primary response to alum precipitated bovine serum albumin in six out of seven female rats (Fig. 1b). On the other hand the administration of this material 4–52 hr after antigenic challenge did not cause immunosuppression (Fig. 1d). Normal horse IgG exerted no significant effect whether given before or after the alum precipitated bovine serum albumin (Fig. 1a and c). It should, however, be noted that one rat in each of Groups C and D failed to respond.

Furthermore, as in the experiments reported with sheep erythrocytes and skin homografts, the $F(ab')_2$ preparations from anti-rat lymphocyte IgG (which do not fix complement and lack skin attachment properties) failed to inhibit the primary response of hooded rats to alum precipitated bovine serum albumin.

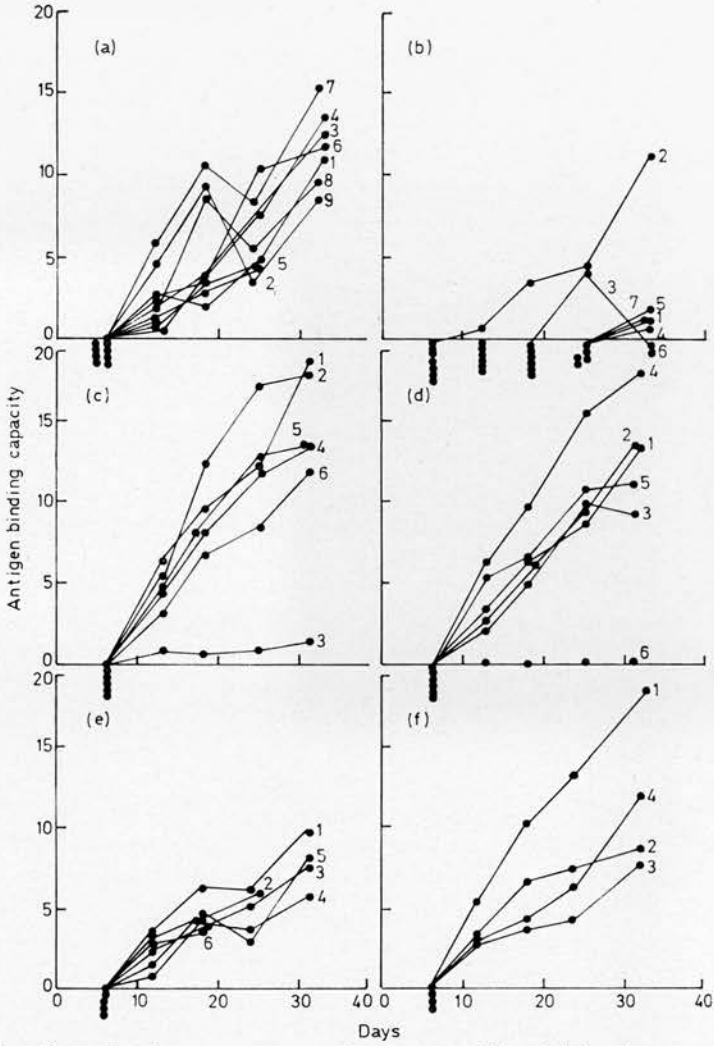

FIG. 1. The effects of various treatments on the response of hooded female rats to alum precipitated bovine serum albumin.

Rats receiving: (a) Normal horse IgG prior to BSA injection; (b) anti-lymphocytic IgG prior to BSA injection; (c) normal horse IgG treatment commencing 4 hr after BSA injection; (d) anti-lymphocytic IgG treatment commencing 4 hr after BSA injection; (e) $F(ab')_2$ from horse anti-lymphocytic IgG prior to BSA injection; and (f) $F(ab')_2$ from rabbit anti-lymphocytic IgG prior to BSA injection. For further details see Table 1 and text.

DISCUSSION

The inability of anti-lymphocytic antibody to suppress humoral antibody formation in animals which had been injected shortly beforehand with alum precipitated bovine serum albumin would suggest that one of the major effects of this material may be on the sensitizing

('triggering') phase of the immune response. Such a hypothesis would be in agreement with previous results on the effect of anti-lymphocytic antibody on the secondary response in sensitized animals (James & Jubb, 1967). The observations of Levey & Medawar (1966a, b) that this material prolongs homograft survival when administered 3 days after grafting could be due in part to delayed antigenic stimulation (sensitization) in this situation.

The failure of the non-complement binding (and hence non-cytotoxic) $F(ab')_2$ preparations to inhibit the primary response is in agreement with previous observations on this material (James & Anderson, 1967; Anderson *et al.*, 1967). These results in conjunction with those obtained in transformation studies with human lymphocytes (assuming that one can extrapolate from rats to humans and vice versa) suggest that the immunosuppressive properties of anti-lymphocytic antibody might be more readily explained on a basis of its cytotoxicity rather than on its ability to cause 'sterile transformation' of lymphocytes (Levey & Medawar, 1966b). Although the anti-rat $F(ab')_2$ preparation failed to inhibit both cellular and humoral aspects of the immune response, similar preparations from horse anti-human lymphocyte serum caused marked transformation of human lymphocytes (Woodruff, Reid & James, 1967). Suppression resulting from cytotoxicity need not involve the gross depletion of the lymphocyte population but may involve the destruction or inactivation of a sub-population of 'immunologically important' cells (Monaco *et al.*, 1966; Levey & Medawar, 1966a; James & Anderson, 1967; James & Jubb, 1967; Anderson *et al.*, 1967).

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Strain Variations in the Primary Humoral Response of Rats and its Susceptibility to Inhibition by Anti-lymphocytic Antibody

BECAUSE strain dependent differences in the primary humoral response of mice have been reported[1-6], we have examined the primary humoral response of a number of inbred and random bred strains of rats and also determined the effect of anti-lymphocytic antibody on this process. The results of our preliminary studies indicate that, in the various groups of rats studied, marked variations occurred both in the primary immune response and in its susceptibility to anti-lymphocytic antibody inhibition.

Table 1 records details of the age, weight, sex and source of the rats used. The anti-lymphocytic serum was produced using thoracic duct lymphocytes obtained from random bred Wistar rats (group 5, Table 1). The immunization schedule used, and the preparation and properties of the normal horse and anti-rat lymphocyte IgG, were similar to those previously described[7,8].

The animals (5–6 per group) received daily intraperitoneal injections of normal horse or anti-rat lymphocyte IgG on days −3, −2 and −1 (groups 1*A* to 9*A* and 1*B* to 9*B* respectively). Each injection contained 1 ml. of 2 g per cent (w/v) protein per 300 g body weight. On day 0 the animals were injected intraperitoneally with 5 mg of alum precipitated bovine serum albumin and were subsequently bled by cardiac puncture on days 7, 14, 21 and 28.

The μg of bovine serum albumin nitrogen bound by 1 ml. of sera obtained from the various bleeds (the antigen binding capacities) was determined by the ammonium sulphate procedure of Farr using ^{131}I-labelled antigen[8-11]. These values were estimated in a system containing 0·2 μg of bovine serum albumin nitrogen per test, the end point being the dilution of serum which bound 33 per cent of the test antigen. Peak values were observed 21 days after antigenic challenge (Table 1). The significance of the difference between the means has been determined using Student's *t* test and the Bessel correction for small numbers.

Table 1 shows that the various groups of rats exhibited widely differing responses to bovine serum albumin. The mean humoral response in groups 1*A* and 2*A* hooded strain rats (which were isogeneic) were on average two to three times greater than the mean values observed in other hooded rats (groups 3*A* and 4*A*) and up to six times those observed in random bred Wistar strain rats (groups 5*A* and 5*B*). Furthermore, the peak primary response in the Sprague–

Dawley rats (groups 7 to 9*B*) was much less than that observed in groups 1*A* and 2*A* hooded rats. Considerable variation occurred, however, within the various groups.

In addition to these differences there were also marked variations in the immunosuppressive effect of anti-lymphocytic antibody. Significant suppression of the primary immune response was achieved in all the hooded strain rats and in one of the Wistar groups (groups 1*B* to 5*B*) (refs. 8 and 10). By contrast the anti-lymphocytic antibody was ineffective in Sprague–Dawley rats obtained from a number of sources (groups 7*B* to 9*B*). Thus anti-lymphocytic antibody may readily suppress the primary immune response to alum precipitated bovine serum albumin in hyper-responsive rats (groups 1 and 2) but is relatively ineffective in rats exhibiting a less marked response to this antigen (groups 6 to 9).

There are several possible, though untested, explanations of the observed differences. The hyper-reactivity of rats in groups 1 and 2 may be the result of the more efficient clearing and processing of the antigen by the reticuloendothelial system or to quantitative or qualitative differences in the antigen reactive small lymphocytes from the thymus or antibody forming precursor cells[12,13]. Alternatively it may be the result of non-specific stimulation of lymphoid tissue such as that achieved with *Corynebacterium parvum*[14] or that which has been suggested to account for the hyper-responsiveness of NZB mice[6]. It is interesting that the total white cell and lymphocyte content of the peripheral blood of the hyper-reactive rats was much lower than that in the less responsive Wistar and Sprague–Dawley animals (see Table 1). A further examination of the lymphoid organs, and a determination of the thymus derived long lived small lymphocyte content of these animals, may help to explain the observed differences.

Strain specific differences in the effect of anti-lymphocytic antibody on the primary humoral response of rats have previously been noted[7,8,15] and similar differences have also been observed in the cell mediated allograft rejection process in the mouse[16]. In the latter studies these differences were attributed to the limited cross-reactivity of antisera produced against the lymphoid tissue of one strain of mice. We believe, however, that this explanation does not satisfactorily account for our present observations, for the antiserum, which was produced against Wistar rat lymphocytes, was usually more effective in hooded strain rats. Furthermore, on the basis of peripheral blood lymphocyte counts it appeared that the antisera were capable of effectively destroying (cross-reacting with) the peripheral blood lymphocytes of all the animals. It is possible, however, that the differences observed could result from differences in the accessibility of antigen reactive cells[17,18] and in the susceptibility of these cells in particular to anti-lymphocytic globulin inactivation[19–21].

Our experiments are preliminary and further experi-

Table 1. PRIMARY RESPONSE TO ALUM PRECIPITATED BOVINE SERUM ALBUMIN IN RATS PRE-TREATED WITH NORMAL AND ANTI-LYMPHOCYTIC IgG

GP	Strain	Breeding	Av. weight (g)	Source	Pre-treatment	WBC (per mm³) Day −3	WBC (per mm³) Day 0	Lymphocytes (per mm³) Day −3	Lymphocytes (per mm³) Day 0	Antigen binding capacity mean ± S.D.*	Significance of difference between means
1A	Hooded	Inbred	297	Small Animal	Normal IgG	5,020	6,470	3,700 ·	4,600	13·0 ± 10·4	$N = 9$ $t = 2{\cdot}18$
1B			312	Breeding Station, Univ. of Edinburgh	ALS IgG	4,930	3,170	3,400	2,000	2·2 ± 3·1	$P > 0{\cdot}05$ $< 0{\cdot}10$ Just significant at 10 per cent level
2A	Hooded	Inbred	202	Chester Beatty	Normal IgG	4,650	6,440	3,000	4,100	19·4 ± 4·7	$N = 7$ $t = 6{\cdot}27$
2B			354	Research Institute, London, England	ALS IgG	4,760	4,380	2,900	2,400	2·4 ± 0·9	$P < 0{\cdot}001$ Highly significant
3A	Hooded	Inbred	180	Agric. Research	Normal IgG	6,300	6,080	4,700	4,500	9·3 ± 4·5	$N = 8$ $t = 3{\cdot}34$
3B			187	Council, Compton, Nr. Newbury, England	ALS IgG	4,340	3,650	3,000	2,000	1·2 ± 1·7	$P \simeq 0{\cdot}01$ Significant
4A	Hooded "Lister"	Random bred	274	Animal Suppliers (London), Ltd, England	Normal IgG	10,480	8,510	8,500	6,900	6·9 ± 4·8	$N = 9$ $t = 2{\cdot}184$
4B			270		ALS IgG	10,900	6,950	7,700	4,300	1·5(4) ± 1·5(4)	$P > 0{\cdot}05$ $< 0{\cdot}10$ Just significant at 10 per cent level
5A	Wistar	Random bred	361	Small Animal Breeding Station, Univ. of Edinburgh	Normal IgG	13,180	14,660	11,600	11,300	2·5 ± 1·8	$N = 10$ $t = 3{\cdot}103$
5B			365		ALS IgG	11,130	8,180	9,100	5,100	No responders†	$P < 0{\cdot}02$ $> 0{\cdot}01$ Significant
6A	Wistar	Random bred	325	Animal Suppliers (London), Ltd, England	Normal IgG	8,880	11,860	7,500	9,500	4·0 ± 2·9	$N = 7$ $t = 1{\cdot}48$
6B			322		ALS IgG	9,430	6,070	7,900	4,300	1·6 ± 1·1	$P \simeq 0{\cdot}20$ Insignificant
7A	Sprague–Dawley	Random bred	427	Small Animal Breeding Station, Univ. of Edinburgh	Normal IgG	12,680	10,240	9,100	7,600	4·2 ± 2·9	$N = 8$ $t = 0{\cdot}3292$
7B			404		ALS IgG	12,630	11,580	8,200	6,000	3·9 ± 6·4	$P < 0{\cdot}80$ $> 0{\cdot}70$ Highly insignificant
8A	Sprague–Dawley	Random bred	411	Animal Suppliers (London), Ltd, England	Normal IgG	10,640	11,030	8,000	8,300	6·7 ± 6·6	$N = 9$ $t = 0{\cdot}4033$
8B			407		ALS IgG	11,010	8,120	8,600	5,400	5·2 ± 4·9	$P < 0{\cdot}70$ $> 0{\cdot}60$ Highly insignificant
9A	Sprague–Dawley	Random bred	435	Carworth Europe, Alconbury, England	Normal IgG	9,250	10,480	7,800	8,400	8·2 ± 6·5	$N = 7$ $t = 0{\cdot}0763$
9B			402		ALS IgG	7,980	6,210	6,400	4,400	6·8 ± 5·5	$P < 0{\cdot}95$ $> 0{\cdot}90$ Highly insignificant

All the animals used were 3 to 7 months old male rats with the exception of those in groups 2A, 3A and 3B which were female.

* The large standard deviations observed in some of the groups were due to the inclusion of the occasional rat which failed to respond to antigen challenge.

† All the animals in this group failed to bind 33 per cent of the test antigen and so ABC values could not be determined in the conditions of assay.

ments will have to be performed to determine if the variations in the primary response and susceptibility to anti-lymphocytic antibody are antigen and adjuvant dependent[5] and if they specifically affect certain immunoglobulin classes and sub-classes[5,6]. Furthermore, the effect of antigenic competition resulting from pre-treatment with normal and anti-lymphocytic IgG will have to be considered. Nevertheless these studies indicate the difficulties which can arise when attempting to compare the ability of reagents to suppress the primary humoral response in rats from different sources.

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In vivo and *in vitro* Effect of Anti-lymphocytic IgG on Sensitized Spleen Cells

IT has been shown that treatment of a number of animals with species specific anti-lymphocytic antibody suppresses their primary immune response to a variety of antigens[1]. Cell transfer experiments have also shown that "non-sensitized" lymphoid tissue from mice treated with anti-lymphocytic antibody can exhibit a reduced immunological potential when transferred to suitably prepared recipients[2–5]. Furthermore, suppression of suspensions of sensitized mouse and rabbit lymphoid cells can also be achieved by incubating them *in vitro* with anti-lymphocytic antibody before transferring them to test recipients[6,7]. In order to confirm and extend these previous cell transfer results, preliminary experiments have been undertaken to determine the effect of anti-rat lymphocyte antibody treatment (*in vivo* and *in vitro*) on the humoral antibody producing potential of sensitized rat spleen cells on subsequent transfer to irradiated recipients.

The suspensions of sensitized spleen cells were prepared from female rats of a hooded strain which had received two intraperitoneal injections of 5 mg of alum precipitated bovine serum albumin (alum BSA), 6 weeks and 2 weeks before the spleen was removed. The preparation and properties of the horse anti-rat lymphocyte IgG and normal horse IgG have been reported elsewhere[8–10].

The *in vitro* treatment of suspensions of the sensitized spleen cells consisted of incubating them for 1 h at 37° C in sterile 0·15 molar solutions of sodium chloride containing normal horse or anti-lymphocytic IgG (1 mg of protein/ml.). No extraneous complement was added to the mixture[6]. Each incubation mixture (total volume 20 ml.) contained cells from six spleens. The *in vivo* treatment consisted of injecting (intraperitoneally) the sensitized animals daily with 2 ml. of a 1 g per cent solution of the normal or the anti-lymphocytic IgG on the 3 days before the spleen was removed. These cells were incubated in sterile saline alone. All suspensions were washed twice following incubation and were made up to a volume of 7 ml. (equivalent to six spleens).

The recipients of the various suspensions of sensitized spleen cells were 3–6 month old male rats of the hooded strain weighing from 200–390 g. They all received terramycin in their drinking water (1 mg/100 ml.) commencing 2 days before receiving 600 rads of whole body X-irradiation. This dose of radiation had previously been shown to suppress completely the primary response of

Table 1. DETAILS OF THE TRANSFERRED SPLEEN CELL SUSPENSIONS

Experimental group	Treatment of sensitized spleen cells	Number of cells transferred	Percentage viable
A	Normal horse IgG *in vitro*	200×10^6	50
B	Horse anti-rat lymphocyte IgG *in vitro*	150×10^6	40
C	Normal horse IgG *in vivo*	200×10^6	80
D	Horse anti-rat lymphocyte IgG *in vivo*	150×10^6	54

hooded male rats to alum BSA. Twenty-four hours after irradiation the rats received an intraperitoneal injection of 1 ml. of one of the suspensions of spleen cells (see Table 1). A further 24 h after cell transfer the animals were injected intraperitoneally with 5 mg of alum BSA. The animals were bled 7, 14, 25 and 33 days after injection of antigen and the serum antibodies were determined by the Farr procedure[11] using an iodine-131 labelled BSA preparation containing 0·2 μg of nitrogen in each test. Further details on the preparation of labelled antigen[12] and the Farr technique are recorded elsewhere[13].

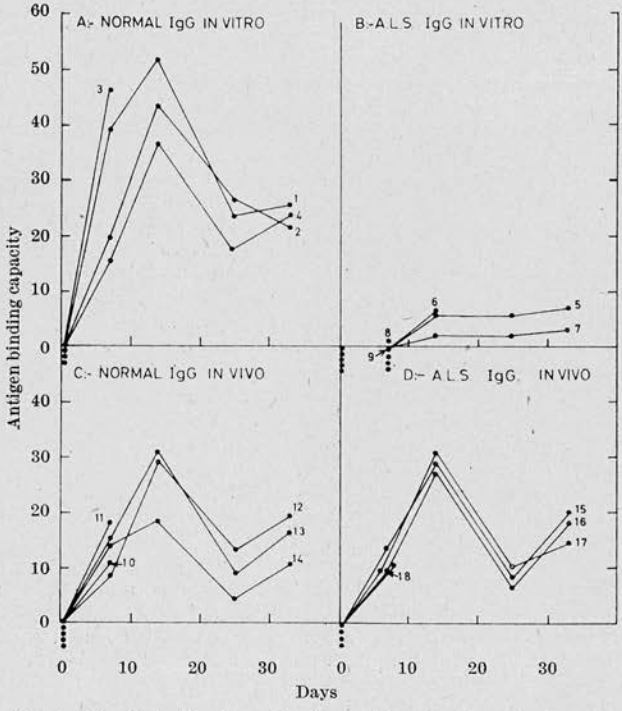

Fig. 1. The effect of *in vivo* and *in vitro* anti-lymphocytic antibody treatment of sensitized spleen cells on their subsequent response to sensitizing antigen (BSA) following transfer to irradiated recipients. For further details of the treatment of the sensitized spleen cells see the text. Note that several animals died during the course of the experiment.

It is apparent from Fig. 1*B* that the treatment of sensitized spleen cells *in vitro* with anti-lymphocytic IgG markedly inhibits their subsequent response to alum BSA following transfer to X-irradiated recipients. In contrast, the animals (Fig. 1*A*, *C* and *D*) which received the other suspensions of spleen cells showed a characteristic secondary response to the bovine serum albumin[9].

The suppression of the humoral antibody response of sensitized lymphoid cells by *in vitro* treatment with anti-lymphocytic antibody confirms previous results in other species[6,7]. These observations suggest that this form of treatment could be capable of erasing immunological memory, presumably as a result of direct interaction between memory cells and antibody. It is appreciated, however, that alternative explanations exist including the rapid destruction of transferred antibody coated cells, their poor "seeding" and reduced proliferation. Furthermore, one also has to take into consideration the fact that the animals in group *B* received fewer viable cells than those in group *A* although a comparison of the results between groups *C* and *D* would suggest that this was not a principal factor.

The *in vivo* results support previous observations in rats[9,10] where a similar dose of anti-lymphocytic antibody did not have a marked effect on the secondary response of rats to alum BSA (ref. 9) and also failed to suppress the primary response if given a short while after the antigen[10]. It has been suggested that *in vivo* the anti-lymphocytic IgG has limited access to central lymphoid tissue such as the spleen and lymph nodes[7] and that its effect is primarily on peripheral lymphocytes[5]. Thus the antibody is unable to inactivate memory cells or prevent plasma cell proliferation in these sites. In contrast, however, it should be emphasized that studies on skin allograft rejection[5] and the graft versus host phenomenon[2–4] indicate that *in vivo* treatment with antilymphocytic antibody can significantly modify the subsequent immunological response of non-sensitized lymphoid tissue.

Further experiments are now under way to confirm these preliminary observations using larger numbers of animals and transferring identical numbers of viable cells.

I thank Dr N. F. Anderson for irradiating the rats, Mrs V. James, Miss E. Nelson and Mr A. Wood for technical assistance, and Mr C. Shepley for preparing the diagram. I also thank Professor M. F. A. Woodruff and Dr R. N. Pinckard for their advice and interest in this work.

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The effect of anti-lymphocytic IgG on unsensitized and sensitized spleen cells *in vivo* and *in vitro*

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THE EFFECT OF ANTI-LYMPHOCYTIC IgG ON UNSENSITIZED AND SENSITIZED SPLEEN CELLS *IN VIVO* AND *IN VITRO*

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(*Received* 27 *June* 1968)

SUMMARY

Rat spleen cells sensitized to bovine serum albumin and treated *in vitro* with anti-lymphocytic IgG are unable to transfer secondary immunological (humoral) responsiveness to irradiated isogeneic rats. Similar *in vitro* treatment of unsensitized spleen cells has a significant, though less marked effect, upon their ability to confer primary humoral responsiveness to irradiated recipients.

INTRODUCTION

The ability of anti-lymphocytic antibody to suppress both cellular and humoral immune responses in animals treated with this material is well documented (see reviews James, 1967a, 1968a). Cell transfer experiments have also shown that lymphoid cell suspensions prepared from animals treated with anti-lymphocytic antibody, or lymphoid cells which have been treated *in vitro* with this material, exhibit reduced immunological activity when transferred to suitable recipients. For example, such treated cells are less capable of eliciting graft-*versus*-host reactions (Boak, Fox & Wilson, 1967; Monaco *et al.*, 1967; van Bekkum *et al.*, 1967; Brent, Courtenay & Gowland, 1967; Naysmith & James, 1968) rejecting skin allografts (Levey & Medawar, 1967) or producing the normal or sensitized lymphocyte transfer phenomena (Levey & Medawar, 1966, 1967). Furthermore recent studies in mice have shown that anti-lymphocytic antibody treatment of isogeneic bone marrow cells destroys their haemopoietic stem cell function (Demeester, Anderson & Shaffer, 1968).

In addition to modifying cell mediated immune processes, cell transfer experiments have also revealed that the treatment of sensitized lymphoid cells *in vitro* with anti-lymphocytic serum suppresses their ability to transfer humoral responsiveness to irradiated recipients. This effect was originally observed by Harris & Harris (1966) who found that the ability of popliteal lymph node cells from rabbits sensitized to horse spleen ferritin to confer immunological responsiveness to irradiated rabbits was greatly reduced if the cells had been treated *in vitro* with anti-lymphocytic antibody prior to transfer. Similar *in vitro*

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treatment of spleen cells from adult NZB mice with stong positive antiglobulin reactions also suppressed the development of Coombs positivity in 1-month-old mice receiving these cells (Denman, Denman & Holborow, 1967). Finally preliminary investigations in rats in our own department have indicated that comparable *in vitro* treatment markedly reduced the ability of spleen cells obtained from animals sensitized to bovine serum albumin to transfer secondary humoral responsiveness to irradiated recipients (James, 1968b). The present series of experiments were performed to confirm our previous preliminary observations and to determine the effect of anti-lymphocytic antibody treatment (*in vivo* or *in vitro*) on the humoral antibody producing potential of transferred non-sensitized spleen cells.

MATERIALS AND METHODS

Antisera production

The anti-lymphocytic serum was produced in a horse by the intravenous injection of thoracic duct lymphocytes obtained by cannulation of hooded strain male rats. The horse was injected on days 0, 7, 14 and 28 with between 1·07 and $1{\cdot}3 \times 10^9$ lymphocytes (95% viable) and was bled out on day 38.

Preparation of normal and anti-lymphocytic globulin

Immunoglobulin G preparations were obtained from heat inactivated (56°C for 30 min) normal horse and horse anti-rat lymphocyte sera by the following procedure. The initial step involved the slow addition of 1 volume of 28% (w/v) sodium sulphate to 1 volume of serum. The precipitate was harvested, re-dissolved in physiological saline and re-precipitated as above. This precipitate was then dissolved in and dialysed against 0·02 M-phosphate buffer, pH 6·5, and then further purified by diethylaminoethyl cellulose batch chromatography on a Whatman DE11 cellulose exchanger with an exchange capacity of 1·0 mEq/g. The final product was concentrated by lyophilization, reconstituted in phosphate buffered saline (pH 7·2, 0·06 M-phosphate containing 0·15 M-sodium chloride) and sterilized by filtration through a 0·22 mμ Millipore filter.

Antigen

Crystalline bovine serum albumin supplied by Armour Pharmaceutical Company (list No. 3200) was used throughout these studies. This preparation was reported to contain less than 0·01% contaminating globulin.

Spleen cell donors

The sensitized spleen cells were obtained from 3–6-month-old female rats of an inbred hooded strain which had received intraperitoneal injections of 5 mg of alum precipitated bovine serum albumin, 6 weeks and 2 weeks prior to spleen removal. The normal spleen cells were obtained from 3–6-month-old untreated female rats of the hooded strain. Spleen cell suspensions were obtained by disrupting these organs in cold sterile physiological saline in a ground glass homogenizer followed by filtration through fine mesh stainless steel sieves and washing ($\times 3$) with cold sterile saline.

In vitro treatment of spleen cells

Spleen cell suspensions were incubated for 1 hr at 37°C in sterile physiological saline containing normal horse or anti-lymphocytic IgG (4 mg protein/ml). Each incubation mixture (total volume 20 ml) contained cells from eight spleens, and no extraneous complement was added.

In vivo treatment of spleen cells

Spleen cell donors were injected intraperitoneally with 1 ml of a 2 g/100 ml solution of the appropriate IgG preparation on the 3 consecutive days immediately prior to spleen removal. These cells were incubated in sterile saline alone and all the suspensions were washed three times in this solution prior to transfer.

The viability of the various spleen cell preparations was assessed by their ability to exclude 0·05% Trypan blue and varied between 54 and 88%, the lowest values being observed where the cells had been incubated *in vitro* with anti-lymphocytic IgG.

The preparation and challenge of spleen cell recipients

The recipients of the various suspensions of spleen cells were 3–6-month-old male rats of an inbred hooded strain weighing 220–380 g. All these animals received terramycin in their drinking water (1 mg/100 ml) commencing 2 days before receiving 500 rad of whole body X-irradiation. The irradiation was performed with a 250 kVp Westinghouse therapy unit run at 15 mA. Other radiation factors were 230 kV, constant potential and added filtration of 0·5 mm Cu and 1 mm Al giving a half value layer of 1·2 mm Cu. The final effective dose rate was 61·09 rad/min at a focal skin distance of 75 mm. This dose of radiation had previously been shown to suppress completely the primary response of hooded strain male rats to alum precipitated bovine serum albumin, the antibody assay procedure (see later) revealing that serum samples obtained up to 21 days after antigenic challenge failed to bind low dilutions of test antigen (0·02 μg protein nitrogen/test). Twenty-four hours later the rats were injected intraperitoneally with 150×10^6 viable spleen cells. A further 24 hr after cell transfer the animals were challenged with an intraperitoneal injection of 5 mg of alum precipitated bovine serum albumin. The animals were bled at weekly intervals by cardiac puncture and the circulating antibodies were estimated as follows.

Assay of humoral antibody formation

The antigen binding capacities of the rat sera were determined by the ammonium sulphate procedure of Farr (1958) using ^{131}I-labelled bovine serum albumin. The bovine serum albumin was labelled by the chloramine T technique of Hunter & Greenwood (1962) and more than 96% of the radioactivity was precipitated with 10% trichloroacetic acid. The end point of the antibody titrations was that serum dilution which bound 33% of the antigen (containing 0·2 μg of protein nitrogen) and the antigen binding capacities calculated from this value were expressed as the μg of bovine serum albumin nitrogen bound by 1 ml of undiluted rat serum. All the estimations were performed in duplicate and normal rat serum and standard rat antisera to bovine serum albumins were employed as controls.

The effect of anti-lymphocytic IgG on unsensitized spleen cells was statistically assessed by means of the Student's *t*-test using the Bessell correction for small numbers. The antigen binding capacities of the sera obtained on day 28 from animals receiving normal spleen

cells treated *in vitro* with normal horse or anti-lymphocytic IgG were compared and a value of $P<0{\cdot}1$ was taken as significant.

RESULTS

The effect of anti-lymphocytic IgG on sensitized spleen cells in vivo and in vitro

The results of the present series of experiments show that the treatment of sensitized spleen cells *in vitro* with anti-lymphocytic IgG has a marked effect upon their immunological

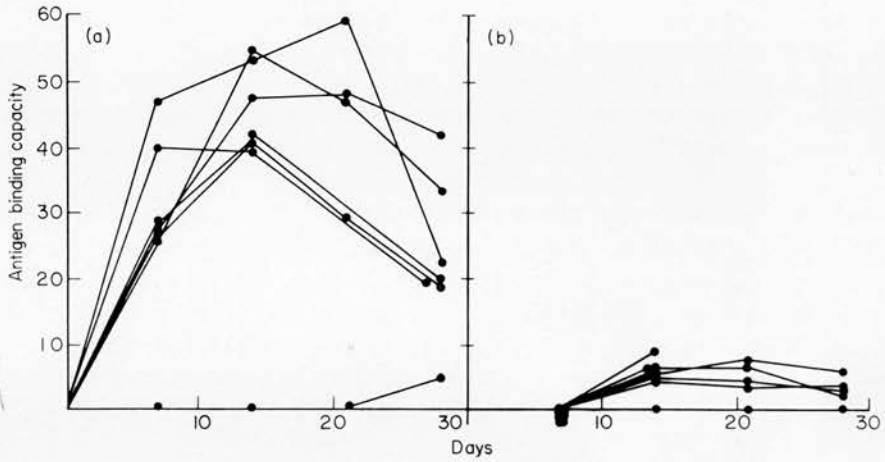

FIG. 1. Cell transfer studies, the effect of anti-lymphocytic IgG on sensitized spleen cells. The figure illustrates the humoral response to alum precipitated bovine serum albumin observed in individual irradiated hooded rats repopulated with isogeneic sensitized spleen cells treated *in vivo* (a) or *in vitro* (b) with anti-lymphocytic IgG. It will be observed that in animals receiving spleen cells treated *in vitro* with anti-lymphocytic IgG the humoral response was greatly reduced. Note also that in many of the early samples the amount of antigen bound was so low that the antigen binding capacity could not be determined with the antigen concentration used. In addition a number of animals died during the investigation period.

activity. Irradiated rats 're-populated' with these treated sensitized cells exhibited a relatively weak humoral response when subsequently challenged with the sensitizing antigen (see Fig. 1b). In contrast a characteristic secondary response was observed following antigenic challenge in irradiated rats which had received sensitized spleen cells from animals treated with a short course of anti-lymphocytic IgG prior to spleen removal (Fig. 1a). The latter response was similar to that previously noted following the transfer of sensitized spleen cells treated *in vivo* or *in vitro* with normal horse IgG (James, 1968b).

The effect of anti-lymphocytic IgG on unsensitized spleen cells in vitro

From Fig. 2 it will be observed that the treatment of unsensitized spleen cells *in vitro* with anti-lymphocytic IgG slightly modifies their subsequent primary humoral response following transfer to irradiated isogeneic recipients. In general there was a delay in the appearance, and a reduction in the quantity of free circulating antibody in animals

receiving these cells (Fig. 2d) when compared to the response observed in animals re-populated with spleen cells treated *in vitro* with normal IgG (Fig. 2b).

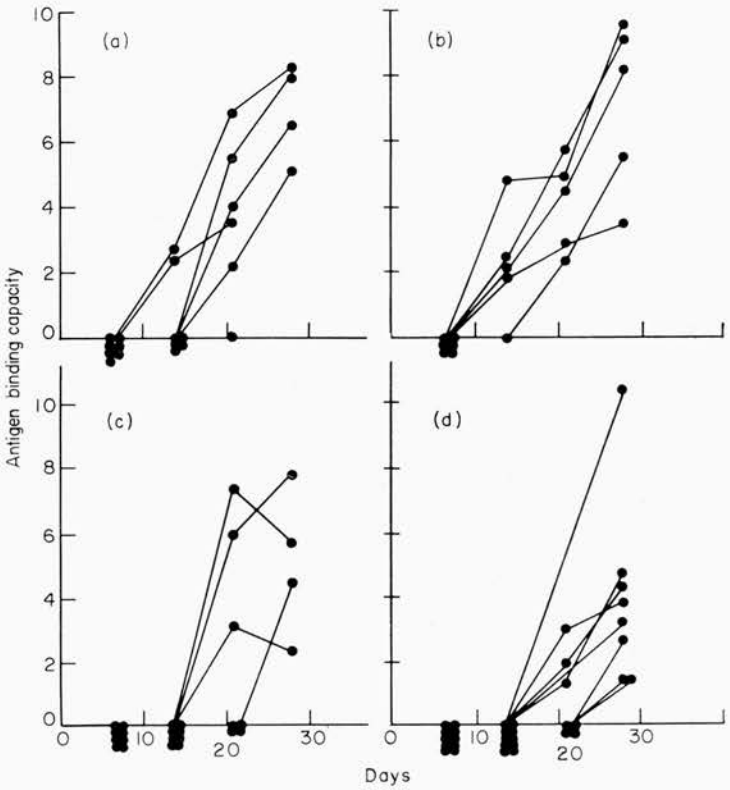

FIG. 2. Cell transfer studies, the effect of normal horse IgG and anti-lymphocytic IgG on unsensitized spleen cells *in vivo* and *in vitro*. The figure illustrates the humoral response to alum precipitated bovine serum albumin in individual irradiated hooded rats re-populated with unsensitized spleen cells treated as follows. (a) Normal IgG *in vivo*, (b) normal IgG *in vitro*, (c) ALS-IgG *in vivo*, and (d) ALS-IgG *in vitro*. It will be observed that the humoral response in (d) was delayed and reduced in comparison to that observed in (b). Note also that in many of the early samples the amount of antigen bound was so low that the antigen binding capacity could not be determined with the antigen concentration used. In addition a number of animals died during the investigation period.

The effect of anti-lymphocytic IgG on sensitized cells in vivo

The *in vivo* treatment of 'unsensitized' spleen cell donors with anti-lymphocytic IgG failed to exert a significant effect upon the immunological activity of the spleen cells following subsequent transfer to X-irradiated recipients. The immune response in animals re-populated with these cells and challenged with alum precipitated bovine serum albumin (Fig. 2c) was similar to that observed in animals receiving spleen cells from donors treated with normal horse IgG (Fig. 2a). In both groups there was a delay in the appearance of free circulating antibodies in a high proportion of the animals.

DISCUSSION

These results clearly confirm the earlier observations from our laboratory and elsewher that sensitized lymphoid cells (isogeneic or allogeneic) treated *in vitro* with small doses c anti-lymphocytic antibody are unable to restore secondary humoral responsiveness t irradiated rats (James, 1968b) or rabbits (Harris & Harris, 1966). Furthermore, the dat suggests that *in vitro* treatment of unsensitized spleen cells may render them less able t confer primary humoral responsiveness. However, in this case the results are less dramatic the peak mean response being just significantly different from that observed in anima receiving normal horse IgG treated spleen cells.

As previously discussed (James, 1968b) these observations suggest that the *in vitr* treatment of sensitized spleen cells with anti-lymphocytic antibody results in the erasur of immunological memory, presumably as a consequence of memory cell destructio However, the observed results can be explained on the basis of less selective mechanism since the recent results of Martin & Miller (1967) have shown that lymphoid cells coate *in vitro* with anti-lymphocytic antibody are 'relatively' ineffective at 're-populating' th lymphoid tissue of irradiated isogeneic recipients. The failure of the anti-lymphocytic ant body treated cells to seed in the lymphoid organs following transfer could have been du to their rapid complement mediated destruction, or as a result of the opsonization an sequestration of intact viable cells. Thus the much reduced immune response in anima receiving anti-lymphocytic IgG treated cells, might have been the direct result of th generalized reduction in effective lymphoid cell repopulation (James, 1968b; Field & Gibb 1968).

The ability of sensitized lymphoid cells from animals treated with short courses of ant lymphocytic IgG, to confer secondary immunological responsiveness upon irradiate recipients, supports previous observations in other rat experiments. It has previously bee shown that doses of anti-lymphocytic antibody similar to those used in these experiment were incapable of exerting a marked effect upon the secondary response of rats to alu precipitated bovine serum albumin (James & Jubb, 1967) and also failed to suppress th primary response if administered shortly after the antigen (James, 1967b). All thes observations can be explained on the basis of the limited accessibility of anti-lymphocyti IgG to central lymphoid tissues such as the spleen and lymph nodes, its effect bein primarily upon the peripheral lymphocytes (Denman *et al.*, 1967; Levey & Medawa 1967). Thus the antibody is relatively ineffective in inactivating memory cells or preventin plasma cell proliferation in these sites, though it is feasible that much higher doses than use in these experiments could prove effective.

In contrast to the above, studies on the ability of transferred lymphoid tissue to promot skin allograft rejection and graft-*versus*-host reactions, indicate that *in vivo* treatment wit small doses of anti-lymphocytic antibody can significantly modify the subsequent cellula response of non-sensitized lymphoid tissue (Boak & Wilson, 1968). It was these observa tions on cellular systems which prompted us to investigate the *in vivo* and *in vitro* effect c anti-lymphocytic antibody on the humoral antibody producing potential of unsensitize spleen cells. Whilst the *in vitro* anti-lymphocytic IgG treatment had a slight, yet significan effect on the subsequent immunological activity of unsensitized spleen cells, the effects *i vivo* were not significantly different from those of the controls (Fig. 2). However, in a these groups there was a delay in the appearance of free circulating antibodies whe

compared to those animals receiving cells treated *in vitro* with normal horse IgG (Fig. 2b) or the response in normal rats (James & Jubb, 1967). This apparent delay in the appearance of free circulating antibodies may have been due to a genuine inactivation of mature antigen reactive lymphoid cells, or their immediate descendants, but was most probably a direct reflection of the small number of cells which seeded, and hence could participate in the immune response. In these circumstances, the low levels of antibody originally produced, could have been bound by the antigen thus stimulating a delayed humoral response.

Although the rate of appearance of free circulating antibody in animals receiving sensitized spleen cells treated *in vitro* with anti-lymphocytic IgG was similar to that observed following primary antigenic stimulation (compare Figs. 1b and 2b), the observed response could be a secondary one effected by a reduced number of active sensitized spleen cells. The decrease in circulating antibody observed 21 days following antigenic challenge is suggestive of a decline in the activity or number of antibody producing cells.

In the present series of experiments the precise interpretation of results has been made difficult by the fact that the spleen cell population is extremely heterogeneous. Hence the percentage of antigen-reactive cells in a standard inoculum might be greatly distorted in anti-lymphocytic IgG treated rats by the proliferation of other cell types, even though the absolute number of lymphocytes remains unchanged. However, studies performed with varying numbers of lymphoid cells obtained from different sources and investigations designed to determine the distribution of anti-lymphocytic antibody and antibody coated cells should further our understanding of the effect of anti-lymphocytic antibody on sensitized and normal lymphoid tissue.

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Elimination of Normal Horse IgG labelled with Iodine-131 in Rats receiving Horse Anti-rat Lymphocytic IgG

RECENT reports have indicated that antilymphocytic IgG, in addition to inhibiting the homograft reaction, is capable of suppressing the formation of humoral antibody against a number of antigens[1–4]. Somewhat conflicting results have appeared, however, on the ability of antilymphocytic antibody to suppress the formation of antibody against itself, or against normal IgG globulin obtained from the species in which the antibody was produced[5–7]. We have investigated this problem by studying the effect of prolonged administration of antilymphocytic IgG (horse anti-rat) on the elimination of normal horse IgG, labelled with iodine-131, from rats.

The techniques of raising antilymphocytic serum and preparing purified IgG derivatives have been described previously[3,8]. The labelling with iodine-131 was performed by the method of Hunter and Greenwood[9] and it was found that more than 95 per cent of the radioactivity of the preparation was precipitable with trichloroacetic acid. The rats were females aged 3–4 months (160–210 g) of an inbred hooded strain maintained in this laboratory[10]. Six animals received daily intraperitoneal injections of antilymphocytic IgG in a dosage which had previously been shown to prolong the survival of skin homografts by a factor of about 2·5 (ref. 8) (1 ml. of 2 g per cent solution daily for 7 days followed by 0·5 ml. daily for 14 days) Six animals received a similar course of normal horse IgG and six others received daily injections of saline. Four days after the last injection of IgG or saline each rat received an intraperitoneal injection of 10 mg normal horse IgG labelled with 10 μc. of iodine-131. Sodium iodide (100 mg/l.) was added to the drinking water of all rats commencing 7 days before the injection of labelled protein and continuing to the end of the experiment.

The fate of the labelled protein was investigated in three ways: by counting serial blood samples; by repeated whole body counting ; and by counting samples of various tissues removed at autopsy. Blood from a tail vein (0·1 ml.) was diluted with heparinized saline (0·9 ml.) and counted in a well-type scintillation spectrometer incorporating a 2 in. sodium iodide crystal. After tissue samples had been weighed and digested in 6 normal potassium hydroxide, they were counted with the same apparatus. Whole body counting was performed in a ring counter consisting of

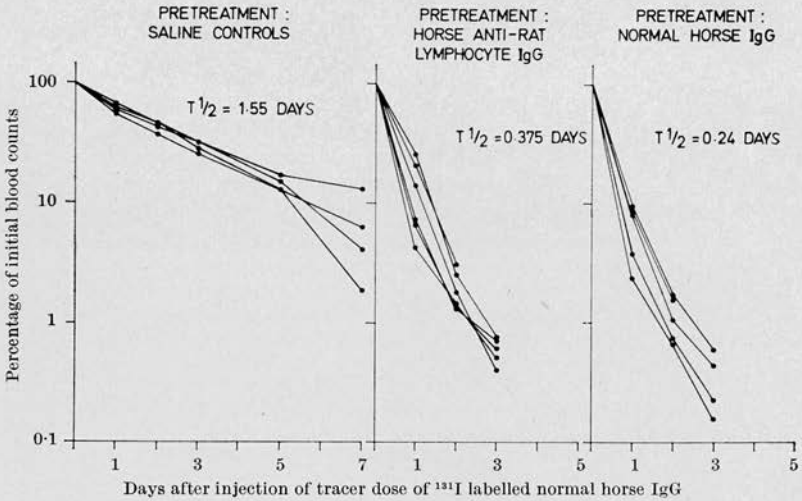

Fig. 1. Clearance of normal horse IgG labelled with iodine-131 from the blood stream of rats pretreated with saline, antilymphocytic IgG and normal horse IgG. Initial counts ranged from 95 to 187/sec. Mean background count 2·5/sec.

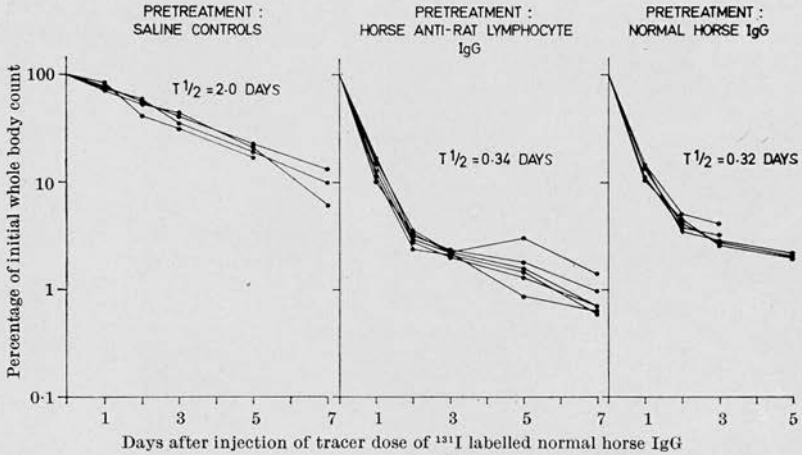

Fig. 2. Elimination of normal horse IgG labelled with iodine-131 as determined by whole body counting in rats pretreated with saline, antilymphocytic IgG and normal horse IgG. Initial counts ranged from 1,572–1,930/sec. Mean background count 15/sec.

twelve J26Pb Geiger–Müller tubes shielded with 1·25 in. lead shot which was kindly provided by Dr P. Tothill of the Department of Medical Physics.

The blood and whole body counts are shown in the accompanying graphs (Figs. 1 and 2). It will be seen that rats pretreated with either antilymphocytic or normal

horse IgG showed greatly accelerated elimination of the radioactive material from the blood stream (mean biological half-life 0·24 day and 0·38 day, respectively) as compared with the rate of elimination in the control animals pretreated with saline (mean biological half-life 1·55 days). Total body counts showed a similar pattern except that after the initial rapid fall some 2–4 per cent of the original activity remained for up to 5 days. Tissue counts showed that this activity resided principally in the liver and kidneys, where it presumably represents either stages in the breakdown of the injected protein or antigen-antibody complexes.

The results suggest that administration of antilymphocytic IgG in this experimental system does not prevent immunization of the treated animals to horse IgG, thus confirming the hypothesis put forward to account for the failure of initial attempts to achieve immunosuppression with antilymphocytic serum (ALS)[11]. It is now apparent that a high degree of general immunosuppression can be achieved despite immune elimination of the ALS (or IgG) itself, but it is pertinent to ask whether still greater immunosuppression would result if the rate of elimination of the material could be slowed down. This might perhaps be achieved by previous induction of specific immunological tolerance of this material as suggested by Lance and Dresser[12], by concurrent administration of chemical immunosuppressants, or by reducing the proportion of non-antilymphocytic IgG in the preparation. These possibilities are being investigated.

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Parallel studies on the effect of anti-lymphocytic antibody on cell mediated and humoral antibody responses in the rat

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PARALLEL STUDIES ON THE EFFECT OF ANTI-LYMPHOCYTIC ANTIBODY ON CELL MEDIATED AND HUMORAL ANTIBODY RESPONSES IN THE RAT

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SUMMARY

The effect of prolonged treatment with anti-lymphocytic IgG raised in horses on cell mediated (homograft rejection) and humoral type immune responses has been investigated simultaneously in the same animal. The rejection of skin homografts precedes the development of circulating antibodies against alum precipitated bovine serum albumin but may follow the formation of agglutinating antibodies against sheep erythrocytes. High levels of antibodies against horse IgG are frequently detected prior to graft rejection.

INTRODUCTION

Recent reports from a number of laboratories have indicated that anti-lymphocytic antibody is capable of suppressing both cellular and humoral aspects of the immune response (James, 1967). However, apart from the work of Jeejeebuoy in thymectomized rats (1965, 1967) few attempts have been made to assess the effect of anti-lymphocytic antibody on both cellular and humoral responses in the same animal. We have, therefore, performed parallel investigations on the effect of antibody to rat lymphocytes on skin homograft survival and at the same time assessed its effect on their humoral response to alum precipitated bovine serum albumin, sheep erythrocytes and to the anti-lymphocytic antibody itself. Previous reports from this laboratory have already described the effect of the anti-rat lymphocyte antibody preparation used on the various antigens under test (Anderson, James & Woodruff, 1967; Clark, James & Woodruff, 1967; James & Anderson, 1967; James & Jubb, 1967).

MATERIALS AND METHODS

Details on the preparation and the properties of the horse anti-rat lymphocyte IgG preparation used in these studies have previously been reported (James & Anderson, 1967).

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These parallel studies on the effect of anti-lymphocytic IgG on both cell mediated and humoral antibody responses were performed in female rats of a hooded strain (weight range 185–255 g). The rats, twelve in number, received daily intraperitoneal injections of the anti-lymphocytic IgG preparation (1 ml of a 2 g/100 ml solution) for seven consecutive days prior to grafting (day -7 to -1). On day 0 skin grafting was performed by the method of Woodruff & Simpson (1955) the donor rats being an inbred Wistar strain. At the time of grafting all the animals were injected intraperitoneally with 5 mg of alum precipitated bovine serum albumin whilst six of the rats (see Fig. 2) also received an intravenous injection of 1×10^9 sheep erythrocytes. The rats continued to receive daily intraperitoneal injections of anti-lymphocytic IgG (0·5 ml of a 2 g/100 ml solution) from days 1 to 14 post grafting. The grafts were examined daily following the removal of the dressing on day 8 and serum samples were obtained at regular intervals for humoral antibody determinations.

The antibody to bovine serum albumin was determined by the Farr procedure (Farr, 1958) using a ^{131}I-labelled bovine serum albumin preparation containing 0·02 μg nitrogen per test. In many of the sera samples, less than 33% of the test antigen was bound and we were unable to estimate the antigen binding capacities. Because of this, the results have been expressed as a percentage of the test antigen bound by 0·5 ml of a 1:10 dilution of the serum. Further details of the procedure and the controls have been reported elsewhere (Pinckard, Weir & McBride, 1967; James & Jubb, 1967).

The production of anti-sheep erythrocyte agglutinins was assessed as previously described from this laboratory (James & Anderson, 1967). The results have been expressed as the reciprocal $\log_{10}$ of the titre.

The antibodies to normal horse IgG were estimated using tanned pyruvic aldehyde preserved sheep erythrocytes (Ling, 1961) sensitized with a 0·1 g/100 ml solution of normal horse IgG. Serial dilutions were performed using the Takatsky microtitrator. Each analysis included normal serum and standard antiserum controls as well as controls using unsensitized sheep erythrocytes. The controls using unsensitized cells indicated that the production of erythrocyte agglutinins in rats receiving sheep erythrocytes was so weak that it did not interfere with this test. The results are again expressed as the reciprocal $\log_{10}$ of the titre.

RESULTS

From Figs. 1 and 2 it can be seen that the anti-lymphocytic IgG treatment delayed the rejection of skin homografts in all but two animals thus confirming previous observations with this material (Anderson *et al.*, 1967). The rapid destruction of grafts observed in animals B and D (Fig. 2) can probably be attributed to a failure in the grafting procedure. It should perhaps be noted that the course of treatment used produced on average only a slight fall (less than 10%) in the peripheral blood lymphocyte count prior to grafting and injecting the test antigens.

As anticipated this prolonged course of anti-lymphocytic antibody treatment delayed and suppressed the primary immune response to bovine serum albumin and sheep erythrocytes. Although all the animals developed antibodies to bovine serum albumin the levels detected at 30–32 days were less than 10% those observed in animals receiving a much shorter course of anti-lymphocytic antibody treatment (James & Jubb, 1967). Of considerable interest is the time of appearance of antibodies to bovine serum albumin in relation to homograft rejection. In general, only low levels of antibody to bovine serum albumin were detected

at the time of complete rejection of the homograft (exception Fig. 1F) and these levels frequently showed a marked increase following the destruction of the graft.

Five out of six of the rats injected with sheep erythrocytes developed low levels of sheep erythrocyte agglutinins. This response was much weaker and later than that previously observed in control animals (James & Anderson, 1967). However, as in previous studies

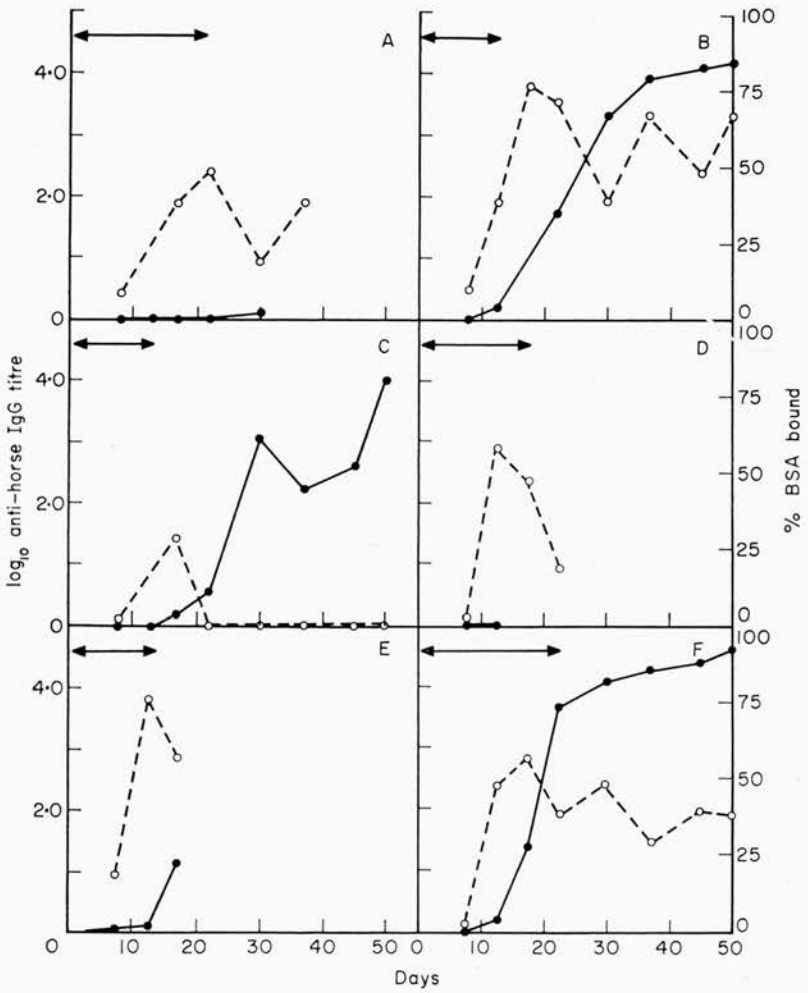

FIG. 1. The effect of anti-lymphocytic IgG on skin homograft survival and humoral antibody formation in hooded rats. Arrows represent graft survival; ●, Humoral antibody formation, antibodies to BSA; ○, humoral antibody formation, antibodies to horse IgG.

maximum erythrocyte agglutinin titres were observed before antibodies to bovine serum albumin could be detected and frequently prior to graft rejection.

All the rats developed antibodies against normal horse IgG. These were first detected about 14 days after commencing anti-lymphocytic IgG treatment and high levels were frequently observed prior to graft rejection. However, there was no apparent correlation

between the titre of antibodies to horse IgG and the time of graft rejection. The peak response usually coincided with the peak response to sheep erythrocytes.

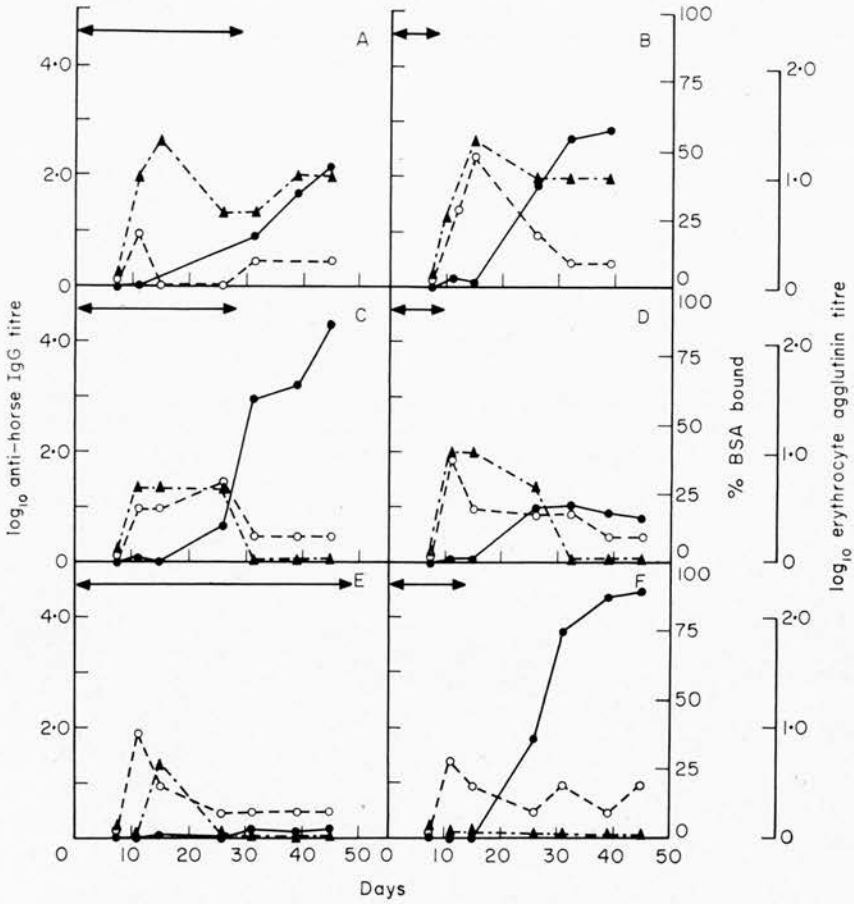

FIG. 2. The effect of anti-lymphocytic IgG on skin homograft survival and humoral antibody formation in hooded rats. Arrows represent graft survival; ●, Humoral antibody formation, antibodies to BSA; ○, humoral antibody formation, antibodies to horse IgG; ▲, humoral antibody formation, agglutinating antibodies to sheep erythrocytes.

DISCUSSION

It is appreciated that in studies such as these the intensity and course of the various immune responses might be influenced strongly by antigenic competition and that the apparent absence of humoral antibody might be a reflection of the insensitivity of the technique of detection or the combination of the antibody *in vivo* with circulating antigen. Nevertheless it is apparent that anti-lymphocytic antibody may readily inhibit both cell mediated and humoral antibody type immune phenomena in one and the same animal. These results do not support the recent suggestion of Levey & Medawar (1967) that

anti-lymphocytic antibody is less able to oppose immunization by mobile antigens which are able to travel directly to the spleen, than to oppose immunization mediated through peripheral mechanisms. The relative effect is, as one might expect, dependent upon the antigen under test.

The results also indicate that even prolonged treatment with anti-lymphocytic antibody does not prevent humoral antibody formation. Thus as in the case of homografts, animals eventually recover their capacity to produce humoral antibodies, presumably due to the subsequent development of immunologically competent cells or to the functional recovery of pre-existing lymphocytes (James & Jubb, 1967). Of interest, however, is the rate at which the animal recovers its capacity to produce antibodies against the various antigens or to reject the homograft. Peak levels of antibodies to sheep erythrocytes (and horse IgG) and the rejection of skin homografts are apparent before the development of significant levels of antibodies to bovine serum albumin. This, however, may only be a reflection of basic differences in the normal immune response to these antigens and not to preferential recovery of specific immunological potential.

The inability of anti-lymphocytic antibody to completely inhibit humoral antibody formation against itself confirms previous observations in rats (Currey & Ziff, 1966; Clark *et al.*, 1967). However, it still remains to be established whether or not this inability directly affects the immunosuppressive efficiency and therapeutic potential of these materials.

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IN VIVO AND *IN VITRO* PROPERTIES OF ANTILYMPHOCYTIC SERUM

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IT has been reported in previous publications from our laboratory (Abaza and Woodruff, 1966; Woodruff, Anderson and Abaza, 1966) and elsewhere (Gray *et al.*, 1966) that antilymphocyte serum which possesses immunosuppressive properties *in vivo* causes *in vitro* agglutination of lymphocytes and lysis in the presence of complement. We are at present engaged in studying and comparing the *in vivo* and *in vitro* properties of various sera, and also of serum fractions and antibody fragments. The present communication is in the nature of a progress report.

Antilymphocytic serum or plasma* (ALS) has been prepared by immunizing rabbits and horses with rat thoracic duct lymphocytes and with cells obtained from human thymus or spleen. Serum fractions and antibody fragments were prepared as described below. These materials, together with comparable material obtained from normal (non-immunized) rabbits and horses, were submitted to some or all of the following tests:

I. Tests of immunosuppressive activity *in vivo*.
   (1) Primary response of rats to sheep erythrocytes.
   (2) Primary response of rats to alum-precipitated bovine serum albumin (BSA).
   (3) Survival of skin homografts in rats.

II. *In vitro* tests.
   (1) Erythrocyte agglutination.
   (2) Lymphocyte agglutination.

* In the interest of brevity *serum* is used in this paper to denote both serum separated in the usual way from clotted blood, and plasma from which the fibrinogen has been removed by heating (56°C for 30 min.) and filtration.

(3) Lymphocyte cytotoxicity (in the presence of complement).
(4) Lymphocyte transformation in culture (stimulation of RNA and DNA synthesis).

A further test based on inhibition of antibody formation *in vitro* is under investigation but as yet we have no results to report.

In addition we have been investigating the capacity of ALS-treated animals to eliminate ^{131}I-labelled IgG of the species in which the ALS was raised.

MATERIALS AND METHODS

Preparation of serum and fractions

Rabbit antirat ALS was prepared by immunizing rabbits by repeated intraperitoneal injection of rat thoracic duct lymphocytes as described by Woodruff and Anderson (1963, 1964).

Horse antirat ALS was prepared by immunizing a horse with four intravenous injections of $1 \cdot 07$–$1 \cdot 03 \times 10^9$ rat thoracic duct lymphocytes and harvesting the serum 11 days after the last injection.

Two types of horse antihuman ALS were studied, both obtained from the same horse. The first was harvested 10 to 24 days after the last of a series of five intravenous injections of $5 \cdot 4$–$16 \cdot 6 \times 10^9$ cells prepared from infant human thymus obtained at autopsy. The proportion of viable cells, as judged by failure to stain with trypan blue, ranged from nil to 90 per cent. The second was harvested eight to ten days after the horse had received two to four additional injections of $8 \cdot 5$–$62 \cdot 2 \times 10^9$ nucleated human spleen cells. The first of these injections was given intravenously, the others were given subcutaneously. The proportion of viable cells ranged from 62 to 84 per cent.

Serum fractions were prepared by gel filtration at 4°C on G200 Sephadex with 0·06M-phosphate buffer, pH 7·2, containing 0·15M-NaCl. Fractions were concentrated to the original serum volume by ultrafiltration. Immunoglobulin G (IgG) was prepared by repeated (twice) sodium sulphate precipitation (using an equal volume of 28 per cent w/v Na_2SO_4) followed by batch chromatography on Whatman DEAE cellulose (DE11) with an exchange capacity of 1·0 m-equiv./g. These preparations were sterilized by Millipore filtration and then stored at −20°C or

yophilized. The purity of the fractions was checked by immuno nd polyacrylamide gel electrophoresis.

The $F(ab')_2$ portion of the IgG molecule was obtained by igestion with pepsin (one part enzyme to 50 parts protein by veight) in 0·1M-acetate buffer, pH 4·0. Digestion was shown to e complete by immunodiffusion and cytotoxic analyses.

The univalent Fab' fragment was obtained by reducing the ivalent $F(ab')_2$ fragment in phosphate buffer (0·06M-phosphate ontaining 0·15M-NaCl, pH 7·2) with 0·1M-cysteine, and then lkylating by dialysis against large volumes of iodoacetamide ·02M) in the same buffer.

gglutination and cytotoxic tests

Agglutination and cytotoxic tests were performed as described y Abaza and Woodruff (1966) except that for tests with human erum lymphocytes were obtained not from thoracic duct lymph ut from heparinized blood, by sedimentation of the erythrocytes vith 3 per cent gelatin and filtration through a column of nylon wool".

ymphocyte transformation tests

Lymphocyte cultures were set up in bijoux bottles and incu- ated at 37°C. Each culture contained approximately 10 million ells in 2 ml. medium 199 plus 0·5 ml. autologous inactivated lasma. To this was added 0·1 or 0·2 ml. of the serum to be ested, except that control cultures were set up either without dded serum or with the addition of inactivated normal whole erum or serum fractions.

The cultures were subdivided into three groups:

Cultures of group 1 were used for morphological studies nly.

Cultures of group 2 were used to study the incorporation of H]uridine (Radiochemical Corporation, Amersham) which was dded to give a concentration of 1·67 μc/ml. when the cultures vere set up. Eighteen hours later the cells were spun down and richloroacetic acid-insoluble material was harvested and counted s described below. Since there is evidence that under the condi- ions described most of the isotope in the trichloroacetic acid- nsoluble material is incorporated in RNA, these cultures consti- ute what will be referred to here as the " RNA synthesis " test.

Cultures of group 3 were used to study the incorporation of [^{3}H]thymidine (Radiochemical Corporation, Amersham). This was added 48 hours after the cultures were set up to give a concentration of 1·67 μc/ml. The cells were spun down after a further 24 hours and washed successively in phosphate-buffered saline (three washes), 5 per cent trichloroacetic acid, phosphate-buffered saline, and absolute methanol. After the final spin the supernatant was discarded and the precipitate was taken up in the minimum possible quantity of hyamine HCl and heated for a few minutes in a water bath at 60°c. After this had been cooled, 10 ml. scintillant (NE 213, Nuclear Enterprises, Edinburgh) were added and counting was performed with a Packard Tricarb Scintillometer. Since there is evidence that under the conditions described most of the isotope in the trichloroacetic acid-insoluble material is incorporated in DNA, the cultures of group 3 constitute what will be referred to here as the "DNA synthesis" test.

Cultures were set up in duplicate or triplicate and the ratio of the mean counts/min. in the treated cultures to that in the control cultures without added serum was calculated. This ratio is referred to here as "relative count".

To avoid error due to variable quenching effects, a known amount of [^{3}H]hexadecane was added to a series of sample cultures as an internal standard, and values were thus obtained for the counting efficiency of each sample. The same samples were then subjected to the external standard channel of the scintillometer and the values obtained were plotted against the counting efficiency as determined by the internal standard. For all the results reported in this paper external standard counts were performed. The variation in these counts was insignificant, from which it was concluded that there was no significant variation in the degree of quenching from culture to culture.

Other procedures

The response of rats to bovine serum albumin (BSA) was investigated by measuring the binding of [^{131}I]BSA by the technique of Farr (1958) after a single intraperitoneal injection of 5 mg. of alum-precipitated BSA.

The response to sheep erythrocytes was investigated by determining the agglutinin titre after a single intravenous injection of 1×10^8 cells.

Skin grafting (rats) was performed by the method of Woodruff and Simpson (1955).

RESULTS

Antirat ALS and fractions

The results of most of the tests are summarized in Table I. Details of the experiments with BSA are shown in Table II. The data concerning the effect of ALS on the primary response to sheep erythrocytes and on the survival of skin homografts is being published elsewhere (James and Anderson, 1967). The following conclusions would seem to emerge:

(1) As reported previously (Abaza and Woodruff, 1966), erythrocyte agglutinins may be absorbed out with but little reduction in lymphocyte agglutinin titre. It is worth mentioning that in relation to the erythrocyte agglutinin titre surprisingly large volumes of erythrocytes were required for the absorption.

(2) The horse antirat serum tested appeared to have slightly less immunosuppressive effect than the rabbit antirat serum.

(3) The immunosuppressive activity *in vivo* and the leucocyte agglutinin and cytotoxic activity *in vitro* are very largely concentrated in the IgG fraction of the sera raised in both the rabbit and the horse. Neither the $F(ab')_2$ nor the Fab' fragment showed significant aetivity in the tests to which they were subjected (i.e. effect on primary response to sheep erythrocytes *in vivo* and leucocyte agglutinin and cytotoxic titres *in vitro*), apart from a lymphocyte agglutinin titre of 1:32 in the divalent fragment, but the IgG from which they were prepared was of rather low potency and we would like to postpone judgmeht concerning both the immunosuppressive capacity and the *in vitro* activity of these fragments until further experiments now being performed have been completed.

(4) The *in vivo* and *in vitro* tests appear to correlate fairly well when sera and fractions originating in the same species are compared.

We have not obtained any results with the DNA synthesis test using rat lymphocytes because the cells did not survive sufficiently long in culture. The RNA synthesis test appears more promising but we have as yet no findings to report.

Studies on the capacity of ALS-treated animals to eliminate the IgG of the species in which the serum was raised are

TABLE I

PROPERTIES OF ALS (ANTIRAT) PREPARATIONS AND CONTROL SERA

		In vitro tests					Immunosuppression in vivo (rats)				
				Reciprocal titres					Effect on skin homograft survival		
Preparation	Absorbed with rat erythrocytes	Protein content (g./100 ml.) Total	IgG	Erythrocyte agglutination	Lymphocyte agglutination	Lymphocyte cytotoxicity	Primary response to sheep erythrocytes*	Primary response to BSA†	Dose schedule‡	Recipient strain	Mean survival treated / mean survival controls
Horse vs. rat inactivated plasma	No		1·2	512	1024						
	Yes (1 vol. erythrocytes)	5·0	1·0	128	512	512	Suppression		1	Hooded	2·5
	Yes (3 vols. erythrocytes)			8							
Horse vs. rat IgG	No	1·0	1·0	<2	64	256	Suppression	Suppression	1	Hooded	2·5
Horse vs. rat F(ab')$_2$ (pepsin digest from above IgG)		1·0		4	32	8	No effect				
Horse vs. rat Fab' (reduced and alkylated preparation of F(ab')$_2$		1·0		<2	4	8	No effect				
Normal horse inactivated serum	No		0·62						1	Hooded	1·0
Normal horse IgG	No	1·0	1·0	<2	<2	8	No effect	No effect	1	White	>4
Rabbit vs. rat lymphocyte inactivated serum	Yes (0·5 vol. erythrocytes)	5·3	1·0	2	128	128	Suppression		2	Hooded	3·5
Rabbit vs. rat lymphocyte IgG	No		1·0						2	Hooded	5
Normal rabbit inactivated serum	No	4·9		4	<8	<8	Partial suppression		1	Hooded	1·0

* Two ml. of IgG or whole serum i.p. on days −3, −2, −1; 1×10^9 sheep erythrocytes on day 0.
† Two ml. of IgG or whole serum i.p. on days −3, −2, −1; 5 mg. alum-precipitated BSA (i.p.) on day 0.
‡ DOSE SCHEDULES:
1. Two ml. days −7, to −1; 1 ml. days +1 to +14.
2. Two ml., days −2 to 0; 1 ml. days +1 to +10.

TABLE II

THE EFFECT OF ALS IgG PREPARATIONS ON THE PRIMARY RESPONSE OF RATS TO ALUM-PRECIPITATED BSA

Exp. no.	No. of rats	Strain	Treatment*	Lymphocyte count / Day −3	Lymphocyte count / Day 0	% Test† Antigen bound	Effect
1	4	Hooded	Antibody IgG	4,100	1,890	11·6	Suppression
	5		Normal IgG	4,500	2,750	67·5	
2	11		Antibody IgG	3,900	2,450	15·3	Suppression
	12		Normal IgG	3,720	4,150	67·5	
3	4		Antibody IgG		5,340	0 +	Suppression
	4		Normal IgG		6,970	46·3+	
4	11	Wistar "line bred"	Antibody IgG	14,375	11,700	35·2	Slight suppression
	11		Fab' from above	12,700	12,700	72·9	Stimulation?
	11		Normal IgG	13,700	12,300	63·1	Stimulation?
	12		No pretreatment			48·3	

* Animals received 2 ml. of 1 g./100 ml. IgG intraperitoneally on days −3, −2 and −1. Alum-precipitated BSA (5 mg.) was injected intraperitoneally on day 0.

† Results expressed as % of test BSA sample (containing 0·02 μg. N) bound by 0·5 ml. of a 1/10 dilution of rat serum obtained on day 12, except with rats marked + where test BSA sample contained 0·2 μg. N.

N.B. These are the results of four separate experiments. The ALS IgG was ineffective in line-bred Wistar rats (exp. 4) which possessed an extremely high lymphocyte count. Similar results were also observed when these rats were injected with sheep erythrocytes (James and Anderson, 1967).

incomplete, but we can at least say that rats treated with rabbit antirat ALS from seven days before to 14 days after they received skin homografts showed immune-type elimination of ^{131}I-labelled rabbit IgG when tested with this material a few days after the grafts had been rejected.

Antihuman ALS and fractions

As we have used antihuman antilymphocyte IgG in only one patient, and then in conjunction with other immunosuppressive agents, we are unable to comment on its immunosuppressive activity *in vivo*, though it can be said that the material was well tolerated and that the only complication observed was the development of painful red swellings at the site of subcutaneous injections.

The results of the *in vitro* tests are summarized in Table III. A full report of the studies on the transforming action of ALS on lymphocytes in culture will be published in detail elsewhere. The following conclusions would seem to emerge.

(1) The serum harvested after the horse had received adult human spleen cells as well as infant human thymus cells showed much greater activity in all tests than serum harvested when the animal had received only thymus cells.

(2) As before, it proved possible to absorb erythrocyte agglutinins without significantly reducing any of the actions of the serum on lymphocytes.

(3) The erythrocyte agglutinin titre of fractions of unabsorbed serum was greatest in the 19S fraction.

(4) The RNA and DNA stimulation tests showed good correlation with the lymphocyte agglutinin titre.

Both, it may be added, showed good correlation with the degree of blast transformation as judged on morphological grounds, though no attempt was made to develop this quantitatively.

DISCUSSION

It is clearly important to develop a method of *in vitro* assay of ALS which reflects its immunosuppressive effect *in vivo*. The present results suggest that stimulation of uridine and thymidine uptake by lymphocytes in culture merits further investigation from this point of view, but much will depend on whether or not it can

TABLE III

HORSE VS. HUMAN ALS AND ALS FRACTIONS: MEAN RESULTS OF *in vitro* TESTS

Method of immunization	*Preparation*	*Whether absorbed with human erythrocytes*	*Reciprocal titres*			*Transforming activity (mean relative count)**			
						RNA test		*DNA test*	
			Erythrocyte agglutination	*Lymphocyte agglutination*	*Lymphocyte cytotoxic*	0·1 ml.	0·2 ml.	0·1 ml.	0·2 ml.
Nil	Inactivated normal horse serum or filtered inactivated normal horse plasma	No	16	<4	<4	0·98	1·06	1·14	2·69
		Yes	<4	<4	<4	0·57		0·83	
Infant thymus (5)	Filtered inactivated plasma	No	1,024	192	32	1·18	2·23	0·20	4·91
		Yes	<4	96	32				
	IgG	No	32						
		Yes	4	256	128				
Infant thymus (5) + adult spleen (2)	Filtered inactivated plasma	No	>1,024			2·14	4·45	5·56	74·0
		Yes	4	2,048	256				
	19S (Sephadex)	No	512				2·17		8·08
		Yes	4	16					
	"10S" (Sephadex)	No	64				2·41		6·29
		Yes	2	128					
	7S (Sephadex)	No	64				11·9		14·8
		Yes	4	512					
	4·5S (Sephadex)	No	16				2·39		9·24
		Yes	2	8					
Infant thymus (5) + adult spleen (3)	Filtered inactivated plasma	No	1,024			6·74	10·80	10·20	15·95
		Yes	8	4,098					

* This denotes the ratio of the mean counts/min. in the cultures to which ALS or normal horse serum was added to the mean counts/min. in cultures without heterologous serum.

be applied successfully to lymphocytes from small laboratory animals such as rats and mice, of which inbred strains are available, since these are virtually essential for some of the corresponding *in vitro* studies.

It is of interest that in the studies with horse antihuman ALS the activity, as judged by all the *in vitro* tests, remained low while the animal was being immunized with infant thymus but increased promptly after the first adult spleen cell injection and continued to increase after subsequent injections. Since chronic immunization in other systems has been reported to result in a falling off in immunosuppressive activity it would be of great interest to repeat the present work with lymphocytes of a species other than man so that parallel estimates of immunosuppressive activity can be made at the same time.

It is noteworthy that horse antirat IgG preparations were active both *in vivo* and *in vitro*, and this correlates well with the observation that among the Sephadex fractions of horse antihuman ALS (which were tested *in vitro* only) the 7S fraction was much the most active. Since much of the erythrocyte agglutinin activity resided in the 19S region it could be largely eliminated by fractionation of the crude serum without the necessity for absorption. It is felt that this might be of great practical importance for the large-scale production of ALS globulin for therapeutic use.

The demonstration that ALS stimulates lymphocyte transformation and uptake of uridine and thymidine *in vitro* would seem at first sight to provide support for the sterile activation theory of ALS action *in vivo*, which Sir Peter Medawar will describe later. It must be remembered, however, that in the *in vitro* tests complement was excluded as far as possible from the system, and when complement was added lysis occurred instead of transformation. It would scarcely be surprising if similar ambivalence were also manifested *in vivo*, where the balance between stimulation and lysis in lymphoid tissue might depend on such factors as the local concentration of ALS and complement, and the degree of anticomplementary activity. The fact that in some of our own experiments (Woodruff and Anderson, 1964; Abaza *et al.*, 1966) prolonged administration of ALS resulted in lymphoid atrophy, whereas in the experiments of Levey and Medawar (1966) lymphoid hyperplasia occurred, suggests that this is indeed the case.

SUMMARY

Horse antirat and horse antihuman sera have been prepared by immunizing horses with rat thoracic duct lymphocytes, and with human thymus and spleen cells, respectively. Serum fractions and antibody fragments have been prepared from these sera by standard procedures.

The antirat preparations have been tested for immunosuppressive activity *in vivo* as judged by the primary response to sheep erythrocytes and alum-precipitated bovine serum albumin and the survival of skin homografts. Both antirat and antihuman preparations have been assayed *in vitro* for erythrocyte agglutinins, lymphocyte agglutinins and lymphocyte cytotoxins. In addition, antihuman preparations have been tested for their effect on the uptake of [^{3}H]uridine and [^{3}H]thymidine by lymphocytes in cultures.

Immunoglobulin G (IgG) preparations and 7S Sephadex fractions appeared to contain most of the material responsible for immunosuppression *in vivo*, and for lymphocyte agglutination, cytotoxicity and stimulation *in vitro*, whereas much of the erythrocyte agglutinin was in the immunoglobulin 19S Sephadex fraction.

Within the limits of the experiment there appeared to be a fairly good correlation between the results of the various *in vitro* tests and the *in vivo* activity when sera and fractions originating in the same species were compared. Possible implications for an understanding of the mode of action of ALS *in vivo* are discussed.

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DISCUSSION

James: The rabbit antisera produced quite a marked lymphopenia, while the horse antirat only produced very little, and I think this may reflect the avidity of the antibodies. The rabbit antibodies really adhere to the lymphocytes and damage them.

Levey: We have been able to confirm Professor Woodruff's and Dr. James' results with bovine serum albumin (BSA). We found that the secondary response, at least as measured by the serum antigen binding capacity, can be inhibited. The maximum inhibition obtained was about 94 or 95 per cent. The animals that have been hyperimmunized with BSA, and then treated with antiserum and challenged, are inhibited; if the challenge dose is given long enough after cessation of ALS treatment these behave as prime rather than as sensitized reactors. There is a difference in the humoral system as compared to the homograft system in that the degree of inhibition of the primary response can be augmented if the animals are pretreated with ALS before stimulation with antigen. With primed cells *in vitro*, it is possible to overcome the inhibition by antiserum of the secondary response by increasing the concentrations of antigen. This suggests that at least in this system, though not necessarily *in vivo*, there is an element of competitive inhibition between antigen and the antibody (ALS).

Humphrey: After you found the inhibition of response to priming with BSA, if you then left the animals without ALS for a while, were they still unresponsive? Is there some specific immunological paralysis or is it just a transient phenomenon?

Levey: We have tested them for about six weeks from cessation of ALS treatment; they give a primary response but not a secondary one.

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MUNKSGAARD

Biological Properties of Antilymphocytic Antibody and Antibody Fragments

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Antilymphocytic sera (ALS) have been prepared by immunizing rabbits and horses with lymphoid tissue from other species including man and rat. From these sera IgG has been prepared by sodium sulphate precipitation followed by batch chromatography, with DEAE cellulose; fractions have also been prepared on G 200 Sephadex columns. Divalent antibody fragment F $(ab')_2$ has been prepared by pepsin digestion of antilymphocytic IgG, and univalent fragment Fab′ by reduction of $F(ab')_2$ with cysteine hydrochloride followed by dialysis against iodoacetamide. These materials have been tested for immunosuppressive properties *in vivo*, using as criteria their effect on homograft survival and on the antibody response of rats to sheep erythrocytes and alum precipitated bovine serum albumin, and for their effect on lymphocytes *in vitro* as judged by agglutination, cytotoxic destruction in the presence of complement, and stimulation of uptake of ^3H uridine and ^3H thymidine.

In addition antilymphocytic and normal IgG have been labelled with ^{131}I, and the capacity of animals which have been treated with normal or antilymphocytic IgG to eliminate these materials has been investigated. The labelled preparations have also been used *in vitro* to determine approximately how many molecules of each are bound by a single lymphocyte under various experimental conditions.

Anti-rat ALS prolonged the survival of skin homografts in rats, and produced a sustained lymphopenia, thus confirming and extending earlier observations from this laboratory (Woodruff & Anderson 1963, 1964). IgG prepared from ALS had a similar effect but $F(ab')_2$ and Fab′ fragments of horse-anti-rat IgG, given in a dosage equal to the dose of IgG which increased homograft survival by a factor of 2.5, had no effect on homograft survival and produced only a transient lymphopenia. Normal horse and rabbit serum had little or no effect (Anderson *et al* 1967).

The effect of antilymphocytic antibody and antibody fragments on the primary response of hooded rats to sheep erythrocytes and alum precipitated bovine serum albumin is summarised in Table 1. It will be seen that only whole ALS or intact antilymphocytic IgG suppressed or delayed the primary response, and this occurred only if the material was administered prior to the test antigen. Destruction of the complement-binding (Fc) portion of the antilymphocytic IgG molecule destroyed its immunosuppressive properties. The results suggest that antilymphocytic antibody may also slightly depress the secondary response of sensitized animals to the same test antigens, but further experiments will have to be performed before any firm conclusion can be drawn on this point.

It is interesting to contrast these findings, some of which have already been reported (James & Anderson 1967, Woodruff *et al* 1967), with the results obtained so far in *in vivo* studies with ^{131}I tagged IgG. Tracer doses of ^{131}I labelled normal horse IgG were given to 3 groups of rats which had previously received either antilymphocytic IgG (20 mg. on Days −24 to −19 and 10 mg. on Days −8 to −5, reckoning the day of the injection of tracer as Day 0), normal IgG (in the same dosage) or normal saline. Total body counts and counts of serial blood samples have shown that the time required to eliminate 90 per cent of the tracer was about 24 hours for the animals treated with either antilymphocytic or normal IgG, but about 7 days in the saline treated controls. It thus appears that horse-anti-rat antilymphocytic IgG does not prevent the formation of antibody against horse IgG. One possible explanation would seem to be that the lymphocytes to which antilymphocytic IgG becomes attached are subsequently phagocytosed by macrophages and these in turn stimulate unaffected lymphocytes to make antibody. In addition the presence of non-antilymphocytic IgG in the preparation may have contributed to the result. As will be seen later there is good evidence that a high proportion of the IgG prepared from our horse-anti-

TABLE I

The effect of anti-lymphocyte antibody on humoral antibody formation (primary response in hooded rats)

Test antigen	Nos. of rats	Preparations* administered	Anti-lymphocytic properties of preparations		Per cent change in peripheral blood lymphocytes	Effect on primary response	Reference
			Agglutination	Cytotoxicity			
1×10^9 Sheep erythrocytes intravenousky	8	Horse ALS	512	512	−63	Suppression	James and Anderson 1967
	14	IgG from above	64	256	−25	Suppression	
	8	$F(ab')_2$ from above	32	8	− 1.5	No effect	
	10	Fab′ from above	4	8		No effect	
	8	Normal horse IgG	<2	8	−11.6	No effect	
	12	Rabbit ALS	512	512	−86.8	Suppression	
	6	Normal rabbit serum	<8	<8	− 8.5	Partial suppression	
5 mg. alum precipitated BSA intraperitoneally	12	Horse ALS IgG	256	256	−37	Suppression	James and Jubb 1967
	12	Normal horse IgG	<2	<8	+12	No effect	
	6	Horse ALS IgG**	256	256	−28	No effect	James 1967
	6	Normal horse IgG**	<2	<8	+28	No effect	
	6	F(ab′) from horse ALS IgG	64	<8	− 3	No effect	
	4	$F(ab')_2$ from rabbit ALS IgG	256	<8	−18	No effect	

* 2 ml. volume of all materials were injected I.P. on days −3, −2, −1 and the test antigen was injected on day 0. The whole sera contained approximately 10 mg. IgG per ml. The isolated IgG and fragments contained 10 mg. protein/ml.

** The IgG preparations were injected 4, 28 and 52 hours after the test antigen.

human ALS is non-antilymphocytic, and it would be surprising if this were not true also of the horse-anti-rat material.

Horse-anti-human ALS and IgG, and $(ab')_2$ prepared therefrom, have been shown to agglutinate human lymphocytes *in vitro*, and to stimulate the uptake of ^3H uridine and ^3H thymidine by human lymphocytes cultured in the absence of complement. In the presence of complement ALS and antilymphocytic IgG do not stimulate uridine and thymidine uptake, and at high serum concentration cause cytolysis. Fab' does not agglutinate, lyse or stimulate lymphocytes, nor does it appear to block stimulation by subsequent exposure to intact antibody, although the possibility that this stimulation results from a homoreactant type of phenomenon has not been ruled out (Woodruff *et al* 1967a).

In further experiments, which will be fully reported elsewhere (Woodruff *et al* 1967b), the mean number of IgG molecules taken up when 3 million to 30 million human lymphocytes are maintained for 1 hour in culture medium containing 1 to 4 mg. IgG prepared from horse-anti-human ALS and subsequently tagged with ^{131}I, has been estimated on the assumption that all IgG molecules in the preparation were labelled equally. This value was found to range from 4 million to 9 million molecules per lymphocyte. Experiments of this kind give no indication of the proportion of IgG molecules in the preparation which are antilymphocytic in the sense that they stimulate transformation. If it is assumed however that the preparation contains only two classes of IgG molecule, namely antilymphocytic molecules which bind to lymphocytes and stimulate them to an equal extent (as judged by increased ^3H uridine uptake), and non-antilymphocytic molecules which neither bind nor stimulate,* then it is possible to estimate the ratio R of antilymphocytic to total IgG molecules in a particular preparation of IgG by combining the results of studies of uptake of ^{131}I tagged IgG with absorption experiments. As a preliminary a dose-response curve is established for the stimulating effect of varying amounts of the preparation in question on a fixed number of lymphocytes from a particular donor, and this is then used to determine the degree of stimulating activity which remains after standard amounts of the same preparation have been absorbed with known numbers of lymphocytes from the same donor. In this way we have obtained estimates for R in our own horse-anti-human antilymphocytic IgG preparations ranging from 0.6 per cent to 2.3 per cent. If these estimates are reliable, and if in addition immunosuppressive activity *in vivo* is correlated with stimulating activity on lymphocytes *in vitro*, it would follow that the great bulk of the protein we are administering is irrelevant so far as immunosuppression is concerned. It might be possible to obtain preparations with higher R values by appropriate selection of the animals whose plasma level of IgG is either naturally low or has been reduced by plasmaphoresis. Alternatively, the same end might be attained by modifying the schedule of immunization. We plan to investigate these possibilities.

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* Other possibilities, e. g. that non-antilymphocytic molecules may bind but do not stimulate, may also be considered.

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Effect of Acid Treatment on the Immunosuppressive Properties of Anti-lymphocytic IgG

THE abolition of the complement binding activity of IgG globulin molecules by exposure to low *p*Hs (refs. 1 and 2) has prompted us to investigate the effect of acid-treated anti-lymphocytic IgG on humoral and cell mediated immune responses in the rat. These studies form part of a larger investigation aimed at elucidating the mode of action of anti-lymphocytic serum and developing less toxic products for therapeutic use.

The anti-rat lymphocyte globulin used in these studies was prepared as described earlier[3]. The preparation contained several sub-classes of IgG globulin and small amounts of IgG(T) globulin and the acid treatment was carried out as follows. The globulin solution (2 per cent in phosphate buffer, *p*H 7·2, 0·06 M containing 0·15 M sodium chloride) was adjusted to *p*H 3·0 by the addition of 0·1 N HCl or 0·1 M citric acid and the acidified mixture was incubated at 37° C for 20 min. After incubation the *p*H of the solution was readjusted to *p*H 7·2 by the addition of 0·1 N NaOH or 0·2 M di-sodium hydrogen *ortho*phosphate and the final product was dialysed against the original phosphate buffered saline. This treatment, which may result in an increased turbidity of the globulin solution and the precipitation of a small amount of protein, was accompanied by alterations in the immunoelectrophoretic and gel filtration properties of the globulin, characteristic of aggregate formation.

The effect of the acid-treated IgG on the primary humoral response was assessed in 5–6 month old male rats of the hooded strain (experiments *A* and *B*). The animals received intraperitoneal injections of untreated or acid-treated anti-lymphocytic IgG on days −3, −2 and −1 (1 ml. of a 2 g per cent solution). On day 0 the animals were injected intraperitoneally with 5 mg of alum precipitated bovine serum albumin. The animals were then bled at weekly intervals and the antibody content of the sera was assessed by the Farr procedure[4] using a ^{131}I-labelled bovine serum albumin preparation. Further details on the Farr procedure and the labelling of the antigen are recorded elsewhere[5,6].

The skin allograft studies were performed in 5–6 month old male and female rats of the hooded strain (experiments *C* and *D*). These animals received intraperitoneal injections of untreated or acid-treated anti-lymphocytic IgG

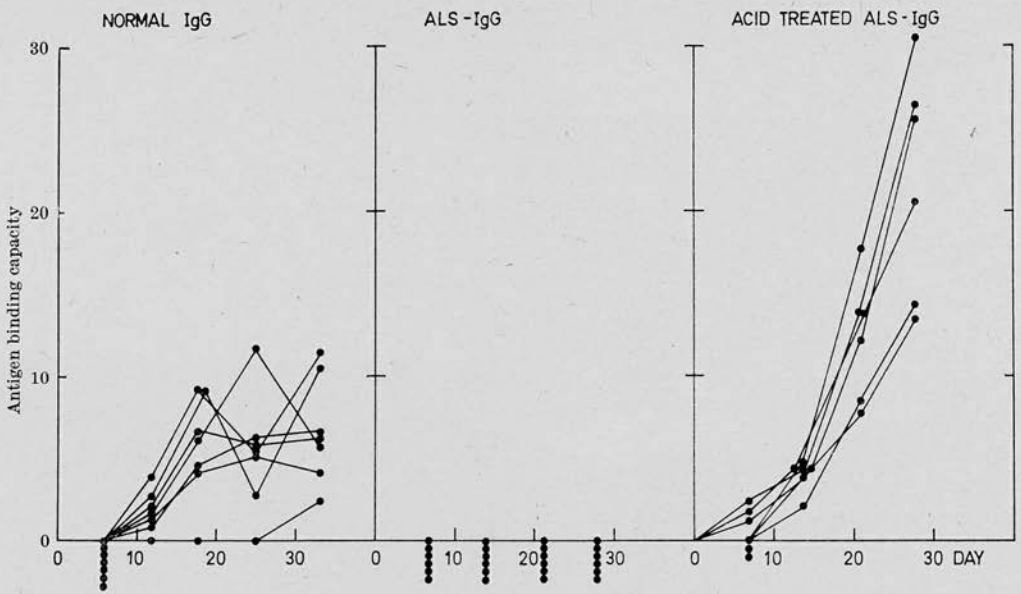

Fig. 1. Effect of acid-treated anti-lymphocytic IgG on the primary response of hooded rats to alum precipitated bovine serum albumin. The humoral response was determined by measuring the antigen binding capacities of the rat sera. These are expressed as μg of bovine serum albumin nitrogen bound by 1 ml. of rat serum. They were determined using 0·2 μg of bovine serum albumin nitrogen per test and taking as the end point the dilution of serum which bound 33 per cent of the antigen. Note the marked response (high antigen binding capacity) in animals receiving acid-treated anti-lymphocytic IgG. In contrast, the sera from rats receiving untreated anti-lymphocytic IgG bound so little antigen that antigen binding capacities could not be measured in our system. Included in the figure for comparison are results previously obtained in rats pretreated with normal horse IgG (ref. 5).

(2 ml. of 1 g per cent globulin) for 7 consecutive days. They were then grafted with skin from an inbred Wistar strain by the method of Woodruff and Simpson[7] and daily treatment with the protein preparations described was continued up to 14 days after grafting (1·0 ml. of 1 g per cent protein per day).

The *in vitro* studies confirmed that the exposure of anti-lymphocytic IgG to low *p*Hs results in a marked loss in lymphocytotoxicity (Table 1). Short courses of this material failed to lower the peripheral blood lymphocyte count (experiment *B*) although more prolonged courses resulted in a significant depression (experiment *D*). In both cases, however, acid treatment was accompanied by a dramatic decrease in immunosuppressive activity, the modified anti-lymphocytic IgG failing to suppress humoral antibody formation (Fig. 1) or to significantly prolong skin allograft survival (see Fig. 2). Indeed, the data presented in Fig. 1 suggest that acid-treated antibody may stimulate the primary humoral response, although this will require critical investigation. These results are similar to those previously observed with anti-lymphocytic antibody fragments[3,8,9], indicating once more that the immunosuppressive properties of this molecule are associated with its cytolytic activity, and confirming that non-cytotoxic products are of little therapeutic value. Furthermore, the inability of "intact" anti-lymphocytic antibody molecules (containing the "antigenic" Fc portion of the molecule) to exert a significant immunosuppressive effect strongly suggests that the immunosuppressive properties of this molecule are not dependent on antigenic competition[10]. Nevertheless, it

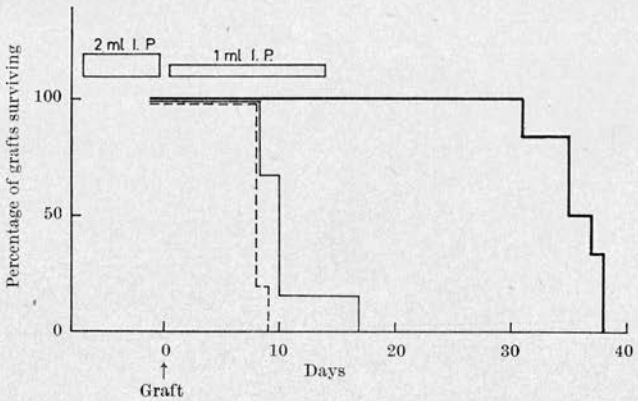

Fig. 2. The effect of acid-treated anti-lymphocytic IgG on skin allograft survival in hooded rats. Note the rapid rejection of Wistar strain skin allografts by hooded rats receiving acid-treated anti-lymphocytic antibody. Treatment: – – –, normal horse serum; ——, acid-treated anti-lymphocytic IgG; ——, untreated anti-lymphocytic IgG.

Table 1. PROPERTIES OF UNTREATED AND ACID-TREATED ANTI-LYMPHOCYTIC IgG

Response investigated		Material injected	*In vitro* effect: Lympho. agg.	*In vitro* effect: Lympho. cytotox.	Effect on peripheral blood lymphocyte count (total and percentage): Initial	Effect on peripheral blood lymphocyte count (total and percentage): Final*	Effect on immune response
Humoral	*A*	Untreated anti-lymphocytic IgG	512	512	4,380 (100)	2,930 (66·9)	Suppression
	B	Acid-treated anti-lymphocytic IgG	256	32	4,780 (100)	5,600 (117)	No suppression
Cellular	*C*	Untreated anti-lymphocytic IgG	512	512	6,970 (100)	3,870 (55·5)	Suppression
	D	Acid-treated anti-lymphocytic IgG	512	32	8,430 (100)	3,420 (40·6)	No suppression

* The count (expressed as cells per cubic mm) is that observed at the time of antigenic challenge (BSA or skin allograft). Counts performed 7 days after grafting indicated that animals in group *D* exhibited a more marked recovery in circulating lymphocytes (6,440) than those animals in group *C* (4,630).

should be borne in mind that the failure to demonstrate immunosuppressive activity with acid-treated globulin could also be due to the more rapid elimination (immune or otherwise) of this material.

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The suppression of experimental thyroiditis in the rat by heterologous anti-lymphocyte globulin

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THE SUPPRESSION OF EXPERIMENTAL THYROIDITIS IN THE RAT BY HETEROLOGOUS ANTI-LYMPHOCYTE GLOBULIN

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SUMMARY

The auto-allergic thyroiditis produced in rats by the injection of rat thyroglobulin in complete Freund's adjuvant can be suppressed by the administration of the IgG immunoglobulin fraction of heterologous (horse) anti-lymphocytic serum (ALS-IgG). ALS-IgG also suppressed the formation of circulating antibodies against rat thyroglobulin.

INTRODUCTION

The ability of anti-lymphocytic serum to suppress a variety of cell mediated and humoral immune responses is well documented and has been recently reviewed (James, 1967, 1968). In particular it has been demonstrated that anti-lymphocytic serum suppresses the development of autoallergic encephalomyelitis (Waksman, Arbouys & Arnason, 1961; Leibowitz, Lessop & Kennedy, 1968), of Freund adjuvant arthritis (Currey & Ziff, 1966, 1968) and of haemolytic anaemia in NZB mice (Denman, Denman & Holborow, 1967). The present paper describes the effect of anti-lymphocytic serum on the development of experimental thyroiditis in rats.

MATERIALS AND METHODS

Animals

Two- to 3-month-old male rats derived from a random bred Wistar strain maintained in the breeding station of the University of Edinburgh were used for immunization.

Preparation of thyroglobulin

Rat thyroids (stored at $-20°C$ prior to use) were minced and then disrupted in 2 volumes of phosphate-buffered saline, pH 7·2, in a ground glass homogenizer. The resultant suspension was centrifuged at 4°C for 10 min at 30,000 *g* and the crude thyroglobulin was precipitated from the supernatant with ammonium sulphate (saturation between 40 and 42%). The precipitate was dialysed and freeze dried.

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Preparation of anti-rat lymphocyte serum and normal horse IgG

The ALS was produced in a horse by the intravenous injection of thoracic duct lymphocytes obtained following the cannulation of random bred Wistar rats. The horse received six injections at weekly intervals followed by a seventh injection after a further 2 weeks. Each injection consisted of $1{\cdot}2–1{\cdot}5 \times 10^9$ cells of which 87–95% were viable. Two weeks after the last injection, the animal was bled and the serum obtained was inactivated by incubation at 56°C for 30 min.

Immunoglobulin G fractions were prepared from this antiserum and from normal horse serum by a combined salt precipitation and chromatographic procedure. The initial step involved the precipitation of a crude globulin concentrate by the addition of 1 volume of 28% (w/v) sodium sulphate. The precipitate was re-dissolved in 0·15 M-sodium chloride and re-precipitated as above. This material was then dissolved in and dialysed against 0·02 M-phosphate buffer, pH 6·5, and then batched as Whatman DE11 diethyl-aminoethyl cellulose exchanger with an ion exchange capacity of 1·0 mEq/g. The final products were concentrated by lyophilization and reconstituted when required in 0·06 M-phosphate buffered saline, pH 7·2, containing 0·15 M-sodium chloride. The 1 g/100 ml anti-lymphocytic IgG solution so obtained was then absorbed with one-quarter its volume of rat erythrocytes to remove erythrocyte agglutinins. Both preparations of the ALS-IgG and normal horse IgG (NH-IgG) were finally sterilized by filtration through an 0·22 mμ millipore filter. Immunoelectrophoretic analysis using a rabbit anti-horse serum indicated that the products contained several IgG components. The reciprocal lympho-agglutination and lymphocytoxic titres of the anti-rat lymphocyte IgG were both 256 while the normal horse IgG possessed negligible amounts of anti-lymphocyte activity (Abaza & Woodruff, 1966).

Immunization schedule

Each of the thirty rats was injected intracutaneously into the base of the tail with 0·05 ml emulsion containing 2 mg of the rat thyroglobulin preparation in complete Freund's adjuvant (CFA). The injection was repeated 21 days later. Ten control rats were immunized with 0·05 ml saline in CFA and injections were also repeated 21 days later. The animals were killed after 4 weeks and serum stored at −20°C.

Treatment schedule

The thirty test animals were divided into three equal groups. Taking the time of the first sensitization to rat thyroglobulin as day 0, the first group of ten animals was injected with 3 ml of 1 g/100 ml ALS-IgG solution intraperitoneally on days −3, −2 and −1. From day 2 on and on every 2nd day thereafter, these animals were given 2 ml ALS-IgG solution intraperitoneally. The second group of test animals (ten animals) were treated with 1 g/100 ml NH-IgG solution following the same schedule. The third group of ten test animals received no treatment. One of these animals died. A further group of ten control rats received saline in CFA but were not treated with ALS-IgG or NH-IgG (Table 1).

Detection of circulating antibody

Tanned sheep red cells were sensitized with one of the following antigens: (1) 2 mg/ml rat thyroglobulin, (2) 5 mg/ml solution of ALS-IgG, or (3) 5 mg/ml solution of NH-IgG, and incubated overnight at room temperature with doubling dilutions of the sera from the test and control animals (Herbert, 1968).

The indirect Coombs test was also employed to detect antibody to thyroglobulin. Goat anti-rat γ-globulin (Hyland Division, Travenol Laboratories Inc., Los Angeles) was conjugated with fluorescein isothiocyanate and incubated with cryostat sections of rat thyroid 4–6 μ thick and fixed in methanol at 56°C for 3 min. In order to test for complement fixation, fixed thyroid sections were incubated with inactivated test sera and guinea-pig complement and then with anti-guinea-pig complement serum conjugated with fluorescein isothiocyanate. Both of these immunofluorescence methods were also employed to unfixed sections of rat thyroid to detect thyrocytoplasmic antibody in the test sera.

Histology

The thyroid gland, thymus, lymph nodes and spleen were fixed overnight in Carnoy solution and the sections were stained by haemotoxlin and eosin (H & E), methyl-green–pyronine, and PAS. The kidneys were fixed in formol saline (4%) and stained with H & E alone.

Scoring of thyroiditis

The degree of thyroiditis was scored as follows:

$-$ = Normal thyroid structure.
$+$ = Focal thyroiditis.
$++$ = More severe thyroiditis.
$+++$ = Diffuse thyroiditis with altered thyroid structure.
$++++$ = Severe thyroiditis with goitre.

The arithmetic mean score for each group of animals was obtained by totalling the number of '+'s for the thyroid lesions and dividing by the number of animals. The thyroid sections were randomized before they were read.

Absolute lymphocyte counts were obtained from total white cell haemocytometer counts and differential counts on Pappenheim stained blood smears and are expressed as counts per cubic millimeter blood.

RESULTS

Treatment with ALS-IgG prevented the development of thyroiditis in response to sensitization with thyroglobulin in CFA. Rats sensitized with rat-thyroglobulin in CFA and treated with saline developed histological evidence of thyroiditis with the mean score of 1·4, and a similar degree was seen in thyroglobulin immunized rats treated with NH-IgG (mean score 1·0) (Fig. 1). In contrast, the thyroid of animals treated with ALS-IgG closely resembled those of animals given only saline in CFA (Table 1).

Furthermore, ALS-IgG also prevented the development of antibody to thyroglobulin in rats subsequently sensitized with rat thyroglobulin in CFA. NH-IgG had no such effect (Table 2). The tanned red cell titres in the animals sensitized with rat thyroglobulin CFA that were not given any heterologous globulin ranged up to 1:1280 and, in those animals given NH-IgG, the titres ranged up to 1:640. None of the tanned red cell titres was positive in those immunized rats that received ALS-IgG treatment.

The findings with the indirect immunofluorescence test using fixed rat thyroid sections

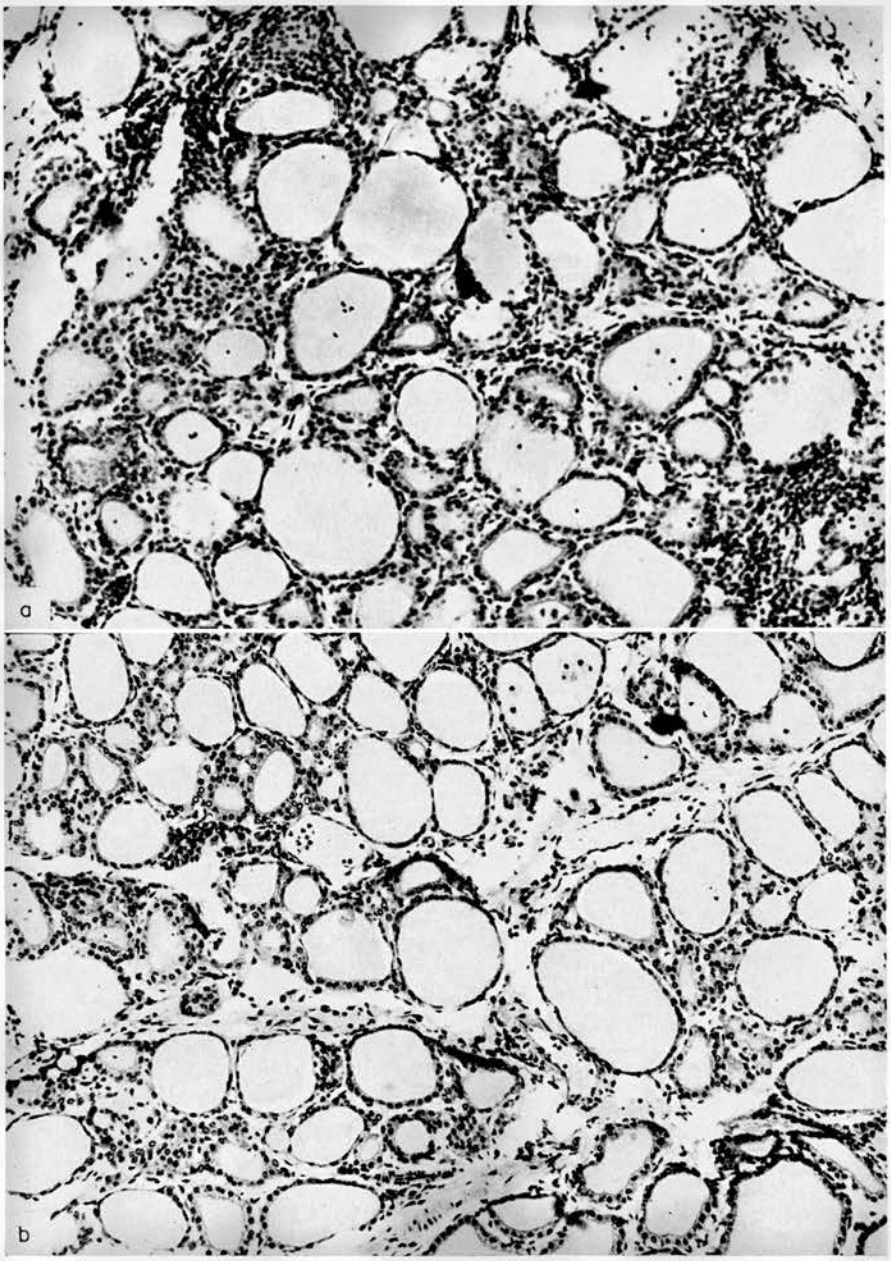

FIG. 1. (a) Thyroid of a rat treated with normal horse IgG and subsequently sensitized to rat thyroglobulin in CFA. Note the inflammatory reaction with involvement and destruction of some follicles. H & E, ×85. (b) Thyroid of a rat treated with horse anti-rat lymphocytic IgG and subsequently sensitized to rat thyroglobulin in CFA. The thyroid shows an intact structure. H & E, ×85.

confirmed the results of the tanned cell method insofar that staining of the colloid was not seen in the animals treated with ALS-IgG, except in one doubtful case.

Immunofluorescence using unfixed thyroid sections did not detect any antibody against rat thyroid cytoplasm whether or not the sensitized animals had been treated with ALS-IgG.

TABLE 1. The effect of anti-rat-lymphocytic serum (ALS-IgG) on the production of experimental thyroiditis in the rat by sensitization with rat thyroglobulin in complete Freund's adjuvant

Sensitization	Treatment	No. of rats	Severity of thyroiditis*					Mean† score
			−	+	++	+++	++++	
Rat thyroglobulin +CFA	Saline	9	3	2	2	1	1	1·4
Rat thyroglobulin +CFA	NH-IgG‡	10	4	3	2	1	0	1·0
Rat thyroglobulin +CFA	ALS-IgG§	10	9	1	0	0	0	0·1
Saline+CFA	None	10	10	0	0	0	0	0

* Gradation of thyroid lesions as described under 'Materials and methods'.
† Lesions were scored as follows: − = 0; + = 1; ++ = 2; +++ = 3; ++++ = 4.
‡ Normal horse IgG.
§ Anti-rat-lymphocytic serum.

Likewise, indirect immunofluorescence methods did not demonstrate any fixation of complement in the control and sensitized animals either with regard to the colloid or to the thyroid cytoplasm.

Fig. 2 shows the fall in the absolute peripheral lymphocyte count in animals treated with ALS-IgG in contrast to the animals given no treatment or only normal horse serum. Four

TABLE 2. The effect of ALS-IgG on the production of antibody to thyroglobulin in rats subsequently sensitized with rat thyroglobulin in CFA

No. of rats	Sensitization	Treatment	Thyroglobulin antibody	
			Tanned cell titre ⩾1/20	Immunofluorescent
9	Rat thyroglobulin +CFA	None	6	8
10	Rat thyroglobulin +CFA	NH-IgG	5	6
10	Rat thyroglobulin +CFA	ALS-IgG	0	(1)
10	Saline+CFA	None	0	0

hours after a single injection of ALS-IgG, there was a significant fall in the peripheral lymphocyte count and this depression was maintained with continuous treatment for approximately 3 weeks after which it rose to almost normal levels in spite of continuing ALS-IgG injection. A slight depression was initially observed in the control groups.

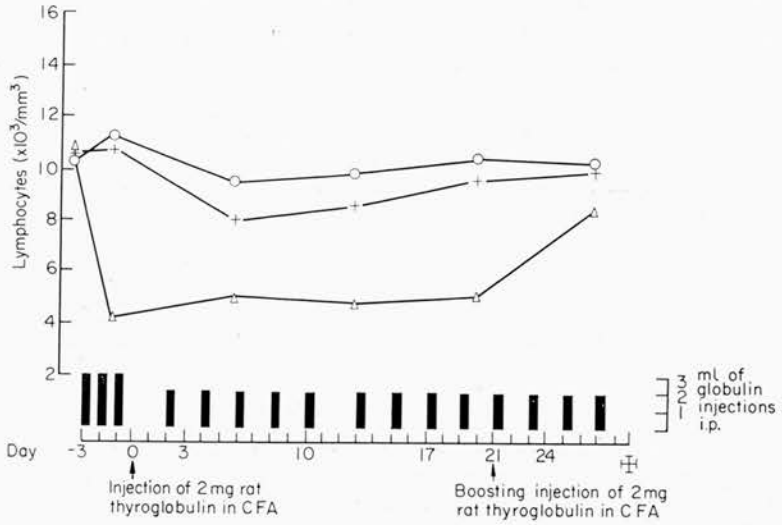

FIG. 2. The effect of various treatments on the peripheral lymphocyte count in immunized rats. Note the rapid initial fall in the animal group receiving anti-lymphocytic IgG and its eventual recovery. △, Horse anti-lymphocyte globulin; +, normal horse IgG; ○, no treatment.

Although the ALS-IgG suppressed the formation of anti-thyroglobulin antibodies, it failed to suppress the development of circulating antibodies against itself. The antibody titres against horse immunoglobulin determined by the tanned red cell method were equally high in rats treated with ALS-IgG or NH-IgG (Table 3).

TABLE 3. Tanned cell titres of antibodies to horse immunoglobulin in rats after 4 weeks treatment with NH-IgG or ALS-IgG

No. of rats	Treatment	No. of rats and titre of antibodies			
		<1:20	1:20	1:1280	1:2560
10	NH-IgG			10	
10	ALS-IgG			9	1
19	None	18	1		

The spleen and lymph nodes of one of the ALS-IgG treated rats showed lymphocytic depletion and a decrease in the number of germinal centres. Histological examination of the kidneys showed no abnormalities in any of the experimental animals.

DISCUSSION

Chronic thyroiditis in man is closely associated with autoimmune phenomena in which the mechanisms of humoral antibody formation and of delayed hypersensitivity are involved (Glynn & Holborow, 1965). In animals, thyroiditis can be produced experimentally by sensitization with thyroglobulin in complete Freund's adjuvant (Witebsky & Rose, 1956; Terplan *et al.*, 1960; Jones & Roitt, 1961).

Anti-lymphocytic serum is known to suppress the production of delayed hypersensitivity and of primary antibody formation (reviewed by James, 1967, 1968). The observations reported in the present paper confirm that this is so in relation to autoimmune experimental thyroiditis in rats. These observations are in keeping with studies previously reported on autoimmune allergic encephalomyelitis in guinea-pigs (Waksman *et al.*, 1961; Leibowitz *et al.*, 1968). ALS has also been shown to prevent the development of Coombs positivity in NZB mice if treatment is commenced at 2 months of age (Denman *et al.*, 1967). In contrast, however, these investigators also showed that ALS was ineffective in suppressing Coombs positivity in NZB mice once this has developed. Furthermore, anti-lymphocytic-globulin treatment also failed to prevent the onset of renal disease in (NZB × NZW) F_1 hybrid mice (Denman, Denman & Holborow, 1966). Indeed, in animals treated with ALS, the albuminuria was more pronounced than in control animals receiving normal IgG. However, as suggested by Denman *et al.* (1966) it is possible that ALS treatment commencing at an earlier age than 3 months could prove to be effective. ALS has also been shown to prevent the development of Freund's adjuvant arthritis in rats (Currey & Ziff, 1966, 1968) but again was relatively ineffective in suppression of already established disease.

Studies are now in progress in our laboratory to determine whether ALS has any significant effect on previously induced experimental thyroiditis in rats. It may be anticipated from the known properties of ALS that it might have relatively little effect on circulating thyroglobulin antibody titres. In this context, it is of particular interest that Leibowitz *et al.* (1968) noted that the clinical signs of autoimmune allergic encephalomyelitis could be suppressed by the administration of ALS, but there was no change in the appearance of the brain lesions including perivascular infiltration of inflammatory cells. The same authors were also able to demonstrate that treatment of already paralysed animals with ALS was capable of prolonging the survival time in four out of eleven animals with marked improvement of the physical manifestations. Again there was no improvement of the brain lesions.

The rapid initial fall in the peripheral lymphocyte count of animals following ALS administration and subsequent recovery, even during prolonged treatment, has been widely observed (reviewed by James, 1968). Recently these observations have been attributed to repopulation of the lymphocyte pool by short-lived small lymphocytes, the number of long-lived immunologically completent lymphocytes remaining markedly depleted (Denman, Denman & Embling, 1968).

The rat treated by ALS-IgG or NH-IgG developed antibodies against horse IgG but these antibodies had no effect on the immunosuppressive properties of ALS-IgG. In spite of the high antibody titres against horse immunoglobulin, there were no signs of serum sickness or of anaphylactic reactions during the course of treatment. No histological changes were observed under light microscopy in the kidneys of the ALS-IgG treated rats.

In man, ALS treatment is associated with a number of local and systemic complications, the most troublesome being severe pain at the site of the injection and thrombocytopenia

(Starzl *et al.*, 1967; Kashiwagi *et al.*, 1968) and in dogs there is evidence of nephrotoxicity (Iwasaki *et al.*, 1967). Therefore, the use of ALS in patients with autoimmune diseases of the organ-specific type (such as chronic thyroiditis) should await the further study of its application in established experimental thyroiditis in animals and the development of less toxic and more specific ALS preparations.

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PRELIMINARY OBSERVATIONS ON THE EFFECT OF HETEROLOGOUS ANTI-LYMPHOCYTIC GLOBULIN ON AUTOLOGOUS IMMUNE COMPLEX NEPHRITIS IN RATS

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SUMMARY

The development of autologous immune complex nephritis induced by repeated intraperitoneal injections of chemically modified homologous kidney mitochondria in complete Freund's adjuvant in an inbred strain of rats can be delayed in its onset and greatly reduced in its severity by the administration of heterologous anti-lymphocytic globulin.

INTRODUCTION

The ability of anti-lymphocytic globulin (ALG) to suppress a variety of experimentally induced autoimmune diseases in a number of species is well documented. For example ALG has been shown to suppress the development of allergic encephalomyelitis in the guinea-pig (Waksman, Arbouys & Arnason, 1961; Leibowitz, Lessof & Kennedy, 1968), experimentally induced thyroiditis in the rat (Kalden *et al.*, 1968) and adjuvant induced polyarthritis in the rat (Currey & Ziff, 1966, 1968). In addition to these experimental autoimmune diseases, ALG has been shown to inhibit the development of the spontaneously occurring Coombs-positive haemolytic anaemia observed in NZB mice (Denman, Denman & Holborow, 1967). Such preparations however failed to prevent the onset of the naturally occurring autoimmune renal disease observed in B/W mice (NZB × NZW) F_1 (Denman, Denman & Holborow, 1966).

In this preliminary communication we report the effect of anti-lymphocytic globulin on the pathogenesis of another experimentally induced autoimmune disease, namely autologous immune complex (AIC) nephritis in the rat (Heyman *et al.*, 1959; Edgington, Glassock & Dixon, 1968; Glassock *et al.*, 1968; Barabas & Lannigan, 1969).

MATERIALS AND METHODS

Preparation of renal tubular antigen

Kidneys from Wistar strain rats were homogenized and the mitochondrial fraction was

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separated by differential centrifugation. An azo-mitochondrial complex was prepared according to the method described by Vogel (1951). Full details of the preparatory procedures are described elsewhere (Lannigan *et al.*, 1969).

Preparation of horse anti-rat lymphocytic IgG

The preparation and properties of the ALG used in these experiments have been previously described (James & Anderson 1967; Anderson, James & Woodruff, 1968).

Induction of autologous immune complex nephritis

Adult female rats of an inbred hooded strain received three weekly intraperitoneal injections of 2 mg azo-mitochondrial protein in complete Freund's adjuvant and a fourth injection was given at 7 weeks.

Three groups of animals were used:

Group I: twelve animals received a single injection of 0·75 ml ALG (2 g/100 ml) on the day before and the day after the first injection of homologous tubular antigen. The animals were given ALG twice weekly in doses of 0·75 ml for 7 weeks and thereafter a dose of 0·5 ml was given twice weekly until the end of the experiment.

Group II: Five animals received normal horse IgG in dosage similar to the ALG in Group I.

Group III: ten animals received the azo-mitochondrial complex and no other treatment.

The animals were fed on standard diets and 24-hr specimens of urine were collected at weekly intervals. Urinary protein estimations were carried out by the Weichselbaum (1946) biuret technique.

The animals were killed at 19 weeks and tissues were prepared for light and electron microscopy.

Light microscopy

Blocks of kidney were fixed in 10% formol–saline and embedded in paraffin. Four-micron sections were stained with haematoxylin and eosin, the periodic acid–Schiff (PAS) reaction and the methenamine silver method.

Electron microscopy

Blocks of cortex (1 mm^3) were fixed in gluteraldehyde (5% in cacodylate buffer) for $1\frac{1}{2}$ hr, washed in buffered sucrose for 2 days, post-fixed in Caulfields' osmium tetroxide fixative for 1 hr and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined on uncoated grids.

Fluorescent microscopy

Frozen cryostat sections of rat kidney were treated with fluorescein labelled rabbit anti-rat IgG and by the sandwich technique using rabbit anti-horse IgG and fluorescent sheep anti-rabbit γ-globulin. Appropriate controls for each test were included.

Antibody assays

Circulating antibodies to horse IgG were measured using tanned pyruvic aldehyde preserved sheep erythrocytes sensitized with an 0·1 g/100 ml solution of normal horse IgG

(Ling, 1961). Serial dilutions were performed using the Takatsky microtitrator. Each analysis included normal serum and standard antiserum controls as well as unsensitized sheep erythrocyte controls.

RESULTS

During the experimental period a number of animals died as a result of technical failures and this seriously depleted Group II. Nevertheless, recognizing these limitations several important points emerged and these are as follows:

Proteinuria

In these determinations a urinary protein excretion rate of greater than 10 mg/day was considered to be indicative of proteinuria. On this basis the onset and intensity of the

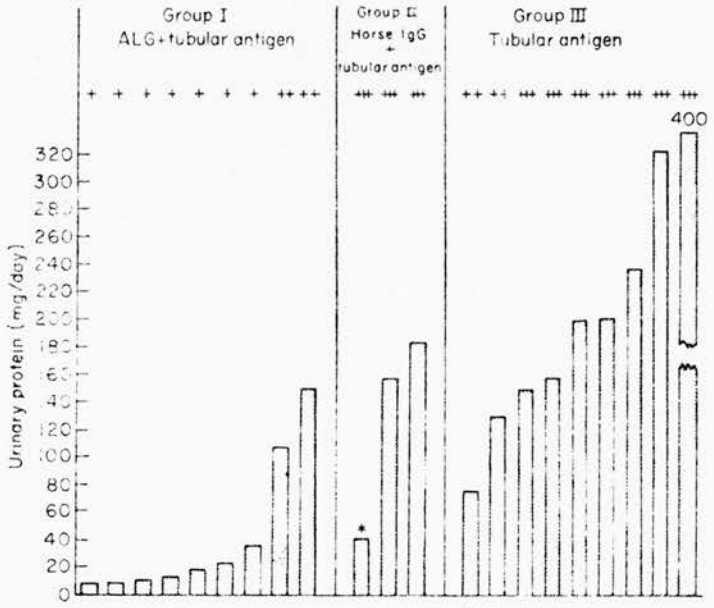

FIG. 1. Levels of proteinuria at the end of experiment, each column representing one animal. Most of the ALG treated animals had low urinary protein values. Grading of glomerular lesions by electronmicroscopy is indicated. + = one to two deposits per open capillary loop; + + = three to five deposits per open capillary loop; + + + = five deposits per open capilliary loop.
* This animal had proteinuria of 195 mg/day on the previous week.

proteinuria in ALG treated rats was less marked than in the other groups of animals studied. For example in the ALG treated group (Group I), one animal developed proteinuria at 8 weeks, one at 10 weeks, 4 at 15 weeks, and three had no proteinuria at 19 weeks. In contrast all the control animals (Groups II and III) had proteinuria within 8–9 weeks after the initial injection of tubular antigen and the level progressively increased, often reaching very high values. The level of proteinuria at the end of the experiment is shown for each animal in Fig. 1. It is evident that most of the ALG treated rats had considerably lower proteinuria than the controls.

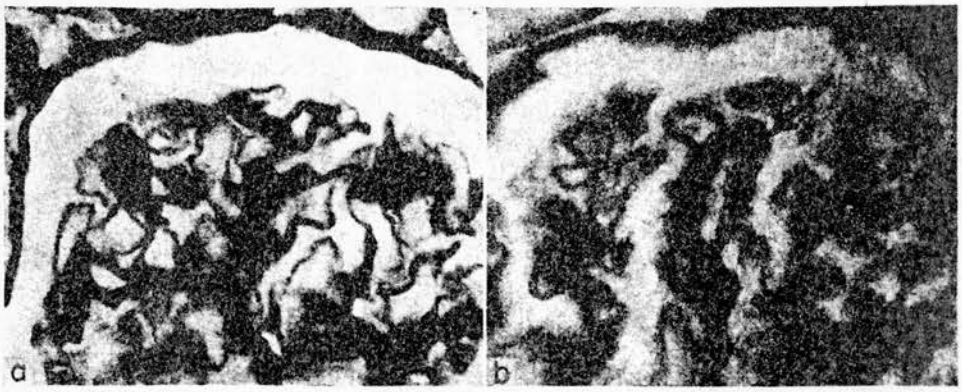

FIG. 2. (a) ALG treated animal, with no proteinuria (8 mg/day). Portion of glomerulus showing no histological abnormality. Methenamine silver, ×1080. (b) ALG treated animal, with proteinuria (108 mg/day). Portion of glomerulus with a few silver positive projections on the epithelial side of the basement membrane (arrow). Methenamine silver, ×1080.

Light microscopy

Significant histological differences between the ALG treated group (I) and the other groups (II and III) were observed by light microscopy. In the ALG treated group the picture was variable ranging from normal looking glomeruli in those animals with no proteinuria (Fig.2a) to moderately damaged glomeruli in those with more severe protein loss. In the latter animals the lesions were best shown by the methanamine silver stain, the capillary basement membrane being slightly thickened and a few silver positive projections being noted on its outer border (Fig. 2b). On the other hand the capillary loops remained patent and there was no cellular proliferation.

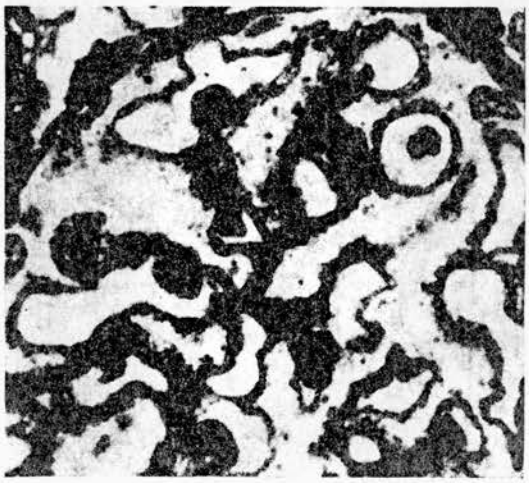

FIG. 3. Untreated animal with proteinuria (240 mg/day). Portion of glomerulus showing thickening of the capillary basement membrane and large numbers of silver positive projections on its outer surface. All animals in Groups II and III showed severe lesions. Methenamine silver, ×1200.

In contrast to this, all the animals in Groups II and III showed well developed lesions. With haematoxylin and eosin stains the capillary walls were thickened and irregular small deposits were noted on the outer border of the basement membrane with the PAS reaction. Furthermore, the methanamine silver stain revealed numerous silver positive projections on the epithelial side of the capillary basement membrane (Fig. 3). In rats with the highest level of proteinuria the lumina of the capillaries were sometimes reduced to narrow slits. As in the ALG treated group there was no evidence of cellular proliferation.

Electron microscopy

Although lesions were not detected by light microscopy in some of the ALG treated animals, characteristic lesions were observed in all animals by means of the electron microscope, even in those with no proteinuria. These changes were similar in quality in all groups but there were considerable quantitative differences.

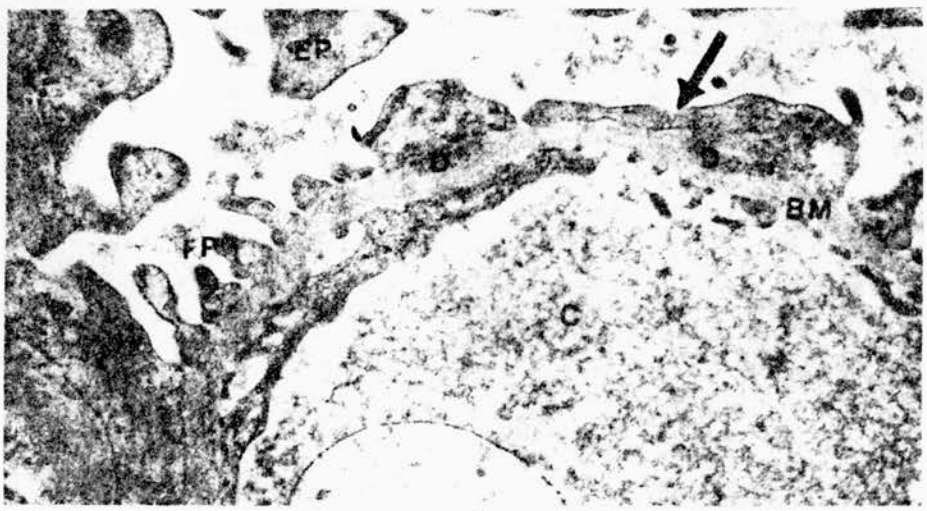

FIG. 4. ALG treated animal with no proteinuria (8 mg/day). Portion of a glomerular capillary loop showing occasional osmiophilic deposits (D). Some of the foot processes (FP) are preserved but in other areas are fused (arrow). EP, epithelial cell; BM, basement membrane; C, capillary lumen. ×6000.

In those animals in Group I in which proteinuria was not detected, the glomerular basement membrane was normal in thickness, an occasional deposit was present on the outer border of the basement membrane or rarely within the membrane (Fig. 4) and the foot process layer was preserved in most capillaries although occasional small area of fusion were seen. On the other hand, in the more proteinuric animals the number of deposits was increased and a greater loss of the foot process layer was observed. The cytoplasm of the epithelial cells often showed osmiophilia in the area adjacent to the deposits (Fig. 5) and in some areas small projections of basement membrane could be identified apparently in the process of surrounding the osmiophilic deposits. No changes were noted in the endothelial or axial cells.

However, all electron micrographs from Groups II and III showed more marked changes

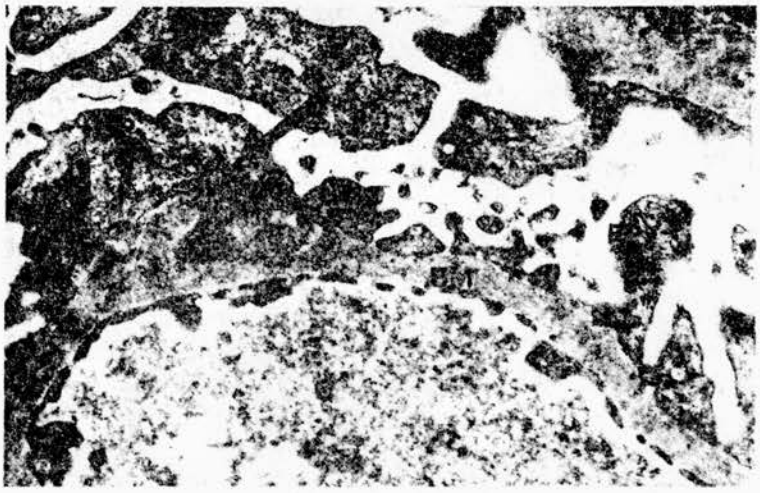

FIG. 5. ALG treated animal with proteinuria (35 mg/day). Portion of a glomerular capillary showing osmiophilic deposits (D) partially enclosed by spurs of basement membrane (BM). Occasional distorted foot processes (FP) are present. Osmiophilic areas are present in epithelial cytoplasm adjacent to deposits (arrow). C, Capillary lumen; EP, epithelial cell. ×20,000.

Large numbers of osmiophilic deposits were present on the outer borders of the basemen membranes and within the membranes. Numerous irregular projections of basement mem brane partially or completely surrounded the deposits (Fig. 6) There was extensive loss c the foot process layer and osmiophilia of the epithelial cytoplasm was more marked. A approximate evalution of the number of deposits is shown in Fig. 1 where a considerabl difference is evident between the ALG treated group and the untreated group.

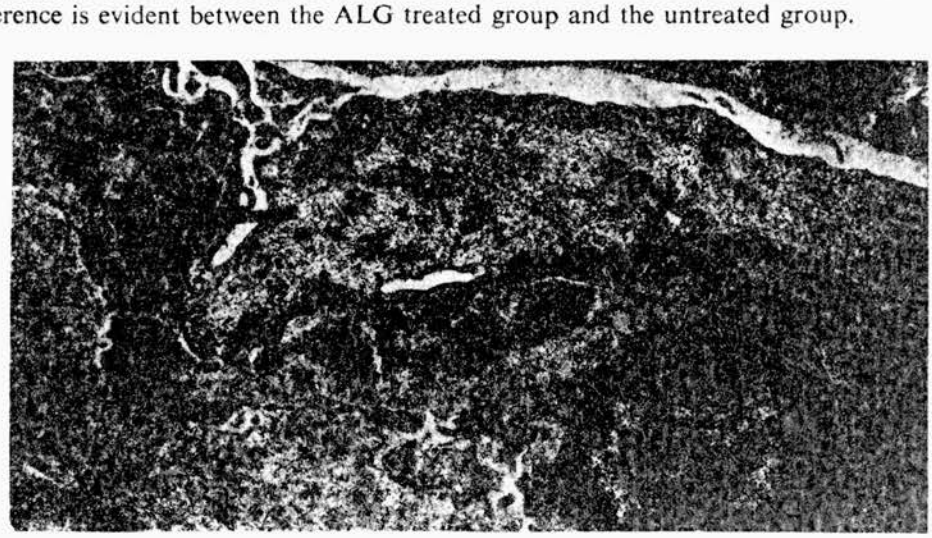

FIG. 6. Untreated animal with proteinuria (240 mg/day). Portion of a capillary showing extensive numbers of deposits (D). Projections of basement membrane appear to be surrounding some of them (arrows). There is complete loss of the discrete foot process layer. C, Capillary lumen; EP, epithelial cell; AX, axial cell. ×6000.

Fluorescent microscopy

Rat IgG was demonstrated in the kidneys of all animals. Its distribution was in a beaded fashion in relation to the glomerular capillary walls and varied in amount from animal to animal. In general those with heavy proteinuria had considerably more fluorescence than those with low proteinuria.

Horse γ-globulin was also demonstrated in all animals in Groups I and II, i.e. animals receiving normal horse IgG or horse ALG. The distribution of the fluorescence was uneven within glomeruli and varied from glomerulus to glomerulus. In some loops or even in parts of a loop there was intense diffuse fluorescence. In other regions of the glomerulus less intense fluorescence was sometimes noted.

Antibody determinations

Free circulating antibodies to horse IgG were observed in all the animals treated with normal horse and anti-lymphoytic IgG. These antibodies were detected within one week of starting the experiment and the titres rose sharply during the subsequent weeks remaining at high (though fluctuating) levels throughout the entire experimental period. There was some suggestion that the level of these antibodies in the ALG treated group was less than in the normal IgG treated group but because of the small number of animals in the latter group the true significance of these results is difficult to assess.

Attempts were also made to measure complement fixation against kidney homogenates but because of the high anti-complementary activity of the sera of animals treated with heterologous IgG the results could not be interpreted.

DISCUSSION

The anti-lymphocytic globulin treatment used in this experiment significantly delayed and reduced the proteinuria which results from the intraperitoneal injection of chemical modified homologous kidney mitochondria in complete Freund's adjuvant. It is possible, however, that proteinuria would have progressed to the same degree as in controls if the animals had been allowed to survive for a longer period and specially if ALG administration had been terminated.

The value of electron microscopy in a study of glomerular lesions is well illustrated by the fact that if light microscopy alone had been used many of the ALG treated animals would have been considered as normal. However, electron microscopy revealed that these animals exhibited lesions qualitatively similar to those observed in untreated groups. The sub-epithelial deposits noted in these experiments have also been observed in other animals and in human patients treated with anti-lymphocytic globulin (Monaco *et al.*, 1967; Traeger *et al.*, 1968; Starzl *et al.*, 1967; Iwasaki *et al.*, 1967). In contrast to previous observations no sub-endothelial deposits were observed in the present experiments (Starzl *et al.*, 1967; Iwasaki *et al.*, 1967).

The presence of rat IgG and horse IgG was demonstrated by fluorescent microscopy in the glomeruli of all animals receiving heterologous IgG. However, the distributions of the heterologous and autologous γ-globulin were different and the reasons for this have still to be ascertained. In this respect the influence of the immune complex disease and the autologous phase nephrotoxic nephritis which may accompany anti-lymphocytic antibody administration will have to be considered (Guttman *et al.*, 1967a, b).

The pathogenesis of this type of experimental autoimmune nephritis is not well understood. γ-Globulin, complement and renal tubular antigen have been demonstrated in the glomeruli and the disease is believed to be an example of immune complex nephritis, the source of the antigen being in the renal tubules. Edgington *et al.* (1968) have prepared a purified antigen, a lipoprotein, from the renal tubules and one injection of this is sufficient to produce the disease. It seems unlikely that this injected antigen would persist in sufficient quantity to permit the progressive disease to occur and the possibility of a change over to an autologous source of antigen has to be considered. In previous experiments (Barabas & Lannigan, 1969) it has been shown that a transient mild proteinuria occurs at around 3–4 weeks at a time when both specific anti-kidney IgG and IgM antibodies can be detected by a complement fixation test (Barabas, Elson & Weir, 1969). It is possible that antibody to the homologous antigen may be released into the lumen of the tubules during this phase of proteinuria and some of it be reabsorbed by the proximal convoluted tubules. Within the cytoplasm of tubular cells the antibody, presumably directed against the homologous antigen, may cross react with some component of the cell, producing damage with release of autologous tubular antigen and subsequent formation of autoantibody. Glassock *et al.* (1968) have shown that when tubular antigen prepared from human kidney is used to produce the disease in rats, a rat tubular antigen can be demonstrated in the glomerular lesions, a finding suggesting a switch from a heterologous to an autologous source of antigen. If this hypothesis is correct then ALG might be acting either by delaying the initial phase of antibody production to homologous antigen, or the second phase of autoantibody formation, or both.

Further experiments are in progress to determine if larger doses of ALG will prevent the development of the disease and to see if delay in the administration of ALG until the stage of initial transient proteinuria, will have a similar effect. The possibility of lesions induced by normal horse γ-globulin or anti-lymphocytic IgG is also being studied (Guttman *et al* 1967a, b).

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ANTI-LYMPHOCYTIC ANTIBODY—A REVIEW

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INTRODUCTION

The *in vitro* leucocytotoxic properties of antisera to lymphoid and other tissues have long been recognized, and most of the initial observations in this field have already been summarized (Gray *et al.*, 1966). These initial *in vitro* observations were extended by the work of Chew & Lawrence (1937) and Cruickshank (1941) who demonstrated that anti-lymphocytic sera could also produce a marked lymphopaenia *in vivo*. Stimulated by these findings and by the growing awareness of the involvement of lymphocytes in the homograft reaction, attempts were made to prolong homograft survival in rats with anti-lymphocytic serum, but were unsuccessful (Woodruff, 1960). In 1956 Interbitzen demonstrated that anti-lymphocytic serum could inhibit tuberculin sensitivity in guinea-pigs. These observations were confirmed and extended by Wilhelm, Fisher & Cooke (1958) and Waksman, Arbuoys & Arnason (1961). Later Woodruff & Anderson (1963, 1964) did achieve significant prolongation of skin homografts in rats using a rabbit antiserum to rat lymphocytes. During the last 2 years anti-lymphocytic antibody has been found to influence a wide number of immunological phenomena in a variety of species. These results indicate that anti-lymphocytic antibody has considerable potential both as an immunosuppressant and as a tool for investigating immunological phenomena. Because of the obvious importance of this reagent an attempt has been made to review this rapidly developing field.

THE PREPARATION OF ANTI-LYMPHOCYTIC SERUM

The present review is restricted to the more recently described anti-lymphocytic sera for these alone have been shown to exert a definite immunosuppressive effect. Such antisera have now been raised to a wide range of species (see Table 1). These include mouse, rat, guinea-pig, dog, monkey and human. A variety of immunizing antigens have been used to prepare these anti-lymphocytic sera. These include viable cell suspensions of lymph nodes, thymus and spleen, peripheral blood lymphocytes, thoracic duct lymphocytes and subcellular fractions of lymphoid and non-lymphoid origin.

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TABLE 1. A summary of effective anti-lymphocytic sera

Donor	Antigen	Recipient	System in which immunosuppression demonstrated	References
Mouse	Inguinal and axillary lymph node suspension in Freund's adjuvant	Rabbit	Skin homograft survival and humoral antibody formation	Gray *et al.* (1964, 1966) Monaco *et al.* (1966b)
	(1) Thymocytes (2) Fractioned thymocytes (3) Epidermal cells (4) L cells	Rabbit	Skin homograft survival	Levey & Medawar (1966a, b)
	Thymocytes	Horse	Skin homograft survival	James & Medawar (1967)
	Small lymphocytes from thymus and lymph nodes with complete Freund's adjuvant	Rabbit	Humoral antibody formation	Denman *et al.* (1967a, b)
	Inguinal and axillary lymph node suspension in Freund's adjuvant	Rabbit	Graft versus host reaction	Boak, Fox & Wilson (1967)
Rat	Thoracic duct lymphocytes	Rabbit	Skin homograft survival and antibody formation	Woodruff & Anderson (1963, 1964) Woodruff *et al.* (1967a) James & Anderson (1967) Anderson *et al.* (1967)
	Thoracic duct lymphocytes	Horse		James & Jubb (1967)
	Thymocyte or lymphocytes from mesenteric lymph nodes in complete Freund's adjuvant	Rabbit	Skin homograft survival	Nagaya & Sieker (1965)
	Mesenteric and thoracic lymph nodes	Rabbit	Skin homograft survival and humoral antibody formation in thymectomized rats	Jeejeebhoy (1965)
	Thymocytes	Rabbit	Renal plasma flow following renal homograft	Guttman *et al.* (1967)
	Thymocytes and mesenteric lymph nodes	Rabbit	No immunosuppressive studies but detailed account of *in vitro* and *in vivo* properties	Sacks *et al.* (1964)

Guinea-pig	Pooled lymph nodes	Rabbit	Tuberculin and contact allergic reactions delayed hypersensitivity to diphtheria toxoid and allergic encephalomyelitis	Waksman *et al.* (1961)
	Thymocytes and mesenteric lymph node cells	Rabbit	Lymphocyte transfer reaction	Levey & Medawar (1966b)
	Thymocytes and cell free extract guinea-pig lymph nodes	Rabbit	Contact sensitivity	Turk & Willoughby (1967)
Rabbit	Peripheral blood leucocytes	Rabbit	Transfer of anti-lymphocytic antibody treated sensitized cells to X-irradiated recipients	Harris & Harris (1966)
Dog	Thoracic duct lymphocytes	Horse	Renal homografts	Abaza *et al.* (1966)
	Mesenteric lymph node cells with adjuvant	Horse	Renal homografts	Monaco *et al.* (1966a)
	Lymph nodes and spleen	Horse	Renal homografts	Iwasaki *et al.* (1967)
	Spleen	Horse	Renal homografts	Iwasaki *et al.* (1967)
	Spleen	Horse	Renal homografts	Clunie & Nolan personal communication (1967)
Monkey	Thymocytes or lymphocytes	Monkey and rabbit	Skin homograft survival	Balner & Dersjant (1967)
Human	Peripheral blood lymphocytes	Rabbit	Lymphocyte transformation	Gräsbeck *et al.* (1964)
	Peripheral blood lymphocytes	Rabbit	Lymphocyte transformation	Holt *et al.* (1966)
	Thymus and spleen	Horse	Lymphocyte transformation	Woodruff *et al.* (1967a, b)
	Lymph nodes, thymus and spleen	Horse	Renal homografts	Iwasaki *et al.* (1967)
	Spleen	Horse		
	Lymph nodes	Rabbit	Skin homografts and delayed hypersensitivity	Monaco *et al.* (1967)

The antisera have been produced in a number of animals, the most commonly used being rabbit and horse. The antigen, dose and route of administration have varied considerably and some investigators have incorporated the antigen in adjuvant. Further details on most of the anti-lymphocytic sera recently described are summarized in Table 1 together with the appropriate references.

THE IMMUNOSUPPRESSIVE PROPERTIES OF ANTI-LYMPHOCYTIC SERA

Skin homograft survival

Although attempts had previously been made to prolong skin homograft survival with anti-lymphocytic serum (Waksman *et al.*, 1961) the first really significant results were those reported by Woodruff & Anderson (1963, 1964). They were able to demonstrate prolonged survival of skin homografts from an inbred albino strain on an inbred strain of hooded rats, following treatment with rabbit antiserum to thoracic duct lymphocytes from hooded rats. This treatment was most effective if commenced prior to grafting. Similar observations have since been reported by Nagaya & Sieker (1965) who also found that antiserum to thymocytes was more effective than antiserum to mesenteric lymph nodes. Furthermore, Jeejeebhoy (1965) described an anti-lymphocytic serum which was ineffective on its own, but in conjunction with thymectomy exhibited immunosuppressive properties.

The results originally obtained with rats have since been observed in mice (Gray, Monaco & Russell, 1964; Monaco *et al.*, 1966b; Levey & Medawar, 1966a, b). These investigators have also reported that anti-lymphocytic serum also causes considerable prolongation of second-set skin grafts and heterografts (Levey & Medawar, 1966a; Monaco *et al.*, 1966b).

Recent studies have also indicated that anti-lymphocytic antibody is capable of prolonging skin homograft survival in monkeys (Balner & Dersjant, 1967) and in humans (Monaco *et al.*, 1967).

Renal transplants

Anti-lymphocytic antibody alone has been demonstrated to delay the rapid destruction of renal homotransplants in both rats (Guttman *et al.*, 1967) and dogs (Abaza *et al.*, 1966; Monaco *et al.*, 1966a; Clunie & Nolan, 1967, personal communication). Investigations in humans with renal transplants, indicate that anti-lymphocytic antibody in conjunction with greatly reduced doses of steroids and other immunosuppressants can effectively maintain normal kidney function (Iwasaki *et al.*, 1967; Starzl *et al.*, 1967).

Humoral antibody formation

Anti-lymphocytic antibody has now been shown to suppress humoral antibody formation to a variety of antigens in a number of species. Heterologous anti-lymphocytic sera have been used to suppress the primary immune response of both mice (Gray *et al.*, 1964; Monaco *et al.*, 1966b; Denman, Denman & Holborow, 1966) and rats to sheep erythrocytes (Currey & Ziff, 1966; Woodruff *et al.*, 1967a; James & Anderson, 1967). Anti-lymphocytic antibody is also capable of suppressing the primary response of mice to *Salmonella* II antigen incorporated into Freund's adjuvant (Gray *et al.*, 1964) and of rats to alum precipitated bovine serum albumin (Woodruff *et al.*, 1967a; James & Jubb, 1967) and to diphtheria toxoid preparations (Waksman *et al.*, 1961; Parke, 1967 personal communication).

Harris & Harris (1966) have also demonstrated that rabbit anti-rabbit leucocyte serum is capable of inactivating sensitized lymph node suspensions; X-irradiated rabbits receiving such treated cells were unable to produce antibody following subsequent challenge with the original sensitizing antigen. It would appear, at least in some systems, that the anti-lymphocytic antibody is only effective if given prior to challenge with the antigen (James, 1967).

The results to date on the effect of anti-lymphocytic antibody on humoral antibody formation in sensitized animals, indicate that this material does not have such a marked effect on the secondary response (Gray *et al.*, 1964; Monaco *et al.*, 1966b; James & Anderson, 1967; James & Jubb, 1967). However, it has been suggested that anti-lymphocytic antibody may suppress the secondary response of rats to soluble diphtheria toxoid (Parke, 1967 personal communication).

Heterologous anti-lymphocytic globulin is in itself an antigen. Investigations have, therefore, been performed to determine if this material could suppress humoral antibody formation against itself or against globulin preparations from non-immunized members of the same species in which the antiserum was produced. Although Gray *et al.* (1966) have reported that mice treated with rabbit anti-lymphocytic globulin make antibody to rabbit IgG less readily than those receiving normal rabbit IgG, other investigators have failed to observe this. Currey & Ziff (1966) found increased levels of antibodies to rabbit globulins in rats which had been pretreated with rabbit anti-rat lymphocyte globulins. Other investigators have also observed the rapid immune elimination of isotopically labelled normal and anti-lymphocytic IgG preparations in animals which have received intensive courses of treatment with anti-lymphocytic antibody (Guttman *et al.*, 1967; Clark, James & Woodruff, 1967; Lance & Dresser, 1967). Furthermore, Iwasaki *et al.* (1967) have demonstrated precipitin formation in the sera of dogs receiving chronic treatment with anti-lymphocytic globulin and have also observed the deposition of immune complexes in transplanted kidneys.

The effect on other immune phenomena

Interbitzen (1956) and Waksman *et al.* (1961) both found that anti-lymphocytic serum suppressed the tuberculin reaction in guinea-pigs. Waksman *et al.* (1961) also demonstrated that anti-lymphocytic serum suppressed contact allergy to 2,4 dinitrochlorobenzene, the delayed skin reaction to purified diphtheria toxoid and exerted a relative effect on allergic encephalomyelitis. The material slightly reduced the non-specific reaction to turpentine and dinitrochlorobenzene but had no effect on either passive cutaneous anaphylaxis or the reversed passive Arthus reaction. Turk & Willoughby (1967) have demonstrated that antithymic serum is able to suppress both specific and non-specific aspects of the contact allergic reaction in guinea-pigs. Recent reports also indicate that anti-human lymphocytic sera will prevent the expression of previously established delayed hypersensitivities in humans to a number of antigens including trychophyton and mumps (Starzl *et al.*, 1967; Monaco *et al.*, 1967).

Levey & Medawar (1966a) have found that anti-lymphocytic serum completely inhibits all components of the lymphocyte transfer phenomenon in guinea-pigs. In addition Boak, Fox & Wilson (1967) have demonstrated that lymphoid tissue from mice treated with anti-lymphocytic serum was unable to induce a graft versus host reaction in F_1 hybrid recipients.

Anti-lymphocytic serum also appears to affect a number of other immune phenomena. It is effective in suppressing Freund adjuvant polyarthritis in rats (Currey & Ziff, 1966)

and Coombs positive haemolytic anaemia in NZB mice (Denman, Denman & Holborow, 1967a). However, it fails to suppress the onset of renal disease in B/W mice (NZB × NZW)F_1 (Denman *et al.*, 1966). Recent reports indicate that anti-lymphocytic serum will also protect mice from the lymphocytic choriomeningitis virus (Gledhill, 1967) and reduce the inflammatory lesions resulting from acute bacterial infection (Morris & Burke, 1967).

THE POTENTIATION OF THE IMMUNOSUPPRESSIVE EFFECT OF ANTI-LYMPHOCYTIC ANTIBODY

The immunosuppressive effect of anti-lymphocytic antibody has been potentiated by a number of procedures as previously noted (Woodruff, 1967). The effective prolongation of skin homografts in rats has been increased by lymphoid depletion through a thoracic duct fistula, prior to grafting (Woodruff & Anderson, 1963, 1964). Jeejeebhoy (1965) described an anti-lymphocytic serum which was effective only in thymectomized rats. A variety of procedures have been used in attempts to improve the effect of anti-lymphocytic serum on skin homograft survival in mice. The results on the effect of thymectomy have been conflicting. Monaco, Wood & Russell (1965) observed potentiation of the anti-lymphocytic effect whilst Levey & Medawar (1966b) failed to confirm this. However, as suggested by Jeejeebhoy (1965) and Woodruff (1967) this might be due to differences in experimental protocols. Nevertheless, Levey and Medawar were able to potentiate graft prolongation by whole body irradiation before administration of anti-lymphocytic antibody (Levey & Medawar, 1966a) and by administration of hydrocortisone acetate (Levy & Medawar, 1966b). However, whole body irradiation, following anti-lymphocytic antibody treatment, and adrenalectomy both diminished the ability of this material to prolong skin homograft survival (Levey & Medawar, 1966b).

THE EFFECT OF ANTI-LYMPHOCYTIC ANTIBODY ON LYMPHOCYTES AND LYMPHOID TISSUE *IN VIVO* AND *IN VITRO*

All reports to date indicate that so called anti-lymphocytic antibody agglutinates lymphocytes *in vitro* and in the presence of complement causes lysis of such cells. The specific uptake of anti-lymphocytic antibody has been demonstrated by immunofluorescent (Woodruff, Anderson & Abaza, 1966) and isotopic procedures (Levey & Medawar, 1966b; Woodruff, Reid & James, 1967b). Many of the antisera have also been shown to react strongly with donor erythrocytes, serum and tissue proteins (Sacks, Filippone & Hume, 1964; Gray *et al.*, 1966). It would appear, however, that absorption with donor strain erythrocytes or serum does not affect the anti-lymphocytic properties of such preparations. Woodruff *et al.* (1966) have also reported that anti-lymphocytic antibody exhibits a considerable degree of strain and cell specificity as determined by cytotoxicity. It should be noted that Levey & Medawar (1966b) have reported that antisera to non-lymphoid tissues are also capable of prolonging homograft survival and presumably react with lymphocytes.

As well as agglutinating lymphocytes and causing their lysis (in the presence of complement), anti-lymphocytic antibody has been shown to induce blast transformation and mitosis of human lymphocytes *in vitro*. Blast transformation induced by intact anti-lymphocytic antibody is only observed in the absence of complement and it is difficult to demonstrate

in other species (e.g. rat and mouse) due to problems of culturing these lymphocytes *in vitro*. The transformation of lymphocytes has been demonstrated by morphological examination of cultures (Gräsbeck, Nordman & de la Chapelle, 1964) and by the incorporation of isotopically labelled (^{3}H and ^{14}C) uridine and thymidine into lymphocyte RNA and DNA, respectively (Holt, Ling & Stanworth, 1966; Woodruff *et al.*, 1967a; Woodruff, Reid & James, 1967c). Further details on the transforming properties of Sephadex G-200 fractions of anti-lymphocytic serum and an isolated IgG and IgG fragments will be discussed later.

The reported *in vivo* effects of anti-lymphocytic antibody have been somewhat inconsistent. This is no doubt due to the differences in immunization schedules used in producing the antisera, the courses of anti-lymphocytic treatment, the ability of various antibodies to fix complement, as well as possible species and strain differences (Iwasaki *et al.*, 1967).

Levey & Medawar (1966a) have shown that in CBA mice a single injection of their anti-lymphocytic serum caused only a moderate and transient fall in the lymphocyte count with no apparent histological changes in the thymus. Following chronic treatment with anti-lymphocytic serum blast transformation of peripheral blood lymphocytes was observed together with hypertrophy of lymphoid tissue (Levey & Medawar, 1966a). On the other hand, Gray *et al.* (1966) observed that a single, much smaller dose of their material (raised using adjuvant), caused a profound and sustained lymphopaenia. Prolonged treatment caused depletion of most lymphoid tissues. The thymuses were depleted of lymphoid cells and the Peyer's patches showed decreased numbers of lymphocytes. The most pronounced changes were observed in the lymph nodes and the spleen where there was a marked diminution in the number and size of germinal centres and evidence of tissue necrosis.

Turk & Willoughby (1967) have also described in some detail the effect of their anti-thymocyte serum and anti-lymph node permeability factor (anti-LNPF) serum on lymphoid tissue. In guinea-pigs prolonged treatment (6 days) with the anti-thymocyte serum caused a 90% drop in circulating small lymphocytes whilst similar treatment with their anti-LNPF produced only a 30% drop. The central effect of the anti-thymocyte serum was limited to the paracortical area or thymus dependent area of the lymph nodes, where small lymphocytes were absent. There was no depletion of lymphocytes from lymphoid follicles, or plasma cells at the cortico-medullary junction and in the medullary cords. Both antisera had the same effect on large pyroninophilic cells. The number of these cells in the paracortical areas of the draining lymph nodes was reduced 4 days after sensitization with oxazolone although mitosis was still observed. There was no evidence of lymphoid hypertrophy after treatment with the anti-thymocyte serum. However, evidence of lymphoid hyperplasia was found in the paracortical and germinal centres of the mesenteric lymph nodes following intraperitoneal injection of normal rabbit serum and the anti-LNPF serum. Finally they also observed a pronounced depletion of lymphocytes in the thymus cortex of animals receiving anti-thymocyte serum.

Several investigators have reported that rabbit anti-sera to rat lymphocytes and lymphoid tissue cause a substantial fall in the peripheral blood lymphocyte count. This depression was not always maintained, however, during prolonged treatment (Woodruff & Anderson, 1964; Sacks *et al.*, 1964; Nagaya & Sieker, 1965; Anderson, James & Woodruff, 1967). Nagaya & Sieker (1965) have demonstrated that antiserum to thymus may produce a more effective lymphopaenia than antiserum to mesenteric lymph nodes. Histological examination of rat spleens following 7 days' treatment with anti-lymphocytic antibody has shown

them to be enlarged with few malphigian bodies which were depleted of lymphocytes. The red pulp of the spleen was packed with histiocytes and large pyroninophilic cells whilst the lymph nodes showed some depletion (Woodruff & Anderson, 1964).

In dogs the results have again been conflicting. Iwasaki *et al.* (1967) observed lymphopaenia in their chronically treated dogs but this was not as marked, or as sustained as that observed by Monaco *et al.* (1966a) who again used Freund's adjuvant when raising their antisera. Marked differences were also observed on histological investigation. Iwasaki *et al.* (1967) observed that the lymphocytes of lymphoid tissue were replaced by large and medium sized cells with pyroninophilic cytoplasm and germinal centre formation was common. In contrast Monaco *et al.* (1966a) found a marked depletion of both large and small lymphocytes and germinal centres were totally absent from lymph nodes and spleen. In addition the spleens were markedly reduced in weight with depletion. However, only slight depletion of the Peyer's patches was noted. The studies in humans with anti-lymphocytic serum have naturally been very limited but this material has been observed to cause a fall in peripheral blood lymphocyte count (Iwasaki *et al.*, 1967). A number of laboratories are now investigating the tissue distribution of anti-lymphocytic antibody using radioisotopic and fluorescent procedures for investigations such as these may be of importance in relation to anticipated therapeutic applications. Hintz & Webber (1965) have already shown that their ^{131}I-labelled anti-mouse thymus IgG reacted strongly with thymus gland, but also showed some reactivity with lymph nodes, bone, striated muscle and the gastrointestinal tract.

THE CHARACTERIZATION OF ANTI-LYMPHOCYTIC ANTIBODY

Several investigators have shown that crude immune globulin preparations obtained by ammonium sulphate precipitation of anti-lymphocytic sera are capable of causing lymphocyte destruction and immunosuppression (Waksman *et al.*, 1961; Monaco *et al.*, 1966b; Curry & Ziff, 1966; Denman *et al.*, 1967a). Furthermore, investigations using Sephadex G-200 fractions of a variety of anti-lymphocytic sera and purified IgG preparations have shown that the major part of the anti-lymphocytic activity in many sera is located in the IgG fraction (see Table 2). The erythrocyte agglutinins, on the other hand, were predominantly of the IgM type. However, it should be stressed that these observations were obtained with a limited number of antisera and it is possible that in other antisera the major part of the activity may be associated with immunoglobulins other than IgG. The exact distribution no doubt depends upon all the factors known to influence antiserum production. However, the results do indicate that gel filtration on Sephadex G-200 (or gradient ultracentrifugation) may provide valuable data on which subsequent absorption and fractionation procedures can be designed and the possible advantages of using fractionated material have previously been stressed (James & Anderson, 1967). As well as overcoming the need for extensive absorption with erythrocytes to remove haemagglutinins, it also avoids the administration of large amounts of inactive extraneous protein. Furthermore, recent absorption studies with ^{131}I-labelled IgG suggest the need for more sophisticated fractionation procedures for more than 95% of the IgG in our preparations failed to combine with lymphocytes (Woodruff *et al.*, 1967b) and presumably was ineffective as an immunosuppressant.

TABLE 2. The molecular characteristics of anti-lymphocytic antibody

Antiserum		Property tested	Sephadex G-200 fractions				IgG pre-parations	References
Donor	Recipient		19S	'10S'	7S	4SS		
Mouse	Rabbit 1	Prolongation of tail skin homografts	T	T	+++	T		James & Medawar (1967)
	Rabbit 2		T	T	+++	T	+++	
	Horse		T	T	+++	T	+++	
Rat	Rabbit 1	Lymphoagglutination and lympho-cytotoxicity	T		+++	—		James & Anderson (1967)
	Rabbit 2		T		+++	—		
	Rabbit	Homograft survival					+++	Anderson *et al.* (1967)
	Horse	Lymphoagglutination and lympho-cytotoxicity	+++	++	+++	—		James & Anderson (1967)
	Horse	Erythrocyte agglutination	+++	+	T	T		
	Horse	Homograft survival					+++	Anderson *et al.* (1967)
	Rabbit	Effect on renal plasma flow of transplanted kidney					+++	Guttman *et al.* (1967)
	Horse	Humoral antibody formation (a) Sheep erythrocytes					+++	James & Anderson (1967)
		Humoral antibody formation (b) Alum precipitated bovine serum albumin					+++	James & Jubb (1967)
Human	Horse	Lymphoagglutination	T	+	+++	—	+++	Woodruff *et al.* (1967)
		Lymphocytotoxicity	T	+	+++	—	+++	
		Erythrocyte agglutination	+++	+	+	—	+	
		Lymphocyte transformation (a) RNA synthesis	+	+	+++	+	+++	
		Lymphocyte transformation (b) DNA synthesis	+	+	+++	+	+++	
	Horse	Renal homograft survival					+++	Iwasaki *et al.* (1967)
Human	Rabbit	Skin homograft survival and inhibition of delayed hypersensitivity reactions					+++	Monaco *et al.* (1957)

Note: All the Sephadex G-200 fractions (except '10S') were concentrated to the original serum volume prior to assay. The '10S' fraction was concentrated to half the original serum volume. In the studies reported from our laboratories the IgG concentration was similar to that in the original antiserum (approximately 1 g/100 ml).

* DEAE cellulose chromatography also revealed leucoagglutinating antibody with β-globulin mobility presumably IgM or IgT.

THE IMMUNOSUPPRESSIVE AND LYMPHOCYTE TRANSFORMING PROPERTIES OF ANTI-LYMPHOCYTIC ANTIBODY FRAGMENTS

The Porter model of the structure of IgG globulin is now well established (Porter, 1963). Based on this model it is accepted that pepsin digestion (Nisonoff *et al.*, 1960) results in the partial degradation of the Fc portion of the molecule. This region of the molecule is responsible for complement binding, skin attachment, membrane transmission and contains the major species specific antigenic determinants. The remaining $F(ab')_2$ portion contains both the antibody combining sites and hence can combine with and agglutinate antigen, in this case the lymphocyte. However, as pepsin treated IgG lacks the complement binding Fc portion it is non-cytotoxic. The univalent Fab′ moiety, on the other hand, obtained by reducing and alkylating the $F(ab')_2$ fragment is capable of binding to the lymphocyte but neither agglutinates or lyses this cell.

TABLE 3. The immunosuppressive and lymphocyte transforming properties of anti-lymphocytic antibody fragments

Antibody preparation	Effect on lymphocytes *in vivo*		Test system	Effect	Reference
	Agglutination	Lysis			
Intact IgG	+	+	Prolongation of skin homografts	+	Anderson *et al.* (1967)
$F(ab')_2$	+	−	in rats	−	
Fab′	−	−		−	
Intact IgG	+	+	Inhibition of primary response	+	James & Anderson
$F(ab')_2$	+	−	of rats to sheep erythrocytes	−	(1967)
Fab′	−	−		−	
Intact IgG	+	+	Inhibition of primary response	+	James (1967)
$F(ab')_2$	+	−	of rats to alum precipitated	−	
$F(ab')_2$*	+	−	bovine serum albumin	−	
Intact IgG	+	+	*In vivo* transformation of human	+	Woodruff *et al.*
$F(ab')_2$	+	−	lymphocytes	+	(1967c)
Fab′	−	−		−	

All the preparations described above were obtained from horse antisera with the exception of * which was obtained from a rabbit antiserum. In all the studies similar amounts of intact antibody and antibody fragments were used.

On the basis of this model, studies were performed with antibody fragments in an attempt to gain a better understanding of the mode of action of this material and with the aim of obtaining products with distinct therapeutic advantages (less antigenic). The ability of antibody fragments to prolong homograft survival and inhibit humoral antibody formation in rats has been studied. In addition the lymphocyte transforming properties of antibody fragments from horse anti-human lymphocyte IgG have been investigated. The results have been summarized in Table 3 together with the appropriate references.

From Table 3 it will be observed that intact anti-lymphocytic IgG alone could prolong

homograft survival or inhibit the primary response of rats to sheep erythrocytes or alum precipitated bovine serum albumin. As would be expected, the intact molecule alone exhibited lymphocytotoxic properties. It should also be noted that papain fragments of rabbit anti-rabbit leucocyte antibody failed to suppress humoral antibody formation as demonstrated *in vitro* by the antibody plaque test (Harris & Harris, 1966).

In vitro studies with antibody fragments to human lymphocytes indicated that the non-agglutinating Fab′ moiety did not cause transformation whilst the agglutinating (non-cytotoxic) $F(ab')_2$ portion was effective. Sell (1967) however has shown that both the $F(ab')_2$ and Fab fragments of sheep antibody to rabbit IgG will transform rabbit lymphocytes. The importance of these observations in relationship to the mode of action of anti-lymphocytic antibody will be discussed in the next section.

MODE OF ACTION OF ANTI-LYMPHOCYTIC ANTIBODY

A number of hypotheses have been postulated to explain the immunosuppressive properties of anti-lymphocytic antibody. These have been discussed in some detail by Levey & Medawar (1966a, b) and Woodruff (1967). The conclusion of Levey & Medawar (1966a) that no one hypothesis adequately explains the mode of action and that no theories are mutually exclusive is still basically correct. Indeed it is accepted that the mode of action may vary from experiment to experiment being dependent on the factors previously discussed (page 621) as well as on the nature of the antigenic stimulus. Nevertheless, an attempt has been made to discuss the major theories proposed to date in the light of available knowledge. These are as follows:

The cytotoxic theory

The original presumption was that anti-lymphocytic antibody acted by causing a marked reduction in peripheral blood lymphocytes with or without causing depletion of lymphoid tissue. However, it has now been shown that the immunosuppression achieved is not dependent upon a marked or sustained lymphopaenia (Levey & Medawar, 1966a, b; Woodruff, 1967; Iwasaki *et al.*, 1967; James & Anderson, 1967). Moreover the degree of immunosuppression achieved by chronic drainage through a thoracic duct fistula is not as marked as that produced by anti-lymphocytic antibody. Nevertheless, the failure to suppress humoral antibody formation or prolong homograft survival in rats with non-cytotoxic antibody fragments suggests that a certain degree of cytotoxicty may be important (James & Anderson, 1967; Anderson *et al.*, 1967; Woodruff *et al.*, 1967; James, 1967). Additional support for the cytotoxicity concept is also suggested by the work of Harris & Harris (1966). However, as previously stated, immunosuppression may not be dependent upon gross destruction of lymphocytes but could be achieved by the selective destruction, preferably of lymphocytes in a state of readiness to undertake immune responses (Levey & Medawar, 1966a; Monaco *et al.*, 1966b; James & Anderson, 1967; James & Jubb, 1967).

In contrast to the cytotoxic theory there are several alternative theories which are based upon the ability of anti-lymphocytic antibody to combine with lymphoid tissue without producing lysis. That such coating of lymphocytes does indeed occur has been demonstrated *in vitro* by fluorescent and isotopic procedures (Woodruff *et al.*, 1966; Levey & Medawar, 1966a; Woodruff *et al.*, 1967b). The theories referred to are known as the blindfolding, the competitive antigen and the sterile inactivation theories and they overlap to some extent.

The blindfolding theory

According to this theory the anti-lymphocytic antibody coats lymphocytes in such a way so as to occlude (prevent access to) their combining sites or recognition units (Levey & Medawar, 1966a). Thus the coated ('blindfolded') lymphocyte is unable to recognize the antigen or respond to the appropriate immune stimulus from the macrophage.

The failure of antibody fragments [$F(ab')_2$ and Fab'] to cause immunosuppression would appear to be irreconcilable with a 'blindfolding' theory, whether of lymphocytes or graft (see below). The fragments described have been shown to bind firmly to lymphocytes *in vitro*. Furthermore the suggestion of Levey & Medawar (1966b) that the descendants of affected cells remain unreactive for one or more cell generations would also suggest that 'blindfolding' of lymphocytes is not a major cause of immunosuppression. Guttman *et al.* (1967) have suggested another variant of the 'blindfolding' theory which involves coating of the graft tissue. This is believed to occur as the lymphocyte and graft share histocompatibility antigens. According to this hypothesis the graft bound antibody could mask histocompatiblity antigens, prevent lymphocyte sensitization and/or contact, and could also bind antigen released from the kidney. Thus the operative mechanism would be similar to immunological enhancement. The observations that anti-lymphocytic antibody cross reacts with a number of tissues, including kidney would lend support to such a hypothesis in relationship to homograft survival (Sacks *et al.*, 1964; Levey & Medawar, 1966b; Iwasaki *et al.*, 1967). It would not, however, explain the ability of anti-lymphocytic antibody to suppress humoral antibody formation against antigens such as alum precipitated bovine serum albumin. In addition it would not account for the failure of antibody fragments to prolong skin homograft survival (Anderson *et al.*, 1967).

The competitive antigen theory

This theory is based upon the rapid and preferential uptake of anti-lymphocytic antibody by lymphoid tissues. It may be safely assumed that antibody to lymphocytes is quickly taken up by lymphoid cells. As the anti-lymphocytic antibody is itself potentially antigenic, it preoccupies, and indeed may sensitize, lymphoid cells. Such preoccupied cells would be unable to respond to subsequent stimuli with other less favoured antigens, i.e. non-anti-lymphocytic antibody. In contrast to the blindfolding theory it involves the specific immunological commitment of the lymphoid cells rather than a non-specific prevention of antigenic stimulation by impeding access of antigen. Levey & Medawar (1966a) believe that the chief obstacle to this theory is the ability of the antisera to nullify the action of sensitized cells; however, this property does not appear to be a universal one (James & Anderson, 1967; James & Jubb, 1967). Furthermore, on the basis of this theory one might also have expected antibody fragments to exert some effect, even although they are less antigenic.

Although the competitive antigenic theory does not satisfactorily explain the ability of anti-lymphocytic antibody to suppress humoral antibody formation or prolong homograft survival, it may explain the apparent inability of this material to effectively suppress humoral antibody against itself or against normal IgG obtained from the species in which the antisera was produced (Currey & Ziff, 1966; Guttman *et al.*, 1967; Iwasaki *et al.*, 1967; Clark *et al.*, 1967). Lymphocytes coated with anti-lymphocyte antibody will presumably be aggregated, lysed or opsonized and so prone to phagocytosis by macrophages, thus sensitizing the recipient to lymphocyte bound IgG (or other immunoglobulins). It is possible that

the therapeutic problem of humoral antibody formation against anti-lymphocytic IgG (and its normal IgG content) might be overcome by administering larger doses of high titre anti-lymphocytic antibody and by use of conventional immunosuppressants. It should also be stressed that phagocytosis of lysed and non-lysed (opsonized) cells following anti-lymphocytic antibody treatment could possibly explain the immunosuppressive properties of this material.

The sterile inactivation theory

The observation that chronic anti-lymphocytic antibody treatment may result in lymphoid hypertrophy accompanied by the formation of blast cells (Levey & Medawar, 1966b) has led these investigators to propose the 'sterile inactivation' theory. According to this theory anti-lymphocytic antibody causes a generalized sterile activation of lymphoid cells, which forestalls or supplants all other immunological commitments. This may or may not be analogous to the transformation produced by phytohaemagglutinin. The transformation of the lymphocytes is believed to result in a loss of immunological potential. The ability of anti-leucocytic sera to transform human lymphocytes *in vitro* would be in agreement with such a hypothesis (Gräsbeck *et al.*, 1964; Holt *et al.*, 1966; Woodruff *et al.*, 1967a; Reid & James, 1967c). However, Woodruff *et al.* (1967c) have shown that the divalent non-cytotoxic $F(ab')_2$ fragment will also transform lymphocytes. Similar fragments of anti-rat lymphocyte antibody do not inhibit the immune response in rats (Table 3). Assuming one can extrapolate from species to species it would seem that transformation of lymphocytes is not the sole answer. Furthermore, it should be mentioned that lymphoid hyperplasia and blast cell formation is not always observed in animals treated with anti-lymphocytic antibody (Gray *et al.*, 1966).

Other theories to explain the mode of action of anti-lymphocytic serum are as follows:

Anti-lymphocytic antibody acts through the thymus

Investigations have shown that anti-serum to thymus cells is a more effective immunosuppressant than antiserum raised against other lymphoid tissue (Nagaya & Sieker, 1965). This has led Levey & Medawar (1966a) to suggest that anti-lymphocytic antibody might be working by neutralizing the thymus humoral factor. They concluded that the action on the thymus may be on event in a complex and many sided action.

Anti-lymphocytic antibody antagonizes the lymph node permeability factor

Turk & Willoughby (1967) have recently discussed the effect of anti-thymocytic serum on the central and peripheral aspects of cell mediated immunity. The peripheral effect is shown by the suppression of the inflammation associated with delayed hypersensitivity in guinea-pigs, and is not entirely specific. They suggest that the peripheral effect of anti-lymphocytic antibody could be due to the antagonism of the lymph node permeability factor described by Willoughby, Spector & Boughton (1964). Morris & Burke (1967) have suggested that such a property might explain the ability of anti-lymphocytic antibody to diminish the development of acute lesions following bacterial infection. The central effect is believed to be specifically directed against the area of lymphoid tissue concerned in the development of specific cell mediated immunity (delayed hypersensitivity).

FURTHER COMMENTS ON THE MODE OF ACTION OF ANTI-LYMPHOCYTIC ANTIBODY

Whatever the mode of action of anti-lymphocytic antibody it is evident that this material alone does not produce tolerance although it may do so in conjunction with thymectomy (Monaco, Wood & Russell, 1966c). It has been shown that rats treated with anti-lymphocytic antibody eventually recover and respond to alum precipitated bovine serum albumin (James & Jubb, 1967) and that rats and mice recover their capacity to reject skin homografts (Levey & Medawar, 1966a; Monaco *et al.*, 1966b; Woodruff, 1967). This indicates that the treated animals recover their immunological capacity, presumably as a result of the development of immunologically competent cells. In this respect Gray *et al.* (1966) observed that the lymph nodes and spleen of mice receiving chronic treatment with anti-lymphocytic antibody showed evidence of recovery within 2 weeks of terminating this treatment. These organs showed evidence of repopulation with lymphoid cells and the appearance of germinal centres. Thus it would appear that one of the major effects of anti-lymphocytic antibody is one of delaying the immune response.

The work on humoral antibody formation in rats (James, 1967) suggests that anti-lymphocytic antibody is probably affecting the sensitizing or triggering phase of the immune response. This has been demonstrated in experiments in which anti-lymphocytic antibody was administered shortly after antigen and was found to be ineffective. The observation that anti-lymphocytic antibody prolongs homograft survival in mice even when administered after grafting (Levey & Medawar, 1966a, b) is probably due to the delay in antigenic stimulation in this system.

Further differences in the effect of anti-lymphocytic antibody on humoral and cellular immune processes have also been reported in relation to secondary stimulation. Levey & Medawar (1966a, b) and Monaco *et al.* (1966b) have shown that anti-lymphocytic antibody delays the rejection of second set grafts. Anti-lymphocytic antibody, however, does not have a profound effect on the secondary humoral antibody response (Monaco *et al.*, 1966b; James & Anderson, 1967; James & Jubb, 1967). However, it is recognized that much larger doses might cause significant suppression (Monaco *et al.*, 1966b) and that the doses previously used might be effective with other antigens. As previously suggested the differences between the effect on homograft survival and humoral antibody formation is probably due to time differences in antigenic stimulation and also to possible differences in immune mechanisms. A number of theories have been proposed to explain the absence of a profound effect on the secondary humoral antibody response. Monaco *et al.* (1966b), suggested that it may be due to a decreased susceptibility to anti-lymphocytic antibody of the lymphocytes involved in the secondary response, or simply due to the greater number of cells involved in this process. Denman *et al.* (1967a), on the other hand, have suggested that the anti-lymphocytic antibody has a limited access to the spleen and lymph nodes and hence does not suppress plasma cell proliferation in these sites.

CONCLUSION

Although the mode of action of anti-lymphocytic antibody is still uncertain it is apparent that this material is a most effective immunosuppressant. It would appear to have considerable potential both as a research tool and a therapeutic agent. The material has already been

used with some success in human renal transplantation (Iwasaki *et al.*, 1967) and has enabled a considerable reduction in the dose of other immunosuppressants. However, animal experiments have indicated that a number of complications may arise including serum sickness and viral infections (Abaza *et al.*, 1966) and renal lesions associated with the deposition of antigen–antibody complexes in the kidney (Iwasaki *et al.*, 1967). Such observations suggest caution in the use of this material. Nevertheless, it is believed that improved methods of preparing and administering this material together with a greater understanding of its mode of action will enable some of the previous difficulties encountered in renal transplantation to be overcome. Furthermore such a product may also be of therapeutic importance in certain autoimmune diseases and allergic disorders.

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ANTI-LYMPHOCYTE SERUM

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INTRODUCTION

The main forms of immunosuppressive therapy currently used in organ transplantation include whole body or local irradiation and chemotherapeutic measures using steroid derivatives, anti-metabolites, alkylating agents and antibiotics[1,2]. Almost all these forms of therapy are associated with undesirable side effects and attempts are therefore continually being made to develop alternate less toxic forms of immunosuppression. During the last 2–3 years considerable attention has been centred on the potential value of anti-lymphocytic serum as an additional (adjunct) form of immunotherapy in human transplantation. This interest was originally stimulated by the observations that anti-lymphocytic sera inhibited delayed hypersensitivity[3–5] reactions in guinea pigs and also significantly prolonged the survival of skin allograft in rats across a marked histocompatibility barrier[6,7]. More recent observations have shown that anti-lymphocytic antibody will suppress a variety of cell mediated and humoral antibody responses[8,9] (Table I). In certain situations this material has been more effective than conventional immunosuppressants[10,11].

TABLE I

SOME OF THE PROPERTIES OF ANTI-LYMPHOCYTIC SERA

Property	*Species demonstrated in*
Delays the rejection of 1st set skin allografts	Mouse, rat, guinea pig, monkey, pig and human
Delays the rejection of 2nd set skin allografts and xenografts	Mouse
Delays renal allograft rejection	Rat, dog, human
Delays liver allograft rejection	Dog, human
Prevents graft versus host disease	Mouse, rat, monkey
Facilitates production of lymphoid chimaeras	Mouse
Suppresses the normal and immune lymphocyte transfer reaction	Guinea pig
Suppresses immediate and delayed hypersensitivities	Mouse, guinea pig, human
Transforms lymphocytes *in vitro*	Dog, human
Reduces inflammatory lesions	Guinea pig
Suppresses autoimmune phenomena	Mouse, rat, guinea pig
Suppresses the primary humoral antibody response	Mouse, rat, rabbit
In conjunction with thymectomy suppresses the secondary antibody response	Mouse, rat
Enhances tumour growth	Mouse
Protects from lymphocytic chorio-meningitis virus and the yellow fever virus	Mouse
Potentiates polyoma and adenovirus oncogenesis	Mouse

The results to date indicate that anti-lymphocytic antibody has considerable potential both as an immunosuppressant and as a reagent for investigating immunological phenomena. However as very few comprehensive reports have appeared on the use of this material in human transplantation, most of the data to be presented will be based on animal investigations. The effects of anti-lymphocytic antibody on both cell mediated and humoral antibody formation will be discussed and illustrated with results obtained in the author's department. However results similar to those presented have frequently been observed in other laboratories and for a more extensive coverage the reader should consult previous reviews[8,9].

THE PREPARATION, CHARACTERIZATION AND ISOLATION OF ANTI-LYMPHOCYTIC ANTIBODIES

Most of the antisera described to date have been of the heterologous type and these have been raised using a variety of antigens including thoracic duct lymphocytes, peripheral blood leukocytes, cell suspensions from thymus lymph node and spleen, as well as subcellular fractions of lymphoid cells. These antigens have been administered with and without adjuvants, the dose and routes of injection varying from laboratory to laboratory[8,9].

The anti-lymphocytic antibodies produced are generally species specific but not strain specific, furthermore they frequently cross react *in vivo* and *in vitro* with other formed elements of the blood, especially erythrocytes and platelets[8,9]. It is therefore essential that these toxic cross reacting antibodies are removed by absorption or fractionation procedures. The fractionation procedures also offer other distinct advantages permitting the administration of large doses of antibody in a small dose

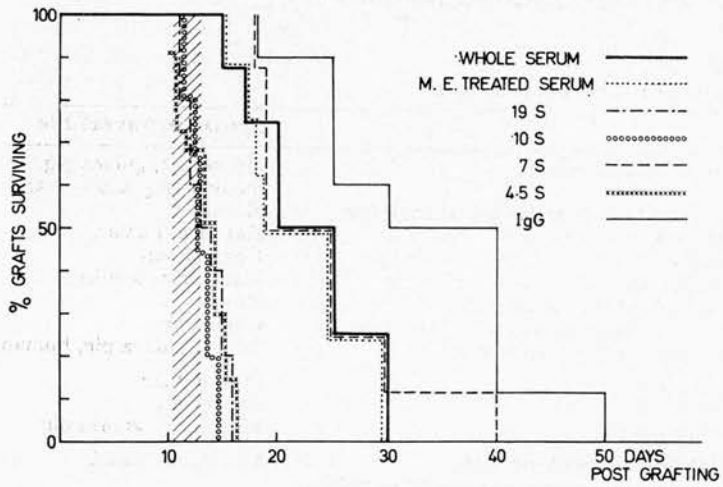

Fig. 1. The effect of anti-lymphocytic serum and serum fractions on skin allograft survival in mice (A → CBA). The hatched area represents the survival time of A strain tail skin allografts on untreated CBA mice. Note that the graft prolonging activity is located in the 7S region and is mercaptoethanol resistant suggesting that the molecule is of the IgG type. This was confirmed in experiments using isolated IgG preparations[12].

as well as avoiding the injection of large amounts of antigenic non-antibody protein which may also possess undesirable anti-enzyme activity *i.e.* anti-plasmin, anti-thrombin etc.

However before the fractionation of anti-lymphocytic sera is attempted it is advisable to ascertain the precise distribution of antibody activities. This can be most readily achieved by preparative ultracentrifugation or by gel filtration on G 200 Sephadex, procedures which enable the resolution of 19S IgM and 7S IgG class antibodies. The characterization of rabbit anti-mouse thymocyte antibody by fractionation on G 200 Sephadex is shown in Fig. 1. It will be observed that the graft prolonging (anti-lymphocytic activity) of the serum is located in the 7S fraction and that the antibody is mercaptoethanol resistant (0.1 *M*) and possibly of the IgG type. This was confirmed in experiments using isolated IgG preparations[12]. Similar studies with horse anti-human lymphocyte sera have also revealed that the bulk of the lymphocyte transforming activity is associated with the IgG fraction of serum[13].

Based on these and other observations many investigators routinely use anti-lymphocytic globulin (IgG) preparations in their immunosuppressive studies. In the author's laboratory these are obtained by twice precipitating the inactivated anti-lymphocytic antisera with 28% sodium sulphate (final concentration 14%). The crude globulin obtained is further purified by batch chromatography on DEAE cellulose*. The final product is concentrated by lyophilization, reconstituted in and dialyzed against phosphate buffered saline, absorbed with erythrocytes if necessary, and finally sterilized.

THE IMMUNOSUPPRESSIVE PROPERTIES OF ANTI-LYMPHOCYTIC ANTIBODY

Although this section of the conference is primarily concerned with the inhibition of cell mediated graft rejection processes, considerable attention will also be paid to the effect of anti-lymphocytic antibody on humoral antibody formation. This approach has been adopted for both theoretical and practical reasons. In the first instance it is essential to study the effect of anti-lymphocytic antibody on both types of immune response in order to elucidate its mode of action. Furthermore, for practical reasons, it is desirable to ascertain its effect on the humoral response, to bacterial and other antigens, including the heterologous anti-lymphocytic globulin.

These studies have been performed using intact anti-lymphocytic antibody and antibody fragments. The experiments were designed with the aim of developing less antigenic (hence less toxic) products for therapeutic use, and in order to elucidate the mode of action of this material. The practical and theoretical importance of studies of this type has been emphasized in the opening lecture on the chemistry and the function of immunoglobulins. The fragments used have been the divalent $F(ab')_2$ component obtained on the pepsin digestion of anti-lymphocytic IgG globulin[14] and the univalent product (Fab') obtained following its reduction and alkylation[15]. The $F(ab')_2$ fragment agglutinates lymphocytes but as it lacks the complement binding (Fc) portion of the molecule it fails to cause their lysis. Furthermore as the Fc portion of the molecule contains the major species specific antigenic determinants, the $F(ab')_2$

* In our earlier studies Whatman DE 11 cellulose was used, however recent investigations suggest that the high capacity advanced ion exchange cellulose (DE 52) offers distinct advantages.

fraction is less antigenic than the intact molecule[16]. The univalent Fab' component will neither agglutinate or lyse lymphocytes.

IMMUNOSUPPRESSIVE STUDIES IN ANIMALS

The effect on cell mediated immune responses

(a) Skin allograft survival

The ability of anti-lymphocytic serum to significantly prolong the survival of skin allografts across a marked histocompatibility barrier was first observed in rats by Woodruff and Anderson[6,7]. Similar results have since been reported in mice, pigs, monkeys and humans[8,9]. In addition, in certain donor recipient combinations anti-lymphocytic serum has been found to delay the rejection of second set skin allografts and xenografts[10,17]. Some of the results obtained in our own laboratories are shown

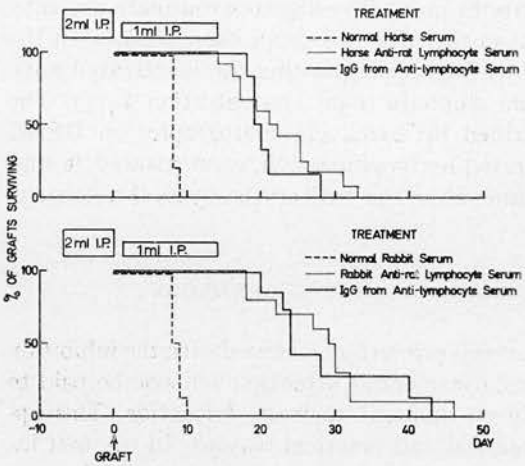

Fig. 2. Effect of anti-lymphocyte antibody on skin allograft survival in rats (albino strain grafts to hooded recipients). All the preparations contained approximately 10 mg of IgG per ml and the volume administered and length of the course of treatment are indicated in the figures. Note that both antisera and their IgG fractions prolong skin allograft survival[18].

in Fig. 2 where it will be noted that horse and rabbit antisera to rat lymphocytes, and IgG preparations from such sera, delay the rejection of albino strain skin allografts on hooded strain recipients[18]. In contrast however the non cytotoxic antibody fragments failed to demonstrate immunosuppressive properties (see Fig. 3).

(b) Whole organ transplants

Having demonstrated that anti-lymphocytic antibody delayed the rejection of skin allografts, the next step was to investigate the effect of these products on whole organ allotransplants possessing vascular anastomosis. These studies revealed that anti-lymphocytic antibody delayed the rapid rejection of renal allografts in both rats and dogs and liver allotransplants in dogs[9]. The recent results obtained following renal transplantation in dogs in our own department are presented in Fig. 4. It will be observed that effective immunosuppression is achieved in those dogs in which anti-lymphocytic antibody treatment was commenced prior to renal transplantation[19].

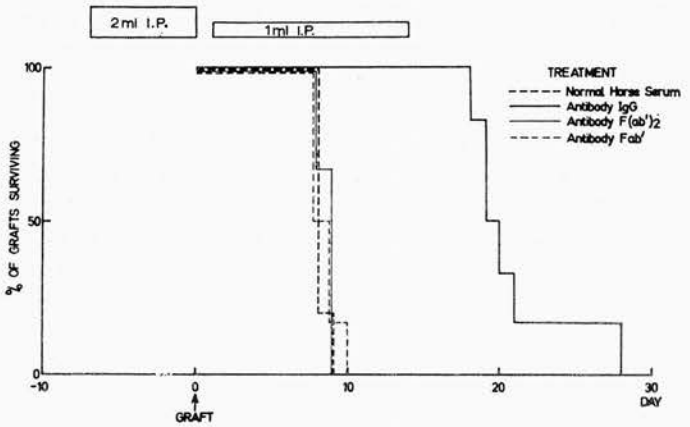

Fig. 3. The effect of anti-rat lymphocyte IgG and IgG fragments on skin allograft survivals (albino strain to hooded). All the preparations contained approximately 10 mg of protein per ml. Note that the non-cytotoxic $F(ab')_2$ and Fab' fragments failed to prolong skin allograft survival indicating that the Fc portion of the molecule is required for immunosuppression[18].

(c) Effect on other cell mediated immune phenomena

In addition to suppressing the cell mediated processes associated with graft rejection, anti-lymphocytic sera also suppress or completely inhibit several other important immune phenomena. These include the normal and sensitized lymphocyte transfer reaction, delayed hypersensitivities, graft versus host phenomena and certain autoimmune diseases (see refs. 8, 9). Furthermore the administration of anti-

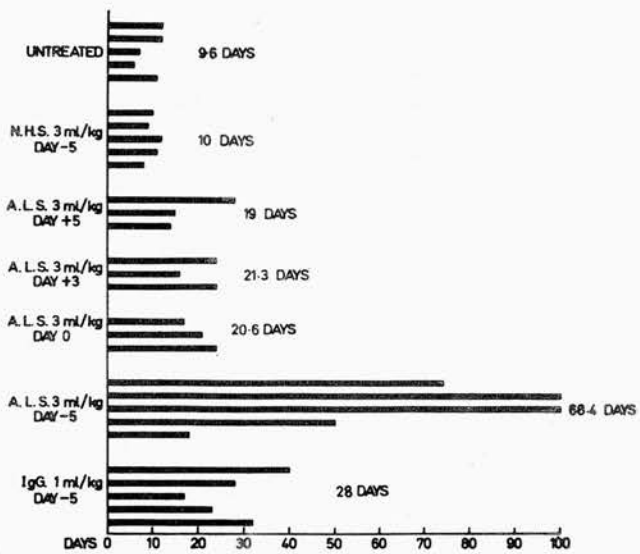

Fig. 4. The effect of anti-lymphocytic antibody on the survival of dogs with renal transplants. The transplantations were performed between beagle dogs. The anti-lymphocytic serum contained approximately 10 mg of IgG per ml and the IgG preparation 30 mg; both preparations were administered subcutaneously, the course of treatment being described elsewhere. Note that the anti-lymphocytic serum was most effective if given prior to transplantation[19].

lymphocytic serum in conjunction with thymectomy has permitted the production of immunological tolerance and lymphoid chimaeras in mice[20].

The effect on humoral antibody formation

Anti-lymphocytic antibody has been shown to suppress the primary humoral antibody response to a number of antigens (see Table II). Its ability to suppress the primary response of hooded rats to alum precipitated bovine serum albumin is illustrated in Fig. 4 (group B compare group A). Additional data presented in this figure indicates that anti-lymphocytic antibody is not effective if given after the antigen (group D compare group B) and that antibody fragments are ineffective (groups E and F compare group B). Furthermore antilymphocytic antibody fails to cause a marked suppression of the secondary humoral[17,21] antibody response, or to inactivate sensitized lymphoid cells *in vivo*[22]. In contrast however cell transfer experiments reveal that anti-lymphocytic antibody is capable of inactivating sensitized spleen cells *in vitro* in the absence of complement[22].

TABLE II

THE EFFECT OF ANTI-LYMPHOCYTIC SERA ON THE PRIMARY HUMORAL ANTIBODY RESPONSE

Donor species	*Antigen*	*Produced in*	*Suppresses primary response to*
Mouse	Lymph nodes	Rabbit	Salmonella II antigen and sheep erythrocytes
	Thymocytes	Rabbit	Sheep erythrocytes, BSA
	Thymocytes and lymph nodes	Rabbit	Sheep erythrocytes
Rat	Thoracic duct lymphocytes	Rabbit and horse	Sheep erythrocytes Alum precipitated BSA
	Mesenteric and thoracic lymph node cells	Rabbit	Sheep erythrocytes
	Lymph node thymus and spleen cells	Rabbit	Sheep erythrocytes
Dog	Thoracic duct lymphocytes	Horse	Sheep erythrocytes

For appropriate references see review[9].

The majority of immune precipitin, passive haemagglutination and immune elimination studies have revealed that anti-lymphocytic antibody (IgG) does not exert a marked anti-dotal effect, indeed there is some evidence that it may be more immunogenic than its normal IgG counterpart[9]. In our own laboratory the development of circulating antibodies against anti-lymphocytic IgG has been shown by all the above procedures[23,24]. This response is illustrated in Fig. 5 summarizing the results of experiments designed to simultaneously assess the effect of anti-lymphocytic antibody on a variety of humoral responses and on cell mediated immune processes. In general high levels of circulating antibodies to anti-lymphocytic IgG and sheep erythrocytes were detected prior to graft rejection and the termination of anti-lymphocytic antibody therapy. On the other hand antibodies to bovine serum albumin were generally detected following skin allograft rejection[24].

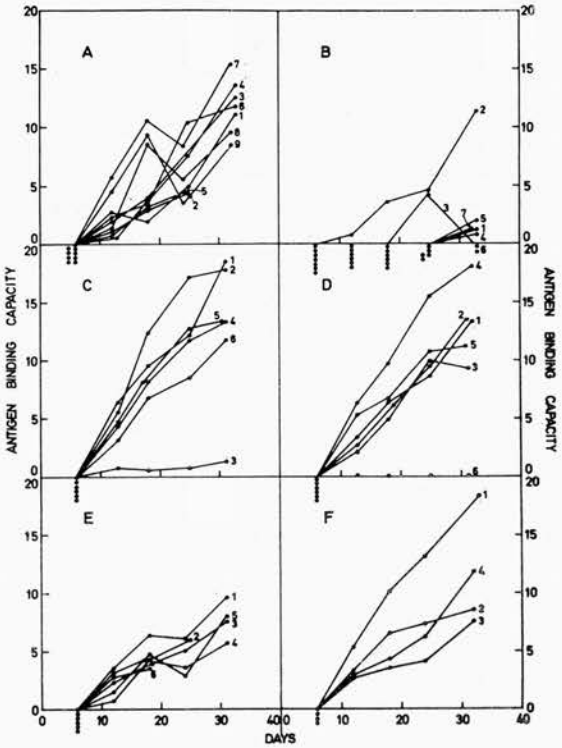

Fig. 5. The effect of anti-lymphocytic IgG and antibody fragments of the primary humoral response of hooded rats to alum precipitated bovine serum albumin (BSA). The rats were treated with the following preparations a) normal horse IgG; b) anti-lymphocytic IgG; c) normal horse IgG; d) anti-lymphocytic IgG; e) $F(ab')_2$ from horse anti-lymphocytic IgG and f) $F(ab')_2$ from rabbit anti-lymphocytic IgG. In groups a, b, e and f the rats were injected with 20 mg (2 ml) of the above preparations on days -3, -2 and -1 and the test antigen (5 mg alum precipitated BSA) was injected intraperitoneally on day 0. In groups c and d the preparations were injected 4, 28 and 52 hrs after the test antigen. Antibody formation was assessed as previously described[21]. Note that suppression of humoral antibody formation occurs only in group b[34]. (Reproduced by kind permission of the Editor of Clinical and Experimental Immunology).

IMMUNOSUPPRESSIVE STUDIES IN HUMANS

At the present time it is difficult to give a critical assessment of the value of anti-lymphocytic antibody in human transplantation, for relatively few patients have received this material and then only in conjunction with other forms of immunosuppression. Furthermore the long term effects of this material have still to be determined. Nevertheless the data currently available suggests that anti-lymphocytic antibody could prove a valuable immunosuppressant providing it could be made less toxic.

The use of anti-lymphocytic serum in human transplantation

The most extensive clinical reports have been those of Starzl and his co-workers who have now used this material in 53 patients receiving renal allografts[25–27].

However the data presented will be restricted to the earlier patients receiving treatment between June and December 1966 for these have survived a sufficient period of time to enable reasonable assessment of the efficacy of this material. The antilymphocytic globulin (volume 1–5 ml) was generally administered daily commencing 5 to 6 days before transplantation and continuing up to 10 to 14 days postoperatively. The amount of protein administered during this period was 14–50 mg/kg/week. Thereafter the material was administered on alternate days for two weeks followed by twice a week for two months and finally once a week for one month. Occasionally treatment had to be terminated prematurely because of toxic reactions. Azathioprine was given daily commencing the evening before transplantation and continued indefinitely thereafter whilst prednisone was used as sparingly as possible.

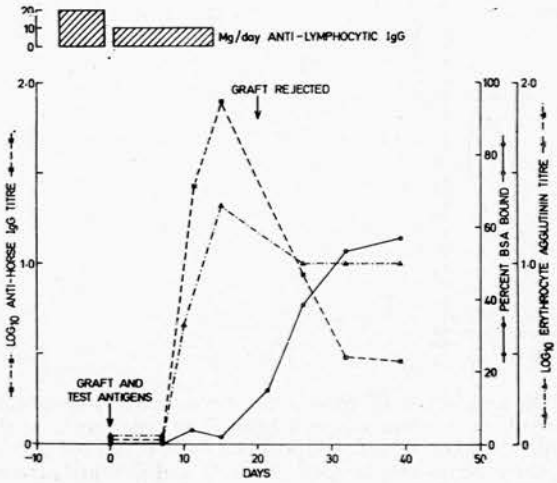

Fig. 6. The response of anti-lymphocytic antibody treated rats to multiple antigenic challenge. A schematic representation of the effect of anti-lymphocytic IgG on cell mediated and humoral antibody responses in the same rat. On day 0 the rats received a skin allograft and were injected (I.V.) with 1×10^9 sheep erythrocytes and (I.P.) with 5 mg of alum precipitated BSA. Further details of these experiments are recorded elsewhere[24].

In an attempt to assess the value of anti-lymphocytic antibody Starzl and his co-workers have compared their results with those they previously obtained in other groups receiving consanguineous transplants. Retrospective and prospective antigenic typing revealed that the anti-lymphocytic antibody treated patients did not possess the added advantage of better matching. Further details on the patients, donors and the course of treatment are recorded elsewhere[25–27].

The preliminary results indicate that the early course of patients with renal allotransplants can be greatly improved by the addition of anti-lymphocytic globulin to the therapeutic protocol (see Fig. 7). This has been achieved with about half the usual dose of prednisone and with a reduction in the amount of azathioprine, and has not been obtained at the expense of renal function. It should perhaps be mentioned that additional reports have recently appeared on the use of anti-lymphocytic antibody in human renal transplantation[28,29].

Other investigations have indicated that anti-lymphocytic antibody might

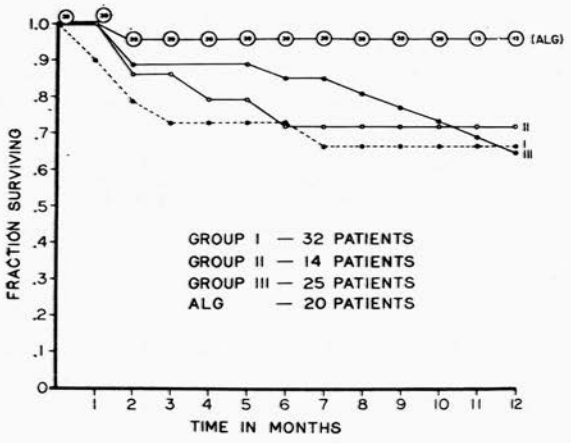

Fig. 7. Mortality data in patients receiving anti-lymphocytic globulin. In group 1 prednisone was withheld until a rejection crisis occurred and then it was administered in large doses. Patients in groups 2 and 3 received 3 mg per kg and 0.5 to 1.0 mg per kg per day respectively commencing the day before transplantation. In all groups azathioprine treatment commenced several days before transplantation. The numbers in the anti-lymphocytic globulin series refers to the patients at risk at the times indicated. Further details of the patients and courses of treatment are reported elsewhere[25–27]. (Reproduced by kind permission of the authors and the Editor of Surgery, Gynecology and Obstetrics).

also be of value in the treatment of threatened renal allograft rejection[9], and in prolonging the survival of liver[30] and skin allografts[31].

Other immunosuppressive studies in humans

As in the previously reported animal studies, anti-human lymphocyte sera have been shown to suppress, or indeed completely abolish pre-existing delayed hypersensitivities to trychophyton, monilia, mumps, toxoplasmin and purified protein derivative[25,29,31]. Furthermore recent investigations suggest that this material may be of value in the treatment of certain autoimmune disorders[32].

Side effects of anti-lymphocytic antibody therapy

There are as anticipated a number of complications (both local and systemic) arising from the use of anti-lymphocytic antibody, and these are at the moment limiting its application (see Table III). In addition to the problem of local pain there

TABLE III

SOME SIDE EFFECTS OF ANTI-LYMPHOCYTIC ANTIBODY TREATMENT

Local	*Erythema and induration associated with pain, itching and tenderness* *Abscesses (sterile and non sterile)*
Systemic	Fever with associated chills and occasional tachycardia Hypotension and air hunger Hives, rashes, generalized pruritis arthralgia Anaphylactic shock Serum sickness and associated nephritis Leukopenia and thrombocytopenia

For further details see review[9].

are problems associated with the use of a cross reacting antibody which can cause thrombocytopenia, and the immunological complications resulting from the administration of heterologous protein. As in the animal studies (see Fig. 6) all Starzl's patients developed fairly high levels of circulating antibody against the heterologous anti-lymphocytic globulin and in a high proportion of patients anaphylactic reactions occurred and clinical manifestations of serum sickness were observed[25,33]. However in those patients where renal biopsies were performed (109–145 days after transplantation) there was no evidence of serum sickness of Masugi type nephritis. Nevertheless histological changes have been observed in the transplanted kidneys, namely thickening of the sub-endothelial basement membranes and fusion of the epithelial foot processes together with the deposition of human IgM globulin[25].

THE MODE OF ACTION OF ANTI-LYMPHOCYTIC ANTIBODY

An attempt will not be made to explain the various theories (see Table IV) which have been advanced to explain the mode of action of anti-lymphocytic antibody for these have been reviewed elsewhere[8,9,10]. However factors influencing the immunosuppressive activity will be discussed and the major theories will be reconsidered in the light of recent experimental evidence.

TABLE IV

THEORIES OF THE MODE OF ACTION OF ANTI-LYMPHOCYTIC ANTIBODY

1. The general or specific destruction of lymphocytes.
2. Antigenic competition by lymphocyte bound antibody.
3. Blindfolding of lymphocytes preventing antigenic recognition.
4. Blindfolding of homograft preventing lymphocyte sensitization and/or contact.
5. Sterile inactivation of lymphocytes.
6. Neutralization of thymus humoral factor.
7. Antagonization of lymph node permeability factor.

For further details of these theories see refs, 8, 9 and 10.

From allograft survival studies it appears that maximum immunosuppression is achieved if treatment is commenced prior to transplantation[19,26]. Furthermore pretreatment is essential for the suppression of the primary humoral antibody response[34,35]. These results suggest that antilymphocytic antibody critically affects the sensitizing or "triggering" phase of the immune response.

The apparently conflicting observations that anti-lymphocytic antibody may suppress secondary cell mediated immune responses[10,17] whilst having little effect on secondary humoral responses[17,21] can now be readily explained on the basis of recent suggestions that this material has limited access to central lymphoid organs[36]. Thus the anti-lymphocytic antibody cannot readily inactivate memory cells in these sites and so prevent the secondary humoral response to mobile antigens such as bovine serum albumin[21]. On the other hand as its prime effect is on peripheral lymphocytes[37] it is able to inactivate lymphocytes involved in the inductive and effective phases of allograft rejection. However recent observations[22] that anti-lymphocytic antibody will inactivate sensitized spleen cells *in vitro* (sensitizing antigen BSA), indicate that

this reagent is capable of destroying memory cells* and support the view that its relative ineffectiveness *in vitro* may be due to limited access[36]. It is therefore possible that much higher doses may prove effective in suppressing secondary humoral responses if only by stimulating the liberation of lymphoid cells into the peripheral circulation and then destroying them[37].

The results obtained with anti-lymphocytic antibody fragments (see Table V) strongly suggest that the mode of action of anti-lymphocytic antibody is indeed a cytotoxic one as originally suggested[10]. This need not involve the gross destruction

TABLE V

IMMUNOLOGICAL PROPERTIES OF ANTI-LYMPHOCYTIC ANTIBODY AND ANTIBODY FRAGMENTS

	Property	*Intact IgG*	*F(ab')$_2$*	*Fab'*
In vitro	Lymphoagglutinating	Yes	Yes	No
	Lymphocytotoxic	Yes	No	No
	Transform lymphocytes*	Yes	Yes	No
In vivo	Prolongs skin allograft survival (1st set)	Yes	No	No
	Suppresses GVH reaction	Yes	No	No
	Suppresses humoral antibody formation (primary response)	Yes	No	No

* Lymphocyte transformation demonstrated with antibody to human lymphocytes. All other investigations performed with antibody to rat or mouse lymphocytes.

of lymphocytes but a select inactivation of those in a state of readiness to undertake immune responses[8,9,10]. These cells may be the so called antigen reactive small lymphocytes described by Martin and Miller[9,39]. These observations do not however completely exclude other mechanisms such as antigenic competition[8,9,10] for as these fragments lack the antigenic Fc portion of the molecule they will be less effective competitive antigens than the intact antilymphocytic IgG. Nevertheless recent results in our own laboratory have shown that strongly agglutinating, non cytotoxic preparations of intact horse anti-rat lymphocyte IgG do not inhibit the primary humoral response. This data offers further support to the cytotoxic theory in preference to others which could be explained on the basis of properties associated with the Fc portion of the anti-lymphocytic antibody molecule.

FUTURE DEVELOPMENTS IN RELATION TO IMMUNOSUPPRESSION IN HUMANS

The impressive immunosuppressive effects observed in animals together with the encouraging results obtained in humans to date, confirm that anti-lymphocytic antibody has considerable potential as a basic form of immunosuppression in human transplantation. Nevertheless a considerable amount of basic research is essential in order to develop less toxic and more potent products. There are at least three possible levels at which the toxicity problem may be approached, all of these are worthy of attention and they are as follows:

(a) Antiserum production

Less toxic antisera will most probably be produced by the use of "purer" anti-

* This inactivation was demonstrated in cell transfer experiments and could also be explained by limited seeding of the transferred cells[22,38,39].

genic material such as thoracic duct and peripheral blood lymphocytes or cell fractions therefrom. As recently indicated a number of the toxic reactions currently observed might be attributable to the use of complex antigenic mixtures such as spleen cell suspensions[40,41]. Furthermore the choice of animal and course of immunization which produce the most potent and least toxic antisera have still to be ascertained[9].

(b) Further purification of anti-lymphocytic antibody

Recent experiments in our own laboratory using ^{131}I-labelled anti-lymphocytic IgG have shown that less than 5% of the IgG molecules in our preparations is anti-lymphocytic[42]. From previous studies on the nature of horse anti-hapten antibodies[43] it is highly probable that this 5% represents a complex mixture of immuno-globulins with considerable variation in properties. It is quite possible therefore that further resolution of anti-lymphocytic IgG preparations will yield products with distinct therapeutic advantages. However because of the limited amounts of protein which can be processed by selective immunoabsorption procedures it may be necessary to resort to less selective electrophoretic and chromatographic procedures. It should perhaps be stressed at this stage that some of the side effects observed could have been due to changes occurring in the serum proteins during fractionation and therefore attempts should be made to limit denaturation during processing.

(c) Administration

Until less toxic preparations are available it is essential that the use of this material is strictly controlled. In addition to the routine biochemical and haematological investigations, immunological assays should also be performed for these are frequently of considerable prognostic value[32]. The observations in both animals and humans that high levels of circulating antibodies to horse IgG are not observed until approximately three weeks after administration[24,25,33] of anti-lymphocytic antibody would suggest that short intensive course of anti-lymphocytic antibody might be advantageous[9]. Such a course could commence 5 to 7 days prior to transplantation and be terminated 10–14 days post-operatively thus reducing the risks of anaphylactic shock and serum sickness that accompany more prolonged courses.

Finally, it is hoped that as a result of investigations similar to those described that the transplantation surgeon will be provided with a highly specific non toxic immunosuppressant.

ACKNOWLEDGMENTS

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The Preparation and Properties of Anti-Lymphocytic Sera

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The Preparation and Properties of Anti-Lymphocytic Sera[1]

K. James

Contents

I. Introduction

The *in vitro* and *in vivo* lymphagglutinating and lymphocytotoxic properties of heterologous anti-lymphocyte sera have long been recognized and the original investigations in this field have already been summarized [GRAY, MONACO, WOOD and RUSSELL, 1966; RUSSELL and MONACO, 1967]. However, it is only during recent years that people have become interested in the immunosuppressive properties of this material. This interest was initially stimulated by the growing awareness of the role of the lymphocyte in allograft rejection and prompted WOODRUFF [1960] to investigate the effect of anti lymphoid sera on skin allograft survival in the rat. Although these original attempts proved unsuccessful, the subsequent in-

[1] A number of important papers on this subject have appeared since this article was submitted in February 1968.

vestigations of INDERBITZIN [1956] revealed that anti-lymphocytic serum could suppress the cell mediated tuberculin reaction and these observations were later confirmed and extended by WILHELM, FISHER and COOKE [1958] and WAKSMAN, ARBUOYS and ARNASON [1961]. More recently WOODRUFF and ANDERSON [1963, 1964] were able to convincingly demonstrate that anti-lymphocytic antibody could also significantly delay the rejection of first set skin allografts in rats across a marked histocompatibility barrier. Since this date, anti-lymphocytic sera have been found to influence a wide number of cellular and humoral immune responses in a variety of species (see table I). These results indicate that this material may have considerable potential as an immunosuppressant in human transplantation and that it is a most valuable tool for investigating basic immunological phenomena including the homograft rejection processes [see previous short reviews WOODRUFF, 1967; JAMES, 1967a and PICHLMAYR, 1967b].

The aim of the present review is to acquaint the reader with the techniques of preparing, characterizing and purifying anti-lymphocytic antibody, and to outline its *in vitro* and *in vivo* properties. Considerable attention will also be paid to the effect of this material on both cell mediated and humoral immune response in animals, and the preliminary investigations in humans will be outlined. In the final section the possible modes of action of this important immunosuppressant will be discussed.

It is hoped that this review will help the surgeon to appreciate the problems and the potential of anti-lymphocytic antibody and this will result in more controlled and efficient use of this material in human transplantation and in animal experimentation.

II. The Preparation of Anti-Lymphocytic Sera

A. Introduction

A large number of effective species specific anti-lymphocytic sera have been described to date. The methods of production have, however, varied quite considerably. An attempt will, therefore, be made to outline some of the principles involved in their production and a number of the more important methods will be discussed in detail.

Table I. A survey of the preparation and properties of anti-lymphocytic sera

Anti-lymphoid sera	Antigen used	Species produced in	Immunological properties	Some key references
Mice	1. Thymocytes	1. Rabbit	1. Delays rejection of 1st and 2nd set skin allografts and 1st set xenografts.	LEVEY and MEDAWAR [1966a and b]; MONACO *et al.* [1966c]
	2. Subcellular fractions of thymocytes	2. Horse	2. Suppresses delayed hypersensitivity reactions.	RUSSE and CROWLE [1965]
	3. Lymph node with and without Freund's complete adjuvant		3. Suppresses the graft versus host reaction and repopulation of X-irradiated animals with isogeneic spleen cells and may suppress the ensuing secondary disease.	BOAK *et al.* [1967a and b]; VAN BEKKUM *et al.* [1967]; BRENT *et al.* [1967a and b]; LEVEY and MEDAWAR [1967]; MONACO *et al.* [1967b]; NAYSMITH and JAMES [1968]
			4. In conjunction with thymectomy enables production of lymphoid chimaeras in adult mice.	MONACO *et al.* [1966b]
			5. Supresses auto-immune diseases.	DENMAN *et al.* [1967a]; RANLØV [1967]
			6. Suppresses primary and possibly secondary humoral antibody response.	MONACO *et al.* [1965b, 1966c]; BERENBAUM [1967]
			7. Enhances tumour growth.	
			8. Protects from lymphocytic chorio-meningitis virus.	GLEDHILL [1967]
Rat	1. Thoracic duct lymphocytes	1. Rabbit	1. Delays rejection of 1st set skin and renal allografts.	WOODRUFF and ANDERSON [1963, 1964]; ANDERSON *et al.* [1967]; NAGAYA and SIEKER *et al.* [1965a]; JEEJEEBHOY [1965a and b, 1967]; GUTTMAN *et al.* [1967a, b, c and d]
	2. Thymocytes	2. Horse	2. Suppresses graft versus host reaction and inactivates haemopoetic stem cells	FIELD and GIBBS [1968]
	3. Mesenteric lymph node cells		3. Suppresses Freund's adjuvant induced polyarthritis.	CURREY and ZIFF [1966]
	4. Spleen cells		4. Suppress primary humoral antibody response.	JAMES and ANDERSON [1967, 1968]; JAMES and JUBB [1967]; JAMES [1967b]; JEEJEEBHOY [1965a and b, 1967]
	5. Subcellular lymph node fractions		5. Inactivates sensitized spleen cells *in vitro*.	JAMES [1968]
Guinea pig	1. Thymocytes	1. Rabbit	1. Abolishes all components of normal and immune lymphocyte transfer reactions.	LEVEY and MEDAWAR [1966a, 1967]
	2. Lymph node		2. Suppresses tuberculin reaction.	INTERBITZEN [1966]; WILHELM *et al.* [1958]; WAKSMAN *et al.* [1961]
			3. Suppresses delayed hypersensitivity reactions.	WAKSMAN *et al.* [1961]

			5. Suppresses non-specific inflammation.	Waksman *et al.* [1961]; Turk and Willoughby [1967]; Morris and Burke [1967]
Rabbit	1. Peripheral blood leukocytes	1. Rabbit	1. *In vitro* treatment of sensitized spleen cells inhibits plaque forming activity in Jerne plate.	
			2. Suppresses antibody forming potential following transfer to X-irradiated recipients.	Harris and Harris [1966a and b]
Dog	1. Thoracic duct lymphocytes	1. Rabbit	1. Delays rejection of renal allografts.	Abaza *et al.* [1966]; Monaco *et al.* [1966a]; Starzl *et al.* [1967a]; Pichlmayr [1967a]
	2. Mesenteric lymph nodes	2. Sheep	2. Delays rejection of liver allotransplants.	Starzl *et al.* [1966, 1967a]; Pichlmayr *et al.* [1967e and f]
	3. Spleen cells	3. Horse	3. Suppresses primary humoral antibody response.	Pichlmayr *et al.* [1967c and e]
		4. Cow	4. Transforms dog lymphocytes *in vitro*.	Kauffman *et al.* [1967]
Pig	1. Mesenteric lymph nodes	1. Horse	1. Delays skin allograft rejection.	Lucke *et al.* [1968]
Monkey (rhesus)	1. Thymocytes	1. Rabbit	1. Delays rejection of 1st set skin allografts.	Balner and Dersjant [1967]
	2. Lymph node	2. Monkey (rhesus and cynomolgus)	2. Suppresses secondary disease.	Van Bekkum *et al.* [1967]
Human	1. Thymocytes	1. Rabbit	1. Delays rejection of skin allografts.	Monaco *et al.* [1967a and b]
	2. Lymph node	2. Horse	2. Enables maintenance of renal allografts with reduced doses of steroids.	Starzl *et al.* [1967a, b and c, 1968]; Traeger *et al.* [1968]; Carraz *et al.* [1967]
	3. Spleen cells		3. May reverse threatened rejection of renal allografts.	Starzl *et al.* [1967b, 1968]; Woodruff *et al.* [1968]; Calne *et al.* [1968]; Russell *et al.* [1968]
	4. Peripheral blood leukocytes		4. May delay rejection of liver allografts.	Starzl *et al.* [1967c]
	5. Thoracic duct lymphocytes		5. Inhibits a number of delayed hypersensitivity reactions.	Monaco *et al.* [1967a and b]; Starzl [1967b]; Carraz *et al.* [1967]; Traeger *et al.* [1968]
			6. May be of value in autoimmune diseases.	Pichlmayr *et al.* [1968]
			7. Transforms human lymphocytes *in vitro*.	Grasbeck *et al.* [1964]; Holtz *et al.* [1966]; Woodruff *et al.* [1967a, b and c]; Greaves *et al.* [1967]; Carraz *et al.* [1968]

B. The preparation of antigen

Anti-lymphocytic sera have been produced using a number of antigens (see table I). These antigens include thoracic duct lymphocytes, peripheral blood leukocytes and viable cell suspensions of thymus, lymph nodes and spleen and have been used separately or as mixtures comprising lymphoid cells from a number of lymphoid tissues.

All the antigens used require a certain amount of processing prior to injection. Circulating lymphoid cells such as thoracic duct lymphocytes and peripheral blood leukocytes require washing (at least three times) in chilled (0 to 4°C) physiological saline or suitable buffer to remove contaminating lymph and serum proteins.

When the lymphoid cell suspension is prepared from organs such as dog and human spleen it is advisable to perfuse the organ prior to homogenization. In the case of the human spleen this can be achieved by perfusing the organ through the splenic artery with 2–6 litres of chilled saline or buffer [IWASAKI *et al.*, 1967]. Cell suspensions from these and other lymphoid organs may be obtained by a number of procedures. Small organs can be disrupted in chilled buffered solution using a ground glass or suitable mechanical homogenizer. The fibrous and other unwanted debris should then be removed by filtration through a wet stainless steel screen or several layers of gauze. Alternatively the lymphoid tissue can be pressed through a 60 gauge stainless steel mesh [GRAY *et al.*, 1966]. When preparing material from large lymphoid organs such as dog and human spleen, it is advisable to pass the homogenized tissue through progressively finer stainless steel screens, the final dernier being 40 [IWASAKI *et al.*, 1967]. Further processing of such suspensions involving filtration through glass wool columns may provide preparations rich in small lymphocytes [DENMAN *et al.*, 1967 b and c].

All of the cell suspensions have then to be washed several times to remove contaminating soluble proteins and in addition pharmacologically active products which may be liberated on the disruption of cells. Failure to wash the cells thoroughly at this stage may lead to great discomfort and even death of the animal into which the suspension is injected.

Throughout all these procedures it is advisable to use cold buffers or saline, to observe sterile precautions if at all possible, and to use reasonably fresh antigenic material. Observing these precautions

it is possible to obtain suspensions of high viability and consisting almost entirely of small lymphocytes (greater than 90%)[2].

Finally it should perhaps be mentioned that active anti-lymphocytic sera have also been prepared using subcellular fractions of mouse and rat lymphoid tissue and preparations of similar nature may be the antigen of choice in the future (see page 71) [LEVEY and MEDAWAR, 1966b; HINTZ and WEBBER, 1965; MOYNIHAN, GROGAN and HARDY, 1967].

C. Immunization procedures

Most of the antisera described have been of the heterologous type being produced in rabbits and horses and data from our own and other establishments [PICHLMAYR *et al.*, 1967a, d and e] suggest that antisera produced in these species are more effective than those raised by similar procedures in cows and sheep. There has been, as one might anticipate, considerable variations in the dose of antigen used, the route of administration and the length of the immunisation course. LEVEY and MEDAWAR [1966b] have advocated a two pulse procedure for raising antisera to mouse lymphoid tissue, and this has been adopted by a number of investigators [LANCE, 1967; LANCE and DRESSER, 1967, and BERENBAUM, 1967]. The procedure involves injecting (intravenously) New Zealand white rabbits on two successive occasions 14 days apart with 10^9 living thymocytes, or other lymphoid cells, and bleeding the rabbits 7 days later. Antisera prepared by this procedure offers several distinct advantages for they rarely require absorption with erythrocytes of the antigen donor species and should contain fewer antibodies against minor irrelevant, and perhaps undesirable 'contaminants' of the immunizing cells. However, antisera produced by a similar two pulse procedure, in the author's department, have not always been effective in prolonging skin allografts and so the following procedures have been adopted.

The antisera to rat lymphoid tissue have been prepared by immunizing rabbits and horses with thoracic duct lymphocytes obtained following thoracic duct cannulation. A similar source of antigen has been used to prepare anti-lymphocytic sera for use in dogs [ABAZA *et al.*, 1966; HERMAN and SCHLOERB, 1967; LAWSON, ELLIS, KIRCHEIM and DODGES, 1967]. The rabbits received three intraperitoneal

[2] For methods of isolating lymphocytes see GESNER and HOWARD [1967].

injections each containing 2×10^8 lymphocytes at weekly intervals and were then bled 10 days after the last injection. The rabbits were also bled again on several occasions 10 days after they had received a booster injection of 1 to 2×10^8 thoracic duct lymphocytes.

Horse antiserum to rat lymphocytes was prepared by immunizing a horse with three intravenous injections of 1.07 to 1.3×10^9 thoracic duct lymphocytes (95% viable) at weekly intervals, followed by a fourth injection 2 weeks later. The horse was bled out 11 days after the last injection. On some occasions a more prolonged course of immunization may be required.

The anti-dog sera currently used in our department have been produced by a procedure similar to that described by IWASAKI *et al.* [1967]. This involves immunizing the horses with massive doses of spleen cells (by the subcutaneous route) and results in the rapid production of potent antisera.

D. The use of adjuvants

Several investigators have used complete FREUND's adjuvant when preparing antisera against the lymphoid tissues of the mouse [GRAY *et al.*, 1966], rat [NAGAYA and SIEKER, 1965a and b; DENMAN *et al.*, 1967b and c; GUTTMAN *et al.*, 1967a, b, c and d], dog [MONACO *et al.*, 1966a] and man [MONACO *et al.*, 1967a; CARRAZ *et al.*, 1967; TRAEGER *et al.*, 1968].

The adjuvant procedure originally described by GRAY *et al.* [1966] to produce antisera to mouse lymphoid tissue has now been successfully adopted by other investigators [BOAK, FOX and WILSON, 1967a and b; KINNE and SIMMONS, 1967]. Suspensions of inguinal and axillary lymph nodes were emulsified with an equal volume of FREUND's complete adjuvant. Rabbits received 0.2 ml of this emulsion into each foot pad to give a total of approximately 100×10^6 lymph node cells per animal. Four weeks later intravenous booster injections of cell suspensions (100×10^6 cells) without adjuvant were given on three successive days. The animals were then bled 7 days after the last injection.

The antisera against dog lymphoid tissue were prepared using mesenteric lymph nodes obtained from exsanguinated dogs. The lymph node cell suspension (5×10^8 cells in 10 ml saline) were emulsified in 10 ml of FREUND's complete adjuvant. This material

was injected into numerous subcutaneous sites in a horse. Three weeks later the horse received thrice weekly intravenous injections of 5×10^9 lymphocytes without FREUND's adjuvant for 7 consecutive weeks. Two weeks after the last booster injection the animal was exsanguinated.

E. The preparation of antisera to human lymphoid tissues

The immunization schedules used in producing antisera to human lymphoid tissue have been somewhat erratic due mainly to the difficulty in obtaining regular supplies of satisfactory antigenic material. The antisera used in our own department have been produced using spleen cell suspensions as originally described by IWASAKI *et al.* [1967]. The horses have usually received four intramuscular injections over a period of 3 to 4 months, each consisting of the cells from a single spleen (8.5×10^9 to 62.2×10^9 cells, 62–80% of which were viable). A week after the last injection the titre of the antiserum was assessed by *in vitro* agglutination or transformation procedures and if suitable the horse was bled once weekly for three successive weeks (10 litres). The erythrocytes and plasma were separated by aseptic procedures and the erythrocytes removed after each bleed were re-infused into the horse immediately following the next bleed. By re-infusing the erythrocytes one can bleed the horse at regular intervals without any untoward side effects. After allowing the animal two to three months to recover it then received further booster injections and bleedings.

In contrast to the above MONACO *et al.* [1967a] have produced their anti-human lymphocyte sera in rabbits using axillary, mesenteric and periaortic lymph nodes obtained from cadavers. The rabbits were injected intradermally into the foot pads with 200×10^6 lymphoid cells (volume 1 ml) emulsified in an equal volume of FREUND's complete adjuvant. A month later the animals received four consecutive daily intravenous injections of lymphocytes in saline (50 to 100×10^6 cells per injection) without adjuvant. After one month, the intravenous injections were repeated and the animals bled a week after the last injection. Other investigators have also used FREUND's adjuvant in preparing antisera against human thoracic duct lymphocytes [CARRAZ *et al.*, 1967; TRAEGER *et al.*, 1968].

Recently SHORTER, SPENCER and HALLBENBECK [1967] have described an antisera to human thymocytes which was prepared by a

two pulse procedure. The horses received intravenous injections of 1×10^9 viable human thymic cells on two occasions, 2 weeks apart, and were bled 1 week later.

F. The possible use of stored antigen

The process of producing antisera to human lymphoid tissues may be greatly simplified by using stored cell suspensions for this would permit regular and standardised injection schedules. In this respect the procedure described by SYMES and RIDDELL [1966] involving controlled cooling with liquid nitrogen of spleen cells suspensions in dimethylsulphoxide could prove extremely valuable for this and other antigens such as peripheral blood lymphocytes. Human spleen cells have been stored at –196°C for up to 50 days by this procedure and still responded to phytohaemagglutinin stimulation and incorporated tritiated thymidine into nuclear DNA and would most probably be suitable for antibody production [SYMES, MEEK and RIDDELL, 1966; MEEK, SYMES and RIDDELL, 1967]. Recent reports indicate that effective antisera have been produced using thoracic duct lymphocytes stored under these conditions [CARRAZ *et al.*, 1967; TRAEGER *et al.*, 1968].

There are, however, alternative ways of overcoming the supply problem. As already indicated satisfactory antisera may be produced using subcellular fractions of lymphoid tissues (see page 145). It is feasible that such preparations could be stored in frozen or freeze dried form without great changes in antigenicity. Furthermore, it is also reasonable to assume that in the forseeable future antisera may be produced using freeze dried 'purified' histocompatibility antigen preparations similar to those described by DAVIES [1967].

G. Absorption and sterilization of anti-lymphocytic serum

Anti-lymphocytic sera usually contain antibodies against antigens other than lymphocytes or other leukocytes. High titres of erythrocyte agglutinins and lysins may be produced even when one uses extremely pure preparations such as rat thoracic duct lymphocytes (see page 161). In addition, antibodies against serum proteins are frequently detected. As antibodies to both these antigens could lead

to serious side effects upon injection, they have to be removed as follows.

The serum or plasma is first inactivated by incubating at 56°C for 30 min. This usually results in the precipitation of an appreciable amount of fibrinogen from the plasma samples which is removed by centrifugation. To the inactivated antiserum is then added washed homologous erythrocytes the volume used being dependent upon the erythrocyte agglutinin titre of the serum being absorbed. The absorptions may be performed at temperatures from 0 to 37°C. In our own department, horse antiserum to human lymphocytes are now routinely absorbed with stroma preparations obtained from packed outdated AB blood.[3] Absorption is performed at 4°C for 1 to 2 h with frequent agitation of the absorption mixture. The stroma can be removed by centrifugation without the problem of lysis which frequently accompanies absorption with outdated AB erythrocytes.

In addition to absorption of their antisera with erythrocytes, IWASAKI *et al.* [1967] frequently absorb their antisera with inactivated whole serum of the lymphoid antigen donor species (see later). Absorption is achieved by incubating 1 volume of inactivated normal serum with ten parts of immune horse serum for 12 h at 4°C. The insoluble antigen antibody complexes which are formed are then removed by centrifugation at 6000 r.p.m. for 30 min. However, in order to remove soluble antigen antibody complexes which could prove dangerous if injected intravenously it is advisable to use much higher centrifuged speeds (equivalent to 30,000 G for 30 min to 1 h). Immediately following this high speed centrifugation the protein should be sterilized by Seitz or Millipore filtration (0.2 mμ pore size) for the centrifugation step removes aggregated protein which could block the filters. Centrifugation is preferable to pre-filtration which frequently leads to further aggregation and denaturation, at least of purified proteins.

Following sterilization, all preparations should be stored at –20°C until required as marked structural changes may occur in IgG globulin preparations stored at 4°C or higher [JAMES, HENNEY and STANWORTH, 1964]. Such changes could possibly lead to the loss of immunosuppressive activity.

[3] These preparations are generously provided by Dr. R. A. Cumming, Regional Director of the Scottish National Blood Transfusion Service.

III. The Characterization of Anti-Lymphocytic Antibody

A. Advantages of using purified products

The advantages of using 'purified' anti-lymphocytic antibody preparations, especially in humans, have previously been stressed [JAMES and ANDERSON, 1967; JAMES, 1967a]. In the first place it avoids administering large amounts of extraneous non-antibody, as yet potentially toxic protein. In addition to being extremely immunogenic some of the serum proteins possess anti-enzyme activity inhibiting enzymes such as plasmin, trypsin, thrombin and so could possibly interfere with fibrinolytic or other physiological processes if administered in large amounts. Furthermore, by using concentrated (5–10 g per 100 ml) 'purified' antibody preparations one can administer much greater amounts of anti-lymphocytic antibody in a standard volume. This is particularly important when administering this material by the subcutaneous or intramuscular route. Finally, in a number of investigations it has been found that by means of fractionation the anti-lymphocytic activity can often be resolved from the bulk of the erythroagglutinating activity so reducing the need for extensive absorption [JAMES and ANDERSON, 1967; WOODRUFF *et al.*, 1967a].

B. Disadvantages of using purified products

There are, however, disadvantages of using purified products. Apart from the labour and capital involved in this process and the risks of bacterial contamination, fractionation can lead to denaturation of proteins including antibodies. This may result in the loss of anti-lymphocytic activity and the release of pharmacologically active molecules such as bradykinin which could cause undesirable side reactions upon injection into patients. Nevertheless, it is felt that the advantages outweigh the disadvantages, and provided strict precautions are observed, the use of 'purified' products is recommended.

C. Characterisation of the antibodies involved

As previously emphasized the distribution of antibody activity in the various antisera is dependent upon the many factors known to influence the immune response [JAMES, 1967a]. These factors include

the nature and amount of antigen used, the route and course of immunization, species and individual variation and so on. It is, therefore, desirable wherever practical to characterize each particular batch or pool of antiserum prior to large scale fractionation.

This may be achieved by a number of standard procedures nearly all of which involve a preliminary separation of the serum into a small number of well characterized serum fractions in which the antibody distribution can be accurately assessed. The principles involved in these procedures are outlined as follows but for further details the reader should consult SCHULTZE and HEREMANS [1966].

By gradient ultracentrifugation and gel filtration on G200 sephadex serum proteins can be resolved into three major distinct molecular weight classes, the 19S, 7S and 4.5S fractions. The IgM antibodies (molecular weight approximately 1,000,000) occur in the 19S fraction and the major IgG component (molecular weight approximately 160,000) in the 7S region. The IgA component, which may exist in a number of polymeric forms occurs mainly in the region between the major 19S and 7S fractions, known as the '10S' region. In certain species especially the horse antibody distribution in IgA like proteins may be extremely important (see later).

In order to illustrate this, the results obtained with G200 sephadex fractions of a horse anti-human lymphocyte serum and a rabbit anti-mouse serum are shown in figure 1 and 2. From figure 1 it can be seen that the major part of the lymphoagglutinating, lymphocytoxicity and lymphocyte transforming activity is located in the 7S region. In contrast the bulk of the erythrocyte agglutinating activity was found in the 19S region [WOODRUFF *et al.*, 1967a]. Furthermore, from figure 2 it is apparent that the 19S, '10S' and 4.5S fractions of the rabbit anti-mouse lymphocyte serum are incapable of prolonging the survival of A strain skin allografts on CBA strain mice. On the other hand, the 7S fraction proved as effective as the original antiserum. Thus, in these particular antisera, the bulk of the so-called anti-lymphocytic activity, as assessed by *in vitro* and immunosuppressive properties, was located in the 7S region. A suitable ultracentrifugal procedure for achieving a similar resolution has been described by STANWORTH, JAMES and SQUIRE [1961]. In this particular method the exact distribution of the 19S, 7S and 4.5S proteins is determined by use of dye-labelled marker proteins.

Other fractionation procedures which can, and indeed have been used, to characterize anti-lymphocytic antibody, are ion exchange

procedures using diethylaminoethyl cellulose (DEAE) and diethylaminoethyl sephadex [IWASAKI *et al.*, 1967; PICHLMAYR, 1967a]. Resolution of the proteins in these procedures are achieved mainly through the differences in surface charge of the protein molecules, though size may also contribute. However, these procedures are more laborious and time consuming to perform than gel-filtration.

Another method of determining the molecular weight of the antibodies is based upon the observation that treatment of 19S or IgM type antibodies with disulfide splitting agents, such as 2-mercaptoethanol, dissociates the molecule into 7S sub-units, with the accompanying loss of antibody activity [FLEISCHMAN, PAIN and PORTER, 1962]. Most IgG antibodies are, however, unaffected by this kind of treatment. This is again illustrated in figure 2. In these investigations rabbit anti-mouse lymphocyte serum was reduced with 0.1 molar 2-mercaptoethanol for 2 h at 20°C. The reduced material was alkylated (stabilized) by dialysis against 100 volumes of 0.06 molar phosphate buffer pH 7.2 containing 0.15 molar sodium chloride and 0.02 molar sodium iodoacetamide. The reduced and alkylated material was then dialyzed against phosphate buffered saline to remove the excess iodoacetamide. This treatment had no effect upon the immunosuppressive properties of this rabbit anti-mouse lymphocyte serum suggesting that the effective antibodies were mercaptoethanol resistant and presumably of the 7S variety [JAMES and MEDAWAR, 1967a]. Results similar to these have also been obtained by MONACO *et al.* [1967a] with their rabbit anti-human lymphocyte serum. However, it must be stressed that all 7S anti-lymphocytic antibodies are not mercaptoethanol resistant [JAMES and MEDAWAR, 1967a].

IV. Methods of Isolating Anti-Lymphocyte Antibody

A. Introduction

Observations to date have shown that the bulk of the anti-lymphocytic activity of a number of sera has been located in the IgG fraction [WOODRUFF *et al.*, 1967a; JAMES and ANDERSON, 1967; JAMES and MEDAWAR, 1967a; IWASAKI *et al.*, 1967; MONACO *et al.*, 1967a]. This is of great practical importance for the IgG protein is easier to prepare and is usually more stable than the other immunoglobulins.

B. Possible method of isolating IgG globulin

In the literature there are a number of established procedures for isolating IgG. These include:

a) salt precipitation using sodium sulphate, ammonium sulphate and aluminium chloride;

b) precipitation procedures using organic solvents such as ethanol (the Cohn technique) and ether;

c) chromatographic procedures using DEAE cellulose and DEAE sephadex or

d) precipitation procedures using acridine bases such as rivanol (2 ethoxy-6-9-diaminoacridine lactate).

Some of the fractionation procedures utilize a combination of the above techniques such as salt precipitation followed by chromatographic separation. For further details of these and other methods of fractionation the reader should consult SCHULTZE and HEREMANS [1966]. In the present review the only techniques considered will be those currently used in preparing anti-lymphocytic globulin and those which could be of importance in the near future. These techniques are as follows:

C. Salt precipitation

Having investigated the purity, recovery and properties of several protein preparations IWASAKI *et al.* [1967] now routinely prepare their material for human use by a 2-stage precipitation procedure using ammonium sulphate. To the inactivated absorbed serum was added saturated ammonium sulphate to give a final concentration of 40% (volume to volume with respect to saturated ammonium sulphate). The precipitate which formed was removed by centrifugation, redissolved and the above precipitation process repeated. The second precipitate was redissolved in saline and dialysed against tap water and then saline to remove excess ammonium sulphate. The final product was stored in the lyophilized form. This material was found to contain IgG and IgT globulins and traces of B globulin and approximately 33% of the antibody activity was recovered [IWASAKI *et al.*, 1967]. Other investigators have also prepared their anti-human lymphocyte globulin by this procedure [CARRAZ *et al.*, 1967; TRAEGER *et al.*, 1968].

D. Combined salt precipitation and chromatographic procedures

The materials used to date in the authors department for both human and animal studies have been prepared by a three-stage procedure [JAMES and ANDERSON, 1967; CLUNIE *et al.*, 1968].
These stages are as follows:

1. To 1 volume of unabsorbed inactivated serum or plasma was added slowly with mixing 1 volume of 28% (w/v) sodium sulphate (final sodium sulphate conc 14%). The precipitate was recovered by centrifugation and redissolved in half of its original volume of 0.15 molar sodium chloride.

2. The redissolved precipitate was re-precipitated by addition of an equal volume of 28% sodium sulphate (final sodium sulphate conc again 14%). The precipitate was again recovered by centrifugation and then dissolved in and dialyzed against 0.02 M phosphate buffer pH 6.6.

3. The final step involves batch chromatography of the crude globulin precipitate on Whatman DEAE 11 exchanger (ion exchange capacity 1.0 milliequivalents/g) which also was equilibrated against 0.02 M phosphate buffer pH 6.6. Further details of the batch procedure are recorded elsewhere [STANWORTH, 1960; JAMES and STANWORTH, 1964].

The final products were concentrated by lyophilization or ultrafiltration, dialyzed against physiological saline, or phosphate buffered saline (pH 7.2 0.06 M phosphate) absorbed with stroma and finally sterilized. Immunoelectrophoretic analysis using rabbit anti-horse serum and polyacrylamide gel electrophoresis has revealed that these products are extremely pure. However, as in the previous procedure described by IWASAKI *et al.* [1967], the recoveries of antibody activity frequently leave much to be desired. Recent experiments in our laboratories have indicated that better recoveries can be obtained by omitting the second salt precipitation and by using small amounts of new high capacity advanced ion exchanger celluloses (Whatman DE52). These increased recoveries are not achieved at the expense of purity.

MONACO, WOOD and RUSSELL [1967a] have also utilized a combined precipitation and chromatographic procedure to purify their anti-human lymphocytic IgG. Their method involves precipitation of a crude globulin fraction by adding saturated ammonium sulphate to give a final concentration of 33%. The crude globulin was then

purified on DEAE cellulose by the single step column procedure of LEVEY and SOBER [1960].

Procedures similar to the above are also being performed in some laboratories using DEAE sephadex instead of DEAE cellulose.

E. Batch chromatography on DEAE-sephadex

Recently a rapid method of purifying horse antiserum to goat lymphocytes had been described using DEAE sephadex [PERPER *et al.*, 1967]. This technique could be of importance in preparing material for human use. The procedure involves mixing serum and equilibrated DEAE-sephadex A50 (equilibrated with 0.05 M phosphate pH 7.85) for 1 h at 4°C. The supernatant containing the IgG globulin was then separated by passage through a Buchner funnel and further purified by two additional exposures to fresh aliquots of DEAE sephadex. A total of 100 g dry weight of exchanger was used to purify the IgG from 1 litre of serum. This process is rapid to perform and recoveries appear to be good.

F. Other methods of fractionation of anti-lymphocyte globulin

Additional methods of fractionation of anti-lymphocyte IgG are currently under investigation in Edinburgh [WATT, 1968]. One such procedure is the already well established Cohn fractionation procedure involving alcohol precipitation of the IgG under controlled conditions at low temperature. This procedure allows large scale production of IgG and is the most common method of preparing normal human IgG for therapeutic use (Cohn fraction II). The other technique under investigation is the so-called selective plasmphoresis procedure made possible by the development of the force flow electrophoresis machine of BIER [1959]. This apparatus allows the fractionation of large volumes of blood. The blood is withdrawn from the donor animal, mixed with buffer and an anti-coagulant and then separated electrophoretically in the forced-flow electrophoretic apparatus. The gamma globulin fraction is retained, while the rest of the blood is returned to the donor. Experiments in sheep have shown that the total circulating blood volume can be processed daily permitting the isolation of about 30–40% of the total circulating gamma globulin. Using

recently scaled up equipment it should be possible to process the complete blood volume of an immunized horse in four hours. This process could be repeated at fairly frequent intervals without any serious effects upon the horse. By this technique it should be possible to rapidly isolate antibody from large volumes of blood. One disadvantage of this procedure, however, is the cost of such equipment and the skill and manpower required in its operation.

G. General comments of fractionation

Throughout all these procedures the emphasis should be on speed and, whenever possible, the fractionations should be performed in the cold (0–4°C). Such steps will reduce the risk of denaturation and bacterial contamination. This in turn will minimise the release of pharmacologically reactive polypeptides, such as bradykinin, and the production of pyrogens.

The purity of the products should be determined by standard electrophoretic (paper, cellulose acetate, gel electrophoresis) and immunoelectrophoretic techniques using polyvalent and specific antisera. It is also advisable to determine the pH of the products and their tonicity by using a conductivity meter. *In vitro* tests should also be performed, such as lymphoagglutination, lymphocytoxicity and in the case of anti-human lymphocyte products, the lymphocyte transforming activity should be determined [WOODRUFF *et al.*, 1967b].

Tissue transplantation nomenclature

Old nomenclature	New nomenclature	New adjective	Definition
Autograft	Autograft	Autologous	Graft in which the donor is also the recipient.
Isograft	Isograft	Isogeneic, syngeneic	Graft between genetically similar individuals (that is possessing identical histocompatibility antigens).
Homograft	Allograft	Allogeneic	Grafts between genetically dissimilar members of the same species.
Heterograft	Xenograft	Xenogeneic	Grafts between different species.

The above nomenclature was recommended by GORER *et al.* Nature *189:* 1024 (1961).

Immunoglobulin nomenclature

Old nomenclature	New nomenclature	Molecular weight class ($S_{20}W$)	Approximate molecular weight
γ, 7S γ, 6.6S γ, γ_2 or γ' SS	γG or IgG	7S	160,000
	γD or IgD	7S	
β_2A or γ_1A	γA or IgA	7, 10, 13, 15, 17S etc.	160,000 and multiples thereof
γ M, β_2M, 19S γ or γ macroglobulin	γM or IgM	19S	1,000,000

The above nomenclature applies specifically to the human immunoglobulins but is generally applicable to the immunoglobulins of other species and is recommended in the Bulletin of the World Health Organisation *30:* 447–450 (1964). This bulletin also contains the accepted nomenclature for immunoglobulin fragments (see below).

H. The preparation of anti-lymphocyte antibody fragments

During the last ten years there has been a marked increase in our knowledge of the structure of the IgG molecule and this in turn has led to greater understanding of its function. The four chain polypeptide model based on the work of PORTER and EDELMAN and their colleagues is now well authenticated [PORTER, 1963; for later reviews on structure see STANWORTH and PARDOE, 1967]. Treatment of the IgG molecule with pepsin results in the partial degradation of the so-called Fc portion of the molecule[4]. This region of the molecule incorporates the complement binding, skin attaching and membrane transmission sites as well as containing the major species specific antigenic determinants. The portion that remains following pepsin digestion is known as the divalent $F(ab')_2$ fragment, and contains the two antibody combining sites present on the original molecule. Hence, this fragment is able to combine with and agglutinate the original antigen, in this case the lymphocyte. However, as it lacks the complement binding Fc portion of the molecule it is non-cytotoxic. Further treatment of the divalent moiety with reducing agents produces a univalent Fab′ derivative which contains only one

[4] For the accepted nomenclature for immunoglobulins and their components see Bulletin of the World Health Organization (1964), *30:* 447–450.

antibody combining site. This product, whilst capable of combining with the lymphocyte is incapable of agglutinating or lysing the same.

It has been known for a long time that the toxic reactions produced in man following injection of horse antibody to bacterial antigens could be greatly diminished by pepsin treatment of the purified globulin. This treatment is the basis of the manufacture of so-called 'refined' antisera. As the current immunosuppressive protocols in humans utilizing anti-lymphocytic IgG require large amounts of this material and frequently give rise to severe toxic reactions [STARZL *et al.*, 1967a; KASHIWAGI *et al.*, 1968], it seemed desirable to determine if anti-lymphocytic IgG could be rendered less toxic by pepsin treatment, without destroying its immunosuppressive properties. Experiments have, therefore, been performed in animals to assess the immunosuppressive activity of antibody fragments. In addition, it was anticipated that experiments performed with these fragments would increase our understanding of the mode of action of anti-lymphocytic antibody and that this in turn would lead to a more efficient clinical application.

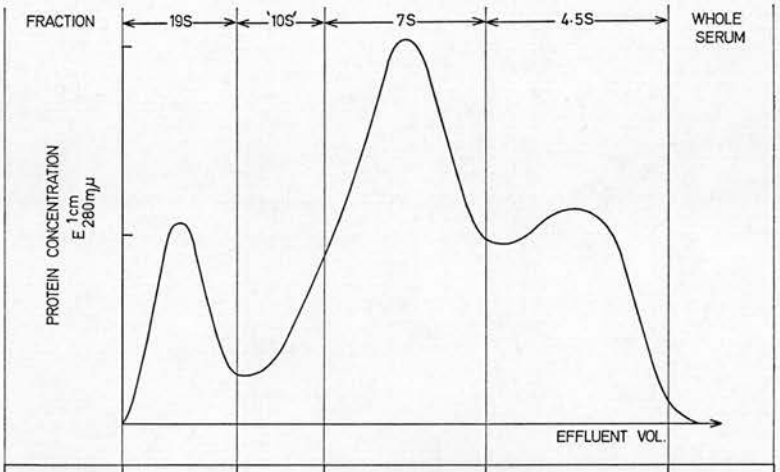
The antibody fragments used in these studies have been prepared by both pepsin [NISONOFF *et al.*, 1960] and papain digestion [PORTER, 1959]. The latter procedure, however, does not cause the extensive degradation of the Fc portion of the molecule.

V. The in vitro *Effects of Anti-Lymphocytic Antibodies*

A. The effect on donor lymphocytes

All the reports to date have demonstrated that anti-lymphocytic antibody combines with lymphocytes *in vitro* and causes their agglutination. In the presence of complement the lymphocytes are lysed. The specific uptake of the anti-lymphocyte antibody has been demonstrated by immunofluorescence [WOODRUFF, ANDERSON and ABAZA, 1966; LEVEY and MEDAWAR, 1966a; RUSSELL and MONACO, 1967] and by isotopic procedures [WOODRUFF *et al.*, 1967c].

In addition, the combination of the antibody with lymphocytes *in vitro* in the absence of complement, has been shown to cause the transformation of the lymphocytes and this observation is of importance in relation to the mode of action of this material [see LEVEY and MEDAWAR, 1966b and page 204]. This has been observed *in vitro*



LYMPHOCYTE AGGLUTINATION	16	128	512	8	2048
LYMPHO-CYTOTOXICITY			32•		256•
RNA STIMULATION	+	+	+++	+	+++
DNA STIMULATION	+	+	+++	+	+++

Fig. 1. The G200 sephadex distribution of lymphocyte transforming activity in horse anti-human lymphocyte serum.
The values displayed were obtained with G200 sephadex fractions which had been concentrated to the original serum volume. Note that the bulk of the transforming activity (as indicated by RNA and DNA stimulation) and the lymphoagglutinating activity were in the 7S region. The erythrocyte agglutinating activity was mainly in the 19S region. For further details see WOODRUFF *et al.* [1967a].

using dog and human lymphocytes but has been difficult to demonstrate in other species (e.g. rat and mouse) due to the problem of maintaining cultures of these lymphocytes *in vitro* for a sufficient length of time. The lymphocyte transformation has been demonstrated by the morphological examination of antibody treated cultures [GRASBECK, NORDMAN and DE LA CHAPELLE, 1964; HUMPHREY, KAUFFMAN and DUNN, 1967, dog lymphocytes] and by measuring the incorporation of isotopically labelled (^{3}H and ^{14}C) uridine and thymidine into lymphocyte RNA and DNA respectively [HOLT, LING and STANWORTH, 1966; WOODRUFF *et al.*, 1967a, b and c; GREAVES *et al.*, 1967; CARRAZ *et al.*, 1967].

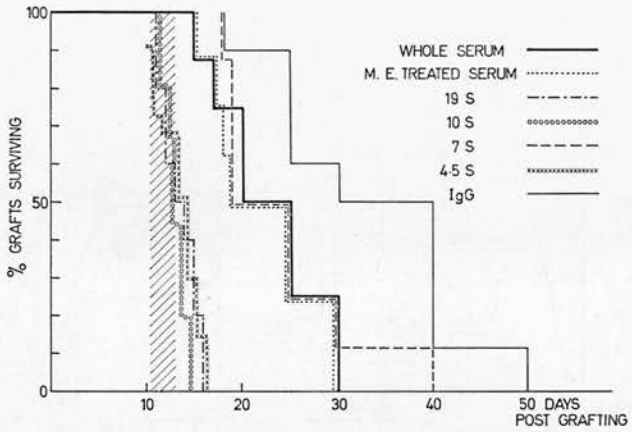


Fig. 2. The effect of anti-lymphocytic serum and serum fractions on skin allograft survival in mice (A strain grafts to CBA recipients).
The hatched area represents the survival time of A strain tail skin allografts on untreated CBA mice. Note the 7S is the only G200 sephadex fraction that delays allograft rejection. The results with mercaptoethanol treated serum also suggest that the molecule is of the 7S type. This was confirmed by the results with the IgG fraction of this rabbit anti-rat thymocyte serum. [For further details see text and JAMES and MEDAWAR, 1967a.]

Studies in our laboratories with G200 sephadex fractions of horse anti-human lymphocyte sera have shown that the major part of the transforming activity is in the 7S region of the serum (fig. 1). Further tests with isolated IgG indicated that this transforming activity is associated with the IgG component of the 7S fraction (fig. 2). In addition the transforming properties of antibody fragments have been investigated. These studies revealed that the non-agglutinating Fab′ moiety failed to stimulate lymphocyte transformation whilst the agglutinating, non-cytotoxic $F(ab')_2$ derivative was active in this respect (see fig. 3). These observations are of great importance in relation to the mode of action of anti-lymphocytic antibody (see later) and have recently been confirmed in a rodent system [RIETHMÜLLER *et al.*, 1968b].

B. Cross reactivity with other lymphocytes

Several investigators have shown that anti-lymphocytic sera are essentially species specific but are not strain specific. This has been demonstrated with antisera against the lymphoid tissues of the mouse [GRAY *et al.*, 1966], the rat and the dog [WOODRUFF, ANDERSON and

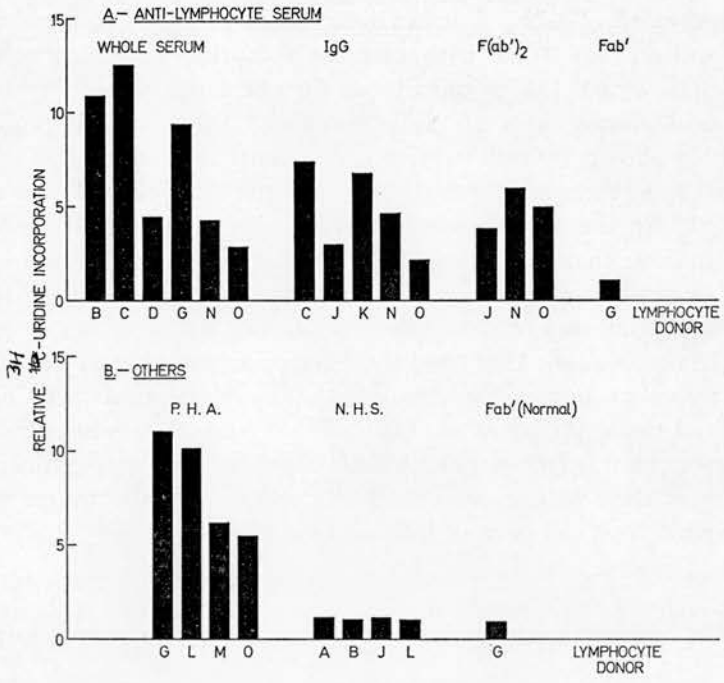


Fig. 3. The lymphocyte transforming properties of various materials as judged by their ability to stimulate ^{3}H-uridine incorporation into nuclear RNA.
Note that anti-human lymphocyte IgG and its $F(ab')_2$ fragment transform human lymphocytes *in vitro* but the non agglutinating Fab' moiety is ineffective. These tests were performed with lymphocytes from a number of donors [see text and WOODRUFF *et al.*, 1967b].

ABAZA, 1966]. In contrast IWASAKI *et al.* [1967] claim that intraspecies variations may occur as well as species cross reactivity. However, this observed cross reactivity may be a reflection of the increased sensitivity of their assay procedures.

C. Cross reactivity with non-lymphoid cells

The antigens used in immunization have been extremely heterogeneous and the individual cells constituting the mixture possess histocompatibility antigens in common with other cells. It is not surprising, therefore, that so-called anti-lymphocytic sera will react *in vitro* with a variety of cells including erythrocytes, other lymphoid cells [PICHLMAYR, 1967a; PICHLMAYR *et al.*, 1967a and d] and platelets

[STARZL *et al.*, 1967b]. Furthermore, limited studies indicate that such antisera will react with mast cells causing histamine release [GUTTMAN *et al.*, 1967b and c] but do not cause marked lysis of peritoneal macrophages [WOODRUFF *et al.*, 1966]. Recently, it has also been shown by cell transfer experiments that anti-lymphocytic serum cross reacts *in vitro* with haemopoietic stem cells [FIELD and GIBBS, 1968]. On the other hand, investigations with non-lymphoid tissue indicate that anti-lymphocytic sera usually do not possess high titres of antibody against the histocompatibility antigens of such tissues. The antisera fail to agglutinate kidney and liver cell suspensions [LAWSON *et al.*, 1967] and their lymphocytotoxic and immunosuppressive properties are not affected by absorption with non-lymphoid tissue [GRAY *et al.*, 1966; LEVEY and MEDAWAR, 1966a]. However, IWASAKI *et al.* [1967] claim that the lymphoagglutinating activity of their antisera was markedly reduced by absorption with the parenchymal cell pack of liver and kidney.

D. Cross reactivity with saline extracts of cells and with serum proteins

Gel diffusion precipitin studies have shown that antisera to mouse, rat and dog lymphoid tissues will cross react with saline extracts of lymphoid and non-lymphoid tissue such as liver, kidney, skeletal muscle and gut mucosa [SACKS *et al.*, 1964; GRAY *et al.*, 1966; LAWSON *et al.*, 1967]; however, as suggested by LAWSON *et al.* [1967], some of this cross reactivity may be due to the presence of serum proteins in the saline extracts for anti-lymphocytic antisera are known to give several lines on immunoelectrophoresis with sera of the lymphoid tissue donor [GRAY *et al.*, 1966; IWASAKI *et al.*, 1967]. It is for this reason that IWASAKI *et al.* [1967] routinely absorb their anti-human lymphocyte sera with normal human serum (see page 149).

VI. The in vivo *Effects of Anti-Lymphocytic Antibodies*

A. The effect on circulating lymphocytes

In general, a single injection of a small volume of antilymphocytic serum causes a marked, though transient lymphopenia, and may be accompanied by polymorphonuclear cytosis. This lymphopenia has

also been observed to be associated with a significant fall in the complement level of the blood of the treated animal [WAKSMAN *et al.*, 1961; GUTTMAN *et al.*, 1967c].

A more profound and sustained lymphopenia is produced, as would be expected, by repeated injections of anti-lymphocytic serum. However, even with prolonged courses of treatment the peripheral blood lymphocyte count often show signs of recovery during the course of treatment or shortly afterwards [WOODRUFF and ANDERSON, 1964; NAGAYA and SIEKER, 1965b; CLUNIE *et al.*, 1968].

A more effective lymphopenia may be produced by using anti-thymocyte sera [NAGAYA and SIEKER, 1965a] or antisera produced with the aid of adjuvants. [GRAY *et al.*, 1966; KINNE and SIMMONS, 1967]. Furthermore, the lymphopenic effect can be enhanced by administering this material to thymectomized animals [MONACO *et al.*, 1965b] or to animals rendered tolerant to the injected anti-lymphocytic globulin [DENMAN and FRENKEL, 1967b].

B. The effect on other blood cells

The studies of PICHLMAYR and colleagues in dogs have shown that anti-lymphocytic sera may also have a transient effect on granulocytes eosinophils, monocytes and thrombocytes [PICHLMAYR, 1967a; PICHLMAYR *et al.*, 1967b and e]. Thrombocytopenia has also been observed in some monkeys [BALNER and DERSJANT, 1967] and humans receiving anti-lymphocytic globulin [STARZL *et al.*, 1967b; KASHIWAGI *et al.*, 1968; WOODRUFF *et al.*, 1968; CALNE *et al.*, 1968]. Others, however, have failed to detect thrombocytopenia in guinea pigs [WAKSMAN *et al.*, 1961] or in humans receiving this material [MONACO *et al.*, 1967; PICHLMAYR *et al.*, 1968].

C. The effect on lymphoid and other organs

The reported *in vivo* effects of anti-lymphocytic antibody on lymphoid organs have been somewhat inconsistent. The variability is no doubt due to factors such as differences in the immunization schedules used in producing the antisera, the courses of anti-lymphocytic antibody treatment, the ability of various antibodies to fix complement, as well as to possible species and strain differences [IWASAKI *et al.*, 1967; JAMES, 1967a].

Studies using isotopically labelled anti-lymphocytic antibody preparations have shown the preferential uptake of this material by some tissues. For example HINTZ and WEBBER [1965] demonstrated that ^{131}I-labelled anti-mouse thymus IgG was preferentially taken up by the peripheral blood leukocytes and the thymus. In contrast GUTTMAN *et al.* [1967a, b and c] observed that their ^{131}I-labelled anti-rat thymus IgG was preferentially absorbed by the cytoplasm of the proximal tubules of the kidney [1967c]. This preferential uptake by the kidney was believed at one stage to explain the apparent immunosuppressive properties of the antibody [GUTTMAN *et al.*, 1967a and b] but now it is believed to be an undesirable toxic side effect [GUTTMAN *et al.*, 1967c; see also page 177]. Similar studies recently reported by DENMAN and FRENKEL [1968a] suggest that anti-lymphocytic antibody has limited access to central lymphoid tissue.

The histological changes observed in the lymphoid organs have varied from hypertrophy to marked depletion. Following chronic treatment in mice LEVEY and MEDAWAR [1966b] observed blast transformation of peripheral blood lymphocytes together with a certain degree of hypertrophy of lymphoid tissue. In contrast GRAY *et al.* [1966] reported that chronic treatment with their antiserum to mouse lymphocytes caused depletion of most lymphoid tissues. The thymus was depleted of lymphoid cells but epithelial elements were preserved. The Peyer's patches showed decreased numbers of lymphocytes which were replaced by histiocytes and macrophages. The most pronounced changes were observed in the lymph nodes and the spleen where there was a marked diminution in the number and size of the germinal centres and evidence of tissue necrosis. Two weeks after termination of serum treatment the lymph nodes and spleen showed evidence of recovery with repopulation by lymphoid cells and the reappearance of germinal centres. Throughout these investigations there was no microscopic evidence of damage to the liver, kidneys and lung in animals chronically treated with anti-lymphocytic serum. It was also found that animals thymectomized prior to anti-lymphocytic serum treatment showed a more marked depletion of small lymphocytes in the lymph nodes and that the repopulation following termination of treatment was much slower [MONACO *et al.*, 1965b]. In contrast to the observations of GRAY *et al.* [1966], RUSSE and CROWLE [1965] observed anatomically intact thymuses in mice which had received rabbit anti-mouse thymus serum for 84 days. There were, however, histological changes in other lymphoid tissues, which

were decreased in size and the germinal centres in the spleen were reduced.

Histological changes have also been reported in lymphoid and non-lymphoid organs of rats receiving anti-lymphocytic antibody. In experiments using antisera to thoracic duct lymphocytes WOODRUFF and ANDERSON [1964] found that after 7 days treatment with this material the spleen was enlarged, and possessed few malphigian bodies or lymphocytes. The red pulp of the spleen was packed with histiocytes and large pyroninophilic cells whilst the lymph nodes also showed some depletion. Although NAGAYA and SIEKER [1956b] reported that antisera to lymph node cells had no effect upon the thymus, antisera to thymus cells depleted this organ of small lymphocytes. A similar effect upon the thymus was also reported by GUTTMAN *et al.* [1967b] who also noted that antisera to thymus also produced changes in the spleen consistant with lymphoid depletion. A number of experiments have also been performed with rabbit anti-lymphocytic antibody in animals which have been rendered tolerant to normal rabbit IgG. In these animals the histological changes were more pronounced than those produced in a non-tolerant group [DENMAN and FRENKEL, 1967b]. The thymus weight decreased with continued treatment but lymphopoiesis was not inhibited in either this organ or the spleen [DENMAN *et al.*, 1967c; DENMAN and FRENKEL, 1968b]. The spleen and the lymph nodes, however, showed a loss of normal architecture, the small lymphocyte population being depleted and furthermore, there was evidence of reticulum cell proliferation and plasmocytosis [DENMAN and FRENKEL, 1967b and 1968b].

TURK and WILLOUGHBY [1967] have also investigated the effect of anti-thymocyte sera on the lymphoid tissue of the guinea pig. They observed a marked depletion of small lymphocytes in the thymus cortex and in the paracortical areas of the lymph nodes but there was no evidence of lymphoid hypertrophy.

In dogs the results have again been conflicting. IWASAKI *et al.* [1967] observed that the lymphocytes in lymphoid tissue were replaced by large and medium size cell with pyroninophilic cytoplasm and germinal cell formation was common. In contrast MONACO *et al.* [1966a] observed that germinal centres were totally absent from lymph nodes and spleen and there was an accompanying marked depletion of both large and small lymphocytes. The depletion on the Peyer's patches on the other hand was only slight. Other investigators have also observed changes in follicular structure and a

marked depletion of the small lymphocyte content of the nodes of anti-lymphocytic antibody treated dogs [ABAZA *et al.*, 1966; PICHLMAYR *et al.*, 1967e; LAWSON *et al.*, 1967]. Histological changes in non-lymphoid tissue, however, appear to have been minimal [IWASAKI *et al.*, 1967; PICHLMAYR *et al.*, 1967e; LAWSON *et al.*, 1967]. In the few cases where focal myocardial and hepatic necrosis were observed, these have attributed to terminal infection and not to a direct toxic effect of anti-lymphocytic antibody [IWASAKI *et al.*, 1967].

VII. The Immunosuppressive Effects of Anti-Lymphocyte Sera in Animals

A. Introduction

Studies in animals have shown that anti-lymphocytic sera inhibit both cellular and humoral immune responses to a variety of antigens. The antibody delays the rejection of first and second set skin allografts and first set renal allografts and also inhibits other cell mediated phenomen such as graft versus host responses, lymphocyte transfer reactions, delayed hypersensitivities and various autoimmune processes. In addition, anti-lymphocytic sera also suppresses the primary humoral antibody response to both soluble and cellular antigens.

Although the reader of this article will be primarily concerned with the prevention of graft rejection and hence inhibition of cellular responses, the present article deals with the effect of anti-lymphocytic antibody on both cell mediated and humoral responses. This approach has been taken for it is felt that the mode of action of anti-lymphocytic antibody will not be fully understood without considering its effect upon both types of immune response. In addition, studies on the ability of anti-lymphocytic antibody to inhibit humoral antibody formation against itself are of great importance in relationship to the therapeutic use of this material.

B. Effect on skin allograft survival

1. Studies in rats

Although attempts had previously been made to prolong skin allograft survival in guinea pigs using anti-lymphocytic serum [WAKSMAN *et al.*, 1961], the first really significant results were those

reported by WOODRUFF and ANDERSON [1963, 1964]. These investigators were able to prolong skin allograft survival between two strains of rats with a high degree of histoincompatibility using a rabbit antiserum to thoracic duct lymphocytes from hooded rats (an inbred albino strain graft to an inbred hooded strain). They also found that the treatment was most effective if commenced prior to grafting and later it was shown that similar results could be obtained with horse anti-rat lymphocyte serum and IgG preparations from anti-lymphocytic sera [ANDERSON *et al.*, 1967a; see also fig. 4]. A similar prolongation of skin allograft survival between non-inbred members of an Osborne-Mendel strain has also been reported by NAGAYA and SIEKER [1965a] who also observed that antiserum to rat thymocytes was more effective than antiserum to mesenteric lymph nodes. Furthermore, JEEJEEBHOY [1965a and b, 1967] described antisera which were only effective if administered to thymectomized rats, though in these experiments the course of treatment terminated 10 days prior to grafting. He also observed that antisera produced in dogs, exhibiting similar *in vitro* properties, failed to delay allograft rejection.

2. Studies in mice

The results originally obtained in rats have since been confirmed and extended in mice [GRAY *et al.*, 1964; MONACO *et al.*, 1965a and b; 1966c; LEVEY and MEDAWAR, 1966a and b; BRENT *et al.*, 1967a and b]. The results of MONACO *et al.* [1966c] suggest that the immunosuppressive effect is most marked if the antiserum is given prior to grafting and can be further increased by continuing the treatment after grafting. On the other hand LEVEY and MEDAWAR [1966a] claim that their anti-lymphocytic serum is very effective in their graft system if given after grafting. However, both these groups have demonstrated that anti-lymphocytic serum causes considerable prolongation of second set skin allografts and heterografts [LEVEY and MEDAWAR, 1966a; MONACO *et al.*, 1966c].

3. The specificity of anti-lymphocytic serum

The data obtained on skin allograft survival in rats using horse antiserum to the thoracic duct lymphocytes of hooded rats suggests that this immunosuppressive property is not strain specific [ANDERSON *et al.*, 1967a]. In contrast, however, BRENT *et al.* [1967a] demonstrated that strain specificity may occur. Their rabbit antiserum to CBA

strain mouse lymph node cells, markedly increased the survival of A strain tail skin homografts on CBA recipients but only slightly prolonged the survival of similar grafts on C57 recipients. A similar strain specificity has previously been noted in experiments designed to investigate the effect of anti-lymphocytic serum on humoral antibody production in rats [JAMES and ANDERSON, 1967].

4. Studies in primates

Heterologous and homologous antisera to rhesus monkey lymphoid tissues have also been shown to prolong skin allograft survival in the rhesus monkey [BALNER and DERSJANT, 1967]. Rabbit antisera to monkey thymocytes appeared more effective than rabbit antisera to lymph node cells and both types of antisera were more effective than that produced in the related monkey species (*Macaca cynomolgus*). The IgG globulin fraction of the rabbit anti-thymocyte serum had similar immunosuppressive properties to the original serum but appeared less toxic. Recently it has also been shown that antisera to human lymphoid tissues will prolong skin allograft survival in chimpanzees and monkeys and it has been suggested that these primate models might be most suitable for testing human antisera [BALNER *et al.*, 1968].

5. The effect of anti-lymphocytic antibody fragments on skin allograft survival

As previously discussed in the section on the characterization of anti-lymphocytic antibodies and as illustrated in figure 4 the major part anti-lymphocytic activity of many sera appears to reside in the IgG fraction [JAMES and MEDAWAR, 1967a; ANDERSON *et al.*, 1967a; BALNER and DERSJANT, 1967; IWASAKI *et al.*, 1967; CURREY and ZIFF, 1966; GUTTMAN *et al.*, 1967a, b, c and d; DENMAN *et al.*, 1967a; PICHLMAYR, 1967b]. These results led us to investigate the effects of anti-lymphocytic antibody fragments on skin allograft survival. However, the results obtained indicate that the destruction of the Fc portion of the anti-lymphocytic antibody molecule impairs its immunosuppressive properties, for neither the divalent $F(ab')_2$ nor the univalent Fab' fragments were able to prolong the survival of albino strain skin allografts on hooded recipients. Results similar to these have recently been reported by RIETHMÜLLER *et al.* [1968a and b]. Thus it appears that these less 'antigenic' preparations will

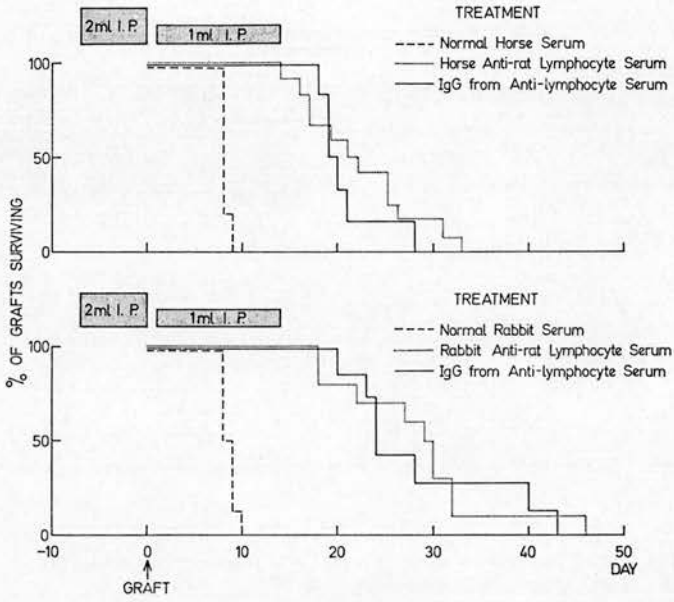


Fig. 4. The effect of anti-lymphocytic antibody on skin allograft survival in rats (albino strain grafts to hooded recipients).
All the preparations used in these experiments contained approximately 1 g% of IgG. Further details of the course are indicated in the figure. Note that both antisera and their IgG components were effective in prolonging skin allograft survival. [For further details see text and ANDERSON *et al.*, 1967a.]

be of little therapeutic value. However, it has been reported that large doses of anti-lymphocytic antibody fragments delay renal allograft rejections [GUTTMAN *et al.*, 1967b and c; see page 176].

6. The potentiation of the immunosuppressive effect

A number of procedures are known to potentiate the immunosuppressive effect of anti-lymphocytic antibody [WOODRUFF, 1967]. Lymphocyte depletion through a thoracic duct cannula leads to a marked prolongation of skin allograft survival in anti-lymphocytic antibody treated rats [WOODRUFF and ANDERSON, 1963, 1964] and thymectomy potentiated the immunosuppressive effect of the course of anti-lymphocytic serum treatment used by JEEJEEBHOY [1965a and b and 1967]. In mice the effects of thymectomy have differed. MONACO *et al.* [1965a and b] observed that it potentiated the effect of

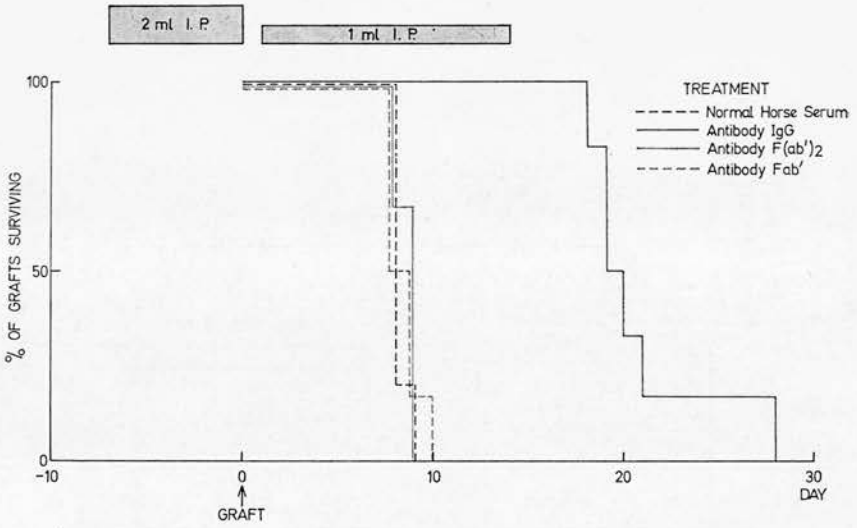


Fig. 5. The effect of anti-lymphocytic IgG and IgG fragments on skin allograft survival in rats (albino strain grafts to hooded recipients).
All the preparations used contained 1 g% of protein. Note that the non-cytotoxic $F(ab')_2$ and Fab' fragments failed to prolong skin allograft survival. [For further details see text and ANDERSON *et al.*, 1967a.]

anti-lymphocytic antibody but LEVEY and MEDAWAR [1966b] failed to observe any potentiation in their system. Whole body irradiation prior to the injection of anti-lymphocytic antibody and hydrocortisone acetate were both found to increase its effects. However, irradiation following the antibody treatment or adrenalectomy diminished its immunosuppressive effect [LEVEY and MEDAWAR, 1966b].

C. Effect on whole organ transplants

1. Renal transplants

Having demonstrated that anti-lymphocytic antibody effectively delayed the rejection of skin allografts, the natural sequel was to investigate the effect of this material on whole organ allotransplants possessing vascular anastomosis. To date a number of models have been investigated, namely the effect of this material on renal allotransplants in rats and on renal and liver allotransplants in dogs. These will be considered in turn.

2. Effect on canine renal allotransplants

The results so far obtained in canine renal allograft studies have been extremely encouraging. Once more, however, there has been considerable variation in the nature of the anti-lymphocytic sera used and in the course of treatment. The lymphoagglutination titres of the preparations have varied from 256–2048, although the individual values are probably influenced by the assay procedure. The amount of material administered has ranged from 0.25 ml to 4 ml per kilogram per day and it has been given by intravenous, intraperitoneal and subcutaneous routes.

Some investigators have given several days pretreatment with anti-lymphocytic antibody prior to transplantation [MONACO *et al.*, 1966a; STARZL *et al.*, 1967a; CLUNIE *et al.*, 1968; see fig. 6]. Others, however, have commenced anti-lymphocytic serum treatment either

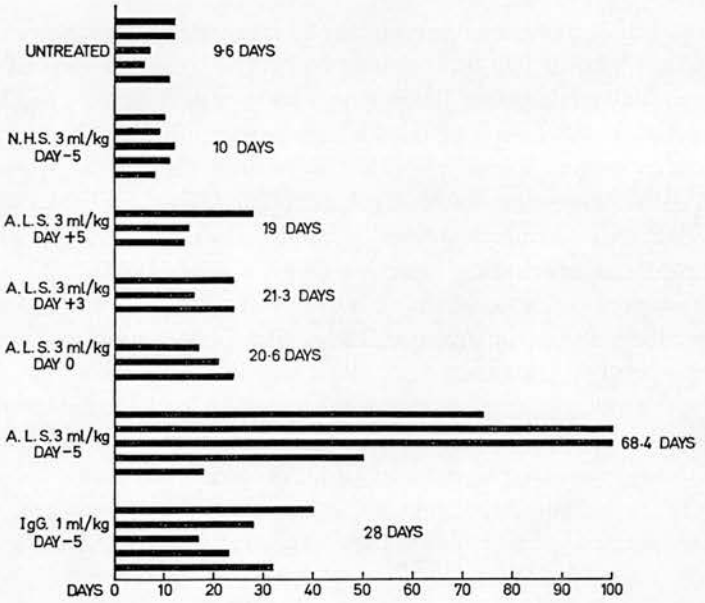


Fig. 6. The effect of anti-lymphocytic antibody on the survival of dogs with renal transplants.

The transplantation was performed between beagle dogs on day 0. The preparations were administered subcutaneously commencing on the day indicated on the left of the figure. The anti-lymphocytic serum contained approximately 1 g% IgG while the IgG preparation was used at a concentration of 3 g%. Note that the anti-lymphocytic serum was most effective if administered prior to transplantation. [For further details see text and CLUNIE *et al.*, 1968] (Reproduced by kind permission of the Editor of Transplantation.)

on the day before or on the actual day of transplantation [ABAZA *et al.*, 1966; PICHLMAYR, 1966, 1967a; PICHLMAYR *et al.*, 1967c, d and e; HERMAN and SCHLOERB, 1967; LAWSON *et al.*, 1967; STARZL *et al.*, 1967a].

3. Transplantation procedure

The majority of the experiments have been performed in bilaterally nephrectomized mongrel and beagle dogs. The exceptions have been in the experiments of ABAZA *et al.* [1966] where one of the recipients own kidneys was removed at the time of transplantation and the other 21 days later, and those of LAWSON *et al.* [1967] where one of the recipients own kidneys was left in place throughout the investigation. Further details of the surgical procedure are recorded in the original papers.

4. Results

In all but one of the investigations so far reported anti-lymphocytic serum or globulin has been found to prolong the survival of dogs with renal allotransplants [exception HERMAN and SCHLOERB, 1967]. The recent results from our own laboratory are illustrated in figure 6. The survival have varied considerably within most groups, even in those using a standard treatment schedule. This variation is due presumably to technical factors, genetic variations and other complicating factors including infection [ABAZA *et al.*, 1966].

As in previous studies on skin allograft survival, crude globulin and purified IgG preparations have also been found to prolong allograft survival [IWASAKI *et al.*, 1967; CLUNIE *et al.*, 1968]. However, there was some suggestion in the studies of CLUNIE *et al.* [1968] that the isolated IgG was less effective (fig. 6).

Although successful prolongation of renal allografts has been achieved in animals in which anti-lymphocytic treatment was commenced on the day before or on the day of grafting [ABAZA *et al.*, 1966; PICHLMAYR 1966, 1967a and PICHLMAYR *et al.*, 1967c and e], several groups have convincingly shown that the maximum immunosuppressive effect occurs if the treatment is commenced several days before grafting (fig. 6, also STARZL *et al.*, 1967a; CLUNIE *et al.*, 1968]. If transplantation is delayed up to 60 days after anti-lymphocytic antibody treatment, then there is no protection of renal allografts [STARZL *et al.*, 1967a]. Attempts have also been made to potentiate the effect of anti-lymphocytic therapy by thymectomization and by

the simultaneous use of azothioprine. However, both these approaches failed to prolong the survival beyond that achieved with anti-lymphocytic serum alone [STARZL *et al.*, 1967a].

5. Histological examination of kidneys

In almost all dogs receiving anti-lymphocytic serum there has been histological evidence of rejection in the kidneys. The most extensive histological studies have been those of STARZL and his coworkers [STARZL *et al.*, 1967a; IWASAKI *et al.*, 1967]. These workers examined 99 renal homografts from dogs treated with anti-lymphoid preparations and the features of rejection were no different than those observed in animals treated with other immunosuppressive agents. The features included infiltration with mononuclear cells of which 20 to 80% had pyroninophilic cytoplasm. Platelet thrombi and intimal fibrous thickening of intertubular and arouate arteries were common. In half of the specimens fibrinoid necrosis of arteriolar and arterial walls was present, and in 27% the glomerular capillary basement membrane was thickened. The only clear difference in their various test groups was a reduction in the degree of cellular infiltration, the number of cells possessing pyroninophilic cytoplasm, and the incidence of mitoses in those homografts from dogs receiving combined azathioprine and anti-lymphocytic globulin therapy. In the latter group the only two kidneys in the whole study which failed to show evidence of rejection were also found. Similar rejection changes to the ones reported above have also been observed by others [ABAZA *et al.*, 1966; MONACO *et al.*, 1966; PICHLMAYR *et al.*, 1967e]. However, in an appreciable proportion of their long surviving allografts these investigators failed to detect histological evidence of rejection.

In addition to the histological changes accompanying rejection there has been evidence of other changes in the kidney directly attributable to the use of heterologous globulin [IWASAKI *et al.*, 1967]. Thickening of the glomerular basement membrane frequently occurs and occasionally glomerular deposits can be observed under the electron microscope. In the long treated animals the above changes were occasionally accompanied by hypercellularity of the tufts and adhesions between tufts and capsules. Ultrastructurally the same dense deposits were present on the subepithelial aspects of the glomerular capillary basement membranes and in the mesangium. Horse gamma globulin, dog IgA and β1C globulin were detected in

the altered basement membrane by immunofluorescent procedures. The highest incidence of lesions occurred in those animals which had been treated for the greatest length of time and were particularly prevalent in animals in which the antiserum had been administered intravenously. Similar changes to the above were also observed in those dogs which had received normal horse globulin.

6. Cause of death

Although in most cases the cause of death has been due to the uraemia associated with the graft rejection, a number of animals have died with healthy grafts. In these situations there was no evidence of rejection and the blood urea level was normal. The cause of death in these cases has been attributed to gastro-intestinal haemorrhage, hepatitis, pneumonia and distemper [ABAZA *et al.*, 1966] and to post-infectious myelitis [PICHLMAYR *et al.*, 1967e]. One of the major causes of death in short term survivors has been intussesception and arterial and venous thrombosis [MONACO *et al.*, 1966a; PICHLMAYR *et al.*, 1967e].

7. Side effects of treatment

The intravenous or intraperitoneal administration of partially absorbed or unabsorbed anti-lymphocytic antibody is poorly tolerated. It causes massive hemolysis, anaemia and bloody diarrhaea and frequently proves fatal [MONACO *et al.*, 1966a; STARZL *et al.*, 1967a]. In contrast, anti-lymphocytic sera which have been extensively absorbed with dog erythrocytes can be administered safely by the intravenous route [ABAZA *et al.*, 1966; PICHLMAYR *et al.*, 1967e]. However, the initial injections may still produce side effects; the dogs begin to salivate, become somewhat ataxic, vomit and defecate [ABAZA *et al.*, 1966]. Similar symptoms were frequently observed 10–20 days after the start of anti-lymphocytic antibody treatment [ABAZA *et al.*, 1966; PICHLMAYR *et al.*, 1967e].

An additional complication of anti-lymphocytic antibody therapy is the high incidence of histologic renal damage observed in animals receiving prolonged courses of treatment with this material. This is attributed to the deposition of antigen antibody complexes in the kidney which results in a Masugi or serum sickness type nephritis. The antigen antibody complexes consist of the heterologous horse protein (the antigen), and dog antibodies to this material, along with components of dog complement. The incidence of these lesions appears to be reduced by the administration of semi-purified material

by the subcutaneous route [IWASAKI *et al.*, 1967]. It has also been observed that the material is less toxic if administered by this route [MONACO *et al.*, 1966a; STARZL *et al.*, 1967a; CLUNIE *et al.*, 1968].

The original observations of ABAZA *et al.* [1966] suggested that this treatment might predispose the recipients to infection and possibly abolish a pre-existing state of immunity. However, recent studies have indicated that this does not constitute a serious problem [MONACO *et al.*, 1966; LAWSON *et al.*, 1967]. Furthermore, it has been shown that animals receiving anti-lymphocytic antibody treatment can still produce high levels of circulating antibodies against dead *Brucella abortus* bacteria [PICHLMAYR *et al.*, 1967e].

8. Effect on canine liver allotransplants

There have been a number of brief reports indicating that anti-lymphocytic serum delays the rejection of orthotopic liver transplants in dogs [STARZL *et al.*, 1966, 1967a and d; PICHLMAYR *et al.*, 1967e and f].

In the experiments reported by STARZL *et al.* [1966 and 1967d] the dogs were injected intraperitoneally with a partially absorbed anti-lymphocytic serum of low titre (32 to 128). The dose used was 1 to 4 ml per kg per day, and this was given for 1–26 days prior to operation. All but one of the dogs continued to receive this material postoperatively. The mean survival times of the anti-lymphocytic antibody treated dogs was 26.8 $\pm$ 26 days, with a limit of maximum survival being taken as 70 days. Two of the dogs were still alive and in good health after 5–6 months. One of these dogs received only 6 injections of antibody over a 26-day period prior to transplantation and then had no further treatment. The other was treated for 1 month prior to operation and for 20 days postoperatively. The mean survival time of untreated dogs with orthotopic liver transplants was 7.0 $\pm$ 3 days.

PICHLMAYR *et al.* [1967e and f] treated their dogs with horse antiserum to thoracic duct lymphocytes. This material had a lympho-agglutination titre of 2000 and was administered intravenously, the dose being 0.5 ml per kg twice daily commencing on the day of operation. The mean survival time in this group was 28 days, but once again there was a considerable range (1 day to 138 days).

The histological changes observed on autopsy of biopsy hepatic allograft specimens were similar to those found in comparable dogs receiving azathioprine [STARZL *et al.*, 1967a]. In the short term

survivors (less than 3 weeks), there was centrizonal and midzonal necrosis, mononuclear cell infiltration around the portal tracts and central veins, and centrilobular cholestasis. The longer surviving allografts (greater than 3 weeks) had variable centrilobular hepatocyte atrophy or reticulin condensation and at a later stage, fibrosis and proliferation of bile ductules in the portal tracts. Mononuclear cell infiltration, though present, was usually less marked. In general, the rejection observed histologically was less marked than that observed in kidney transplants. The results obtained are most encouraging and it is highly probable that they will be improved by extensive pre- and postoperative treatment with more active preparations (high titre).

9. Effect on renal allografts in the rat

The rejection of renal allografts has also been delayed in rats by administration of rabbit antibody ro rat thymus [GUTTMAN *et al.*, 1967a, b, c and d]. The kidneys of (Lewis × BN)F1 hybrid rats were transplanted into Lewis hosts using the microvascular technique of FISHER and LEE [1965]. Allograft rejection was assessed on day 7 following transplantation by morphological examination of the transplant and by measuring the effective renal plasma flow using ^{125}I-labelled hippuran.

These investigations revealed that eight intravenous injections of 1.2 mg of anti-rat thymus IgG over a period of 12 days, delayed the rejection of transplants performed 3 days later. There was only a slight peri-vascular accumulation of mononuclear cells in the kidneys 7 days following transplantation and renal plasma flow was in the normal range. It was also found that treatment of the kidney donors with 4 to 6 intravenous injections (6–15 mg) of the antibody IgG over a period of 5 to 9 days prior to transfer to unmodified Lewis hosts also delayed the onset of rejection, though the effect was not as marked as that achieved by pretreatment of the recipient [GUTTMAN *et al.*, 1967a and c]. Furthermore, it was also observed that antibody fragments delayed the rejection of renal allografts although similar preparations had failed to prolong skin allograft survival in rats [ANDERSON *et al.*, 1967a] and mice [RIETHMÜLLER *et al.*, 1968a and b]. However, in the renal allograft experiments large amounts of fragments were used and in the few animals studied the immunosuppression achieved was not as marked as that observed using much smaller amounts of intact antibody [GUTTMAN *et al.*, 1967c and d].

The anti-lymphocytic antibody appeared to be extremely nephrotoxic producing a nephritis characteristic of immune complex disease and an autologous phase nephrotoxic nephritis. The glomeruli showed hypercellularity increased mesangial matrix and some adhesions and epithelial crescents and glomerular localization of the heterologous antigen (rabbit IgG), host IgG and B_1C were observed histochemically [GUTTMAN *et al.*, 1967b]. Serum sickness type nephritis was eliminated by absorption of the antibody with normal rat serum and by using purified antibody while the nephrotoxic nephritis was reduced by subcutaneous administration, and was attributable to antibody activity against glomerular basement membrane [GUTTMAN *et al.*, 1967c].

D. Effect on other cellular phenomena

In addition to their ability to suppress cell mediated allograft rejection processes, anti-lymphocytic sera have also been shown to inhibit other important cellular immune responses.

LEVEY and MEDAWAR [1966a and 1967] demonstrated that rabbit antibody to guinea pig lymph node cells completely abolished all components of the normal and sensitized lymphocyte transfer reactions. This could be achieved by treating either the donor or the recipient of the lymph node cells. Conventional immunosuppressants such as azathioprine, cyclophosphamide irradiation etc. failed to inhibit these reactions.

It has also been convincingly demonstrated that anti-lymphocytic antibody will suppress the so-called graft versus host reactions (GVH) which result from the immunological interaction between a lymphoid graft and the histocompatibility antigens unique to the host. The severity of graft versus host reaction can be assayed by the resulting increase in the size of the spleen and liver and by increased phagocytic activity. The secondary disease which may ensue in certain systems can be estimated by the degree of runting produced and the premature death of the recipients of the lymphoid cells.

The immunosuppressive effects of anti-lymphocytic antibody have been shown in a number of mice systems which give rise to the GVH reaction or secondary disease. These include the transfer of parental strain spleen, lymph node or thoracic duct cells to normal or lethally irradiated F1 hybrid recipients [MONACO *et al.*, 1967b; BOAK *et al.*,

1967a, b; VAN DER WERF *et al.*, 1967; VAN BEKKUM *et al.*, 1967b; LEVEY and MEDAWAR 1967; NAYSMITH and JAMES, 1968] and the transfer of allogeneic lymphoid cells to neonatal mice [BRENT *et al.*, 1967a and b; MONACO *et al.*, 1967b; VAN DER WERF *et al.*, 1967]. The graft versus host reaction and the secondary disease could be suppressed by treatment of the lymphoid cell donors, or recipients, or by incubating the lymphoid cells *in vitro* with anti-lymphocyte antibody [BRENT *et al.*, 1967a; VAN BEKKUM *et al.*, 1967]. However, treatment of lymphoid cell donors with anti-lymphocytic antibody fragments failed to inhibit the graft versus host reaction [NAYSMITH and JAMES, 1968]. Recently, it has also been shown that treatment of rat spleen cells *in vitro* with anti-lymphocytic serum will also suppress the graft versus host which normally results following transfer to F1 hybrid rats [FIELD and GIBBS, 1968].

Although all the studies described above have been performed in rodents, VAN BEKKUM *et al.* [1967] have also shown that anti-lymphocytic antibody suppresses the secondary disease which follows the transfer of allogeneic bone marrow cells to X-irradiated monkeys. This was achieved by treating the recipients with the antibody and was found to be more effective than any other known chemotherapeutic reagent. However, all the anti-lymphocytic antibody treated animals succumbed to viral infection.

Another important effect of anti-lymphocytic antibody is that in conjunction with thymectomy it has permitted the establishment of specific immunological tolerance [MONACO *et al.*, 1966b; RUSSELL and MONACO 1967]. This was achieved by administration of a small dose of allogeneic donor lymphoid cells to thymectomized adult recipients which had been treated for 7 days with anti-lymphocytic serum (0.25 ml per day). This resulted in a state of lymphoid chimerism and specific immunological tolerance to donor strain skin grafts but failed to impair immunological activity against skin grafts from a third strain of mice.

Several investigators have demonstrated that anti-lymphocytic serum suppresses delayed hypersensitivity to tuberculin in guinea pigs [INDERBITZIN, 1956; WILHELM *et al.*, 1958; WAKSMAN *et al.*, 1961] and in mice [RUSSE and CROWLE, 1965]. This material is also able to delay the onset of immediate and delayed hypersensitivities to ovalbumin and BSA in mice [RUSSE and CROWLE, 1965] and the delayed reaction to purified diphtheria toxoid in guinea pigs [WAKSMAN *et al.*, 1961]. In addition it also inhibits contact hypersensitivity in guinea

pigs to dinitrochloro-benzene and oxazolone and partially suppresses the non-specific reaction to turpentine [WAKSMAN *et al.*, 1961; TURK and WILLOUGHBY, 1967]. It failed, however, to inhibit the humoral antibody mediated passive cutaneous anaphylactic reaction to ovalbumin or the reversed passive Arthus reaction to bovine serum albumin [WAKSMAN *et al.*, 1961]. Recently, it has also been demonstrated that antiserum to human lymphoid tissue will suppress delayed hypersensitivity to dinitrochloro-benzene in chimpanzees [BALNER *et al.*, 1968].

Of great interest is the apparent ability of this material to delay or prevent the onset of a number of autoimmune processes. WAKSMAN *et al.* [1961] originally demonstrated that anti-lymphocytic serum partially suppressed allergic encephalomyelitis in guinea pigs. Since then this material has been shown to effectively suppress Freund adjuvant polyarthritis in rats [CURREY and ZIFF, 1966] and Coombs positive haemolytic anaemia in NZB mice [DENMAN *et al.*, 1967a]. It failed, however, to modify the course of already established haemolytic disease in NZB mice or to prevent the onset of autoimmune renal disease in B/W mice (NZB × NZW)F1 [DENMAN *et al.*, 1966]. Recently, it has also been claimed that rabbit antiserum against mouse lymph node cells reduces the incidence and severity of experimental amyloidosis in mice [RANLØV, 1967].

E. Effect on humoral immune responses

1. Effect on humoral antibody formation against antigens other than anti-lymphocytic globulin

In addition to their effects on cell mediated immune phenomena, anti-lymphocytic sera also suppress or delay primary humoral antibody responses. The systems in which such immunosuppression has been demonstrated are summarized in the accompanying table (tab. II).

By way of illustration the effect of horse anti-rat lymphocyte IgG on the primary response to bovine serum albumin is shown in figure 7. From this figure it will be seen that animals pretreated with normal horse IgG (Gp a) readily responded to a subsequent challenge with alum precipitated bovine serum albumin. Whilst in animals receiving anti-lymphocytic IgG the primary response was suppressed and delayed (Gp b).

Table II. The effect of anti-lymphocytic sera on the primary humoral antibody response

Donor species	Antigen	Produced in	Suppresses primary response to	References
Mouse	Lymph nodes	Rabbit	Salmonella II antigen and sheep erythrocytes	GRAY *et al.* [1964]; MONACO *et al.* [1965]; MONACO *et al.* [1966]
	Thymocytes	Rabbit	Sheep erythrocytes	BERENBAUM [1967]
	Thymocytes and lymph nodes	Rabbit	Sheep erythrocytes	DENMAN *et al.* [1967a]
Rat	Thoracic duct lymphocytes	Rabbit and horse	Sheep erythrocytes Alum precipitated BSA	JAMES and ANDERSON [1967]; JAMES and JUBB [1967]; JAMES [1967b]; JAMES and ANDERSON [1968]
	Mesenteric and thoracic lymph node cells	Rabbit	Sheep erythrocytes	JEEJEEBHOY [1965a, b and 1967]
	Lymph node thymus and spleen cells	Rabbit	Sheep erythrocytes	CURREY and ZIFF [1966]
Dog	Thoracic duct lymphocytes	Horse	Sheep erythrocytes	PICHLMAYR *et al.* [1967c and e]

Several reports have indicated that anti-lymphocytic antibody is most effective if administered prior to the antigen. This has been demonstrated in both mice and dogs using sheep erythrocytes [BERENBAUM, 1967; PICHLMAYR *et al.*, 1967e] and in rats using alum precipitated bovine serum albumin [JAMES, 1967b]. The experiments in rats are again illustrated in figure 7. In group d where anti-lymphocytic antibody treatment commenced 4 hours after antigenic challenge the response was similar to that in controls receiving normal horse IgG before or after challenge with bovine serum albumin (Gps a and c). From these results it would appear that anti-lymphocytic antibody is interfering with the sensitization (triggering) phase of the humoral response and that it has little effect on the proliferative phase [BERENBAUM, 1967; JAMES, 1967a and b].

Anti-lymphocytic antibody fragments failed to suppress the primary response of rats to sheep erythrocytes or to alum precipitated bovine serum albumin supporting previous observations on skin allograft survival [JAMES and ANDERSON, 1967; JAMES, 1967b; ANDERSON *et al.*, 1967a and b]. The effects of both rabbit and horse anti-rat lymphocyte IgG fragments on the primary response to alum precipitated bovine serum albumin are illustrated in figure 7 (groups e and f, compare group b using intact antibody).

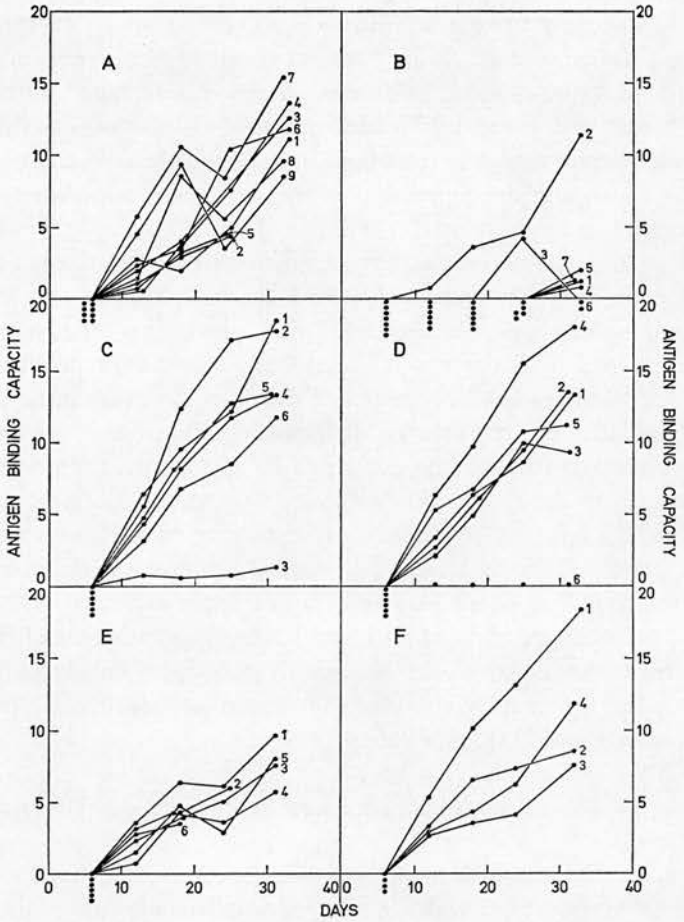


Fig. 7. The effect of anti-lymphocytic IgG and fragments on the primary response of hooded rats to alum precipitated BSA.
Rats received a) normal horse IgG, b) anti-lymphocytic IgG, c) normal horse IgG, d) anti-lymphocytic IgG pretreatment, e) $F(ab')_2$ from horse anti-lymphocytic IgG and f) $F(ab')_2$ from rabbit anti-lymphocytic IgG. In groups a, b, e and f the rats were injected with 2 ml of 1 g% solution on days –3, –2 and –1 and the test antigen (5 mg alum precipitated BSA) was injected intraperitoneally on day 0. In groups c and d the preparations were injected 4, 28 and 52 h after the test antigen. Note that suppression of humoral antibody formation occurs only in group b. [For further details see text and JAMES, 1967b.] (Reproduced by kind permission of the Editor of Clinical and Experimental Immunology.)

The results to date indicate that doses of anti-lymphocytic antibody, which are capable of suppressing the primary response, fail to have a pronounced effect on the secondary response to sheep erythrocytes in mice [MONACO *et al.*, 1966c] and to sheep erythrocytes and alum precipitated bovine serum albumin in rats [JAMES and ANDERSON, 1967; JAMES and JUBB, 1967]. Nevertheless, it has been shown that anti-lymphocytic antibody treatment in conjunction with thymectomy will cause a significant depression of the secondary response to sheep erythrocytes in mice [MONACO *et al.*, 1965b].

Cell transfer experiments have confirmed that the doses of anti-lymphocytic antibody previously used are incapable of inactivating sensitized spleen cells *in vivo* and thus preventing the secondary response, i.e. fail to destroy memory cells or prevent proliferation. However, such studies have revealed that low doses of anti-lymphocytic antibody are capable of inactivating sensitized spleen cells *in vitro* although this may be explained in part by the failure of these cells to seed in the spleen [JAMES, 1968; MARTIN and MILLER, 1967]. The studies referred to involve transferring sensitized spleen or lymph node cells to X-irradiated recipients following the treatment of the cells *in vivo* or *in vitro* with anti-lymphocytic antibody. The animals are challenged later with the sensitizing antigen and the immune response assessed. In studies of this type anti-lymphocytic antibody fragments were also unable to inactivate sensitized lymphoid cells [HARRIS and HARRIS, 1966a].

2. The effect of anti-lymphocytic antibody on humoral antibody formation against itself

Almost all the anti-lymphocytic sera used to date have been of the heterologous type and are therefore extremely antigenic when administered to the species against whose lymphoid tissue they were produced. In addition to the possible reduction in efficacy of this material due to its rapid immune elimination, its antigenicity also constitutes a potential therapeutic hazard. It is well known that deletirious immunological side effects frequently accompany the administration of heterologous protein, including anaphylactic shock and serum sickness nephritis. A number of studies have, therefore, been undertaken to determine the immunogenicity of anti-lymphocytic globulin. In these studies the circulating antibodies to the anti-lymphocytic preparation have been assessed by precipitation, passive haemagglutination and immune elimination procedures.

Although the investigations of MONACO *et al.* [1966c] and IWASAKI *et al.* [1967] indicated that anti-lymphocytic antibody might exert an antidotal effect, others have failed to observe this [CURREY and ZIFF, 1966; ANDERSON *et al.*, 1968b; GUTTMAN *et al.*, 1967a, b and c; CLARK *et al.*, 1967; LANCE and DRESSER, 1967; JAMES and ANDERSON, 1968]. The results of studies performed in our own laboratories to determine the effect of anti-lymphocytic IgG on antibody formation against itself are displayed in figure 8 and 9. Several of these investigations have shown that anti-lymphocytic IgG may be more immunogenic than its normal IgG counterpart [CURREY and ZIFF, 1966; GUTTMAN *et al.*, 1967b and c; LANCE and DRESSER, 1967].

The recent observations of RUSSELL and MONACO [1967] indicate that the antidotal and/or immunogenic effects of anti-lymphocytic antibody are dose dependent. When large amounts of lymphocytic antibody are administered, it is claimed that all the cells which would produce humoral antibody are inactivated or destroyed. With smaller amounts, heterologous protein probably persists in the animal beyond the period of immunosuppression and so antibody formation can occur. Nevertheless, animals receiving large doses of anti-lymphocytic

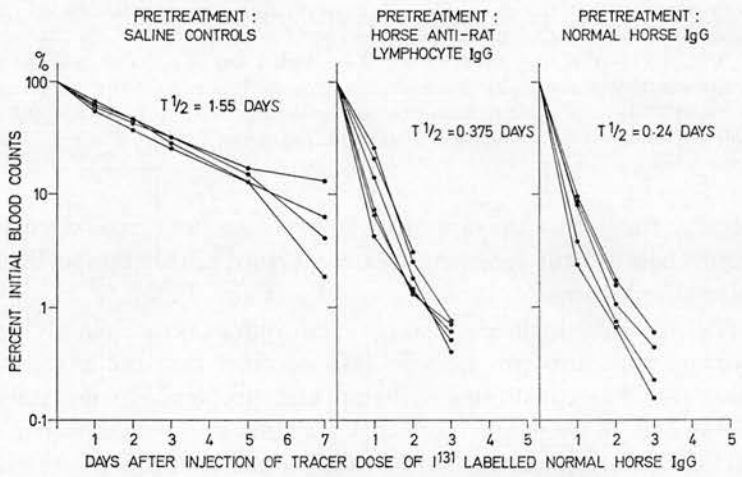


Fig. 8. Immune elimination studies in rats pretreated with normal horse and horse anti-rat lymphocyte IgG.
The course of pretreatment was similar to that illustrated in fig. 4 and 9. Note that rats pretreated with either normal or anti-lymphocytic IgG exhibit marked immune elimination. Similar results were also obtained from whole body counts. [For further details see CLARK *et al.*, 1967.]

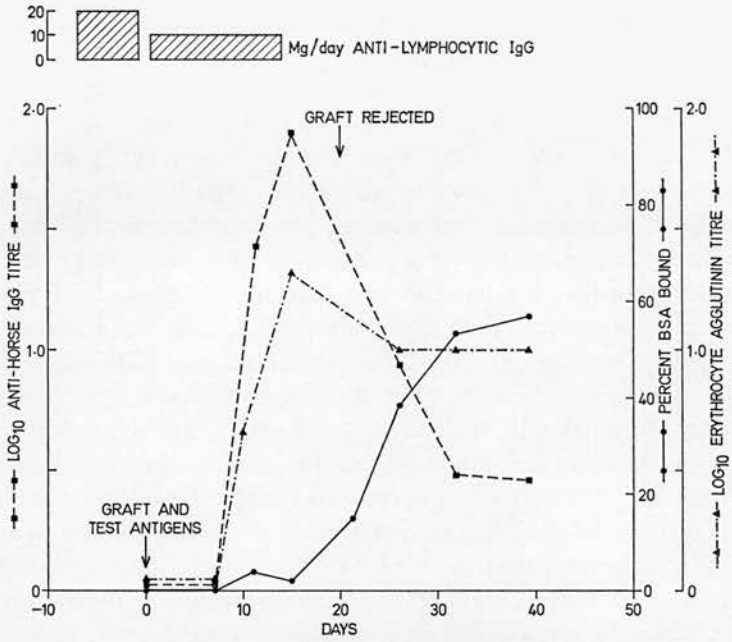


Fig. 9. The response of anti-lymphocytic antibody treated rats to multiple antigenic challenge.
A schematic diagram of the effect of anti-lymphocytic IgG on cell mediated and humoral antibody responses in the same rat. The rats were grafted on day 0 and were also injected (I.V.) with 1×10^9 sheep erythrocytes and (I.P.) with 5 mg of alum precipitated BSA. Note that circulating antibodies to the anti-lymphocytic IgG and the sheep erythrocytes were generally detected prior to graft rejection while antibodies to BSA appeared much later. [For further details see text and JAMES and ANDERSON, 1968.]

antibody may develop appreciable amounts of free circulating antibody whilst still receiving treatment with anti-lymphocytic IgG [JAMES and ANDERSON, 1968, see also fig. 8 and 9].

The results obtained in dogs and humans receiving chronic treatment with anti-lymphocytic IgG confirm that the antigenicity of this material constitutes a therapeutic problem. In dogs serum sickness and Masugi type nephritis may develop as a result of this form of therapy [IWASAKI *et al.*, 1967] while anaphylactic reactions have been observed in 20% of the humans treated with anti-lymphocytic globulin [KASHIWAGI *et al.*, 1968]. It thus appears essential that procedures must be developed to reduce these hazards. In this respect the simultaneous use of low doses of conventional immunosuppressions might be of value in humans (see later). Another

approach to the problem has been tried in rats and mice. These have been made tolerant by injection of large amounts of normal heterologous IgG or by chronic anti-lymphocytic antibody treatment [DENMAN and FRENKEL, 1967b; LANCE and DRESSER, 1967]. However, under these conditions the rats developed a form of haemolytic anaemia and became runted [DENMAN and FRENKEL 1967b].

3. Simultaneous studies of the effect of anti-lymphocytic antibody on cellular and humoral immune responses

Although numerous studies have shown that anti-lymphocytic antibody is capable of suppressing both cellular and humoral aspects of the immune response, there have been few reports in which its effect on both types of immune response have been assessed in one and the same animal. Studies of this kind are important of course if we wish to elucidate the mode of action of anti-lymphocytic antibody and determine what effect it might have on the recipients resistance to bacterial and viral infections.

JEEJEEBHOY [1965a and b, 1967] originally demonstrated that anti-lymphocytic serum treatment in conjunction with thymectomy could prolong skin allograft survival and at the same time inhibit humoral antibody formation against sheep erythrocytes and tetanus toxoid. Other workers have also found that horse antisera to dog lymphoid tissue will simultaneously prolong renal allograft survival and partially suppress antibody formation against sheep erythrocytes. The dogs, however, responded well to dead *Brucella abortus* bacteria [PICHLMAYR *et al.*, 1967c and e].

We have also investigated the simultaneous formation of circulating antibodies against alum precipitated bovine serum albumin, sheep erythrocytes and anti-lymphocytic IgG in hooded rats which had also received an albino strain skin allograft [JAMES and ANDERSON, 1968]. The results obtained are summarized schematically in figure 9. Generalizing it may be said that with the course of anti-lymphocytic IgG used, circulating antibodies against anti-lymphocytic IgG and sheep erythrocytes were detected prior to cessation of anti-lymphocytic antibody treatment and before skin allograft rejection. Circulating antibodies against bovine serum albumin, however, were usually detected following graft rejection.

VIII. Studies in Humans with Anti-Lymphocytic Sera

A. Introduction

The encouraging results obtained in animal experiments have stimulated clinical investigations of the immunosuppressive properties of anti-lymphocytic serum. From the outset many of the potential dangers associated with the administration of heterologous protein were appreciated, including anaphylactic shock and serum sickness type nephritis [STARZL *et al.*, 1967b; RUSSELL and MONACO, 1967]. Nevertheless, this material has now been used in a number of centres as an additional means of immunosuppression in renal and liver allotransplantation and also in the treatment of patients with auto-immune disease. Although the results to date are difficult to assess critically because of the combined therapy used, the relatively small number of cases and the short-term nature of the investigations, it does appear that anti-lymphocytic antibody may have considerable potential as an additional form of immunotherapy in human organ transplantation. However, this material possesses toxic properties in addition to those originally anticipated and, therefore, a considerable amount of basic research will be necessary before it can be used with confidence in transplantation surgery.

B. The use of anti-lymphocytic antibody in human transplantation

1. Effect on skin allograft survival

The only recorded studies on the effect of anti-lymphocytic antibody on skin allograft survival in humans are those of MONACO and co-workers [MONACO *et al.*, 1967a and b]. The anti-serum used was prepared as previously described (see pages 147 and 154) and had a lymphoagglutinin titre of 2048 and a protein concentration of 1 g per 100 ml. The subjects (normal volunteers) were injected (deep subcutaneous) on days 1, 3 and 5 with volumes of 9, 5 and 5 ml respectively and received 1 to 3 skin allografts from different donors on day 2. The controls were 39 allografts performed on normal recipients as part of histocompatibility studies. Within 11 days, 31 out of 39 of the control allografts were rejected, while all the test allografts survived 12 days or more (see tab. III). These preliminary

Table III. The effect of purified rabbit anti-human lymphocyte IgG on human skin allograft survival

Treatment	Nos. of grafts	Days of graft survival											
		8	9	10	11	12	13	14	15	16	17	18	19
None	39	1	16	10	4	2	3			3			
Anti-human lymphocyte IgG	10					1	1	3	2	1	1		1

For further details of the course of anti-lymphocytic IgG treatment see the text. (Published by kind permission of the Authors and the Editor of the Ciba Foundation Study Group No. 29.)

findings on this small number of cases suggests that the brief course of anti-lymphocytic antibody treatment used helped to prolong the survival of skin allografts.

2. The use in renal transplantation

Anti-lymphocytic globulin has now been used as an adjunct to the standard immunosuppressive drugs, azathioprine and prednisone, in the treatment of an appreciable number of renal allograft recipients. It has been used in both newly operated cases and in cases where there was evidence of graft rejection [STARZL *et al.*, 1967a, b and c; STARZL *et al.*, 1968; WOODRUFF *et al.*, 1968; CALNE *et al.*, 1968; RUSSELL *et al.*, 1968; SHORTER *et al.*, 1967; CARRAZ *et al.*, 1967; TRAEGER *et al.*, 1968]. As the major part of the reported clinical work has been undertaken by STARZL and his associates, this section of the review will be centred around the work of this group.

During the last 14 months, STARZL and his workers have treated 53 renal allograft recipients with anti-lymphocytic globulin. However, most of the data presented will be restricted to the patients receiving this treatment between 21st June and 12th December 1966, for these patients have now survived a sufficient period to enable a reasonable assessment of the efficacy of anti-lymphocytic globulin.

The antibody preparations used have been described elsewhere [IWASAKI *et al.*, 1967]. The ammonium sulphate precipitated globulin contained gamma globulin, IgT (the T equine globulin) and a trace of B globulin. The protein content varied from 4.6 to 9.3 g per 100 ml and the leucoagglutinating titre ranged from 4095 to 34,768. This material was administered intramuscularly in doses of 1 to 5 ml

depending upon the weight of the patient and the anti-leukocyte titre of the preparation. In order to standardise their dosage STARZL *et al.* [1967] adopted the following 'unit' system. One ml of an antibody preparation with a titre of 8000 was said to be equivalent to 8000 units. Two ml of such a preparation would contain 16,000 units and so on. Based on this system individual doses were usually about 1000 units/kg/day (range 450 to 2100 units). During the most intensive period of therapy, that is during the early postoperative period, the amount of protein injected per week ranged from 14 to 50 mg/kg. As the authors stressed [STARZL *et al.*, 1967b], this was $^1/_3$ to $^1/_6$ of that which had been used in dogs and which had resulted in glomerular lesions.

In general, the material was given daily commencing 5 or 6 days before the operation and continuing up to 10–14 days postoperatively. Thereafter the material was administered less frequently, injections being given on alternate days for two weeks followed by twice a week for two months and finally once a week for one month (fig. 10). In a number of instances treatment was stopped prematurely because of toxic reactions. Similar observations have terminated anti-lymphocyte antibody treatment in other centres [WOODRUFF *et al.*, 1968; CALNE *et al.*, 1968; RUSSELL *et al.*, 1968].

As originally emphasized, the anti-lymphocytic antibody treatment was administered in conjunction with the more commonly used immunosuppressive agents. Azathioprine was given daily from the evening before transplantation and continued indefinately thereafter. Prednisone was used as sparingly as possible in these studies and in the recent investigations in our own unit [WOODRUFF *et al.*, 1968], it was either withheld until the diagnosis of rejection or was used in much reduced doses commencing on the day of operation and increased if rejection was diagnosed. However, patients who did not receive steroid therapy during the initial two months postoperative period were eventually placed on daily prednisone doses of 0.31 to 1.37 mg/kg/day. In half the cases, 200–400 μg of intravenous actinomycin C and/or local homograft irradiation were used for the treatment of established rejection. Further details on the average daily doses of prednisone and azathioprine are recorded in figure 11 and the treatment used in an individual case is shown in figure 10.

The ages and weights of the patients used ranged from 7 to 47 years and 12 to 75.5 kg respectively. In all but two cases bilateral nephrectomy and splenectomy were carried out at the same time.

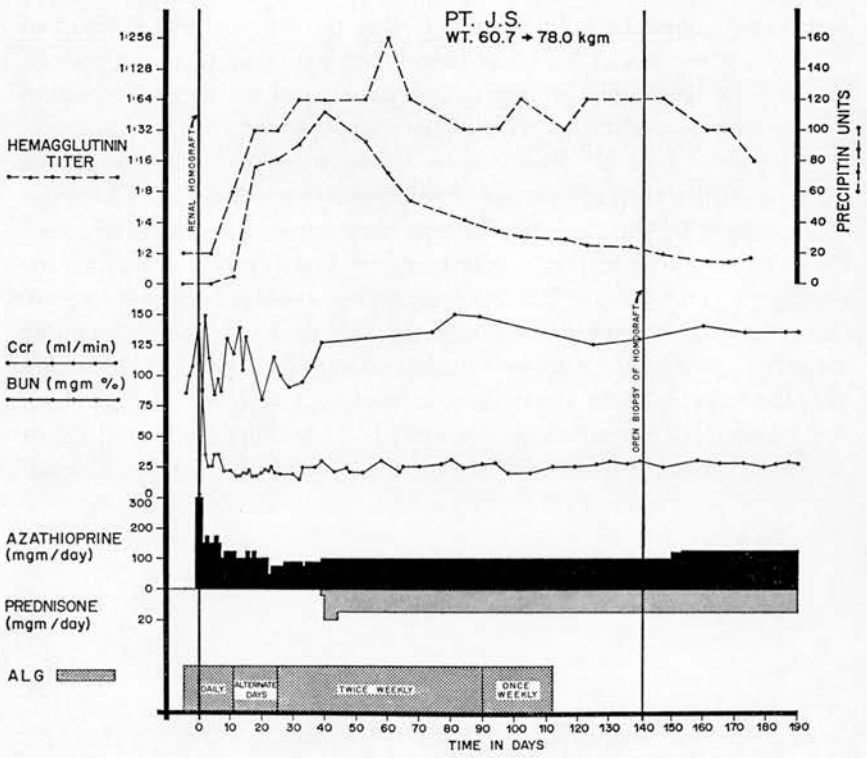


Fig. 10. The course of a patient receiving anti-lymphocyte globulin before and after renal transplantation.
The homograft was provided by a brother and there has been no rejection. A marked rise in haemagglutinating and precipitin titres occurred approximately 14 days after commencing anti-lymphocyte globulin therapy and for this reason prednisone therapy was commenced on day 40. [For further details see text and STARZL *et al.*, 1967b.] (Reproduced by kind permission of the Authors and the Editor of the Ciba Foundation Study Group No. 29.)

Three of the patients had also been thymectomized at an earlier operation. The donors for 19 of the 20 recipients were blood relatives, parents for 5, siblings for 13, and a maternal uncle for one. The remaining patient received a cadaveric homograft. Analysis of donor and recipient lymphocyte antigens indicated that an excellent match was obtained in 4 cases, an average match in 11, and a poor match in five.

The use of anti-lymphocytic antibody in conjunction with other immunosuppressive agents has, of course, complicated evaluation of its effectiveness. Therefore STARZL *et al.* [1967b] have attempted to

assess its therapeutic value by comparing the results in this group of patients with those in other groups of patients. The number of patients in the other groups (designated 1 to 3) were 32, 14 and 25 respectively. Patients in all these groups received azathioprine commencing several days before transplantation and during the ensuing post-transplant period. Prednisone treatment varied between the groups. In group 1 prednisone was withheld until a rejection crisis arose and then it was administered in large doses. Prednisone treatment in the groups 2 and 3 commenced the day before the operation, the patients receiving 3 mg and 0.5 to 1 mg per kg per day respectively. Whenever rejection crisis occurred, the prednisone dosage was increased, and in some cases intravenous actinomycin C and/or local homograft irradiation were used. The average daily dose of azathioprine and prednisone used in all 4 groups over the first 15 post-operative weeks is shown in figure 11.

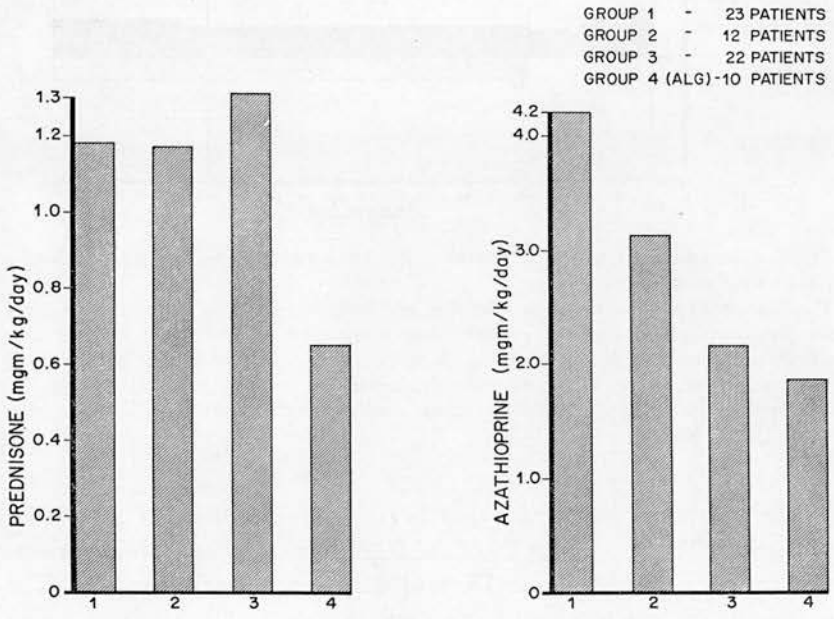


Fig. 11. The amounts of other immunosuppressants used in the various transplant groups. The values expressed are the average daily doses in the first 15 postoperative weeks in various groups of consanguineous homotransplantations performed in Denver compared with the first 10 patients receiving anti-lymphocyte globulin. Note that the anti-lymphocytic antibody treated group (No. 4) received reduced quantities of the 'standard' immunosuppressive agents. [For further details of groups and immunosuppressive therapy see text and STARZL *et al.*, 1967b.] (Reproduced by kind permission of the Authors and the Editor of the Ciba Foundation Study Group No. 29.)

Retrospective antigenic typing was performed in 32 donor-recipient combinations in the randomly paired groups 1 and 2. Prospective antigenic typing was undertaken in control group 3. This data indicated that the patients in group 4 did not have the added advantage of better lymphocyte matching than the other three control groups.

The immunosuppressive properties of the anti-lymphocytic globulin have been assessed by analysing mortality data, renal function, and the azathioprine and steroid requirement. The events in the first 105 postoperative days have been assessed for all patients surviving this time and the following mean values have been determined: blood urea, nitrogen, creatinine, creatinine clearance, white blood count, lymphocyte count and azathioprine and prednisone dosage. This generalized form of analysis provides a composite view of the early course of patients in the various groups, although it does not indicate individual variation and fluctuation. Nevertheless, it does provide a valuable and informative basis on which to make a general assessment of the effectiveness of this material.

In addition to the biochemical and haematological investigations reported above histopathological studies were also performed on biopsies obtained from the first 8 patients and taken 108 to 145 days after allotransplantation. At this time all the patients possessed excellent renal function [see STARZL *et al.*, 1967b]. The sections were investigated by light and electron microscope procedures and by fluorescent techniques.

3. Results

Of the first 20 patients, who received anti-lymphocytic IgG, 19 are still alive and well 13 to 19 months after transplantation. Compared with the other groups, the anti-lymphocytic antibody treated group had the lowest early mortality and deaths appeared to have been reduced in both the acute and chronic phase of convalescence (fig. 12). This reduced mortality was achieved using lower doses of prednisone and azathioprine than was used in the previous control groups and this improvement was not achieved at the expense of renal function (fig. 13). More detailed analysis revealed that deterioration in renal function did not occur during or after discontinuing anti-lymphocytic globulin treatment, on the contrary, renal function improved progressively from the second month onwards.

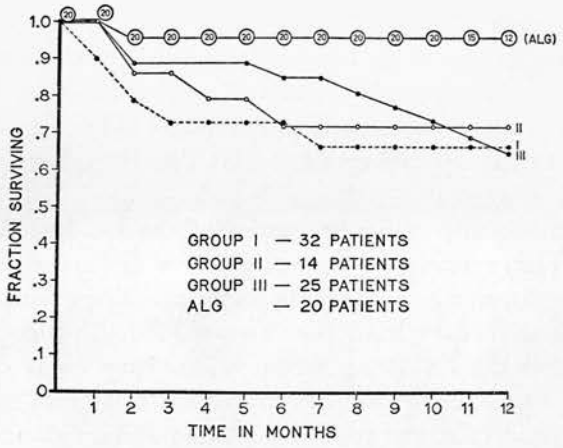


Fig. 12. Mortality data in patients receiving anti-lymphocytic globulin.
Note the reduced mortality in the first 12 months after transplantation in the anti-lymphocytic globulin treated group compared with the control groups. The numbers in the anti-lymphocytic globulin series refer to the patients at risk at the times indicated. [For further details see STARZL *et al.*, 1968.] (Reproduced by kind permission of the Authors and the Editor of Surgery, Gynecology and Obstetrics.)

In eight of the first 20 patients there were no observed rejection crises during the first four postoperative months. Whenever rejection symptoms were observed, the crisis could be averted by increasing the prednisone dosage [STARZL *et al.*, 1967b; STARZL *et al.*, 1968]. In 2 of the patients mild late rejections were observed after the completion of anti-lymphocyte globulin therapy but these were readily controlled by increasing the maintenance dose of steroids [STARZL *et al.*, 1968].

4. The use of anti-lymphocytic antibody in cases of renal allograft rejection

A small number of patients have received anti-lymphocytic IgG during threatened rejection [STARZL *et al.*, 1967b; STARZL *et al.*, 1968; WOODRUFF *et al.*, 1968; CALNE *et al.*, 1968; RUSSELL *et al.*, 1968]. In most of these cases this form of therapy was instituted as it was felt desirable, because of intercurrent infection and other factors, to reduce the azathioprine and prednisone dose. Other patients in this same group were receiving fairly high doses of these immunosuppressants but their renal function was still continuing to deteriorate. In these cases it was considered undesirable to increase the azathioprine or steroid dose.

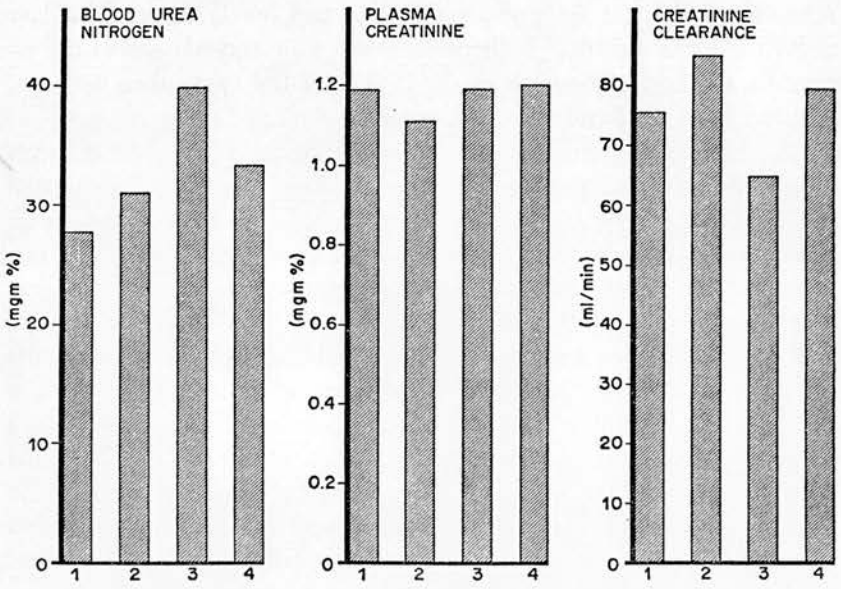


Fig. 13. The renal function in various patients with renal allotransplants.
The values presented are the average weekly renal functions in the first fifteen post-transplant weeks in the various groups shown in the previous figures. The blood urea nitrogen and creatinine values of the anti-lymphocytic globulin treated group (No. 4) were not significantly different than those observed in other groups, indicating that immunosuppression was not achieved at the expense of renal function. [For further details see text and STARZL *et al.*, 1967b.] (Reproduced by kind permission of the Authors and Editor of the Ciba Foundation Study Group No. 29.)

STARZL *et al.* [1967b and 1968] have treated four such patients with a prolonged course of anti-lymphocytic globulin similar to that used in patients receiving this material prior to grafting. In each case the renal function rapidly deteriorated on reducing the dose of prednisone below 0.5 to 1.7 mg/kg/day. A few days after commencing the anti-lymphocytic antibody therapy it was possible to reduce the prednisone dose to 0.23 to 0.3 mg/day in 3 of the patients; the azathioprine was continued. In the remaining patient all immunosuppressive therapy was terminated. A significant fall in the blood urea nitrogen was observed in all four patients but as there was not an accompanying improvement in the creatinine clearance it has been suggested that this fall might have been attributable to a catabolic steroid effect rather than a change in renal function. A recent report has indicated that in 2 of these patients the renal function has remained

relatively stable (at its previous sub-normal level) during the last twelve months and that both patients are now receiving 0.31 mg of prednisone/kg/day [STARZL *et al.*, 1968]. Of the two other patients, one has since died and the other received a second allograft.

A similar suggested improvement in renal function has been observed in a proportion of the patients treated by others [WOODRUFF *et al.*, 1968; CALNE *et al.*, 1968; RUSSELL *et al.*, 1968]. Anti-lymphocytic antibody has been used to treat 5 cases of rejection in our own unit. The course of injections used have varied, the courses usually being terminated due to undesirable side effects. In 2 of the 5 cases there was evidence of an improvement in renal function following anti-lymphocytic IgG treatment. Improvement was also observed in a third patient but in this case increased prednisone was administered together with local X-irradiation. RUSSELL *et al.* [1968] have also observed a slight but significant improvement in a number of their patients who had received anti-lymphocytic antibody treatment during various stages of late rejection. Thus from the limited amount of data available it would appear that anti-lymphocytic IgG may be of value in the treatment of some cases of late rejection.

5. Use in liver allotransplantation

Data on the use of anti-lymphocytic antibody in liver transplantation is still very limited. Nevertheless, as in the canine studies, there is evidence that this material has been of value in children with liver allotransplants [STARZL *et al.*, 1967e].

C. The effect of anti-lymphocytic globulin on delayed hypersensitivity reactions

As in the animal studies antisera to human lymphoid tissues have been shown to suppress, or indeed completely abolish pre-existing delayed hypersensitivities to a number of antigens. MONACO *et al.* [1967a and b] performed intra-dermal tests with several antigens in patients receiving anti-lymphocytic IgG and skin allografts (see page 186). The grade of reaction observation against trichophyton monilia and mumps before, and the day after, a single injection of anti-lymphocytic IgG are shown in figure 14. Eleven out of twelve of the original delayed sensitivity reactions were either markedly reduced or completely abolished. When retested 6 weeks later, most

of the reactions to mumps had returned to the original levels while those to trichophyton and monilia were still reduced.

Similar observations were also made by STARZL and co-workers [1967b]. In the small number of patients they tested there were five delayed hypersensitivity reactions, three to mumps, one to trichophyton and one to purified protein derivative (P.P.D.). Three or four days after commencing anti-lymphocytic antibody therapy all these tests became negative. Recently TRAEGER *et al.* [1968] have also reported that anti-lymphocytic antibody may abolish delayed hypersensitivity to tuberculin and toxoplasmin.

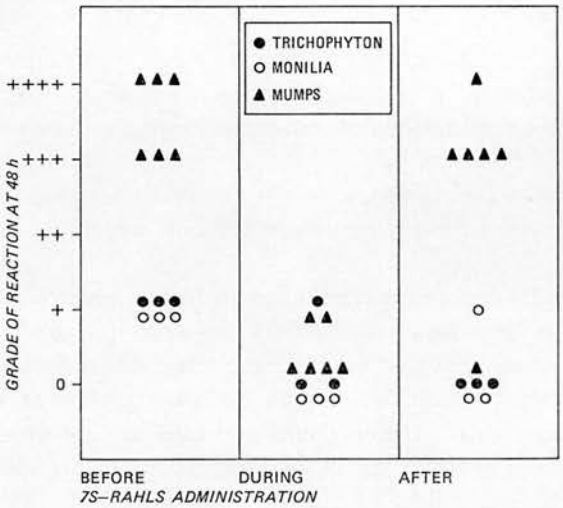


Fig. 14. The effect of rabbit anti-human lymphocyte IgG (7S-RAHLS) on delayed hypersensitivity reactions.
For details of the course of treatment see text and MONACO *et al.* [1967b]. Note that during the course of treatment that 11 of the 12 delayed hypersensitivity reactions were either completely abrogated or greatly reduced in magnitude. (Reproduced by kind permission of the Authors and the Editor of the Ciba Foundation Study Group No. 29.)

D. The use of anti-lymphocytic antibody in treating autoimmune disorders

The ability of anti-lymphocytic antibody to suppress immune responses of the cellular type including certain autoimmune disorders in animals, has stimulated preliminary investigations of the effect of this material on autoimmune diseases in humans [PICHLMAYR *et al.*,

1968]. Exact details of the course of treatment used and the clinical status of these patients is not available as yet, though the authors claim that anti-lymphocytic antibody therapy has been of value in the treatment of dermatomyositis (2 cases), arteriitis temporalis, myasthenia gravis and primary chronic polyarthritis. It is still too soon to assess the potential value of anti-lymphocytic antibody in the treatment of established autoimmune disorders for animal experiments indicate that this material generally fails to exert a marked influence on the course of the already established disease in animals [DENMAN *et al.*, 1967a]. Furthermore, the side effects observed with anti-lymphocytic IgG might prevent prolonged administration of this material.

E. Some complications of anti-lymphocytic antibody therapy

There are as anticipated, a number of complications arising from anti-lymphocytic antibody treatment some of which are quite serious (tab. IV).

Almost all patients experienced pain (often severe) at the site of intramuscular injection and narcotics were required. This pain usually occurred between 3–24 h after injection and was associated with erythema, induration, tenderness and itching at the site of injection. Occasionally the erythema and swelling involved the whole buttock and upper thigh. Several observers have noted that the pain was most marked following the first few injections [STARZL *et al.*, 1967b; RUSSELL *et al.*, 1968].

In addition nearly all the patients have developed fever at some time during the treatment. This has usually been low grade but occasionally more severe fevers have been observed and these were accompanied by tachycardia [KASHIWAGI *et al.*, 1968; WOODRUFF *et al.*, 1968; TRAEGER *et al.*, 1968]. In contrast to the above SHORTER *et al.* [1967] claim that their antiserum to human thymocytes which was produced by a two pulse procedure (see page 147) did not cause local pain or give rise to fever. In a fair number of cases anti-lymphocyte globulin has also caused hypotension and air hunger, hives, rashes and generalized pruritis [STARZL *et al.*, 1967b; KASHIWAGI *et al.*, 1968; WOODRUFF *et al.*, 1968; TRAEGER *et al.*, 1968]. However, perhaps one of the most troublesome side effects has been the rather high incidence of thrombocytopenia (and occasionally leukopenia)

Table IV. Some possible complications of anti-lymphocytic antibody treatment

	Side effects	References
Local	Erythema and induration associated with pain, itching and tenderness	STARZL *et al.* [1967b]; MONACO *et al.* [1967b]; KASHIWAGI *et al.* [1968]; WOODRUFF *et al.* [1968]; RUSSELL *et al.* [1968]; CALNE *et al.* [1968]; CARRAZ *et al.* [1967]; TRAEGER *et al.* [1968].
	Abscesses (sterile and non-sterile)	KASHIWAGI *et al.* [1968]; WOODRUFF *et al.* [1968].
Systemic	Fever with associated chills and occasional tachycardia	STARZL *et al.* [1967b]; MONACO *et al.* [1967b]; KASHIWAGI *et al.* [1968]; WOODRUFF *et al.* [1968]; TRAEGER *et al.* [1968].
	Hypotension and air hunger	STARZL *et al.* [1967b]; KASHIWAGI *et al.* [1968]; WOODRUFF *et al.* [1968]; TRAEGER *et al.* [1968].
	Hives, rashes generalized pruritis, athralgia	STARZL *et al.* [1967b]; KASHIWAGI *et al.* [1968; WOODRUFF *et al.* [1968].
	Anaphylactic shock	KASHIWAGI *et al.* [1968].
	Serum sickness and associated nephritis	KASHIWAGI *et al.* [1968]; MONACO *et al.* [1967b].
	Leukopenia and thrombocytopenia	STARZL *et al.* [1968]; KASHIWAGI *et al.* [1968]; WOODRUFF *et al.* [1968]; CALNE *et al.* [1968].

in patients receiving anti-lymphocytic antibody. This has been observed in 6 of the 11 patients treated with anti-lymphocytic IgG prepared in the authors department [WOODRUFF *et al.*, 1968; CALNE *et al.*, 1968] and in 9 of the patients reported by KASHIWAGI *et al.* [1968]. The observations that termination or reduction in anti-lymphocytic antibody therapy is followed by a recovery in the platelet count [WOODRUFF *et al.*, 1968; KASHIWAGI *et al.*, 1968] and the fact that certain batches of anti-lymphocytic globulin agglutinate platelets *in vitro* [STARZL *et al.*, 1967b] strongly suggests that the thrombocytopenia observed is partially attributable to this material.

As well as the problems outlined above there are the additional ones directly attributable to the administration of heterologous protein. Experiments in animals have clearly shown that anti-lymphocytic antibody will not inhibit antibody formation against all of its constituent proteins (fig. 8 and 9). This has also been observed in humans. STARZL *et al.* [1968] observed that 36 out of 40 of their

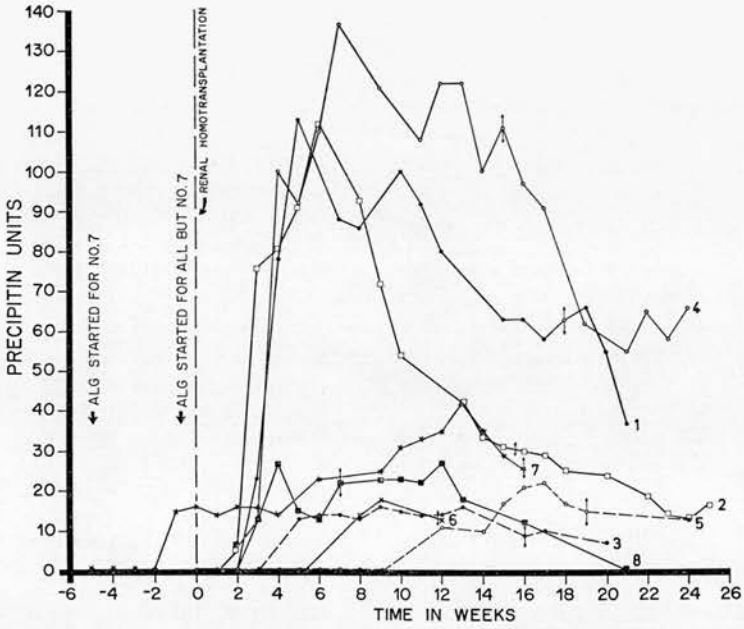


Fig. 15. The development of circulating antibodies to horse serum proteins in patients receiving anti-lymphocyte globulin.
Note that in all the patients high levels of circulating antibodies to horse serum proteins were observed during therapy. The 'free' antibody was usually detected 2 to 3 weeks after commencing anti-lymphocyte globulin treatment and the levels were highest in those patients (No. 1, 2 and 4) who did not receive steroids during the early postoperative period. [For further details see STARZL *et al.*, 1967b; KASHIWAGI *et al.*, 1968.] (Reproduced by kind permission of the Authors and the Editor of the Ciba Foundation Study Group No. 19.)

patients treated with anti-lymphocytic antibody eventually developed antibodies against horse serum proteins. The antibodies formed were detected by tube precipitation procedures and by electro-immunodiffusion in antigen agar plates [STARZL *et al.*, 1967b; KASHIWAGI, 1968]. This response is illustrated in figure 15 where the antibody titres produced in the first 8 patients receiving this material are shown. In 11 of the 40 patients anaphylactic reactions occurred and anti-lymphocytic globulin therapy had to be terminated. The precipitin response appeared to be of significant prognostic value for in patients where this was most prompt, there was a high incidence of anaphylactic reactions. The available data also suggests that early prednisone treatment may suppress the precipitin response. This is again illustrated in figure 15. Patients 1, 2 and 4 who did not receive steroids

until 29 to 43 days after transplantation exhibited high titres to horse serum proteins. In contrast, in those patients receiving steroids earlier, the titres were much lower. Immunoelectrophoretic analysis of the serum of 13 patients has revealed that the antibodies present are directed against the β and α globulin 'contaminants' of the anti-lymphocytic globulin preparations. In only one case were antibodies to gamma globulins detected by this procedure [KASHIWAGI *et al.*, 1968]. Recently, other investigators have also observed the formation of antibodies to horse serum proteins in patients receiving anti-lymphocytic antibody treatment [WOODRUFF *et al.*, 1968; TRAEGER *et al.*, 1968].

In addition a significant number of patients have developed one or more of the clinical manifestations of serum sickness [KASHIWAGI *et al.*, 1968; MONACO *et al.*, 1967]. However, fortunately in the first eight patients in which renal biopsies have been performed there has been no evidence of serum sickness of Masugi type nephritis. Nevertheless, histological changes have been observed in the transplanted kidneys along with the deposition of heterologous and autologous serum proteins. The data obtained on these patients is summarized in table V and the histological changes observed in a patient receiving

Table V. Fluorescent and electron microscopic observations on the glomeruli of eight renal homografts in patients treated with horse anti-human-lymphocyte globulin

	Electron Microscopy Thickening of glomerular capillary basement membranes			Fluorescent microscopy Deposits demonstrated by fluorescent antibody technique					
Patient	Subendothelial	Subepithelial	Fusion of epithelial foot processes	IgM	IgG	Horse globulin	Complement β_1A	Complement β_1C	Fibrinogen
LD 107	±	0	±	+	0	0	0	0	0
LD 108	0	0	±	0	0	0	0	0	0
LD 109	±	0	+	+	0	0	0	0	0
LD 110	+	0	++	+	0	0	++	++	+
LD 111	++	0	±	+	0	0	0	0	0
LD 112	0	0	±	0	0	0	0	0	0
LD 113	+	0	++	±	0	0	0	0	0
LD 114	+	0	±	+	0	0	0	0	0

0 = negative; ± = slight in amount; + = moderate in amount; ++ = marked in amount.
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anti-lymphocytic globulin after transplantation are shown in figures 16 and 17. Histological changes similar to those described above have recently been reported by TRAEGER *et al.* [1968]. Furthermore, these workers also showed that anti-lymphocytic antibody was not grossly nephrotoxic.

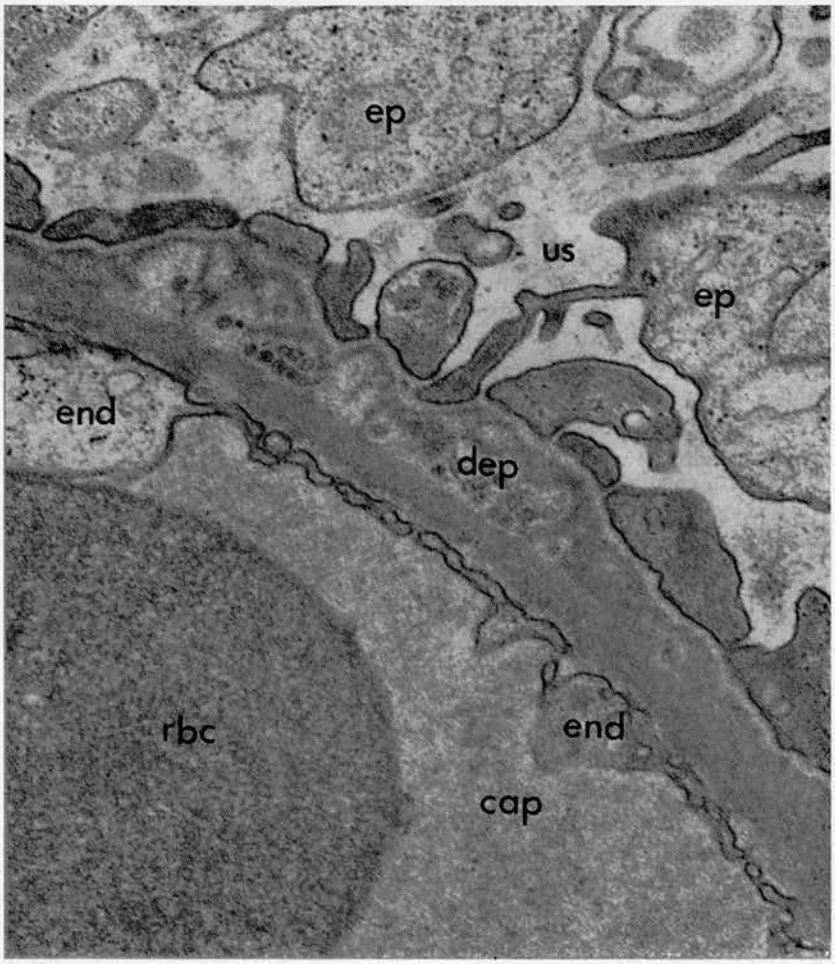

Fig. 16. Histopathological observations in patients treated with anti-lymphocytic antibody. Biopsy of human renal allograft 2 years, 2 months after transplantation. The patient had received a 3 month course of horse anti-human lymphocyte globulin which ended one year before the biopsy was taken. The basement membrane of the glomerular capillary loop is thickened by a subepithelial accumulation of material (dep). Key: cap, glomerular capillary lumen; rbc, red cell; end, endothelial cell lining capillary; ep, visceral epithelial cell; us, urinary space. Electron micrograph stained with lead hydroxide. Magnification ×12,550. [Kindly provided by Professor K. PORTER.]

Examination of 7 spleens and of lymph nodes from some of the patients revealed that the amount of lymphoid tissue was in the normal range. Germinal centres composed of large pale pyroninophilic cells were present in 6 of the 7 spleens and in all the lymph nodes, there was no apparent reduction in the small lymphocytes surrounding these centres [IWASAKI *et al.*, 1967].

Fig. 17. The renal allograft biopsy is the same as that shown in fig. 16. The section was treated with fluorescein labelled goat antibody to equine globulin. There is a granular localization of horse protein in the capillary walls of a glomerulus ×250. [Kindly provided by Professor K. PORTER.]

IX. The Mode of Action of Anti-Lymphocytic Antibody

An understanding of the mode of action of anti-lymphocytic antibody is of interest both to the surgeon and the basic immunologist for such knowledge should lead to a more rational and efficient application of this material, as well as increasing our general understanding of immunological phenomena. A number of theories have been proposed to date but no single one of them offers an entirely satisfactory explanation of the observed effects, indeed it is accepted that the mode of action may vary from experiment to experiment [LEVEY

and MEDAWAR, 1966a and b 1967; WOODRUFF, 1967; JAMES, 1967a]. Nevertheless, an attempt will be made to discuss the major theories in the light of our present knowledge. These are as follows:

A. Cytotoxic theory

The original postulate was that anti-lymphocytic antibody exerted its immunosuppressive effect by causing the gross destruction of peripheral blood lymphocytes, with or without the accompanying depletion of lymphoid tissue. The observations that immunosuppression could be achieved in the absence of marked or sustained lymphopenia, and that the effect was greater than that achieved by lymphocyte depletion through a thoracic duct fistula led to a temporary abandonment of this hypothesis [LEVEY and MEDAWAR, 1966a and b; IWASAKI *et al.*, 1967; JAMES, 1967a]. Nevertheless, the observation that non-cytotoxic anti-lymphocytic antibody fragments generally fail to suppress humoral antibody formation or prolong homograft survival suggests that a certain degree of cytotoxicity may be important [HARRIS and HARRIS, 1966a and b; JAMES and ANDERSON, 1967; ANDERSON *et al.*, 1967; WOODRUFF *et al.*, 1967a; JAMES, 1967a and b]. It is possible (as will be discussed later) that anti-lymphocytic antibody could exert its effect by the selective destruction of a sub-population of lymphocytes in a state of readiness to undertake immune responses [LEVEY and MEDAWAR, 1966a; MONACO *et al.*, 1966c; JAMES and ANDERSON, 1967; JAMES and JUBB, 1967; JAMES, 1967a; DENMAN *et al.*, 1968d].

In contrast to the original cytotoxic theory there are several alternative theories which are based upon the ability of anti-lymphocytic antibody to combine with circulating lymphoid cells or lymphoid tissue without producing lysis or other damage. These theories are referred to as the blindfolding, the competitive antigen and the sterile inactivation theories and they may overlap to some extent.

B. The blindfolding theory

According to this simple hypothesis the anti-lymphocytic antibody coats the lymphocytes so preventing access to their combining sites or recognition units [LEVEY and MEDAWAR, 1966a]. The coated

(blindfolded) lymphocytes are, therefore, unable to recognize the antigen or to respond to the appropriate immune stimulus from the macrophage. Another variation of this theory applicable to renal homografts is that the antibody masks the graft itself [GUTTMAN *et al.*, 1967a and b]. Thus the graft bound antibody would mask histocompatibility antigens preventing lymphocyte sensitization and/or contact and could also bind antigen released from the kidney. In this situation the operative mechanism would be similar to enhancement. However, the failure of antibody fragments (which bind strongly to lymphocytes *in vitro* and *in vivo*) to suppress humoral or cellular immune phenomena (see above) and the observation that antibody to glomerular basement membrane does not protect renal allografts [GUTTMAN *et al.*, 1967c] suggests that the blindfolding theory is inadequate to explain the immunosuppressive properties of anti-lymphocytic antibody.

C. The competitive antigen theory

The competitive antigen theory is based upon the rapid and preferential uptake of anti-lymphocytic antibody by lymphoid cells. Anti-lymphocytic antibody, by definition, has a special affinity for lymphoid cells and as it is potentially antigenic, it preoccupies and indeed may sensitize such cells. Such preoccupied cells would then be unable to respond to subsequent stimuli by other antigens. In contrast to the blindfolding theory, it involves the specific immunological commitment of the lymphoid cells rather than a non-specific prevention of antigenic stimulation through impeding access of antigen. The observations that anti-lymphocytic IgG may be more immunogenic than normal rabbit IgG has led some investigators to favour this theory [GUTTMAN *et al.*, 1967c]. However, there are a number of obstacles. Firstly it would not explain the ability of antisera to nullify the action of sensitized lymphoid cells [LEVEY and MEDAWAR, 1966a, 1967; JAMES, 1968] and secondly, on the basis of this hypothesis, one might have expected antibody fragments to exert some effect, even though they are less antigenic than the intact IgG (as they lack the Fc portion of the molecule which contains the species specific antigenic determinants). Although this theory does not satisfactorily explain the mode of action of anti-lymphocytic antibody it could, like the blindfolding theory, indirectly explain the apparent

inability of this material to effectively suppress humoral antibody formation against itself (see page 182). Lymphocytes coated with anti-lymphocytic antibody will presumably be aggregated, lysed or opsonized and so prone to phagocytosis by macrophages, thus sensitizing the recipient to the lymphocyte bound IgG or other bound protein [see also LANCE and DRESSER, 1967]. Furthermore, it should be stressed that the phagocytosis of lysed and non-lysed (opsonized) cells following anti-lymphocytic antibody treatment could in itself explain the immunosuppressive properties of this material (see later).

D. The sterile inactivation theory

The observation that chronic anti-lymphocytic antibody treatment may result in lymphoid hyperplasia accompanied by the formation of blast cells has led to the proposal of the sterile inactivation theory [LEVEY and MEDAWAR, 1966b]. According to this theory the antibody converts or transforms the lymphocytes into sterile forms (blast cells) which are unable to respond to subsequent antigenic stimuli. The observations that anti-leukocytic sera are capable of transforming lymphocytes *in vitro* lends support to this hypothesis (see page 158) which is favoured by a number of investigators [IWASAKI *et al.*, 1967; DENMAN *et al.*, 1968c]. However, once more there are a number of objections. In the first place lymphoid hyperplasia and blast cell formation are not always observed in animals treated with anti-lymphocytic antibody [GRAY *et al.*, 1966]. In addition, although the divalent anti-lymphocytic antibody fragments namely $F(ab')_2$ are able to transform lymphocytes *in vitro*, they are unable to inhibit cellular or humoral immune responses [ANDERSON *et al.*, 1967a; JAMES, 1967b; JAMES and ANDERSON, 1967; RIETHMÜLLER *et al.*, 1968a and b]. See also pages 160, 168 and 182.

E. Other theories

Other less prominent theories have also been put forward to explain the mode of action of anti-lymphocytic antibody. The apparent immunosuppressive superiority of antisera to thymus cells over those raised against other lymphoid tissues has led to the suggestion that anti-lymphocytic antibody might be exerting its immunosuppressive

effect by neutralizing the thymus humoral factor [NAGAYA and SIEKER, 1965a; LEVEY and MEDAWAR, 1966a]. Alternatively, TURK and WILLOUGHBY [1967] have suggested that the peripheral effect of anti-lymphocytic antibody could be due to the antagonism of the lymph node permeability factor described by WILLOUGHBY *et al.* [1964].

F. Further comments on the mode of action

A number of recent observations indicate that the action of anti-lymphocytic antibody might be quite selective. DENMAN *et al.* [1967a] originally suggested that anti-lymphocytic antibody had a limited access to central lymphoid tissue and, therefore, was incapable of suppressing plasma cell proliferation of these sites [see also JAMES, 1968]. More recently LEVEY and MEDAWAR [1967] have presented data supporting the conclusion that the effect of anti-lymphocytic antibody is primarily upon the peripheral blood lymphocytes and only affects the central lymphoid organs in so much as they liberate cells into and receive them from the peripheral circulation. This might explain in part why doses of anti-lymphocytic antibody which inhibit the primary humoral antibody response in hooded rats were ineffective in Wistar strain rats which have a much higher peripheral blood lymphocyte count [JAMES and ANDERSON, 1967; WOODRUFF *et al.*, 1967a]. MARTIN and MILLER [1967] have restricted the site of action of anti-lymphocytic antibody still further by suggesting that this material specifically eliminates antigen reactive lymphocytes (the long lived small lymphocyte of the thoracic duct) though at the same time it may non-specifically stimulate the proliferation of other lymphoid cells. This hypothesis could also explain the recent observations of DENMAN *et al.* [1968c]. The results of MARTIN and MILLER [1967] also provide direct evidence that the inactivation of lymphoid cells *in vitro* by anti-lymphocytic antibody as indicated in cell transfer experiments, may be explained by the failure of such cells to seed in the lymphoid tissues of the recipient [see also JAMES, 1968; FIELD and GIBB, 1968].

On the basis of these recent observations it would appear that there may be an element of truth in the original suggestions that anti-lymphocytic antibody might function by the select removal of a certain population of lymphoid cells presumably the short lived small lymphocytes [DENMAN *et al.*, 1968d; see also JAMES, 1967].

This could be achieved by the direct destruction of lymphoid cells (cytotoxic theories) or by the indirect mechanisms previously described (page 204).

X. Future Approaches to the Preparation, Purification and Administration of Anti-Human Lymphocyte Antibody

Although the previous data indicates that anti-lymphocytic antibody could be of considerable value in human organ transplantation, it is quite obvious that a great deal of basic research will be necessary in order to develop less toxic and more active preparations. There are a number of possible ways of overcoming these problems. These include more refined methods of antiserum production, further purification of the antibody and strictly controlled administration of this material.

A. Antiserum production

Several factors influencing antiserum production still require thorough investigation. These include the choice of antigen and animal in which the antiserum is produced along with the most effective course of immunization. Recent reports suggest that antiserum against thymocytes or peripheral blood lymphocytes are less toxic, and perhaps more effective than those produced using spleen [SHORTER *et al.*, 1967; BALNER *et al.*, 1968]. It would seem advisable, therefore, to consider the use of antigens such as lymphocytes prepared from the 'buffy coat' of outdated blood or thoracic duct lymphocytes. Furthermore, because of the difficulties frequently experienced in obtaining suitable antigenic material of human origin, the use of stored antigenic preparation deserves further consideration [see page 148 and CARRAZ *et al.*, 1967].

Ideally, the animal in which the antiserum is produced should be one which responds well to the lymphoid antigen but one whose serum does not elicit a marked immune response in humans, i.e. that is not very immunogenic. While this is a theoretical ideal it is not always a practical, or indeed an advisable approach, for BALNER and DERSJANT [1967] demonstrated that antisera produced in cynomolgus monkeys to rhesus monkey lymph node cells were less effective immunosuppressants than those antisera produced in rabbits.

The courses of immunization which produces the most potent and least toxic antisera have still to be ascertained. The recent preliminary results of SHORTER *et al.* [1967] suggest that less toxic (i.e. less painful) antisera may be produced by the 2 pulse procedure advocated by LEVEY and MEDAWAR [1966a and b]. Although it is recognized that the 2 pulse procedure does offer a number of distinct advantages (see page 145) antisera produced by this method have not always proved as effective as those produced by more prolonged immunization procedures. Nevertheless, this aspect of antiserum production is worthy of further consideration.

As previously suggested, approaches similar to those outlined above may also result in an increase in the proportion of the gamma globulin molecules which are specifically anti-lymphocytic [WOODRUFF *et al.*, 1967c]. Alternatively the anti-lymphocytic content may be increased by producing the antibody in animals whose plasma IgG levels are naturally low, or which have been reduced by selective plasmaphoresis prior to immunization.

B. Further purification of anti-lymphocytic antibody

The advantages of using anti-lymphocytic globulin preparations have previously been stressed (page 150) and considerable progress has already been made in this respect. However, recent *in vitro* studies on the uptake of ^{131}I-labelled anti-lymphocytic IgG onto human lymphocytes have demonstrated that less than 5% of the IgG in our preparations was anti-lymphocytic [WOODRUFF *et al.*, 1967c; ANDERSON *et al.*, 1967b]. Furthermore, the observations of KLINMAN *et al.* [1966] on equine anti-hapten antibodies and our own immunoelectrophoretic studies have indicated that gamma globulins of the horse are extremely complex. For example, it would appear that there are at least six antigenically distinct proteins within the so-called IgG and IgA classes. The IgG group consists of the IgG_a, IgG_b and IgG_c proteins which differ in their electrophoretic mobility and ability to fix complement. Thus, even the small amount of lymphocyte binding protein in our preparations may consist of several distinct populations of antibody, some of which are cytotoxic, others which are not. It is highly probable, therefore, that further purification of anti-lymphocytic globulin will provide products with distinct therapeutic advantages. This resolution and enrichment may be achieved

by immunological or physicochemical means. The immuno-absorption of antibody onto lymphocytes and lymphocyte membrane fractions has already been used to effect further purification of antisera to human and rabbit lymphocytes [WOODRUFF, 1968; OGBURN *et al.*, 1967]. However, it is unlikely at the moment that such procedures will permit the fractionation of sufficient material for therapeutic use. In any case the observations of KLINMAN *et al.* [1966] have shown that antibodies obtained from antigen antibody complexes are in themselves still extremely heterogeneous (see above).

A further resolution of such material or of the 'cruder' globulin preparations, may however, be achieved by standard electrophoretic or chromatographic procedures on DEAE cellulose or other suitable ion exchange materials. In this respect KLINMAN *et al.* [1966] have achieved a partial separation of some of the IgG sub-components of anti-hapten antibodies by column chromatography on DEAE cellulose. We also have attempted to 'sub-fractionate' horse anti-mouse lymphocyte globulin on DEAE cellulose and DEAE sephadex, but have not achieved any marked resolution of the anti-lymphocytic antibody, for this appeared to be distributed throughout an heterogeneous population of antibody molecules [JAMES and MEDAWAR, 1967b]. Nevertheless, it is believed that column chromatographic procedures on the new advanced DEAE cellulose exchanges (such as Whatman DE52) will permit further purification of anti-lymphocytic antibody. It should perhaps be stressed once more that whatever techniques are used in purification, attempts should be made to limit denaturation and other changes in the protein preparations, for as previously discussed some of the toxic reactions observed could have been due to the liberation of pharmacologically active peptides during fractionation.

C. Antibody administration

Until relatively non-toxic preparations are available it is essential that the use of this material should be rigorously controlled, and the patients kept under strictest observation. Many of the side effects can be counteracted to some extent [see KASHIWAGI *et al.*, 1968] nevertheless, it is essential to perform frequent immunological and haematological analyses. Antibodies to horse proteins should be assessed by passive haemagglutination, electro-immunodiffusion pro-

cedures or the ultracentrifuge Farr procedure [ROTHBERG and FARR, 1967] for as previously stressed, such estimations often prove to be of considerable prognostic value [KASHIWAGI *et al.*, 1968].

The delay (approximately 3 weeks) in the appearance of appreciable amounts circulating antibodies against horse serum proteins in both rats (fig. 9) and humans (fig. 15) suggests that short intensive courses of anti-lymphocytic antibody treatment in human renal transplantation might have several distinct advantages over protracted courses of treatment [MONACO *et al.*, 1967a; IWASAKI *et al.*, 1967]. Such courses could be given for a few days (5–7) prior to transplantation and then for 10–14 days postoperatively. During this period prednisone could be used in small amounts or completely withheld. The anti-lymphocyte antibody treatment would thus be terminated prior to the formation of high levels of circulating antibody thus reducing the chances of anaphylactic shock and serum sickness. It is also highly probable that much of the anti-lymphocytic globulin administered in the later stages of more prolonged courses, complexes with circulating antibodies and is rapidly removed from the circulation without exerting any significant effect.

Finally it is anticipated, that as a result of investigations currently in progress and those suggested in this review, that highly specific and potent anti-lymphocytic antibody preparations of low toxicity will be developed. Such products will undoubtedly be of great value in human organ transplantation.

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SECTION C - NORMAL AND PATHOLOGICAL HUMAN GAMMA GLOBULINS

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STRUCTURAL CHANGES OCCURRING IN 7S γ-GLOBULINS

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THE stability of isolated γ-globulins is a critical requirement both to experimental immunologists and to clinicians responsible for the prophylactic or therapeutic administration of antibody preparations. Hence, reports of structural changes occurring in the 7*S* γ-globulin molecule following storage are of wide interest.

The 'splitting' effect[1] observed in the immunoelectrophoretic precipitin line formed by 7*S* γ-globulin preparations, which had been stored between 0° C and 4° C, was attributed by Grabar[2] to denaturation of the protein. Following more detailed investigations, Skvaril[3,4] and Augustin and Hayward[5] concluded that structural alterations in the 7*S* γ-globulin molecule resulted from a proteolytic process occurring during storage. In support of this idea Skvaril[1] partially separated a 4·8*S* fragment from stored γ-globulin, by means of ammonium sulphate fractionation.

The investigations reported here form part of a more extensive work on the immunological properties (for example, reactivity with rheumatoid factor) of aggregated and degraded 7*S* γ-globulins. On the basis of the results obtained, a mechanism is formulated to explain how the denaturation of isolated 7*S* γ-globulin can lead to proteolytic hydrolysis of the labile bonds linking the parts of the molecule corresponding to the electrophoretically 'fast' and 'slow' papain digestion pieces.

Physico-chemical and immunological analyses were performed at regular intervals on 1 g per cent (w/v) solutions of purified 7*S* γ-globulin, isolated from the serum of individual donors by 'DEAE'-cellulose chromatography[6]. These preparations (stored at −20° C until ready for use) were incubated at 37° until the characteristic changes in the immunoelectrophoretic line[1,3–5] were observed (Fig. 1).

Structural alterations in the 7*S* γ-globulin molecule appeared to occur in two stages, as is indicated by the

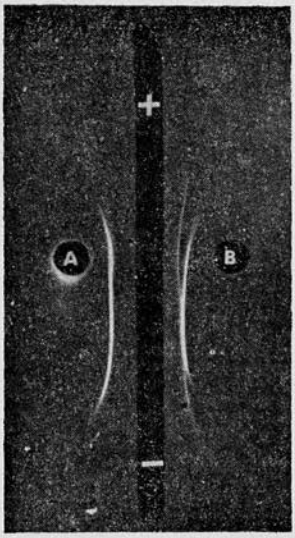

Fig. 1. Immunoelectrophoretic pattern of (*A*) normal and (*B*) altered 7*S* γ-globulin. Antiserum : rabbit anti-whole human serum

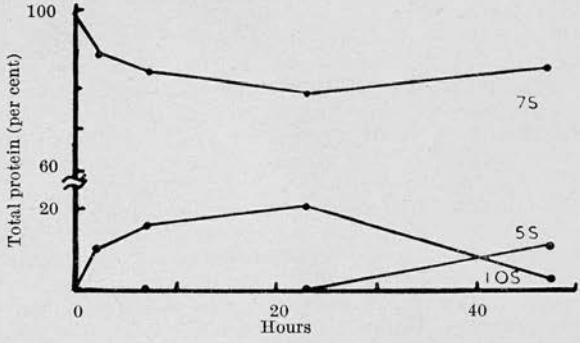

Fig. 2. Ultracentrifugal changes occurring in 7*S* γ-globulin solution stored at 37° C

results of a typical series of ultracentrifugal analyses (represented graphically in Fig. 2). The first stage involved formation of 10*S* component which reached a maximum level of 20 per cent of the total protein. (Small amounts of 10*S* components were, however, sometimes observed in the initial preparations.) This change led to a marked increase in lævorotation (similar to that shown in Fig. 3) which also includes the results of control measurements on a sample of 7*S* γ-globulin which had been stored at −20° C and did not show the splitting of the immunoelectrophoretic precipitin line.

The second stage, which proceeded at a much slower rate than the first, was characterized by the appearance of 5*S* component accompanied by a decrease in the level of 10*S* component (Fig. 2). These changes resulted in only a very slight, if any, increase in lævorotation (Fig. 3). Moreover, the familiar 'splitting' of the immunoelectrophoretic precipitin line was now demonstrable and an additional, faster, component was revealed by urea–starch-gel electrophoresis[7] (Fig. 5).

A similar sequence of changes was observed in 7*S* γ-globulin preparations stored at 4° C, but these occurred at a greatly reduced rate. On the other hand, changes were not demonstrable in human 7*S* γ-globulin preparations which had been maintained for several months at −20° C.

In order to ascertain further the nature of the alteration process two human 7*S* γ-globulin preparations, showing the characteristic physico-chemical and immunological changes, were fractioned by gel-filtration on the same column (75 cm × 2 cm) of 'Sephadex *G*-200'. The preparations gave an identical gel-filtration pattern, illustrated in Fig. 4, together with the pattern obtained by fractionation of papain-digested[8] 7*S* γ-globulin on the same column of 'Sephadex *G*-200'. Ultracentrifugal analysis, urea–starch-gel electrophoresis (Fig. 5), immunoelectrophoresis (Fig. 6) and gel-diffusion precipitin tests, all showed that the first peak comprised intact 7*S* γ-globulin. In contrast, the major portion of the protein recovered in the second peak appeared to be identical to the electrophoretically 'slow' papain digestion piece (Fig. 6), although a small amount of 'fast' component was also detectable. These observations were supported by the results of gel-diffusion precipitin analyses and also by urea–starch-gel electrophoresis (Fig. 5), using as reference substances separated *A* and *B* chains of human 7*S* γ-globulin[9] and the 'slow' component of papain-digested 7*S* γ-globulin[10].

As would be expected, both *A* and *B* chains were demonstrable in the second 'Sephadex *G*-200' fraction of the stored γ-globulin, although this sample appeared to be antigenically deficient with respect to *A* chain. Ultracentrifugal analysis showed this fraction to comprise a single component with a S^0_{20w} value of 3·5*S*. An improved resolution of the 'slow' component observed in starch-gel electrophoresis patterns of peak 2 material following storage at −20° C for several weeks (with intermittent thawings) suggested further degradation of the contaminating 'fast' component.

As predicted from the results of a previous investigation[11], showing that rheumatoid factors react only with the 'fast' papain piece (III) of aggregated γ-globulin, the 'Sephadex' peak 2 proved relatively inactive. This was demonstrated by the addition of 10 mg aliquots of the

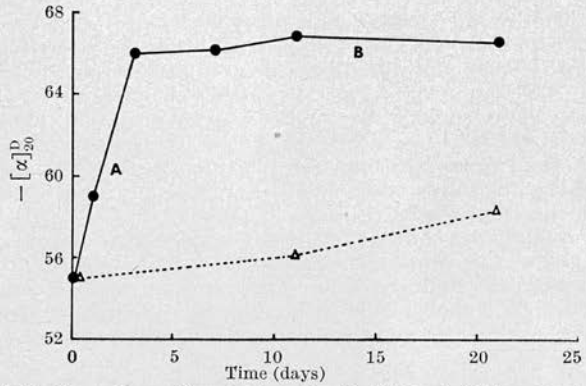

Fig. 3. Changes in specific optical rotation ($\lambda = 589{\cdot}5$ mμ) of a 1 g per cent 7*S* γ-globulin stored at 37° C (●) and −20° C (△). *A*, 10*S* stage ; *B*, 5*S* stage. (N.B. The sample of γ-globulin used to obtain the results was different from that referred to in Fig. 2)

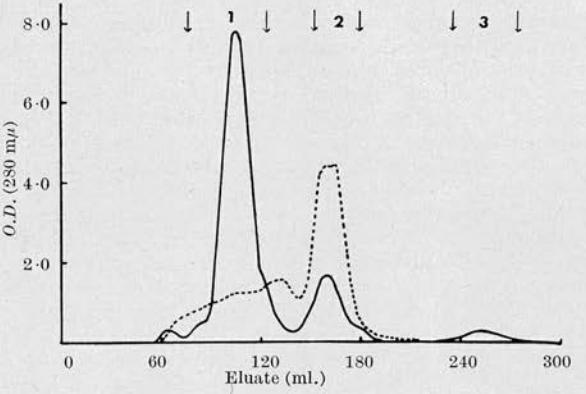

Fig. 4. 'Sephadex *G*-200' fractionation of altered 7*S* γ-globulin solution (——) and papain-digested 7*S* γ-globulin solution (– – – –). 150-mg amounts of each sample applied to column (75 cm × 2 cm)

concentrated 'Sephadex' peaks (1 and 2), before and after heating at 63° C for 10 min, to 0·3-ml. portions of serum from a rheumatoid arthritic patient (Rose-Waaler titre 1024) in a 5-ml. reaction volume, adjusted with 0·15 M saline. After heating the two fractions, only the first—comprising whole 7*S* γ-globulin—formed a precipitate with the rheumatoid factor. These findings provide independent evidence that alterations occurring in isolated human 7*S* γ-globulin preparations lead ultimately to the degradation of the separated 'fast' papain digestion piece (III) of the molecule. Peptides resulting from such a process probably comprise the third, minor, peak observed in the gel-filtration pattern of the stored 7*S* γ-globulin preparation (Fig. 4). A similar peak was not demonstrable

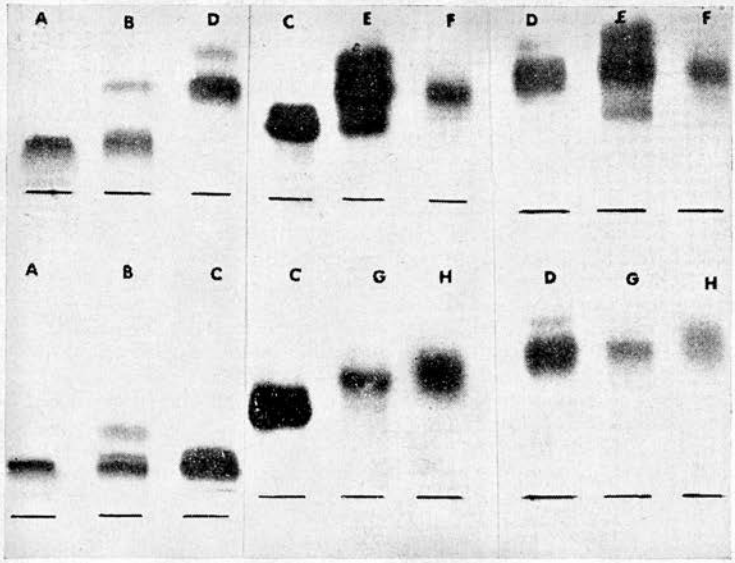

Fig. 5. Urea–starch-gel electrophoretic patterns: (*A*) intact 7*S* γ-globulin; (*B*) altered 7*S* γ-globulin; (*C*) 'Sephadex' peak 1 of altered 7*S* γ-globulin; (*D*) 'Sephadex' peak 2 of altered 7*S* γ-globulin; (*E*) papain-digested 7*S* γ-globulin; (*F*) 'slow' component of papain-digested 7*S* γ-globulin; (*G*) type *A* and (*H*) type *B* polypeptide chains of human 7*S* γ-globulin

in the gel-filtration pattern of 7*S* γ-globulin deliberately degraded by a single digestion with papain. Nevertheless, a second (repeat) direction with papain has been shown to destroy the 'fast' component[12], as revealed by its absence in the immunoelectrophoretic pattern of such 7*S* γ-globulin digests.

A schematic outline of a mechanism which could account for the alterations in isolated 7*S* γ-globulin described here is shown in Fig. 7 (where the Porter structural model[13] has been adopted). The initial stage, characterized by the appearance of 10*S* material, is thought to result from the dimerization of a proportion of the 7*S* γ-globulin molecules (a similar effect has been attributed to the presence of 10*S* material in lyophilized low-temperature ethanol fractions of 7*S* γ-globulin). Such a change would not be expected to lead to the appearance of a separate immunoelectrophoretic line, on testing with rabbit anti-human 7*S* γ-globulin serum. The unfolding of the γ-globulin polypeptide structure resulting from dimerization (revealed by the sharp increase in lævorotation) would, however, expose the labile bonds linking the 'slow' and 'fast' papain digestion pieces (at the position of the dotted line in Fig. 7) to ready attack by proteolytic enzymes. This leads to the fragmenting off and ultimate degradation

of that part of the molecule corresponding to the 'fast' papain digestion piece (III). Hence, the observed loss in reactivity with rheumatoid factor, and the reduced capacity of stored human 7*S* γ-globulin preparations to form precipitate with specific rabbit anti-human serum[1]. In contrast, human antitoxin activity (presumably associated with pieces I and II) was not affected by the alterations occurring during storage[1].

These findings are consistent with those reported by Nisonoff *et al.*[14], who demonstrated that pepsin treatment of human 7*S* γ-globulin produces 5·8*S* fragments, whereas the use of cysteine, in addition to the enzyme, leads to the formation of 3·5*S* pieces. Such digestion products are analogous to the 5*S* 'divalent' and the 3·5*S* 'univalent' fragments of rabbit antibody examined by the same group. After fragmenting off an inactive piece ('fast' component) by treatment with a proteolytic enzyme alone, the more stable 5*S* ('slow') component could be split into two 3·5*S* pieces by the reduction of its interchain disulphide bonds. It would seem that a similar process could occur in isolated human 7*S* γ-globulins during prolonged storage (Fig. 7). The marked lability of the bonds linking the 'fast' and 'slow' papain digestion pieces of the human 7*S* γ-globulin molecule is obviously a key factor in its structural instability when isolated from other serum proteins. Thus, prevention of the denaturation change leading to the exposure of these bonds, by keeping the isolated γ-globulin in 30 per cent (w/v) sucrose solution, for example, or by uninterrupted storage at −20° C, avoids structural alteration. Presumably other protein constituents fulfil a similar function in stored whole serum, in which changes in the 7*S* γ-globulin structure occur less readily. On the other hand, as would be expected, blockage of the second (degradative) state can be accomplished by enzyme inhibitors such as ε-aminocaproic acid[4] which inhibits plasmin.

Investigations of the changes occurring in isolated 7*S* γ-globulins are being continued in the hope of obtaining further insight into the nature of the labile linkages joining the two parts of the molecule. In this connexion, the observation of Skvaril[1] that 7*S* γ-globulins preparations from human placental material are particularly susceptible to alteration, is of interest. The greater resistance to alteration shown by isolated rabbit 7*S* γ-globulin[15], which possessed a more stable papain piece III, as suggested by its ease of crystallization, etc., is also of some significance.

The nature of the structural alterations occurring in isolated 7*S* γ-globulin preparations revealed by this investigation are probably relevant to the clinical use of such materials, especially where repeated injections are given. Such structural changes could result in alterations in antigenicity, which could conceivably be associated

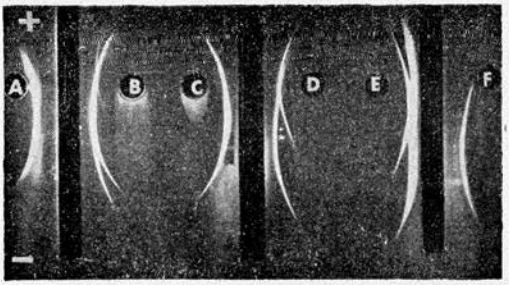

Fig. 6. Immunoelectrophoretic patterns: (*A*) intact 7*S* γ-globulin ; (*B*) altered 7*S* γ-globulin; (*C*) 'Sephadex' peak I of altered 7*S* γ-globulin; (*D*) 'Sephadex' peak 2 of altered 7*S* γ-globulin; (*E*) papain-digested 7*S* γ-globulin; (*F*) 'slow' component of papain-digested 7*S* γ-globulin. Antiserum: rabbit anti-whole human serum

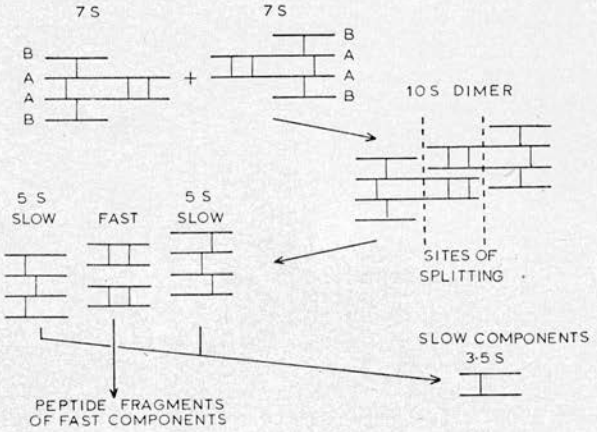

Fig. 7. A hypothetical representation of the changes occurring in the 7*S* γ-globulin molecule during isolation and storage

with deleterious immunological responses or to some relative ineffectiveness owing to accelerated removal from the circulation.

Presumably untoward reactions are usually avoided by the practice (for example, in the treatment of hypo-γ-globulinæmic patients) of administering relatively crude human 7*S* γ-globulin preparations, which possess other serum proteins capable of stabilizing the γ-globulin molecule. Stabilization is probably also enhanced by the use of high protein concentrations (for example, of the order of 13 g/100 ml. in Lister γ-globulin preparations) and by the use of bactericidal reagents such as thiomersalate.

We thank Prof. J. R. Squire for his advice. One of us (C. S. H.) is in receipt of a Medical Research Council studentship for training in research methods.

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Diagnostic Paraprotein in Papular Mucinosis

H. H. FUDENBERG, W. L. EPSTEIN, J. SHUSTER and K. JAMES

Papular mucinosis, also known as lichen myxedematosus, is a rare dermatologic entity characterized by lichenoid papules organized in rows or coalesced into thick plaques [1]. These initially appear on the face and extremities, but later over the entire body. Histologically, the lesions contain masses of large fibroblasts in a mucinous matrix. No consistent pattern of systemic involvement has been reported and the etiology and pathogenesis remain obscure. Two previous reports described the 'co-existence' of papular mucinosis and multiple myeloma, the diagnosis of myeloma being made because of the presence of a spike on serum protein electrophoresis. The first report by PERRY *et al.* suggested that the relation was coincidental [3]. In contrast, other authors suggested that papular mucinosis (hereafter termed P. M.) is basically a variety of multiple myeloma [2].

The data to be presented, obtained in five patients with the disease, and analysis of their serum proteins suggest that P. M. is not a type of multiple myeloma but a unique disease entity with a unique serum protein.

Microzone electrophoresis on cellulose acetate gave good separation of the abnormal protein. All contained a similar homogeneous basic γ-globulin with a characteristic slow mobility in the cathodic region. All five P. M. proteins had this characteristic mobility (Fig. 1). Myeloma proteins vary widely in migration, but less than 1 in 100 random myelomas will have such a slow mobility. Densitometric tracings of the electrophoretic strips from P. M. sera revealed that the amount of P. M. protein is relatively small and characteristically has a basic net charge. Fig. 2 shows a normal serum above, a P. M. serum below. In myeloma the peaks are usually higher and less basic. On alkaline starch gel electro-

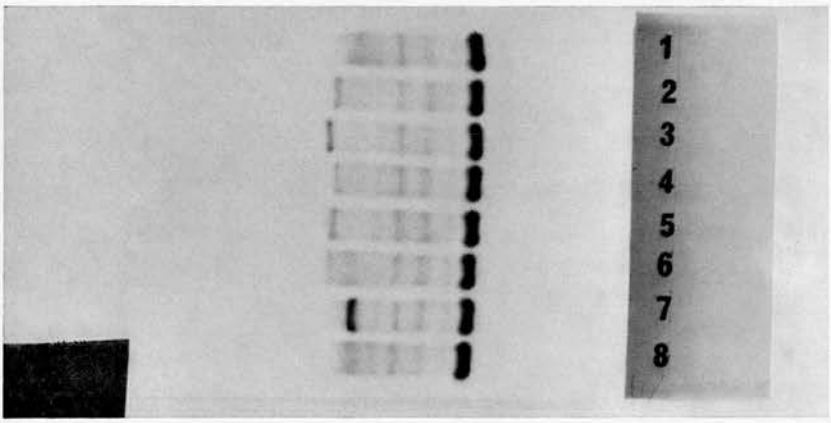

Fig. 1. Cellulose acetate electrophoresis. 1 and 8, normal sera; 7, γG myeloma; 6, diffuse hypergammaglobulinemia; 2, 3, 4, 5, papular mucinosis.

phoresis all five P. M. globulins migrated in the same basic region. In three of the P. M. sera immunoelectrophoresis showed a characteristic spurring in the cathodic region of γG globulin. In the other two sera the P. M. protein precipitated out at the low ionic strength used during immunoelectrophoresis.

The abnormal globulin was isolated from one serum and characterized by immunochemical and physicochemical means. The P. M. protein gave a line of identity with γG using monospecific antisera to γG globulin, and gave no reaction with antisera specific for γA, D, or M globulins. On ultracentrifugation the isolated P. M. protein sedimented at 6.65 and it had optical rotation properties similar to normal γG globulin. We conclude that the abnormal protein is a γG globulin with normal tertiary structure.

The isolated L and H chains and Fab and Fc fragments produced by papain digestion were analyzed. The abnormal L chains also showed marked homogeneity on alkaline urea starch gel electrophoresis with one major and three minor bands as compared with the marked heterogeneity of normal L chains. Of the four antigenic subclasses of the heavy chains of normal human γG globulin, each P. M. protein possessed only one (four were γ_2b, one γ_2c). This antigenic homogeneity was similar to myeloma H chains and different from normal γ-globulin which contains mixtures of molecules of different antigenic subclasses. All five P. M. proteins had light chains only of type lambda, in contradistinction to normal γG globulin which contains molecules with both types of light

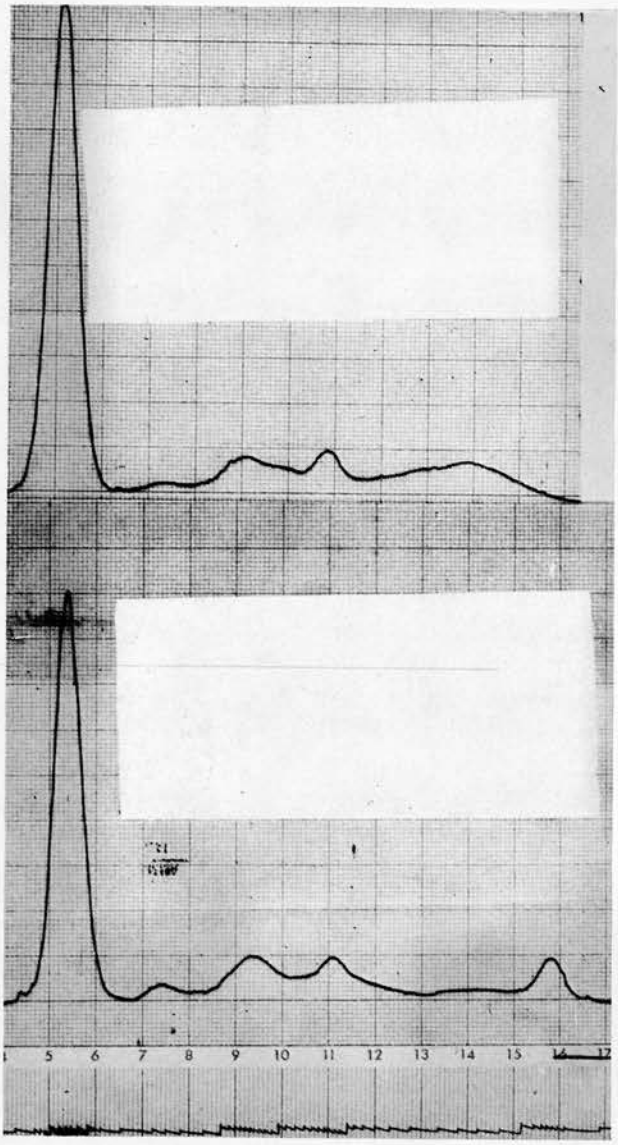

Fig. 2. Densitometric tracings of serum proteins of normal (above) and patient M. C. papular mucinosis (below).

chains, and in contrast to myeloma proteins, some of which have only type K, and others only type lambda light chains. Thus, P. M. globulin and G myeloma protein both are γG globulins, homogeneous on a

variety of supporting media. The L chains are antigenically deficient and homogeneous and the H chains contain only one class of antigenic determinant.

Further analysis indicated the abnormal Fab of the P. M. protein fragment contained antigenic determinants not demonstrated in the intact protein. This indicates these hidden antigenic determinants were located on the abnormal L chain.

The differences between P. M. globulin and myeloma protein can be summarized as follows: First, all P. M. globulins have a basic net charge and slow mobility. Myeloma proteins show much more variability in net charge. Second, P. M. globulin is present in smaller amounts than most myeloma proteins. Third, hidden antigenic determinants have been found in P. M. but not in myeloma proteins to date. Fourth, all P. M. proteins are of type lambda.

Most important, the two diseases differ vastly in clinical appearance and course. P. M. appears to be benign. No evidence of bone lesions, anemia, or renal involvement are seen as in multiple myeloma. The patients have lived as long as 20 years and none, as yet, have shown systemic involvement. We conclude P. M. is basically not a malignancy of plasma cells but rather one of a heterogeneous group of conditions; probably the condition termed 'benign essential monoclonal gammopathy' by WALDENSTRÖM in reality comprises a group of different disorders, out of which P. M. has been delineated as the first disease entity.

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Studies on a unique diagnostic serum globulin in papular mucinosis (lichen myxedematosus)

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STUDIES ON A UNIQUE DIAGNOSTIC SERUM GLOBULIN IN PAPULAR MUCINOSIS (LICHEN MYXEDEMATOSUS)

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SUMMARY

An abnormal γ-globulin present in the sera of five patients with papular mucinosis (PM) (lichen myxedematosus) appears to be characteristic of the disease. The PM protein is closely related to myeloma paraproteins in that it is homogeneous in electrophoretic mobility and homogeneous with respect to light chain antigenic groups. The consistently basic nature of the abnormal γ-globulin was shown by various electrophoretic techniques. In contrast to myeloma paraproteins, the light chains of all five of the PM proteins are L in type. The above characteristics of the PM γ-globulin and the benign clinical course differentiate papular mucinosis from multiple myeloma with cutaneous involvement.

INTRODUCTION

In 1960, Perry, Montgomery & Stickney described a patient with papular mucinosis,* a rare cutaneous disorder, who was thought to have coexistent multiple myeloma on the basis of a homogeneous γ-globulin spike on serum electrophoresis. Several years later, Osserman & Takatsuki (1963) and McCarthy *et al.* (1964) reported an exceptionally basic, electrophoretically homogeneous γG-globulin of the 'myeloma type' in the serum of another patient with generalized papular mucinosis. These investigators concluded that the patient had an unusual type of myeloma, with skin involvement as the primary manifestation, and suggested that the lesions were caused by deposition of complexes of the abnormal globulin and constituents of the connective tissue ground substance.

This report describes the results of electrophoretic and immunologic studies on the sera of five patients with papular mucinosis. A preliminary report of the findings was published elsewhere (Fudenberg *et al.*, 1966). Sufficient serum for isolation and detailed structural

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* We prefer the term papular mucinosis to lichen myxedematosus since extensive investigations have shown no evidence of thyroid dysfunction in such patients.

analysis of the PM protein was available from one of the five patients (McC.). Evidence will be presented to show that although PM proteins are homogeneous, other characteristics of the abnormal γ-globulin and differences in the disease course serve to distinguish papular mucinosis from multiple myeloma.

Clinical findings

All five patients had the characteristic skin lesions of papular mucinosis. These consist of whitish lichenoid macules, which tend to coalesce and form plaques. The lesions are follicular in location, occur bisymmetrically and are distributed in rows; in some areas they are superimposed on thickened skin. Typically, biopsy specimens of skin show proliferation of fibroblasts, particularly in the corium, and stain positively with alcian blue for acid mucopolysaccharide. All five patients are alive 2–8 years after the onset of the disorder.

Case Reports

Case 1

M.McC., a 45-year-old woman, was referred to the University of California Medical Center in August 1965. The clinical findings in this case were reported in detail by Dalton *et al.* (1960). In March 1958 she had first noted 'skin trouble', consisting of erythema over the butterfly area of the face, which soon extended onto the glabella and forehead. Since the eruption was asymptomatic, no treatment was given. Subsequently the cutaneous lesions spread progressively to involve the face, extensor surfaces of arms and legs and most of the trunk. In some areas the lesions eventually coalesced into discrete patches, as is characteristic of papular mucinosis. Local steroid injections resulted in some amelioration of the skin disorder. During the 2 years preceding admission, the patient noticed limitation of jaw motion and symptoms of acrosclerosis, which gradually increased in severity.

At the time of admission, the extensive skin lesions were the most striking physical finding (Fig. 1). The pulse was 72/min, and blood pressure 110/70 mmHg. X-rays of the chest and esophagus and a radiologic skeletal survey showed no abnormalities. An electrocardiogram was normal. Haemoglobin, haematocrit, complete blood count and differential, blood urea nitrogen, serum creatinine, uric acid, cholesterol, acid and alkaline phosphatase, and serum electrolyte levels were within normal limits. Results of thyroid function studies, including PBI, BEI and ^{131}I plasma clearance, and thyroidal uptake, were normal. The basal metabolic rate was normal. Serum total protein, albumin and globulin levels were normal. Urinary excretion of protein was 520 mg/24 hr. Ouchterlony and immunoelectrophoretic analyses showed that the urinary protein consisted chiefly of albumin and low molecular weight γ-globulins. No Bence Jones proteins were detectable by heat test. Serum antinuclear antibodies and antibodies to adrenal tissue, thyroid tissue and gastric mucosa were not demonstrable. An antiglobulin (Coombs') test was negative. The cold agglutinin titre was not elevated. A specimen of bone marrow contained 2% mature plasma cells; no abnormal cells were present. The findings by skin biopsy were characteristic of papular mucinosis. γ-Globulin was not demonstrable in the skin lesions by immunofluorescence.

Case 2

G.Con., a 52-year-old woman, was referred to the University of California Medical Center in 1965. In 1961 a thyroid nodule, noted on routine physical examination, had been removed; histologically it showed changes compatible with thyroiditis. About 1 year later reddish plaques appeared on her arms and legs and subsequently on her face and other parts of her body.

At the time of admission, the only abnormal physical findings were the typical skin lesions of papular mucinosis and a small area of xanthelasma on the upper lid of the left eye. Results of routine laboratory tests, including blood studies, liver function tests, glucose tolerance test and serum electrolyte determinations, were normal. No proteinuria or aminoaciduria was present.

The basal metabolic rate was +15%. Thyroid function studies, including PBI, BEI and red-cell uptake of ^{131}I-triiodothyronine, were normal. Thyroidal uptake of ^{131}I while the patient was receiving thyroid-stimulating hormone, 10 units daily intramuscularly for 2 days, was normal. A thyroid scan showed a normal distribution of radioactivity. Administration of L-triiodothyronine resulted in suppression of thyroidal ^{131}I uptake to 10% in 24 hr. Bioassay of the serum showed no evidence of long-acting thyroid stimulator or of thyroid-stimulating hormone activity (McKenzie & Williamson, 1966). A radiologic bone survey showed no osteolytic lesions or osteoporosis. The bone marrow was normal.

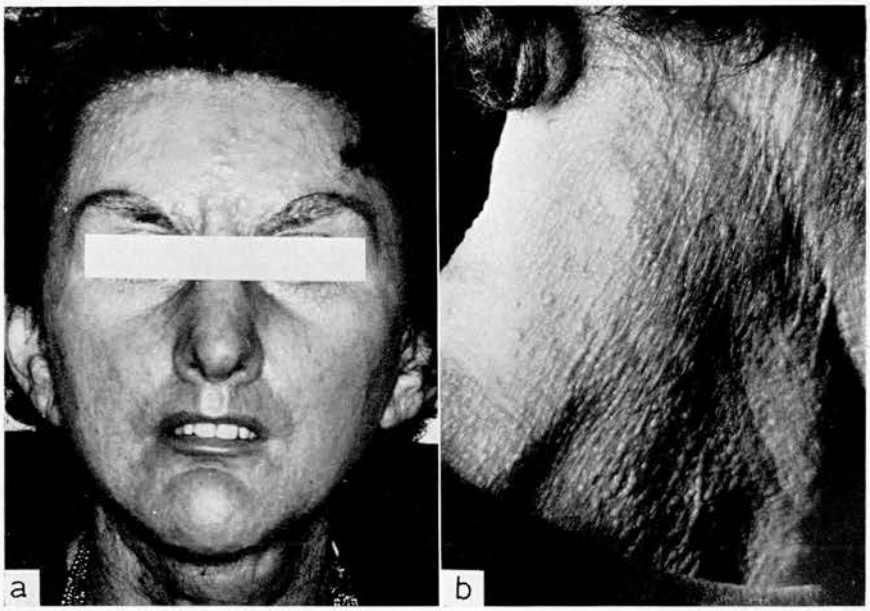

FIG. 1. Patient McC. (a) Face, showing the extensive lesions characteristic of papular mucinosis. Large lichenoid papules, some of which have coalesced, can be seen along the hairline, in the gabella and on the thickened earlobes. (b) Side view of neck, showing the typical linear pattern of the skin lesions, which eventually cover the entire body.

MATERIALS AND METHODS

Fractionation procedures

Large scale isolation of abnormal protein. The PM protein was isolated from the serum of patient McC. as follows. One volume of sodium sulphate solution (28%, w/v) was added slowly with stirring to one volume of serum or defibrinated plasma at room temperature. The resultant precipitate was separated by centrifugation, redissolved in 0·06 M phosphate buffer, pH 6·9, containing 0·15 M sodium chloride, and reprecipitated with the sodium sulphate solution. The precipitate obtained was suspended in 0·01 M phosphate buffer, pH 7·6, and further purified by the batch procedure of Stanworth (1960). The filtrate, containing both normal γG-globulin and the PM protein, was concentrated by ultrafiltration. The concentrate was dialysed against barbitone buffer, pH 8·6, $I = 0{\cdot}05$, and further fractionated by starch block electrophoresis.

Other fractionation procedures. These were performed on small volumes of McC. serum to determine the distribution of the PM protein. The large scale fractionation previously discussed was based upon the results obtained in these studies. Gel filtration was performed on Sephadex G-200 columns as described by Flodin & Killander (1962). γ-Globulin was isolated from the serum by preparative electrophoresis by the method of Kunkel (1954) with

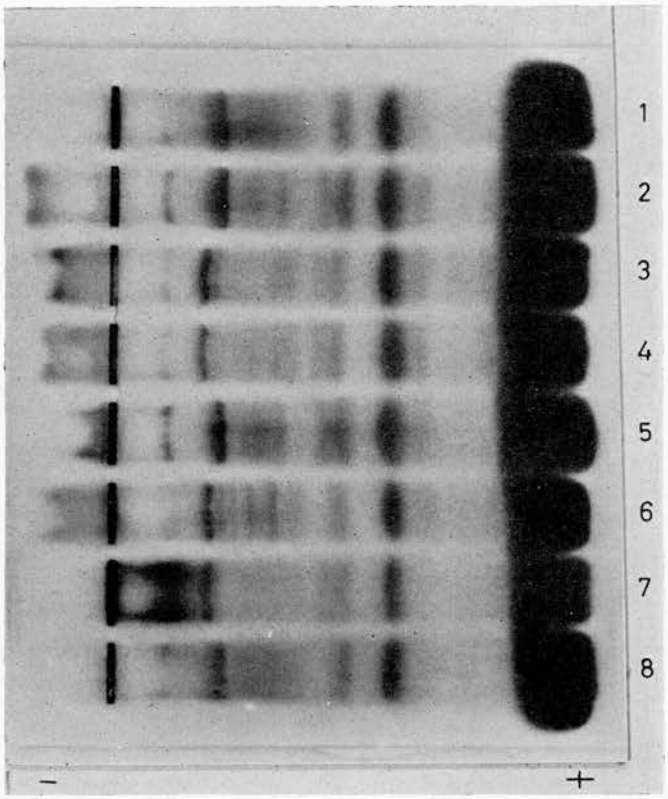

FIG. 2. Starch gel electrophoretic patterns of sera from two normal subjects (1 and 8), five patients with papular mucinosis (PM) (2–6) and one patient with typical multiple myeloma (7). Each PM serum shows a well-defined band near the cathode.

potato starch as the supporting medium. Protein concentrations of the electrophoretic starch-block fractions were determined by the Folin phenol procedure as modified by Lowry *et al.* (1951) with bovine serum albumin as standard. Chromatography was performed on diethylaminoethyl (DEAE)-cellulose columns as described by Peterson & Sober (1956) using a stepwise elution prodecure (James & Stanworth, 1964).

Preparation of chains and fragments. Reduction and alkylation of γ-globulin proteins and separation of heavy and light chains were carried out essentially as described by Fleischman, Pain & Porter (1962). Pepsin and papain proteolytic fragments of γG-globulins and of the PM protein were prepared by the methods of Nisonoff *et al.* (1960) and Porter (1959), respectively.

Immunologic and physicochemical procedures

Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge at 59,780 rev/min at 20°C in 0·06 M phosphate buffer, pH 6·9, containing 0·15 M sodium chloride. Sedimentation velocities were measured at a series of concentrations and the S°_{20w} values were obtained by extrapolation to zero concentration.

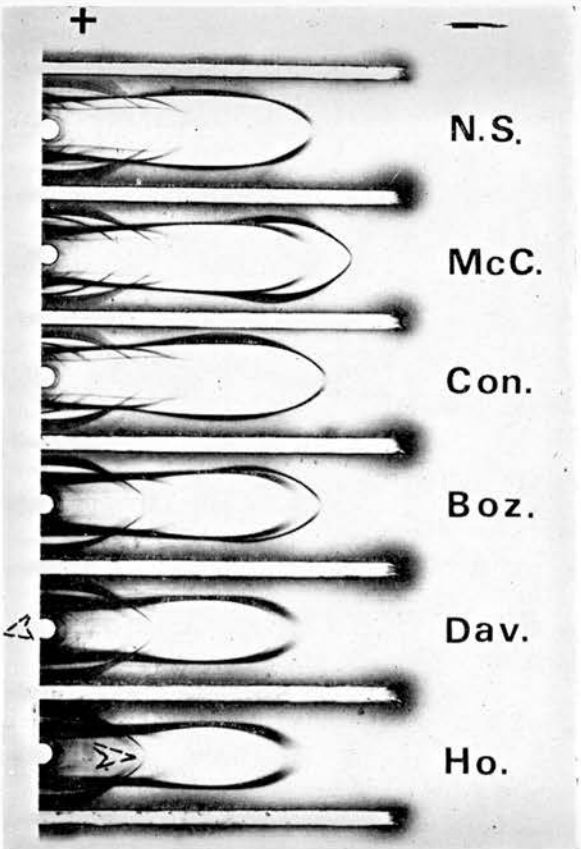

FIG. 3. Immunoelectrophoretic patterns showing the γ-globulin region of normal serum (N.S.) and sera from five patients with papular mucinosis. On unstained slides euglobulins in sera of Dav. and Ho. precipitated out at the ionic strength used, producing a clear area in the region indicated by dotted lines.

Optical rotation measurements were carried out at 27°C in the same phosphate buffer in a Cary model 60 recording spectropolarimeter.

Starch gel electrophoresis of whole sera and γ-globulin preparations was performed with the discontinuous buffer system described by Poulik (1957). Electrophoretic analyses in acid and alkaline urea starch gels were carried out by the techniques of Poulik (1960) and Cohen & Porter (1964), respectively. Agar gel electrophoresis (Wieme, 1959) was performed at ionic strength 0·025, 0·050 and 0·075. Cellulose acetate electrophoresis was carried out

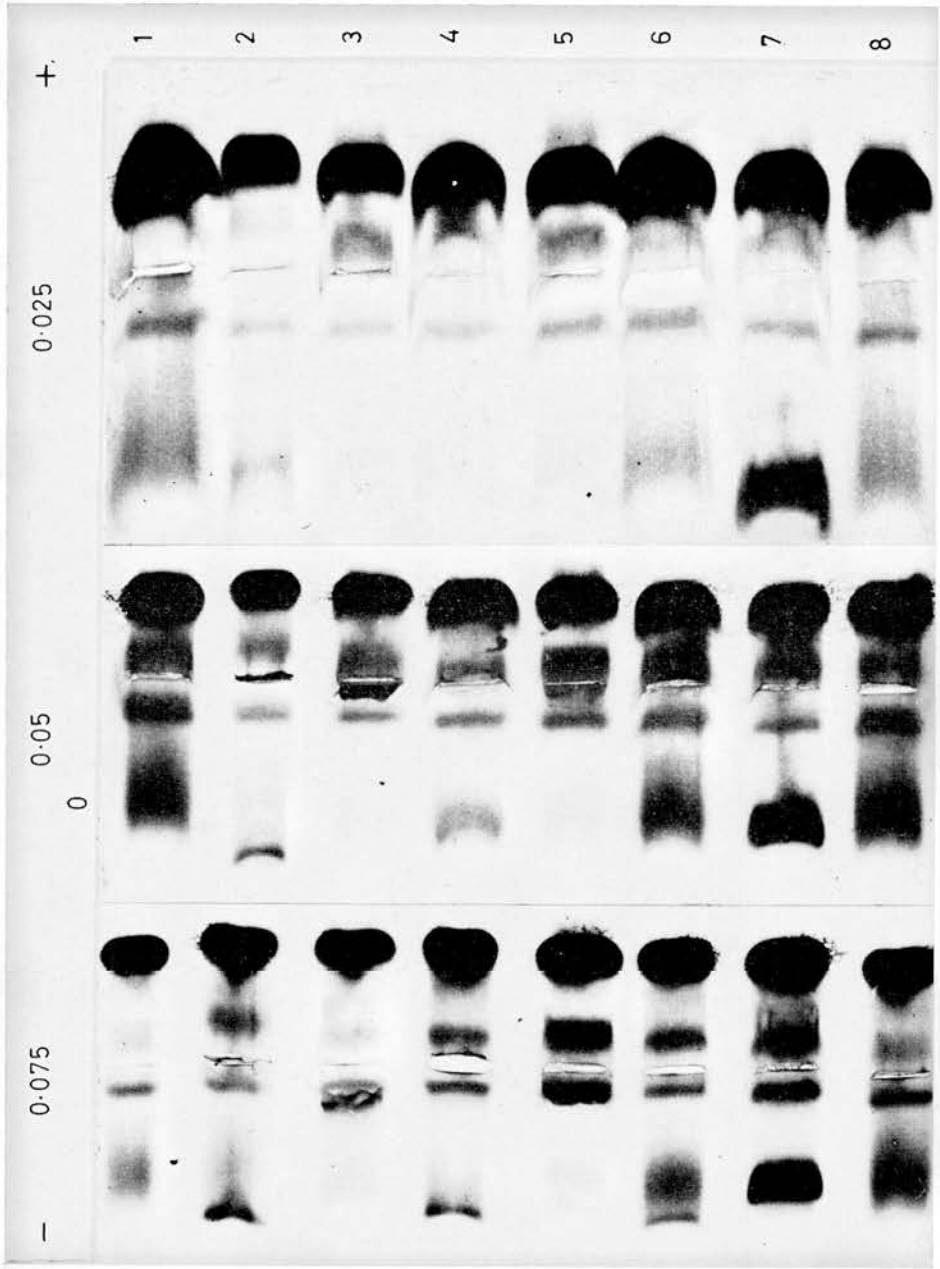

FIG. 4. Wieme agar gel electrophoretic patterns of eight sera defined in Fig. 1, showing effect of ionic strength in sharpening bands of PM sera.

with a Microzone apparatus (Beckman, Inc.) at 0·075 ionic strength, and in some experiments also at ionic strengths of 0·025 and 0·050. Microimmunoelectrophoresis (Scheidegger, 1955) was performed at the same three ionic strengths with a variety of broad-spectrum and monospecific antisera.

Immunodiffusion analyses (Ouchterlony, 1949) were performed with 0·025 M barbital buffer. Thyroglobulin antibodies were estimated by the fluorescent antibody technique of Coons & Kaplan (1950). Serum euglobulin concentrations were measured semiquantitatively by a water dilution turbidity method (Kunkel, 1964, unpublished data); the results were expressed in arbitrary units. Serum immune globulin levels were measured quantitatively by the radial diffusion method of Mancini, Carbonara & Heremans (1965).

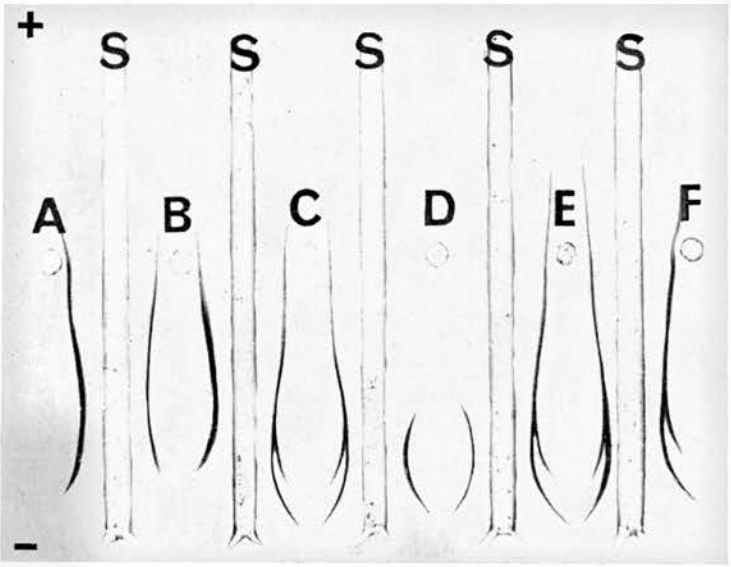

FIG. 5. The distribution of the PM protein of McC. serum in samples obtained by a variety of fractionation procedures. A = Normal γG; B, C and D = successive fractions obtained by starch block electrophoresis; E = the 7S region obtained by G-200 Sephadex gel filtration; F = the protein recovered with 0·01 M phosphate buffer at pH 7·6 on DEAE-cellulose column chromatography; S = specific antiserum to normal γG-globulin.

Gm and Inv typings were performed by previously described techniques (Fudenberg, 1963) with normal saline as the diluent. Haptoglobin and transferrin types were studied by starch gel electrophoresis (Smithies, 1955). Heavy chain antigenic subgroups of the PM protein were typed by immunoelectrophoresis, using specific antisera to γ_{2a}, γ_{2b}, γ_{2c} and γ_{2d} myeloma proteins (Grey & Kunkel, 1964; Terry & Fahey, 1964). The antigenic subgroups on the light chains of the PM proteins were characterized by antiserum specific for Bence Jones protein types L and K.

RESULTS

The sera of all five patients with papular mucinosis demonstrated a homogeneous basic zone by starch gel (Fig. 2), Microzone and Wieme electrophoretic techniques. The basic proteins behaved as euglobulins on immunoelectrophoresis ($\mu = 0{\cdot}05$), where the PM

proteins of patients Dav. and Ho. (Fig. 3) precipitated out. The influence of ionic strength on the euglobulin property of the PM proteins is shown in Fig. 4. At higher ionic strengths the PM protein zones appeared and showed sharpening of boundaries. Sia tests, however, were always negative. The insolubility property presented problems when the PM protein

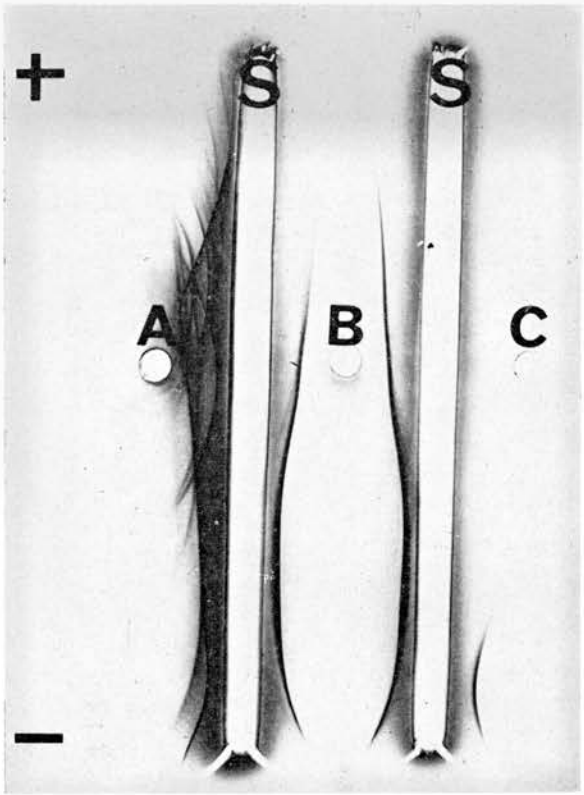

FIG. 6. Immunoelectrophoretic analysis demonstrating purity of isolated PM protein from patient McC. A = Serum of McC; B = normal γG-globulin from Cohn fraction II; C = PM protein from McC. serum prepared by three-stage procedure. Note the restricted precipitin arc of slow electrophoretic mobility in PM protein. Subsequent analyses were carried out on fraction C.

of patient McC. was isolated by DEAE chromatography. No apparent correlation was found between serum protein phenotypes and the occurrence of papular mucinosis (Table 1).

The PM proteins reacted only with antisera to γG-immunoglobulins. They failed to give a band on immunoelectrophoresis with antisera specific for γA-, γM- and γD-globulins. Immunochemical studies confirmed the homogeneous nature of the PM proteins. The PM protein of each of the five sera had only one heavy and one light chain antigenic subgroup (Table 1). In four instances the heavy chain subgroup was γ_{2b}; the light chains of all five were L in type.

The distribution of the PM protein of McC. serum is illustrated in Fig. 5 where it can be seen that the extremely basic protein is also eluted with the bulk of the γG-globulin on G-200 Sephadex gel filtration and DEAE-cellulose chromatography. The basic nature and the purity of the isolated protein obtained by the three stage fractionation procedure are shown in Fig. 6. The isolated pure protein had a sedimentation coefficient of 6·6S when extrapolated to zero concentration and demonstrated a conformation identical with that of the patient's normal γG-globulin and of Cohn fraction II γ-globulin by optical rotary dispersion (Fig. 7). Further evidence of the homogeneous nature of the PM protein is shown in Fig. 8. The light chains of the PM protein demonstrated only a single major and a single

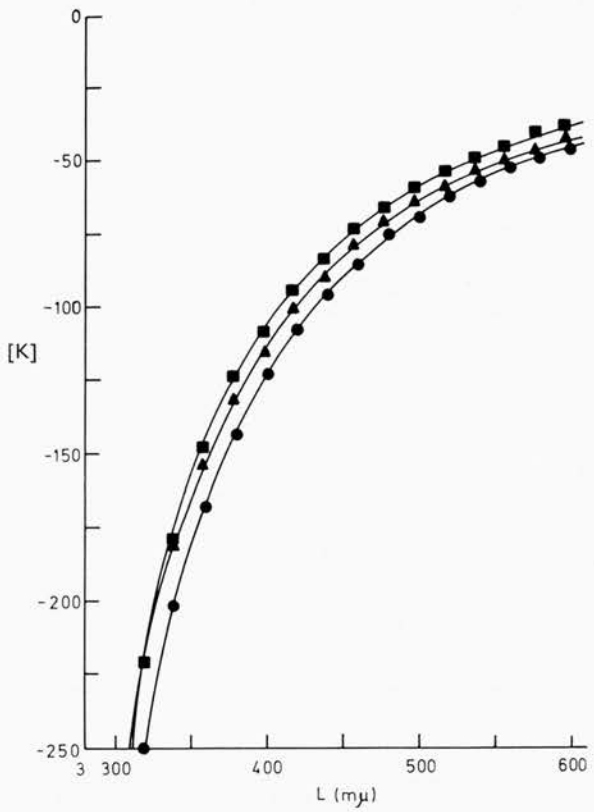

FIG. 7. Optical rotation properties of PM γG-globulin (●) and normal γG-globulin of McC. serum (▲) and Cohn fraction II (■).

minor zone on alkaline urea starch gel electrophoresis in contrast to the nine zones demonstrated by the normal γG-globulin of McC.'s serum. The starch gel electrophoretic pattern of light chains of the PM protein was similar to that obtained with Bence Jones proteins and isolated light chains of myeloma proteins. The isolated light chains of the PM protein possessed only one antigenic group (Fig. 9), as it also seen with myeloma proteins (Fahey, 1962; Mannik & Kunkel, 1962). Comparative immunodiffusion analysis of the isolated PM protein and of Fc and Fab fragments of the patient's normal γG-globulin obtained by

TABLE 1. Immunological findings in five patients with papular mucinosis

Patient and sex	Age (years)		Cutaneous manifestations	Immunoglobulins (mg/ml)			Antigenic subgroups		Serum euglobulin (units)	Thyroglobulin antibodies	Serum protein phenotypes						
	Present	At onset									Gm				Inv (1)*	Haptoglobin	Transferrin
				γG	γM	γA	Light chains	Heavy chains			(1)	(2)	(4)	(5)*			
M.McC F	45	37	Severe	11·0	0·98	1·15	L	γ_{2b}	6·0	Negative	0	0	+	+	0	2–1	CC
G.Con F	52	48	Mild-moderate; rapidly progressing	11·0	1·10	2·14	L	γ_{2b}	1·5	Negative	+	0	+	+	+	2–2	CC
E.Ho M	40	33	Moderate	12·2	0·48	1·40	L	γ_{2b}	0	Negative	0	0	+	+	0	2–1	CC
M.Boz M	57	53	Moderate	6·1	0·82	1·20	L	γ_{2b}	0	Negative	0	0	+	+	0	2–1	CB
W.Dav M	53	37	Severe	10·5	0·63	0·94	L	γ_{2c}	0	Negative	0	0	+	+	0	2–1	CC

* Revised terminology (World Health Organization, 1965): Gm(1) = Gm(a), Gm(2) = Gm(x), Gm(4) = Gm(f), Gm(5) = Gm(b), Inv(1) = Inv(l).

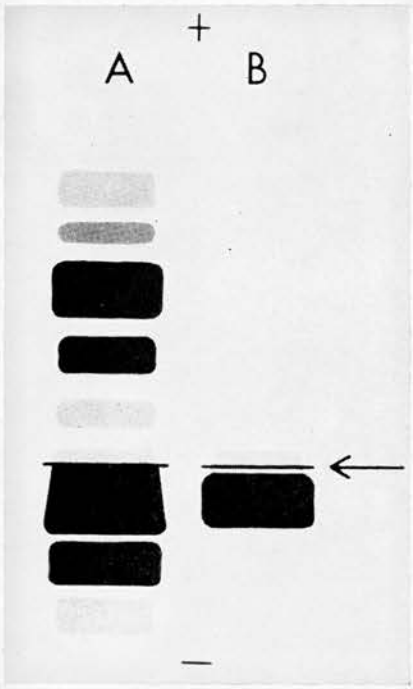

FIG. 8. Alkaline urea starch gel analysis of isolated light polypeptide chains of normal γG-globulin (A) and PM protein (B) of patient McC. Both samples were examined at concentrations of approximately 2 g/100 ml (w/v).

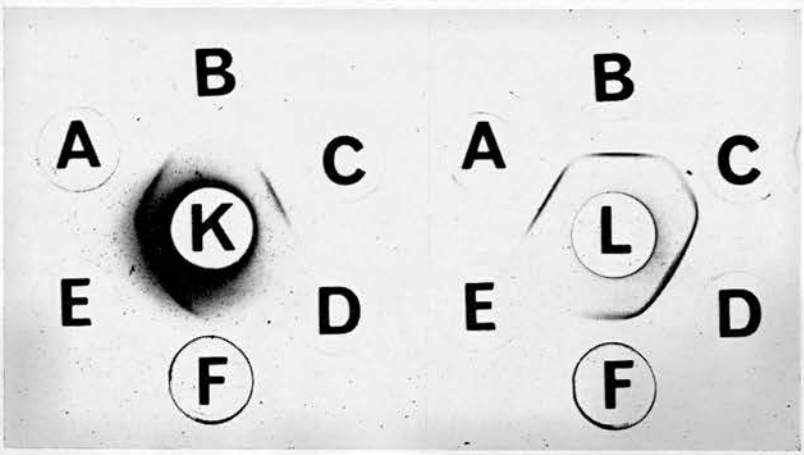

FIG. 9. Immunodiffusion analysis indicating the presence of the K antigenic group on the isolated light polypeptide chain of the PM protein of patient McC. A = Normal γG-globulin from pooled Cohn fraction II; B = PM protein of McC. serum; C = normal γG-globulin of McC. serum; D = type L Bence Jones protein; E = type K Bence Jones protein; F = heavy polypeptide chain of normal γG-globulin; K = rabbit antiserum to type K Bence Jones protein; L = rabbit antiserum to type L Bence Jones protein.

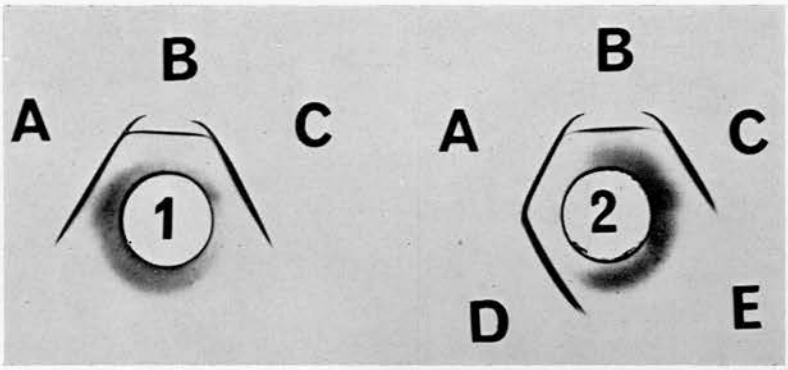

FIG. 10. Immunodiffusion analysis showing antigenic deficiency in the Fab fragment of PM protein. A = Normal γG-globulin from pooled Cohn fraction II; B = PM protein of McC. serum; C = normal γG-globulin of McC. serum; D = Fab fragment of normal γG-globulin; E = Fc fragment of normal γG-globulin. Well 1 contained rabbit antiserum to normal γG-globulin, and well 2 rabbit antiserum to $F(ab)_2$ fragment of γG-globulin.

papain digestion indicated that the PM protein and the Fab portion of normal γG-globulin differ antigenically (Fig. 10). Similar differences existed between the PM protein and the 5S Fab piece obtained by pepsin digestion.

DISCUSSION

Studies on the sera of the five patients with papular mucinosis and on the one isolated PM protein demonstrated several interesting features. The PM protein migrated as a homogeneous zone of characteristic basic mobility. The isolated light chain of PM protein McC. gave a single major zone on alkaline urea starch gel electrophoresis similar to that given by the homogeneous light chains and Bence Jones proteins in multiple myeloma. Heavy and light chain antigenic typings of these molecules confirmed their homogeneous nature. The PM proteins have the properties of γG-globulins as demonstrated by immunochemical methods, ultracentrifugation and optical rotary dispersion.

Although the PM proteins are similar in physical, chemical and immunological characteristics to multiple myeloma paraproteins, other features distinguish papular mucinosis from multiple myeloma. No significant increase in the number of plasma cells and no immature plasma cells are found in the bone marrow of patients with papular mucinosis. The clinical features characteristic of multiple myeloma (bone lesions, anaemia, etc.) were not present in the five patients studied or in those reported on previously (Perry *et al.*, 1960; Osserman & Takatsuki, 1963; McCarthy *et al.*, 1964). The prolonged survival of the patients with papular mucinosis also suggests that this essentially benign disorder is distinct from multiple myeloma. Thus, papular mucinosis appears to be one of a group of clinical disorders which together comprise the benign essential gammopathies (Waldenström, 1960–61). The distinction is further emphasized by the improbability of finding five consecutive PM proteins with L type light chains. Studies of myeloma proteins have shown that only one-third of the total light chains are of the L type (Fahey, 1962; Mannik & Kunkel, 1962) so that the

probability of five consecutive myeloma proteins of the L type is $<0{\cdot}04$ [$(1/3)^5$]. Furthermore, the PM proteins were consistently basic in nature, which is extremely uncommon in myeloma paraproteins. The latter observations are of interest in the light of recent data reported by Sela & Mozes (1966). Their studies showed that the chemical nature of antibodies depends on the net electrical charge of the antigenic stimulus. Thus, a negatively charged antigen tends to elicit the production of more positively charged (basic) antibody molecules. Since the mucopolysaccharides of skin are negatively charged compounds, the acquisition of antigenicity by these cutaneous constituents, either as a primary event or secondary to some other primary degenerative change in the skin, would probably result in a basically charged antibody. This hypothesis is currently being investigated in our laboratory.

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ADDENDUM

Since submitting the paper we have examined sera from three additional patients with papular mucinosis. In each case, the sera contained a very basic monoclonal γG-globulin with light chains of type lambda.

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Etudes sur l'isolement du facteur rhumatismal

RÉSUMÉ

On analysa minutieusement les méthodes employées jusqu'à présent dans les tentatives d'isoler le facteur rhumatismal, telles que le fractionnement par précipitation de l'euglobuline, la chromatographie sur la cellulose DEAE (diéthylaminoéthyl), la centrifugation zonale et le fractionnement à l'éthanol en température basse d'après Cohn.

On détermina la récupération de l'activité sérologique et l'augmentation de l'activité spécifique. Le degré de resolution obtenue par de différents procédés fut étudié par des méthodes immunologiques et ultracentrifuges.

La manière la plus efficace pour préparer le facteur rhumatismal en un état assez pur semble être la précipitation de l'euglobuline par dilution aqueuse, suivie de centrifugation zonale.

Des méthodes impliquant la précipitation de l'euglobuine par une dialyse prolongée à l'eau ou par l'addition de sulfate d'ammonium concentré, la chromatographie sur la cellulose DEAE, et des procédés de concentration, telles que l'ultrafiltration et des procédés au glycol de polyéthylène, aboutirent à une perte considérable d'activité sérologique.

Avec des procédés employés jusqu'à présent il ne fut pas possible d'isoler la facteur rhumatismal et la globuline gamma 19S.

Estudios sobre el aislamiento del factor reumatoide

SUMARIO

Se analizaron detalladamente los métodos empleados hasta ahora en las tentativas de aislar el factor reumatoide, tales como los procedimientos de fraccionamiento por precipitación de la euglobulina, cromatografía sobre celulosa DEAE (dietilaminoetil), centrifugación zonal y fraccionamiento por etanol en temperatura baja según Cohn.

Se determinó la recuperación de la actividad serológica y el aumento de la actividad específica. El grado de resolución por diversos procedimientos fué estudiado por métodos centrífugos e inmunológicos.

La más eficaz manera de preparar el factor reumatoide en un estado bastante puro parece ser la precipitación de euglobulina por dilución acuosa, seguida de centrifugación zonal.

Métodos que implican la precipitación de la euglobina por dialisis prolongada con agua or por añadidura de sulfato de amonio concentrado, la cromatografía sobre celulosa DEAE y procedimientos de concentración, tales como la ultrafiltración o el empleo de glicol de polietileno, ocasionaron una pérdida considerable de la actividad serológica.

Con los procedimientos empleados hasta la hora presente no fué posible resolver el factor reumatoide y la globulina gamma 19S.

could also be attributed to denaturing of the molecule itself or inhibition by aggregated 7S γ globulin, but under the above conditions we should expect both of these effects to be at a minimum.

The special efforts that have been made to measure the amounts of individual proteins present in the solutions by immunological methods have served two purposes. In the first place, it has been useful as a guide to the specific discriminatory powers of special procedures, in particular, euglobulin precipitation. It was also hoped by means of this approach to obtain further information about the nature of the rheumatoid factor, in particular its relationship to 19S γ globulin. In this context it is interesting to note that, while the estimation of 7S component by analytical ultracentrifugation and 7S γ globulin immunologically agreed reasonably well in most of the native sera, comparable analyses of preparations tended to show a considerable discrepancy explicable possibly in terms of the relative concentration of 7S components other than γ globulin. Other possible explanations include the interaction of 19S protein rheumatoid factor with 7S γ globulin thus impeding diffusion of this protein through the agar plate.

Work performed to date has not shown whether or not rheumatoid factor is distinct from 19S γ iso-agglutinins, or is a slightly altered 19S γ globulin. Increases in serological activity have shown no evident parallel with 19S γ concentrations as estimated immunologically. Attempts are now being made to see whether or not it is possible to resolve sheep cell agglutinating activity from iso-agglutinin activity.

Iso-agglutinin preparations from normal sera fail to give positive sheep cell agglutinating activity, although adsorbed by the sensitized sheep cell.

Summary

A detailed analysis has been made of methods previously used in attempts to isolate rheumatoid factor, including euglobulin precipitation, DEAE cellulose chromatography, zone centrifugation, and Cohn type low-temperature ethanol fractionation procedures.

The recovery of serological activity and increases in specific activity have been determined. The degree of resolution of the different techniques has been investigated by ultracentrifugal and immunological methods.

The most efficient way of preparing rheumatoid factor in a fairly pure state appears to be euglobulin precipitation by water dilution followed by zone centrifugation.

Methods involving euglobulin precipitation by prolonged dialysis against water or the addition of saturated ammonium sulphate, DEAE cellulose chromatography, and concentration procedures, such as ultrafiltration and carbowax (polyethylene glycol) techniques, were found to result in a considerable loss of serological activity.

With the techniques used to date it has not been possible to resolve rheumatoid factor and 19S γ globulin.

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lin, 1960), and to the precipitation of protein such as albumin which does not agglutinate sensitized sheep cells. Experience with Sera 5 and 9 would indicate that, with some sera at least, a water-dilution technique is more efficient than dialysis against water. Dilution will certainly have a more profound effect on interionic and intramolecular forces which in sera may tend to solubilize the rheumatoid factor. This technique also prevents the ionic strength from falling to zero level as obtained in complete dialysis and hence reduces the probability of denaturation.

Of the further procedures available for increasing the specific activity of rheumatoid factor preparations, chromatography on DEAE cellulose and zone ultracentrifugation have been most extensively employed. The efficiencies of such procedures may be classified in terms of yield of desired product, concentration of product without further manipulation, and discrimination between individual proteins. Rheumatoid euglobulin prepared by dilution or dialysis was desirable as starting material for zone ultracentrifugation, for it contained little 19S high molecular weight glycoprotein which would sediment with the rheumatoid factor.

In terms of discrimination, zone centrifugation can only be expected to separate proteins of the 19S class and greater molecular weight from other serum proteins, and even this achievement requires special arrangements, such as suitable density gradients and minimum disturbance during the sampling of the tubes. In these experiments zone centrifugation did not achieve complete separation of 19S protein from 7S and 10S material, but the majority of these proteins were removed. Re-centrifugation could have been adopted, but was avoided partly because repeated ultracentrifugation of rheumatoid factor had been shown to decrease its activity (Svartz and others, 1958) and also involved redialysis of the sample.

Column chromatography on DEAE cellulose enables the separation of rheumatoid factor from the bulk of the 7S γ globulin precipitated as euglobulin, but complete separation was not achieved. Losses of protein and activity were always considerable. Products obtained by chromatographic techniques were usually contaminated with 7S γ globulin, albumin if this was present in the euglobulin, traces of α_2 high molecular weight glycoprotein, and β lipoprotein, and contained large amounts of 19S γ globulin. It is thus essential that this step be followed by zone ultracentrifugation to remove low molecular weight contaminants.

Batch chromatography achieved a separation similar to that obtained by the column technique, but total recovery of both protein and units was usually much better. The superiority of the batch chromatographic procedure over the column procedure, in addition to that of speed, may depend on the rheumatoid factor (and other proteins) being attached to the resin for a minimum period of time and so being less likely to be irreversibly denatured.

The lower losses of activity associated with the zone ultracentrifugation procedure could be attributed to the "milder" conditions experienced in this method, which avoids precipitation and adsorption on to resins with subsequent solubilization and elution—procedures which may produce denaturing of the molecule. The use of a sucrose density gradient may help to protect the rheumatoid molecule.

One possible index of denaturing available for the results of these studies lies in the appearance of additional ultracentrifugal components, *e.g.* 27S, 22S, and 10S. Four of the starting sera contained 22S material, and three also contained 10S protein. Whether or not these proteins were originally present in the sera or were produced on storage at −20° C. is not known, but Svartz (1960) believes that the rheumatoid factor is pre-existent in the blood as a globulin with a sedimentation coefficient of 18 to 19S.

The 10S component detected, particularly on euglobulin precipitation and Cohn fractionation, would appear to be produced during the experimental procedure. There is no evidence to suggest that this material resulted from the action of soluble γ globulin aggregates in a manner analogous to the effect of adding soluble γ globulin aggregates to normal human sera followed by incubation at 4° C. (as described by Muller-Eberhard and Kunkel, 1960).

As purification proceeds, the rheumatoid factor becomes more susceptible to denaturing and therefore it is perhaps not justifiable to compare different fractionation procedures unless they were performed on the same starting material. Susceptibility to denaturing could be due to the removal of protective compounds, such as 7S γ globulin which forms 22S derivatives with 19S γ globulin. Experiments performed by dialysing rheumatoid sera against 0·85 per cent. buffered saline and Ringer's solution, with little or no precipitation of euglobulin, results in the loss of a considerable amount of serological activity, suggesting the removal of dialysable activators which could include metal ions or compounds such as cysteine and ascorbic acid which may influence the surface groups of the molecule (*e.g.* sulphydryl groups). Decrease in activity

Zone centrifugation was usually used as a final step in the removal of low molecular weight (7S) γ globulin contaminant from rheumatoid factor, the other 19S component (*i.e.* α_2 glycoprotein) having been previously removed by euglobulin precipitation and DEAE cellulose chromatography. Adoption of zone centrifugation as a preliminary step, as in Fractionation 6, did not prove very satisfactory; a drawback with these large starting volumes is the necessity of using angle rotors in order to be able to process sufficiently large volumes of rheumatoid serum. This has led to the contamination of the 19S zone with low molecular weight serum constituents (as shown in the results in Table VII).

DEAE Cellulose Batch Chromatography

With the aim of increasing the speed of the chromatographic procedure and improving yields, a batch process (Stanworth, 1960) was tried in the isolation of rheumatoid factor. As shown in Table V, protein recoveries of the order of 90 per cent. were obtained when rheumatoid serum was fractionated initially in this way (as in Fractionations 7A and 8). Corresponding improved recoveries (53 to 83 per cent.) of Rose-Waaler activity were also recorded, although this might reflect to some extent the superiority of whole serum as starting material in preference to euglobulin (used in the column procedures in Fractionations 1, 2, 3, 5, and 6). In considering recoveries of both protein and rheumatoid activity, one must bear in mind that in the batch procedure the final eluent was 1·5 M NaCl.

The batch chromatographic technique achieved similar resolution of serum proteins to that obtained by the column procedure, the eluate containing 19S γ globulin being contaminated with 4·5S component and also with relatively small amounts of 7S γ globulin. Further purification was accomplished on these occasions by a subsequent Cohn low-temperature ethanol fractionation procedure, as modified by Dr. K. W. Walton. Rose-Waaler activity was found to be completely precipitated along with the γ globulins, leaving behind in solution both 19S α_2 glycoprotein and 4·5S albumin contaminants.

The poor recovery of activity in Fractionation 8, where the modified Cohn technique was employed as a second step after DEAE cellulose batch chromatography, was probably due to denaturation of either the rheumatoid factor or of traces of 7S γ globulin, which may then inhibit the Rose-Waaler tests (Franklin, 1960), brought about by the addition of relatively large volumes of 0·1 M NaOH solution to adjust the pH to 7·4.

In Fractionation 7A, a repeat batch chromatographic procedure was made after Cohn fractionation, in an attempt to remove the remaining 7S γ globulin. Unfortunately, however, although overall 100 per cent. recovery of protein was achieved, only a 20 per cent. recovery of Rose-Waaler activity was recorded (Table V).

For this reason, zone centrifugation was adopted as the final step in Run 8, resulting in a 35 per cent. recovery of Rose-Waaler activity. This product contained only 19S protein as revealed by ultracentrifugal analysis (see Table II), but immunological analysis revealed 5 mg./100 ml. of 7S γ globulin and traces of α_2 glycoprotein.

Discussion

In this work on the isolation of rheumatoid factor, emphasis has been placed on the quantitative approach, often neglected in earlier studies. For instance, the enrichment of factor after each fractionation has been followed by measuring Rose-Waaler activity and relating this to total protein concentration. On this basis, results have been expressed in terms of "specific activity". Attempts have also been made to determine the yields of active material from the arithmetical products of the specific activities of solutions with their volumes. It is readily admitted that individual quantitative assessments are somewhat approximate because of the inaccuracies inherent in the use of limiting dilution values as indices of Rose-Waaler activity. No other methods of assaying rheumatoid factor activity have been employed.

The ability to precipitate rheumatoid factor as euglobulin varied considerably from serum to serum. This may reflect the properties of the sera or critical differences in the methods of precipitation.

In Table IV, showing experiments performed under identical conditions with the different sera, this variability is clearly seen. It would thus appear that one of the major factors controlling the precipitation of rheumatoid factor by dialysis or dilution lies in the starting serum. Prolonged dialysis of serum results in the precipitation of large amounts of "inactive protein" (Table III, Run 4B), with a considerable drop in specific activity. This drop in specific activity, usually accompanied by an overall loss of units, is probably due to some form of denaturation of rheumatoid factor; it might also, however, be due in part to the production of aggregated 7S γ globulin which is able to neutralize the rheumatoid factor by combining with it (Frank-

concentration of the dilute chromatographic fraction solutions. Various concentrating procedures have been adopted, none of which has proved entirely satisfactory.

For instance, as is shown in Table VI (opposite), losses of activity as high as 95 per cent. were recorded during concentration by negative pressure ultrafiltration through Visking dialysis tubing and by the carbowax (polyethylene glycol) technique described by Kohn (1959), whilst losses of 80 per cent. occurred during ultrafiltration through collodion thimbles accompanied by simultaneous dialysis against buffered saline. Recoveries by ultrafiltration through Visking tubing were improved by massaging adherent protein off the walls of the tubing before pouring off the liquid.

These results, which are similar to those reported by Stanworth (1959) in a study of the effect of various concentration procedures on horse dandruff reagin, suggest that rheumatoid factor is highly susceptible to surface denaturation when purified.

An ultracentrifugal technique employed recently in the concentration of urinary colloid (Rowe and Soothill, 1961) would appear to offer improvements over the procedures just described, all of which involve the rheumatoid factor coming into contact with relatively large surface areas. A modification of this procedure, to avoid pellet formation in the bottom of the tube by using a bottom layer comprised of an inert non-water-miscible solvent (Stanworth and others, 1961), would appear to be advantageous.

In some instances, precipitation of rheumatoid factor from solution by dialysis against water proved a useful concentration procedure, but this was often vitiated by the disadvantage that the products were nearly always redissolved only with difficulty and accompanying denaturation. In order to overcome these difficulties in Runs 8 and 9, techniques were adopted which avoided the need for subsequent concentration.

Zone Centrifugation

Of all the fractionation procedures employed, zone centrifugation is undoubtedly the mildest as far as recovery of Rose-Waaler activity is concerned. This is indicated by the results given in Table VII, where it will be seen that 100 per cent. recoveries of activity are often achieved.

TABLE VII

ACTIVITIES AND COMPOSITIONS OF "RHEUMATOID FACTOR" PREPARATIONS OBTAINED BY ZONE CENTRIFUGATION

Run	Sample	Units Added	Specific Activity	Units Recovered	Specific Activity (Bottom of Tube)	Per cent. Units Recovered	Specific Activity as Percentage of that of Material Applied	Ultracentrifugal Composition (mg./100 ml.) Initial 27S	22S	19S	10S	7S	4·5S	Final 27S	22S	19S	10S	7S	4·5S	Immunological Composition (mg./100 ml.) Initial α_2*	19Sγ*	7Sγ	Final α_2*	19Sγ*	7Sγ
1	Serum	64	1·7	112		175			—	130	—	840	2,730	Not determined						Not determined					
	Euglobulin I	128	22	128	28	100	130	Not determined																	
	Euglobulin II	512	29	353	42·6	69	150																		
3	DEAE Chromatography Fraction†	2,880	116	2,700	Top 330 Bottom 120	94	280 100		130	170	100	280	420	Not determined						6	100	3	Not determined		
	Top of above	1,920	330	680 or 1,280	600 or 1,200	35 or 70	180 or 360	Not determined						—	—	63	—	—	—	Not determined			—	6	0·1
4A	Euglobulin Ia + Ib	2,300	29	1,000	580	44	200		Trace	770	420	1,470	—	170	250	600	80	230	—	4	150	140	1	100	20
B	Euglobulin I + II	2,900	44	8,200	190	280	430		370	240	120	1,400	—	70	450	830	150	530	—	1	100	240	—	200	60
5‡	Euglobulin I	6,400	25	2,200	125	35	350		40	100	55	760	350	—	30	210	—	190	—	3	50	240	3	50	40
6‡	Serum	115,000	63	61,900	380	54	600		—	100	—	1,000	7,000	−9	—	270	—	780	1,010	75	65	1,280	5	10	5
8‡	Cohn Fraction I of "Active" DEAE Batch Fraction	6,550	65	2,050	77	31	120		16	160	130	190	—	—	—	104	—	—	—	1	200	20	1	50	5
9A	Euglobulin	655,000	1,710	615,750	5,120	94	300	280	1,880	1,790	240	1,820	—	115	210	595	30	50	—	4	4,200	1,560	4	600	280
B		—	—	694,700	4,400	—	—							190	910	930	120	340	—				0	1,600	840
10	Euglobulin	126,000	780	176,000	3,820	140	490		235	2,510	355 350 (12S)	1,550	—		15	795	25	85	—	<3	2,000	1,600	<3	700	240

* See p. 370. † Mixing occurred on slicing tube—therefore top centrifuged again. ‡ Zone-centrifugation carried out in 40 angle head rotor at 36,000 r.p.m.

TABLE VI

COMPARISON OF THE EFFICIENCY OF VARIOUS METHODS OF CONCENTRATING CHROMATOGRAPHIC FRACTIONS CONTAINING RHEUMATOID FACTOR

Run	Fraction	Method of Concentration	Concentration Factor	Units Added	Units Recovered	Per cent. of Units Recovered	Per cent. Protein Recovered
1		Ultrafiltration through Visking tube (Grant, Rowe, and Stanworth, 1958)	25	570	320	56	Not determined
2	I	Ultrafiltration through Visking tube (Grant, Rowe, and Stanworth, 1958)	100	2,500	1,300	52	Not determined
3	II	Polyethylene glycol (Kohn, 1959)	3	7,600	380	5	50
5		Ultrafiltration through collodion thimbles with simultaneous dialysis	1	1,300	310	24	Not determined
6	I	Ultrafiltration through collodion thimbles with simultaneous dialysis					
	II	Ultrafiltration through Visking tube with simultaneous dialysis	14	20,000	1,000	5	Not determined
7A		As Run 5	7	2,400	380	16	Not determined
B			7	900	170	19	

Note: Above fractions are final products.

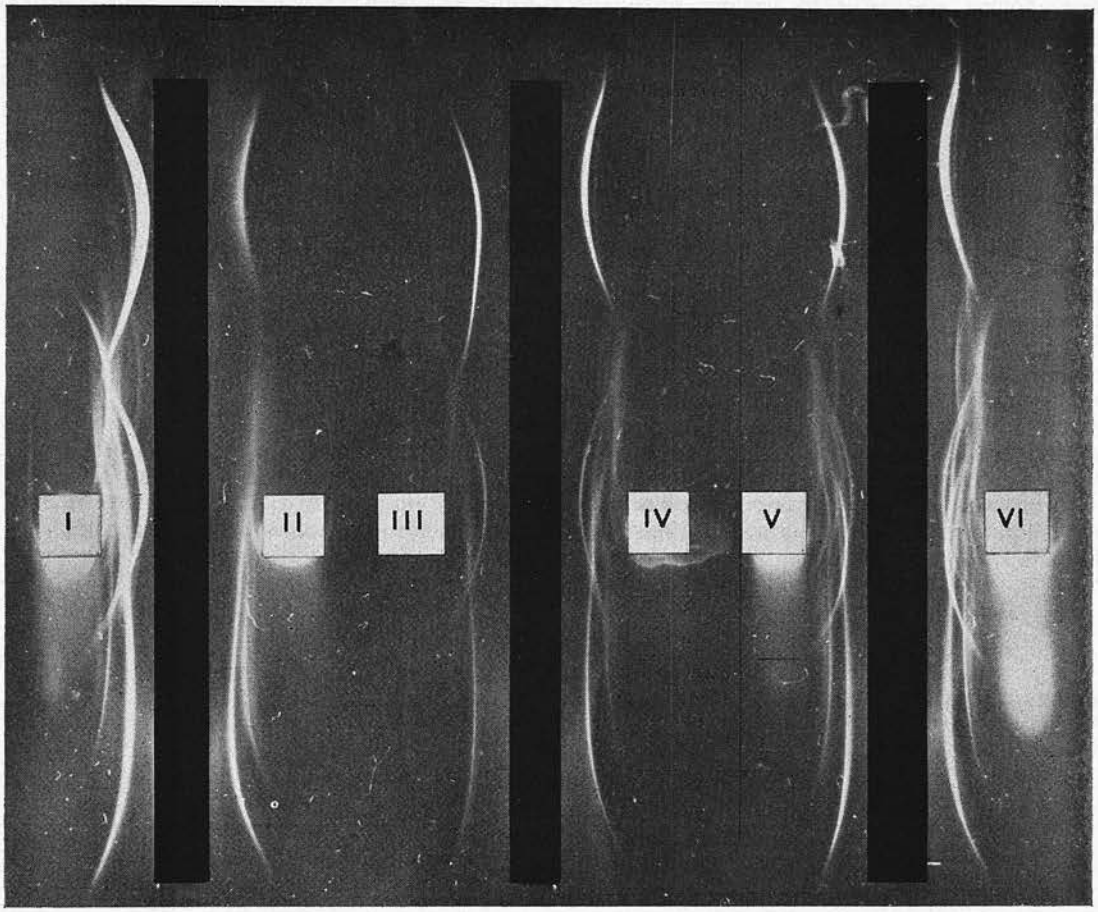

Fig. 2(I).—Rheumatoid serum (No. 9).

Fig. 2(II).—Euglobulin precipitate from (I) above prepared by dilution with de-ionized water (1 : 15).

Fig. 2(III).—Supernatant from (II).

Fig. 2(IV), (V), (VI).—Successive harvestings of euglobulin precipitates after dialysis of rheumatoid serum No. 9 against de-ionized water (15 volumes) with slow stirring for 2, 5, and 23 hrs respectively. Immuno-electrophoresis performed in 0·8 per cent. agar in barbitone buffer (pH 8·6; I=0·05). A current of 15 ma. was passed for 4 hrs.
Antiserum—anti normal human serum.

Note: In γ globulin zone, 7S γ globulin can be seen as a prolonged asymmetric line in all samples. 19S γ globulin appears shorter between this and the cup in all samples except (III).

Euglobulin precipitate obtained by dilution (Sample II) consists mainly of 7S and 19S γ globulins with only small amounts of albumin and α_2 globulin. Supernatant (Sample III) appears substantially free from 19S γ globulin, at least in the dilution tested.

In contrast, euglobulins prepared by dialysis (Samples IV, V, and VI) are immuno-electrophoretically complex, and 19S γ globulin occurs in all these precipitates.

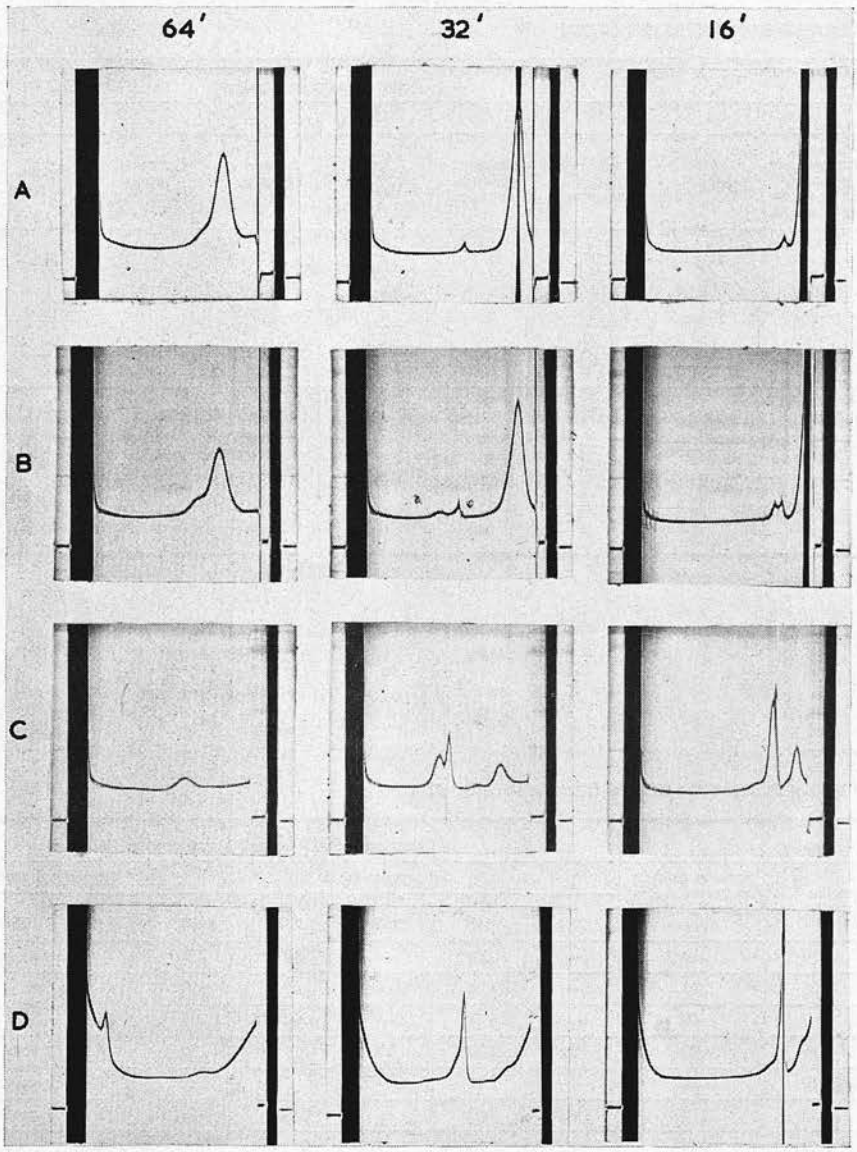

Fig. 1(A).—Normal serum (diluted 1 : 5).

Fig. 1(B).—Rheumatoid serum (No. 9, patient F.A., diluted 1 : 5).

Fig. 1(C).—Euglobin preparation from (B) above (diluted 1 : 5).

Fig. 1(D).—Zone centrifugation, high molecular weight fraction from above.

Solvent: phosphate buffer pH 6·9; M=0·06+0·15M NaCl.

Speed: 60,000 r.p.m.; temperature 20° C.

Note presence of abnormally large amounts of 19S and 22S fractions in (B) and the progressive concentration and purification of the high molecular weight components in (C) and (D).

TABLE IV

EFFICIENCY OF SEPARATION OF RHEUMATOID FACTOR BY VARIOUS METHODS OF EUGLOBULIN PRECIPITATION

Material	Volume (ml.)	Method	Dilution	Supernatant 1 Volume (ml.)	Supernatant 2 1/Titre	Supernatant 1 × 2 Total Units of Activity Recovered	Supernatant 1 × 2 Per cent. of Units Recovered	Supernatant 3 Protein Concentration (mg./ml.)	Supernatant 1 × 3 Total Protein (mg.)	Precipitate (in 2 ml. saline) ⅔ = S Per cent. Protein Recovered	⅔ = S Specific Activity	⅔ = S Specific Activity as per cent. of that of Initial Serum	4 1/Titre	5 Total Units of Activity Recovered	5 Per cent. of Units Recovered	5 Protein Concentration (mg./100 ml.)	5 Total Protein (mg.)	4/5 Per cent. Protein Recovered	4/5 Specific Activity	4/5 Specific Activity as per cent. of that of Initial Serum
"A"	2	Neat serum	—	2	512	1,024	100	97	194	100	5·3	100								
(a)	2	× 2 dilution dialyse H_2O	1/16	4	52	128	12·5	34	136	70	0·9	17	128	256	25	4	8	4·1	32	600
(b)	2	× 2 dilution dialyse 0·01M	1/16	4	32	128	12·5	40	160	83	0·8	16	64	128	12·5	2	4	2·0	32	600
(c)	2	× 2 dilution dialyse 0·05M	1/16	4	64	256	25	35	140	72	1·8	34	8	16	1·5	1·2	2·4	1·2	6·6	120
(d)	2	× 4 dilution dialyse H_2O	1/18	8	4	32	3·2	21	168	87	0·2	4	128	256	25	2·8	5·6	2·9	48·5	920
(e)	2	× 4 dilution dialyse 0·01M	1/18	8	32	256	25	26	208	108	1·2	23	128	256	25	2·6	5·2	2·7	49·1	930
(f)	2	× 4 dilution dialyse 0·05M	1/18	8	64	512	50	20	160	83	3·3	60	8	16	1·5	1·6	3·2	1·6	5	94
(g)	2	Dialyse against N. NaCl	1/15	2·4	256	614	60	69	165	85	3·7	70	4	8	0·8	—	—	—	—	—
(h)	2	Dialyse against Ringer's solution	1/15	2·3	256	588	57	72	163	84	3·6	68	4	8	0·8	2·0	4·0	2·0	2	38
"B"	2	Neat serum	—	2	512	1,024	100	87	174	100	5·9	100								
(a)	2	× 2 dilution dialyse H_2O	1/16	4	64	256	25	39	156	90	1·6	27	256	512	50	3·7	7·4	4·3	67·5	1,150
(b)	2	× 2 dilution dialyse 0·01M	1/16	4	32	128	12·5	32	128	73	1·0	17	512	1,024	100	2·5	5·0	2·9	204	3,460
(c)	2	× 2 dilution dialyse 0·05M	1/16	4	256	1,024	100	32	128	73	8·0	140	8	16	1·5	2·0	4·0	2·3	4	88
(d)	2	× 4 dilution dialyse H_2O	1/18	8·5	32	272	27	19	162	93	1·7	29	512	1,024	100	3·5	7·0	4·0	146	2,500
(e)	2	× 4 dilution dialyse 0·01M	1/18	9	16	144	14	17	153	88	0·9	16	512	1,024	100	2·6	5·2	3·0	197	3,340
(f)	2	× 4 dilution dialyse 0·05M	1/18	8·5	256	2,180	213	23	195	112	11·2	190	8	16	1·5	0·6	1·2	0·7	13·2	200
(g)	2	Dialyse against N. NaCl	1/15	2·4	512	1,230	120	72	170	98	7·1	120	4	8	0·8	0·7	1·4	0·8	5·7	97
(h)	2	Dialyse against Ringer's solution	1/15	2·4	256	615	60	75	180	103	3·4	58	4	8	0·8	0·4	0·8	0·5	10	170

TABLE V

ACTIVITIES AND COMPOSITIONS OF RHEUMATOID FACTOR PREPARATIONS OBTAINED BY DEAE CELLULOSE CHROMATOGRAPHY

Run	Sample	Protein Applied (mg.)	Protein Recovered (mg.)	Per cent. Protein Recovered	Units Applied	Total Units Recovered	Per cent. of Units Recovered	"Active" Fraction Units	"Active" Fraction Specific Activity	"Active" Fraction Specific Activity as percentage of that of Material Applied	Composition of "Active" Fraction (mg./100 ml.) Ultracentrifugal 22S	19S	10S	7S	4·5S	Immunological α_2*	19Sγ*	7Sγ
1	Euglobulin	78	39	50	1,720	330	19	320	50	230	—	20	—	90	210	Not determined		
2	Euglobulin I	115	46	40	8,800	1,230	14	1,200	150	200	—	150	—	25	470	Not determined		
	Euglobulin II	220	40	18	8,800	360	4	210	7·8	40	—	170	—	60	1,090			
3	Euglobulin	81	—	—	38,000	I 2,200	46	2,200	116	20	80	130	70	210	350	6	100	30
						II 15,360		15,360	2,110	430	—	210	—	—	550	Not determined		
5	Euglobulin II + III	395	239	60	24,350	8,100	33	III 2,560	128	210	40	180	—	280	—	3	45	20
								IV 2,560	140	230	30	170	—	260	—	12	35	25
6	Zone Centrifuge Fraction	47	—	—	17,900	1,022	6	II+III 1,022	I 52, II 69	20	—	330	—	90	190	5	10	5
7A	Serum	765	700	91	25,600	21,282	83	15,000	59	180	Not determined							
	Cohn Fraction I of Batched Serum	90	123	137	7,680	1,540	20	900 After concentration 170	110	60	—	250	30	80	—	1	18	40
7B	Cohn Fraction I of Serum	35	38	109	15,360	3,580	23	2,400 After concentration 380	105	120	—	35	50	170	—	3	50	60
8	Serum	6,900	6,700	97	97,000	51,100	53	34,700	64	460	—	45	20	65	370	3	50	40

Roman figures (*e.g.* I and II) denote different chromatographic fractions.

* See p. 370.

"Active Fraction" = Chromatographic fraction containing majority of serological activity.

TABLE III

COMPARISON OF VARIOUS METHODS OF PRECIPITATING EUGLOBULIN

Run Nos.	Fraction	Method of Preparing Euglobulin	Protein Precipitated (mg.)	Recovery		Specific Activity as per cent. of that of Original Serum	Ultracentrifugal Composition (mg./100 ml.)						Immunological Composition (mg./100 ml.)		
				Per cent. Total Protein	Per cent. Total Units Precipitated		27S	22S	19S	10S	7S	4·5S	α_2*	19Sγ*	7Sγ
1	I	80 ml. serum against 2 litres for 48 hrs	99	3·2	40	1,260	Not determined						Not determined		
	II III	Supernatant of I Supernatant of II	20	0·7	10	1,600									
2	I	45 ml. serum (pH adjusted to pH 8 with 0·01 M phosphate buffer)	145	4·2	34	810	—	—	670	—	110	2,100	Not determined		
3	I	25 ml. serum Redialysed against 2 litres for 48 hrs	86	18·5	25	450	—	270	40	70	860	310	1	45	120
4A	I Ia	20 ml. serum diluted × 12 with tap water Distilled water washings of above	118	7·9	75	960	Not determined						Not determined		
	Ib	Washed precipitate in 0·85 saline	63	4·7	25	910									
	Ia + Ib	Ia + Ib redialysed	106	7·1	29	430	—	Trace	770	420	1,470	—	4	150	140
4B	I	Stepwise dialysis 20 ml. serum against tap water stirring	81	5·4	150	2,780	Not determined						Not determined		
	II	Supernatant of I	25	1·7	3·8	225									
	III	Supernatant of II	41	2·7	1·0	35									
	IV	Supernatant of III	35	2·3	3·8	160									
		Precipitates I + II in 0·85 saline redialysed	88	5·9	3·7	640	—	370	240	120	1,400	—	1	100	240
5	I	100 ml. serum against 5 litres tap water for 29 hrs stirring	325	2·9	3	110		40	100	55	760	350	2	50	240
	II	Supernatant I against running tap water for 20 hrs	130	1·2	5	420		110	170	150	1,240	940	1	36	160
9A		96 ml. serum + 1,400 ml. deionized water at 4° C. Centrifuged at 4,000 r.p.m. and 4° C. for 15 min.	482	5·75	70	1,710	280	1,880	1,790	240	1,820	—	4	4,200	1,560
10		100 ml. serum as above	450	5·1	44·4	780		235	2,510	350 355 (12S)	1,550	—	3	2,000	1,600
2	II	Slow addition of saturated ammonium sulphate till solution 33 per cent. saturated	642	19·5	31	210		—	640	—	540	10,500	Not determined		
5	III		414	3·7	8·2	210		50	220	260	3,840	760	200	200	2,560

All dialyses performed against 1 litre glass-distilled water for 24 hrs at 4° C. without stirring, except where stated.

* See p. 370.

method, however, there was generally more euglobulin precipitated and so the increases in specific activity were not as great. The efficiency of euglobulin precipitation was much greater with Serum B than with Serum A, as shown by the greater increase in specific activity. (Averaging the results of Experiments a, b, d, and e with each serum, the concentration achieved was three times greater with B than with A.) Dialysis against 0·05 M phosphate, physiological saline, and Ringer's solution invariably resulted in a considerable loss of units, confirming previous experiments. The specific activities of the euglobulins fell rapidly if the dialysis was continued for longer than 48 hrs.

Ion-exchange Chromatography

Analysis of the fractions obtained by DEAE cellulose chromatography of normal human serum (Stanworth, 1959) has indicated that 19S γ globulin can be readily separated from the major portion of the 7S γ globulin and from the 19S α_2 glycoprotein. This technique would therefore appear to be an ideal successive step to the initial euglobulin precipitation from rheumatoid serum, as it offers a means of freeing the rheumatoid factor from the co-precipitated 7S γ globulin. Such an approach in Fractionations 1, 2, 3, and 5B has provided the results given in Table V.

Alternatively, DEAE cellulose chromatography would be useful in freeing rheumatoid factor from the bulk of 19S α_2 glycoprotein sedimented with it in zone-centrifugation procedures.

As will be observed from Table V, however, a disadvantage of the chromatographic procedure is the relatively poor yield of Rose-Waaler activity (always less than 50 per cent.) recovered from DEAE cellulose columns. This can probably be ascribed to the low recoveries of protein, particularly the macroglobulin. In this connexion, the batch chromatographic procedure (described later) was found to be more useful. As far as purification of rheumatoid factor is concerned, the column chromatographic procedure was found to increase the ratio of 19S to 7S γ globulin, as shown in the results of Fractionation 5 (Table V), although 7S γ globulin was not completely removed.

As would be expected from the analysis of normal serum chromatographic fractions, DEAE cellulose chromatography proved incapable of freeing euglobulin precipitates from contaminating 4·5S components (Table V).

Concentration of Chromatographic Fractions

In ascribing loss of activity to the column chromatographic treatment, it is important to consider the contribution made by the subsequent

TABLE II

COMPOSITION, ACTIVITIES, AND YIELDS OF RHEUMATOID FACTOR PREPARATIONS OBTAINED BY VARIOUS FRACTIONATION PROCEDURES

Run No.	Case No.	Fractionation Procedure (for details of steps see following tables)			Ultracentrifugal Composition (mg./100 ml.)			Product										
		Step 1	Step 2	Step 3	27S	22S	19S	10S	7S	4·5S	Immunological Composition (mg./100 ml.) α_2*	19Sγ*	7Sγ	Protein Concentration (mg./100 ml.)	Total Protein Recovery (mg.)	Per cent. Starting Protein	Total Units	Per cent. Units Recovered	Specific Activity	Specific Activity as per cent. of that of Original Serum
1	1	Euglobulin precipitation 1/I†	DEAE cellulose column chromatography		—	—	20	—	90	210	Not determined			320	6·4	0·3	320	15	50	2,900
2 I	2	I Euglobulin precipitation 2/I	DEAE cellulose column chromatography		—	—	150	—	25	470	Not determined			645	8·7	0·25	1,300	13	150	1,600
II		II Ammonium sulphate precipitation 2/II	DEAE cellulose column chromatography		—	—	70	—	60	1,100				1,230	26·8	0·8	210	3	7·8	90
3	3	Euglobulin precipitation 3/I	DEAE cellulose column chromatography	Zone centrifugation	—	—	63	—	—	—	—	6	0·1	63	0·7	0·04	600	2	600	565
4A	4	A Euglobulin precipitation A/Ia + Ib	Zone centrifugation		170	250	600	80	230	—	1	100	20	1,330	17·2	0·8	1,000	10	58	850
B		B Euglobulin precipitation B/I + II			70	450	830	150	530	—	—	200	60	2,030	26·5	1·3	5,000	49	190	2,800
5A	5	A Euglobulin precipitation 5/I	Zone centrifugation		—	30	210	—	190	—	3	50	40	430	8·8	0·10	640	3	73	300
B		B Euglobulin precipitation / Ammonium sulphate precipitation } 5/II + III	DEAE cellulose column chromatography { F.III		—	40	180	—	280	—	3	45	20	500	20·0	0·20	2,560	10	128	530
			F.IV		—	30	170	—	260	—	12	35	25	460	18·6	0·20	2,560	10	140	580
6	6	Zone centrifugation	DEAE cellulose column chromatography		—	—	330	—	90	190	6	10	5	610	18·0	1·0	670	1·6	56	90
7A	7	DEAE cellulose batch chromatography	Cohn low-temperature ethanol fractionation	DEAE cellulose batch chromatography	—	—	25	30	80	—	1	18	20	135	1·5	0·4	170	1·4	110	330
B		Cohn low-temperature ethanol fractionation	DEAE cellulose batch chromatography		—	—	35	50	170	—	1	35	35	255	3·1	0·8	380	3	125	320
8	8	DEAE cellulose batch chromatography	Cohn low-temperature ethanol fractionation	Zone centrifugation	—	—	104	—	—	—	1	50	5	104	16·8	0·2	1,300	1·4	77	550
9A†	9	Euglobulin precipitation	Zone centrifugation { A		115	210	595	30	50	—	4	600	280	1,000	80·0	0·95	410,000	32	5,120	3,500
B			B		190	910	930	120	340		0	1,600	840	2,490	99·5	1·15	437,000	34	4,400	3,010
10A	10	Euglobulin precipitation	Zone centrifugation			15	795	25	85		<3	700	240	920	84·0	0·95	117,000	36·5	1,390	3,820

* See p. 370. † Euglobulin precipitation. See Table III, *e.g.* 1/I for Run 1, Euglobulin precipitate I.

Euglobulin Precipitation

It has been the practice of various groups engaged in the study of rheumatoid factor to employ euglobulin precipitation as a first step (Svartz and Schlossmann, 1953; Kunkel and others, 1959; Lospalluto and Ziff, 1959). The crude precipitates thus obtained were found to contain most of the Rose-Waaler activity present in the original serum.

Euglobulin would therefore appear to be the most suitable starting material for the subsequent purification of rheumatoid factor. The composition of the euglobulin precipitated varied considerably, as did the efficiency of precipitating the rheumatoid factor without loss of activity (see Table III). These results suggest that there are uncontrolled factors involved in the precipitation of rheumatoid factor as water-insoluble euglobulin. One of these is undoubtedly the variability of the starting sera, all of which were obtained from different patients. In Serum 4B, almost all activity was precipitated after 24 hrs. while in Serum 5 very little had been precipitated after 48 hrs. Experiments recently performed show that, with Serum 5, the rheumatoid factor is efficiently recovered by diluting the serum 15-fold with cold de-ionized water. The most critical factor in these experiments, however, would appear to be the ionic strength to which the rheumatoid sera were finally adjusted.

Trial experiments performed on Sera 9 and 10 showed that, as with Serum 5, Rose-Waaler activity was most efficiently precipitated (80 and 62·5 per cent. of the total) with minimum contamination from other proteins, by diluting the serum 15-fold with cold de-ionized water at 4° C. and centrifuging down the precipitate almost immediately. Precipitates obtained by dialysis on the other hand of these sera against 14 volumes of de-ionized water and centrifuging down the precipitates at 2, 5, and 24 hrs, each time redialysing the supernatant, were very heterogenous as compared with the precipitates obtained by dilution, and the combined precipitate contained only 31 and 12·5 per cent. of the original activity. The greater heterogeneity of the "dialysis" products as compared with the "dilution" products was well shown by immuno-electrophoresis (see Fig. 2). The water-dilution technique was therefore adopted in Fractionations 9 and 10; the euglobulin precipitation, after suspension in the minimum volume of phosphate pH 6·9; M = 0·06 + 0·15 M NaCl, was found to contain 70 and 44·4 per cent. of the original units (Table III). Washing of precipitates was avoided, for, as shown in Run 4 and other unpublished work, quite an appreciable amount of activity may be removed by cold distilled water.

The variability between different euglobulin preparations noted above led to more detailed studies of euglobulin and rheumatoid factor precipitation with a series of tests on only two different sera (Table IV). (In order to measure the activities of the euglobulins, they were dissolved in a known volume of physiological saline, but varying amounts of precipitate remained insoluble as fine suspensions.) The highest recoveries of units were achieved by dialysing either of the water-diluted sera (2- or 4-fold) against 0·01 M phosphate buffer (pH 7·2), although almost as good yields were obtained by dialysis against distilled water. With the latter

TABLE I

PROPERTIES OF RHEUMATOID SERA USED

Case No.	Sex	Protein Concentration (mg./100 ml.)	Specific Activity (ml.‡/mg.)	Ultracentrifugal Composition (mg./100 ml.)					Immunological Composition (mg./100 ml.)		
				22S	19S	10S	7S	4·5S	α_2*	19S γ*	7S γ
1	F	3,700†	1·8	—	130	—	840	2,730	Not determined		
2	F	7,700	9·4	—	150	—	420	7,130	Not determined		
3	M	7,800	106	80	170	120	730	6,700	56	100	750
4	M	7,500	6·8	—	135	—	900	6,460	88	150	960
5	F	10,900	24	—	390	—	1,910	8,600	200	200	4,480
6	M	8,100	63	—	100	—	1,000	7,000	75	60	1,280
7	M	7,700	33	110	180	—	670	6,740	150	150	1,920
8	M	9,200	14	—	250	110	2,700	6,170	75	300	2,560
9	M	8,750	146	450	340	—	1,200	6,760	150	125	1,120
10	M	8,800	36·4	105	105	650	1,350	6,690	200	500	1,280
S.D. Rheumatoid		8,500±1,080			202±107		1,220±700	6,900±690	124±52	198±184	1,794±390
S.D. Normal ..					170±56		1,170±290	6,070±300	107±26	71±30	1,240±250

* See p. 370.
† Serum from Case 1 had been diluted before use.
‡ Specific Activity = Reciprocal Rose-Waaler Titre ÷ Protein Concentration (mg./100 ml.) = (ml./mg.) (see p. 370).
S.D. = Standard Deviation (excluding Serum 1 in the rheumatoid results).
Normal Serum: Ultracentrifugal results from eleven normal sera and average protein content at 7 g. per cent. assumed. Immunological results from twenty normal sera.

composition of products, but it may be noted that there is a general tendency for raised levels of total 7S and 7S γ and probably of 19S γ globulins and high molecular weight α_2 glycoproteins.

Full-scale fractionations were undertaken on ten rheumatoid sera, as summarized in Table II (overleaf), the ninth and tenth being undertaken after a full study of the results of the first eight. Considering Runs 1 to 8, gains in specific activity have been from only 3- to 29-fold (excluding Run 6). Since the rheumatoid factor is a 19S protein, which, even in the serum with highest Rose-Waaler titre, only contributes 4 per cent. of the total protein, it could be expected that efficient zone centrifugation alone would give a 25-fold increase in specific activity and a "pure" 19S protein. However, as will be discussed later, efficient zone centrifugation of 20 to 100 ml. serum is not a practical proposition with the apparatus at our disposal, so some prior method of removing the bulk of contaminating proteins is advantageous.

The recovery of units has varied from 1 to 50 per cent., being below 15 per cent. in any procedure using column chromatography on DEAE cellulose. The final product constituted between 0·11 and 3 per cent. of the starting protein. If the recovery of units had been higher these results would have been more satisfactory. The yield of final product lay between 5 and 30 mg. except in Runs 3 and 7 (only a small-scale fractionation).

Only two preparations were judged free of 7S protein by ultracentrifugal analysis, and these had traces of 7S γ as measured by the very sensitive immunological technique (Runs 3 and 8).

The concentrations of activity obtained in Runs 1 to 8 could quite possibly have been achieved by an efficient euglobulin precipitation with subsequent zone ultracentrifugation, and judging from Run 4, with a large proportion of starting units recovered in the final product. Such a procedure was used in Runs 9A and B and in Run 10 where the gains in specific activity were 35-, 30-, and 38-fold respectively. The recovery of units in the actual products were 32, 34, and 36·5 per cent. respectively (though taking into account samples removed for analysis and less active fractions not included in the product, some 54 to 66 per cent. of the total starting units could be accounted for). The yields of products were 80, 99·5, and 84 mg., which constituted 0·95, 1·15, and 0·95 per cent. of the original protein. These products also contained 7S γ globulin as revealed by immuno-electrophoresis and ultracentrifugal analysis (Figs 1 and 2, overleaf), but the bulk of the protein, *i.e.* 92 per cent. in Run 9A, 81 per cent. in Run 9B and 88 per cent. in Run 10, had a sedimentation coefficient of 19S or greater.

agglutinating dose for sensitization and studying the pattern of sedimentation in plastic cups as the index of agglutination. Units of serological activity have been calculated by multiplying the volume of the sample by the reciprocal of its Rose-Waaler titre. Specific activities are derived by dividing the reciprocal of the Rose-Waaler titre by the protein concentration in mg./ml. and is equivalent to the maximum volume (ml.) to which 1 mg. protein can be diluted and still give a positive Rose-Waaler titre.

Euglobulin Precipitation

Crude protein precipitates rich in rheumatoid factor activity were obtained by lowering the ionic strength of the serum in various ways, involving dilution with, or dialysis against, water (as described in Table III). On two occasions precipitation with 33 per cent. saturated ammonium sulphate was used after failure to precipitate rheumatoid factor activity by dialysis.

Ion Exchange Chromatography

Chromatography in columns (20 cm. high, 1·1 cm. diameter) of DEAE cellulose, prepared according to the method described by Peterson and Sober (1956), was performed by step-wise elution in a manner essentially similar to that employed by those workers (Sober, Gutter, Wyckoff, and Peterson, 1956). DEAE cellulose chromatography was preferred to chromatography on carboxymethyl cellulose (CM), as applied by Fallet, Lospalluto, and Ziff (1958), because of the superior resolving power of the former method in relation to 19Sγ globulin.*

In some cases, however, 0·05 M NaH_2PO_4 solution containing 0·15 M NaCl was used as a final eluent in order to remove the 19S γ globulin in a sharp band.

"Batch" chromatography on DEAE cellulose was carried out by a modification of the method recently described by Stanworth (1960), using filtration instead of centrifugation and with suitable washing (pH greater than 5 and ionic strength 0·05 M) and eluting solutions (1·5 M NaCl).

Cohn Low-temperature Ethanol Fractionation

A small-scale fractionation procedure designed to use 5-ml. volumes of plasma was employed. This was a modification of "Method 10" of Lever, Gurd, Uroma, Brown, Barnes, Schmid, and Schultz (1951), developed by Dr. K. W. Walton. It involved the adjustment of the pH of the sample to 7·4 with 0·01 M NaOH before the initial precipitation, and it provided a rapid means of separating the γ-globulins from other serum protein constituents.

* This is the high molecular weight component of normal serum which is associated with iso-agglutinin activity and is termed B_2M by Grabar and Williams (1955), on the basis of the position of its precipitin line in the serum immuno-electrophoretic pattern. Other synonyms include the term "Iota" protein used by Stanworth (1959) and the γ_1 macroglobulin described by Kunkel (1960). In this paper it will be constantly referred to as 19S γ; it is not suggested that it is identical with rheumatoid factor.

Zone Centrifugation

Fractionations by this procedure were achieved by centrifuging 1-ml. samples in buffered sucrose gradients in lusteroid tubes (5-ml. capacity) at 39,000 r.p.m. and 12·5° C. in a Spinco SW 39 rotor for 7 hrs. This is a modification of the method used by Kunkel and others (1959) in the fractionation of 19S serum proteins (Stanworth, James, and Squire, 1961). Spinco No. 40 angle rotors were also used on two occasions.

Analytical Ultracentrifugation

Analyses were carried out in 12-mm. cells in a Spinco Model E machine at 60,000 r.p.m. and 20° C. Samples (1-ml.) were pre-dialysed for 16 hrs against 1 L. barbitone buffer (pH 8·6; I = 0·05) containing 0·2 M NaCl, or phosphate buffer pH 6·9; M = 0·06 + 15 M NaCl.

Concentration of Protein Solutions

Details of the ultrafiltration and carbowax (polyethylene glycol) methods used are given in Table VI.

Quantitative Gel-diffusion Precipitin Analysis

Estimations were carried out by Dr. J. F. Soothill, using the technique developed by Gell (1957). Three specific antisera were used. One was against 7S γ globulin, and one against high molecular weight α_2 glycoprotein (α_2 macroglobulin of Kunkel). The one against 19S γ globulin was raised with a macroglobulin from the serum of a patient with macroglobulinaemia and was absorbed with 7S γ globulin and hypogammaglobulinaemic serum; this antiserum successfully measures 19S γ globulin. Further details of these antisera are reported by Soothill (to be published). The results for 7S γ are obtained as mg./100 ml. The results for the other two proteins were obtained as a percentage of the concentration in a standard serum from a healthy adult male. For calculation purposes, arbitrary conversion factors to mg./100 ml. were used. These are 100 per cent. α_2 = 100 mg./ml., and 100 per cent. 19S γ = 50 mg./ml.

Immuno-electrophoresis

Analyses were carried out in buffered agar according to the technique of Grabar and Williams (1955).

Results

The properties of the ten rheumatoid sera used for serial fractionation procedures are shown in Table I (opposite).

As already mentioned, the Rose-Waaler titres were variable, so that the specific activity also varied from 1·8 to 146 ml./mg. In Table I the compositions of these sera are also shown in terms of ultracentrifugal and immunological analyses; these are given mainly for comparison with the

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STUDIES ON THE ISOLATION OF RHEUMATOID FACTOR

BY

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The need for the isolation of rheumatoid factor in a high state of purity and in sufficient quantity for antiserum production has led to an appraisal of the various purification methods available.

Ziff, Brown, Lospalluto, Badin, and McEwen (1956) demonstrated that the sensitized sheep cell agglutinating activity of rheumatoid serum was precipitated in the euglobulin fraction, and Svartz and Schlossmann (1954) showed that this factor precipitated in the "cold globulin" fraction and were thus able to obtain a serologically active but heterogenous concentrate.

Later, both these groups of workers applied the cellulose ion exchange resins, diethylaminoethyl and carboyxmethyl cellulose, developed by Peterson and Sober (1956) to further fractionate euglobulin fractions (Lospalluto and Ziff, 1959; Svartz, Carlson, Schlossmann, and Ehrenberg, 1958).

Ultracentrifugal studies of rheumatoid sera and euglobulin fractions showed that the factor circulated as a high molecular weight component of sedimentation coefficient 19 or 22S and this property has been used by Kunkel, Franklin, and Muller-Eberhard (1959) and Heimer, Federico, and Freyberg (1958) to separate rheumatoid factor from lower molecular weight proteins. Most of these workers were able to obtain reasonably pure 19S globulin with a considerable concentration of serological activity. In these investigations, however, little attention was paid to immunochemical analyses of individual proteins, and the relative merits in each step in the concentration of rheumatoid factor were not determined.

In the investigations now reported, an attempt has been made to determine the most efficient procedure available for the isolation of rheumatoid factor in a relatively pure form. The recovery of rheumatoid factor activity has been measured after each procedure by the Rose-Waaler sensitized sheep cell technique, and the fractionation achieved has been assessed by total protein estimation, ultracentrifugal, immunochemical, and immuno-electrophoretic analyses. In this way it has been possible to make a critical comparison of the effectiveness and deficiencies of the different methods of purification. These methods included euglobulin precipitation to obtain a crude concentrate of rheumatoid factor, followed by diethylaminoethyl cellulose chromatography, zone centrifugation, and Cohn low-temperature ethanol fractionations in various orders and combinations.

Some of the difficulties encountered are discussed below; they include serious loss of serological activity as well as low degree of isolation in some protein preparations.

Materials and Methods

Rheumatoid Sera

Sera were obtained from ten patients with classical active rheumatoid disease, selected because they had the highest Rose-Waaler sensitized sheep cell agglutination titre of those available at the time; this titre ranged between 1/1,024 and 1/5,000 in half the patients; exceptionally a serum with a titre as low as 1/64 or as high as 1/12,600 had to be used. There were seven male and three female patients, aged 40 to 67 years, with a 4 to 32-year history of rheumatoid arthritis.

Estimation of Protein Concentration

The modification of the Folin-Ciocalteau method developed by Lowry, Rosebrough, Farr, and Randall (1951) was usually employed, using a bovine serum albumin standard.

Measurement of Rheumatoid Factor Activity

The rheumatoid factor was assayed by Ball's modification of the sensitized sheep cell agglutination test of Rose-Waaler (Ball, 1950), but using one-third of an

* In receipt of a Medical Research Council Studentship for training in research methods.

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SECTION D - HUMAN ALPHA GLOBULINS

A STUDY OF THE ALPHA$_2$-MACROGLOBULIN HOMOLOGUES OF VARIOUS SPECIES

BY

K. JAMES

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A Study of the Alpha$_2$-Macroglobulin Homologues of Various Species

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(*Received 2nd March* 1964)

Summary. The antigenic relationships between the α_2-macroglobulin homologues of a large number of species (predominantly mammalian) have been studied by the gel diffusion precipitin technique using a specific antiserum to human α_2-macroglobulin raised in a rabbit.

This antiserum has been absorbed with eleven different mammalian sera and the ability of the absorbed samples to cross-react with all these sera has again been tested.

From these results an attempt has been made to calculate the minimum number of antigenic determinants on each α_2-macroglobulin homologue recognized by this rabbit antiserum.

INTRODUCTION

The presence of α_2-globulins which cross-react with human serum α_2-macroglobulin has been conclusively demonstrated in the sera of several species by both gel diffusion (Roulet, Gugler, Rosin, Renaud and Hassig, 1960; Picard, Heremans and Vandebroek, 1963) and immunoelectrophoretic techniques (Scheiffarth, Gotz and Soergel, 1957; Williams and Wemyss, 1961; Picard, Heremans and Vandebroek, 1962a, b). In addition to these immunological similarities, practically all mammalian and many non-mammalian sera have been shown to exhibit a characteristic slow α_2 band on starch gel electrophoresis which is believed to be a human α_2-macroglobulin homologue (Smithies, 1959; Engle and Woods, 1960; Picard and Heremans, 1963).

However, apart from the work of Roulet *et al.* (1960) and Picard *et al.* (1963) no detailed studies of the cross-reactivity of the α_2-macroglobulin homologues have been performed using specific antisera. An attempt has therefore been made to confirm and extend these previous observations by studying a greater number of species and by investigating the cross-reactivity of the anti-human α_2-macroglobulin serum following its absorption with various mammalian sera.

MATERIALS AND METHODS

Sera

Most of the sera investigated are listed in Table 1. The other sera studied were rabbit, seal, rat, mouse, tortoise, duck and fowl. All samples were stored at $-20°$ before use.

Antiserum

The antiserum used was raised in a rabbit using as antigen an α_2-macroglobulin sample prepared by zone centrifugation (Müller-Eberhard, Kunkel and Franklin, 1956) of

Cohn Fraction III-0 (Oncley, Melin, Richert, Cameron and Gross, 1949). The immunization courses were similar to that described by Soothill (1962) and the antiserum obtained was rendered specific by absorption with Cohn Fraction II-1,2 (Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, 1946).

Gel Diffusion and Immunoelectrophoretic Analyses

Gel diffusion precipitin analyses were performed in 1 per cent (w/v) barbitone buffered agar (pH 8·6, I = 0·05) by the technique of Ouchterlony (1948), the plates being allowed to develop for 48 hours at room temperature.

RESULTS

STUDIES WITH UNABSORBED SPECIFIC ANTISERUM

Of the nineteen sera studied, eleven gave sufficiently intense precipitin lines on gel diffusion analysis, to allow detailed cross-reactivity studies, the results of which are displayed in Table 1. Many of these analyses were performed several times with the same

TABLE 1

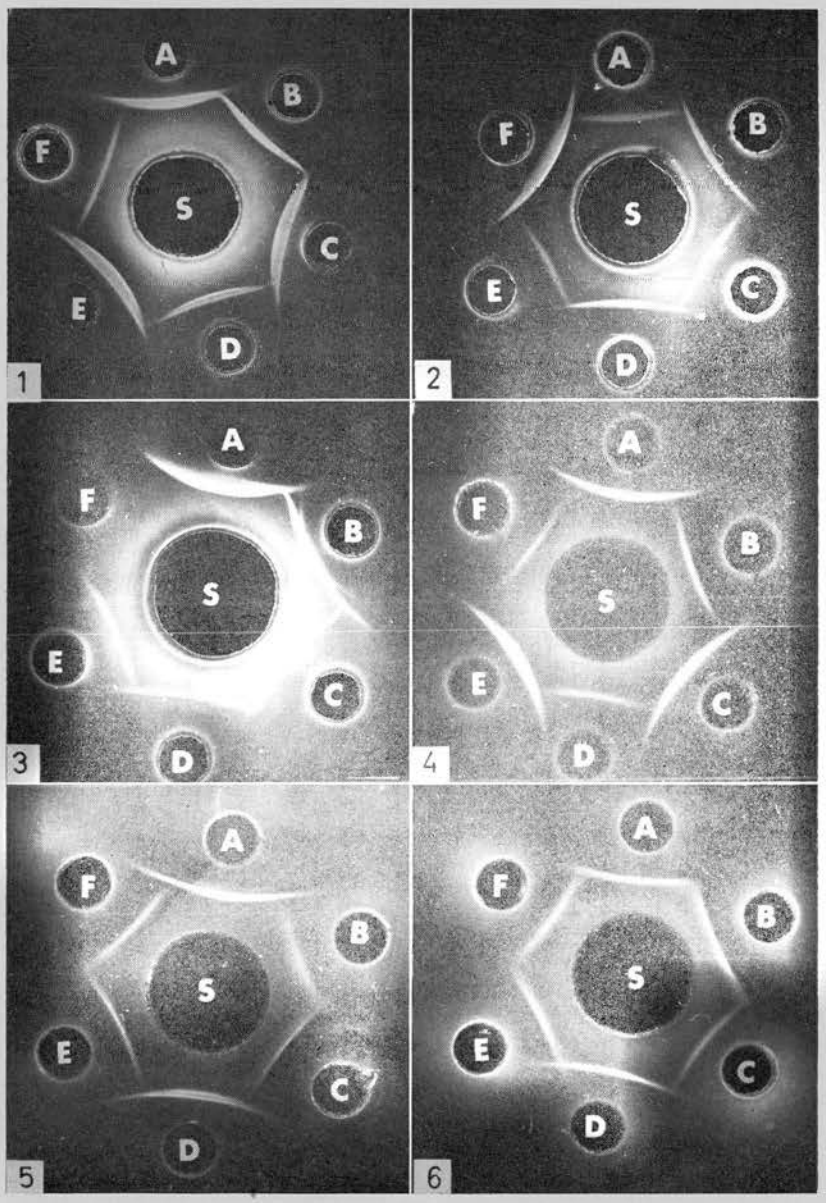
CROSS-REACTIVITY BETWEEN THE α_2-MACROGLOBULIN HOMOLOGUES OF VARIOUS SPECIES

Antiserum—rabbit anti-human α_2-macroglobulin.

Anti-human α_2-macroglobulin	Rhesus monkey	Ox	Sheep	Goat	Donkey	Zebra	Horse	Cat	Dog	Pig	Guinea-pig
Human											
Rhesus monkey											
Ox											
Sheep											
Goat											
Donkey											
Zebra											
Horse											
Cat											
Dog											
Pig											

antiserum and some of the reactions obtained are illustrated in Figs. 1–6. In addition, many of the analyses were repeated with a human α_2-macroglobulin antiserum raised in another rabbit, and the results obtained were similar to those displayed in Table 1.

As was to be expected the antigenic differences were most apparent at both ends of the phylogenetic scale, human and monkey α_2-macroglobulin exhibiting in this system greater complexity than their pig and guinea-pig homologues. The sera of intermediate species studies (see centre of Table 1) did not exhibit the marked mutual spur formation observed



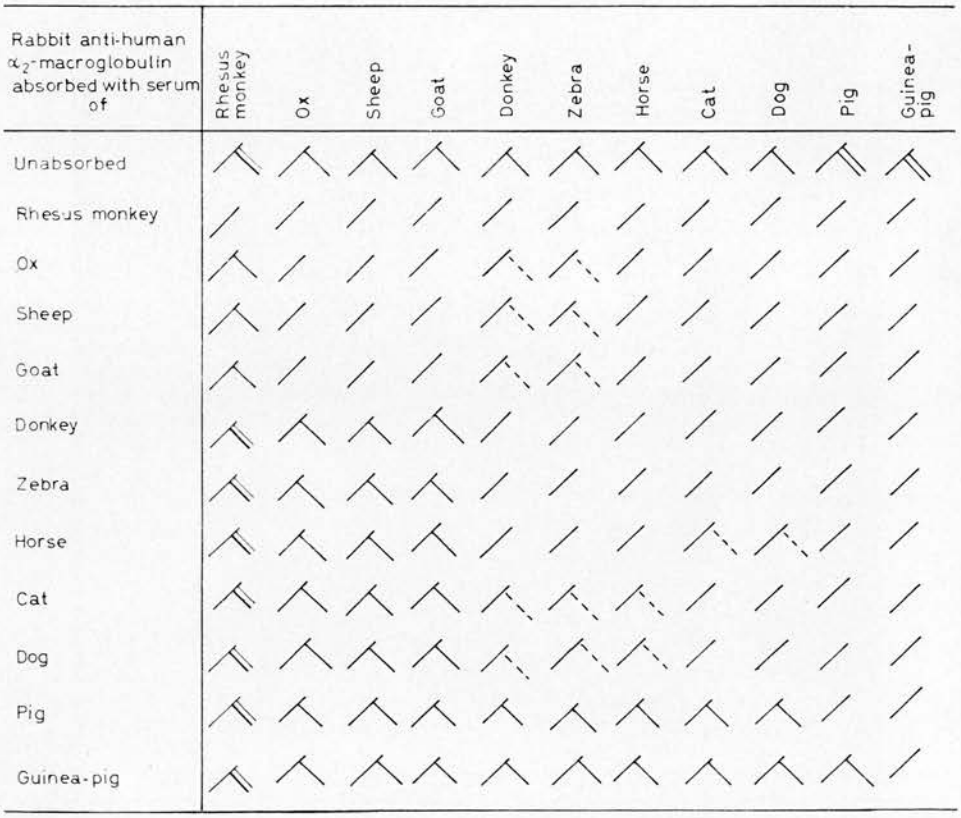
FIGS. 1–6. Gel diffusion precipitin analysis of various sera. Antiserum S—rabbit anti-human α_2-macroglobulin. Fig. 1. Sera: A, human; B, rhesus monkey; C, human; D, ox; E, human; F, horse. Fig. 2. Sera: A, horse; B, ox; C, horse; D, sheep; E, horse; F, rhesus monkey. Fig. 3. Sera: A, human; B, rhesus monkey; C, ox; D, sheep; E, horse; F, rabbit. Fig. 4. Sera: A, human; B, donkey; C, human; D, zebra; E, human; F, horse. Fig. 5. Sera: A, rhesus monkey; B, goat; C, donkey; D, rhesus monkey; E, zebra; F, sheep. Fig. 6. Sera: A, ox; B, goat; C, cat; D, ox; E, donkey; F, zebra.

between them and the α_2-macroglobulins of other more distantly related species. As the phylogenetic differences became less marked, the spurs obtained on gel diffusion precipitin analysis, decreased in size and finally disappeared (see sheep and goat, donkey and zebra, and also dog and cat).

Reactions of 'non identity' were observed between certain of the sera suggesting that the α_2-macroglobulin homologues of these sera possessed antigenic determinants not common to each other. Such pairs of sera included ox and donkey, goat and donkey, goat and zebra.

TABLE 2

THE CROSS-REACTIVITY OF ABSORBED ANTISERUM



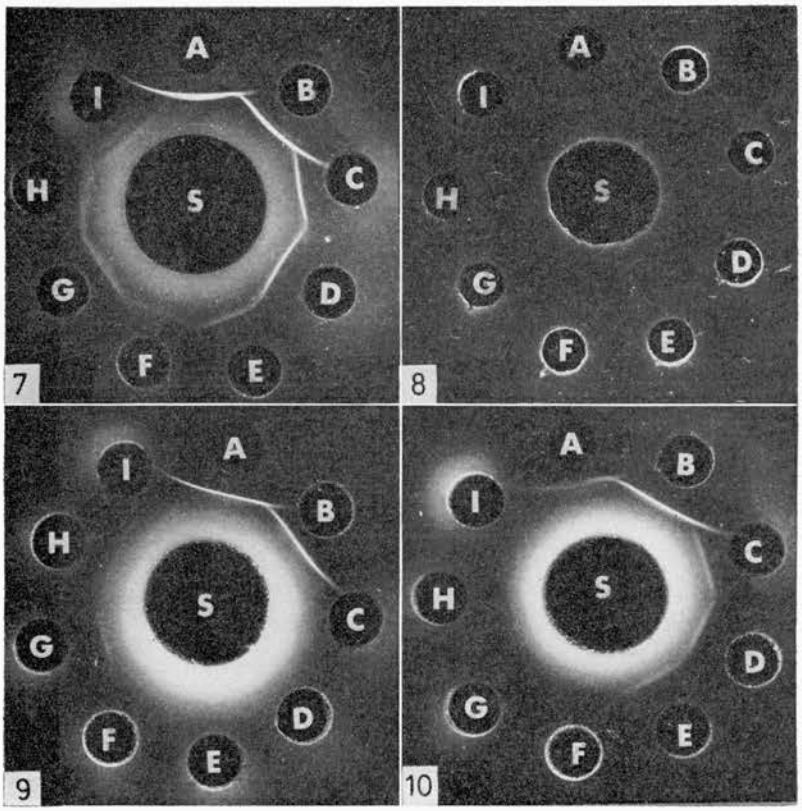
Absorptions performed at 37° for 24 hours with 2 volumes of heterologous serum. The reaction obtained with human serum is recorded on the left of each column. A dotted line indicates a weak reaction.

Double precipitin lines were frequently observed with a number of the sera (see monkey, Figs. 1B, 3B, 7B and 2F and pig, Fig. 7I, and horse) and the significance of this is being investigated. This phenomenon was not apparent in the homologous system.

With the sera of seal, rat and mouse, only weak precipitin lines were obtained, whilst no reactivity was demonstrable with the sera of tortoise, duck and fowl. However, the failure to obtain immunological cross-reactivity with these sera may reflect the limited discriminating power of this particular rabbit anti-human antiserum.

STUDIES WITH ANTI-HUMAN α_2-MACROGLOBULIN SERUM ABSORBED WITH VARIOUS MAMMALIAN SERA

Studies with absorbed antisera were performed in order to provide more precise information on the antigenic relationship between human α_2-macroglobulin and its homologues. The results obtained substantiated those observed using unabsorbed specific antisera, and are represented diagrammatically in Table 2, whilst some of the gel diffusion patterns obtained are shown in Figs. 7–10.



FIGS. 7–10. Cross absorption studies (see Table 2). Sera: A, human; B, rhesus monkey; C, ox; D, sheep; E, goat; F, horse; G, donkey; H, zebra; I, pig. Fig. 7. Antiserum S—rabbit anti-human α_2-macroglobulin. Fig. 8. Antiserum S absorbed with rhesus monkey serum. Fig. 9. Antiserum S absorbed with sheep serum. Fig. 10. Antiserum S absorbed with donkey serum.

Absorption of the antiserum with monkey serum removed its capacity to react with all the other species studied except human. On the other hand, absorption with guinea-pig prevented this serum alone from reacting with the absorbed antiserum. Thus the monkey α_2-macroglobulin homologue possessed, as would be expected, all the antigenic determinants which were demonstrable in the other sera, while guinea-pig sera lacked most of these.

These absorption studies illustrated once more the close antigenic similarities between the α_2-macroglobulin homologues of certain species: especially ox, sheep and goat; donkey, zebra and horse; cat and dog. Moreover, the results also indicated that although

the α_2-macroglobulin homologues of donkey and zebra share fewer determinants with human than do those of ox, sheep and goat, the former homologues do possess antigenic determinants which are absent from the sera of these latter species.

DISCUSSION

This investigation of the cross-reactivity of mammalian α_2-macroglobulins depends upon a detailed analysis of the anti-α_2-macroglobulin determinants in a single antiserum. When analyses were performed with another antiserum, similar results were obtained; however, the shortage of a number of the sera prevented complete characterization of this second antiserum.

These results emphasize once more the value of immunological studies in the determination of protein structure and in phylogenetic serology, for this approach is able to detect differences not apparent by the starch gel technique (Smithies, 1959; Engle and Woods, 1960; Picard and Heremans, 1963). Furthermore, it is apparent that there are certain structural sub-units which are common to all these cross-reacting sera; by applying such studies to enzymatically digested molecules it should be possible to determine the molecular location of cross-reacting antigenic sites. However, it must be emphasized that studies with a single antiserum have limited phylogenetic significance for it is highly probable that antisera raised in other species would reveal a different interspecies distribution of antigenic determinants.

The cross-reactivity studies with the absorbed antisera permit a detailed comparison of the antigenic properties of the various homologues. Using the unabsorbed antiserum, the ox homologue appears to be antigenically more complex than its sheep counterpart (see Fig. 3 C and D) but the immunological reactivity of the antisera absorbed in antigen excess with sera of these two species appeared to be comparable. Thus it would appear that although there are slight differences in antigenic structure these cannot readily be discerned by cross absorption studies. These studies also revealed that although the α_2-macroglobulins of ox, sheep and goat appeared antigenically more complex than their donkey and zebra counterparts, the latter possessed antigenic groupings absent from the more complex homologues.

On the basis of the results described above an attempt can be made to postulate the minimum number of antigenic determinants which are present in various α-globulin homologues and are recognized by the two rabbit antisera used in this study.

Such an analysis indicates that the following antigenic relationship probably exists:

Guinea-pig	A
Pig	AB
Dog	ABC
Cat	ABC
Horse	ABCD
Zebra	ABCD F
Donkey	ABCD F
Goat	ABCDE
Sheep	ABCDE
Ox	ABCDE
Monkey	ABCDEFG
Human	ABCDEFGH

Thus it would appear that there are at least eight major determinants on the human α_2-macroglobulin molecule—a similar number to that observed by Roulet *et al.* (1960). However, there were apparent differences in cross-reactivity, for the present studies, involving cross absorption studies, indicate that the α_2-macroglobulin homologues of the sheep and ox are antigenically more complex in the rabbit–anti-human system than those of horse, dog and cat.

Although limitations in this approach do exist the gel diffusion technique is still a valuable procedure for comparative biochemical studies. Further studies of a similar nature using a greater number of species and various antisera should provide additional valuable phylogenetic and structural data.

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Preliminary Notes

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Preliminary studies of the nature of the papain digestion products of human serum α_2-macroglobulin

Results of investigations of the reductive cleavage products of human serum α_2-macroglobulin employing urea starch gel[1] or ultracentrifugal[2-4] analyses suggested that the molecule comprises several sub-units linked together by disulphide (and possibly other) bonds.

In the preliminary studies described here, a similar degradation pattern of the human serum α_2-macroglobulin molecule was achieved by digestion with papain

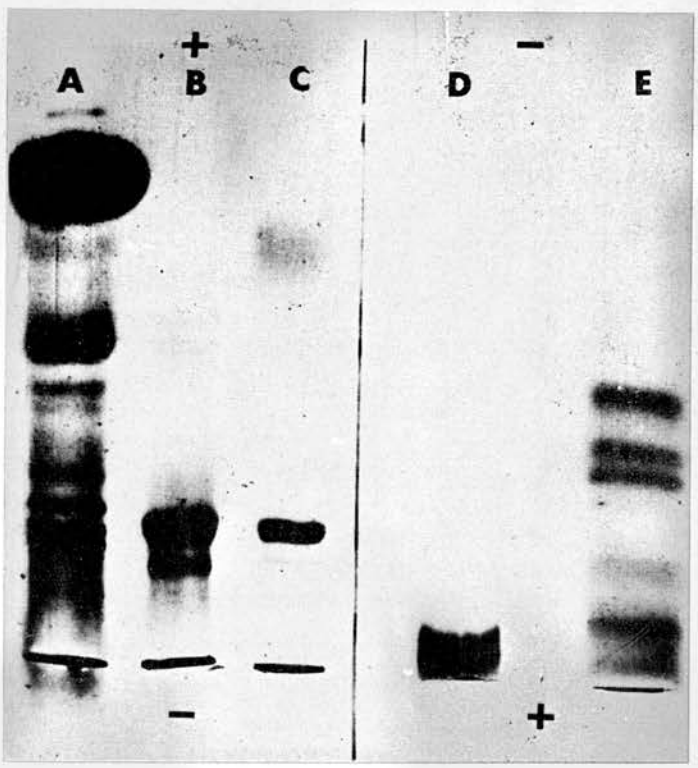

Fig. 1. Starch-gel electrophoresis of human serum α_2-macroglobulin after papain digestion. A, B and C. Horizontal starch-gel electrophoresis using the discontinuous buffer system of POULIK[6]. A, whole human serum; B, intact α_2-macroglobulin; C, papain-digested α_2-macroglobulin. D and E. Horizontal starch-gel electrophoresis in 8 M urea and formate buffer[1]. D, intact α_2-macroglobulin; E, papain-digested α_2-macroglobulin. In both cases electrophoresis was conducted at 20 V/cm for 6 h.

(EC 3.4.4.10) according to the method of PORTER[5] in which a reducing agent (cysteine hydrochloride) is used to activate the enzyme. The enzymic digestion products have been examined by electrophoretic, immunological and ultracentrifugal techniques and an attempt made to resolve them by fractionation on G-200 Sephadex.

Papain digestion was performed on a 2-g% solution of α_2-macroglobulin (obtained from Behringwerke A.G., Marburg/Lahn) which had been equilibrated with phosphate buffer (pH 6.9, 0.06 M) containing 0.15 M NaCl. Starch-gel electrophoresis[6] of the digest revealed the appearance of a band in the post albumin region (Fig. 1C), accompanied by the loss of most of the slower of the two initial bands (Fig. 1B) and a substantial portion of the major α_2 band (corresponding to the predominant 19-S γ peak observed in the ultracentrifugal analysis of the untreated α_2-macroglobulin). In contrast, urea starch-gel electrophoresis[1] demonstrated the presence of several new bands in the digest (Fig. 1E) as compared with two partially resolved bands in the initial α_2-macroglobulin preparation (Fig. 1D). This suggests a more extensive breakdown than that observed by POULIK[1] on reductive cleavage with 0.02 M mercaptoethanol in 8 M urea. The effect could not be achieved by the use of cysteine hydrochloride (0.025 M) alone.

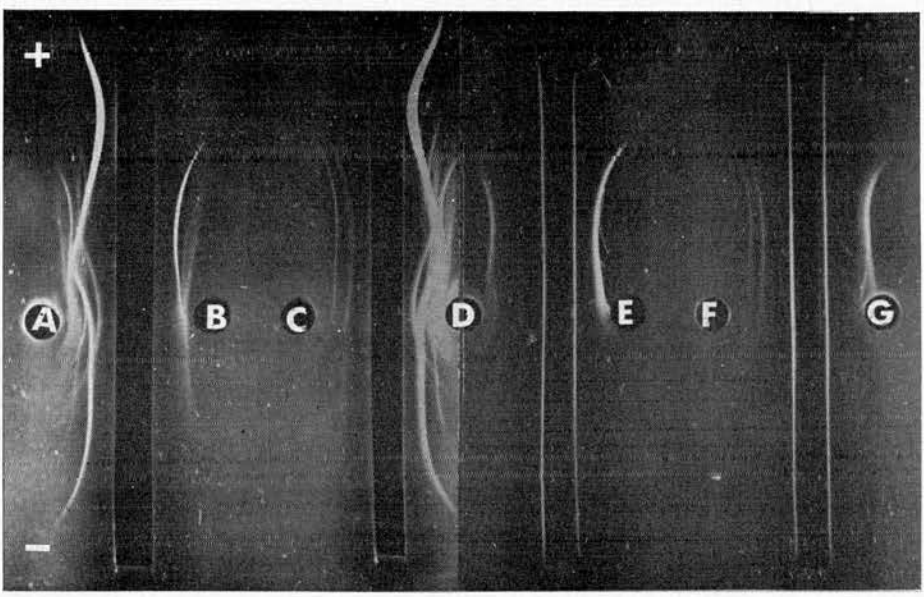

Fig. 2. Immunoelectrophoretic patterns of human serum α_2-macroglobulin after papain digestion. A, D and G, whole serum; B and E, intact α_2-macroglobulin; C and F, papain-digested α_2-macroglobulin. Samples A, B, C and D tested with rabbit anti-whole human serum, D, E, F and G tested with rabbit anti-human α_2-macroglobulin. Electrophoresis conducted at 20 V/cm for 4 h in barbitone buffer (pH 8.6; I 0.05).

Immunoelectrophoretic analysis (Fig. 2) using either a rabbit anti-whole human serum (Fig. 2, A–D), or a rabbit anti human α_2-macroglobulin (Fig. 2, D–G), antiserum demonstrated an additional component in the papain digest (Figs. 2C and 2F), of a similar electrophoretic mobility to that of the parent α_2-macroglobulin (Figs. 2B and 2E) but probably of a smaller molecular size. These observations were supported by

the results of ultracentrifugal analysis, 31% of a component with an $s_{20,w}$ value of 3.5 S being found in the α_2-macroglobulin digest.

Of greater interest, however, are the results of gel-diffusion precipitin analysis using a rabbit anti human α_2-macroglobulin antiserum, where three new antigenic components were detected in the papain-treated α_2-macroglobulin (Fig. 3B). Three additional components were also revealed by immunoelectrophoretic analysis of digests which had been subjected to several freezings and thawings. Although each of the three new components detected showed a reaction of only partial identity with untreated α_2-macroglobulin, one showed a reaction of complete identity with the α_2-macroglobulin of horse serum (see Fig. 3). Hence, it appears that one of the sub-units produced by papain digestion of human α_2-macroglobulin carries antigenic determinants responsible for its cross-reactivity with horse α_2-macroglobulin.

The isolation and characterization of this cross-reacting sub-unit is now being undertaken. Initial gel-filtration studies using G-200 Sephadex have resulted in the separation of a fraction, comprising the various sub-units, from the undegraded α_2-macroglobulin. This composite low-molecular-weight fraction was found to possess more than six times the hexose content of the parent glycoprotein molecule, suggesting

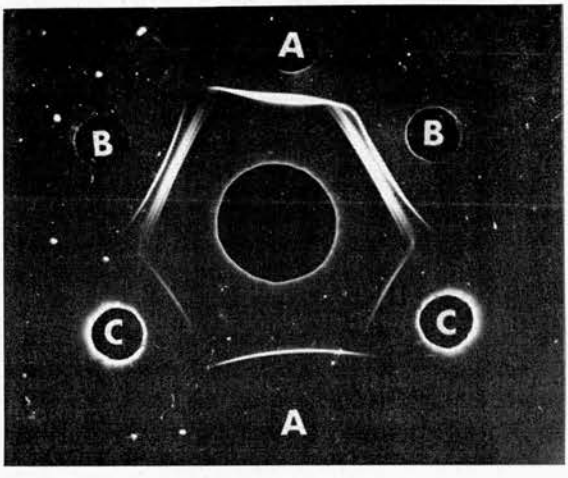

Fig. 3. Gel-diffusion precipitin analyses. A, intact human serum α_2-macroglobulin; B, papain-digested human serum α_2-macroglobulin; C, horse serum. Antiserum: rabbit anti-human α_2-macroglobulin. Diffusion in 0.8% agar in barbitone buffer (pH 8.6; I 0.05).

that carbohydrate-rich fragments connected with immunological specificity are split off by papain digestion.

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THE PAPAIN DIGESTION OF HUMAN SERUM α_2-MACROGLOBULIN

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(Received May 28th, 1964)

SUMMARY

Human α_2-macroglobulin has been digested with papain (EC 3.4.4.10) at pH values of 5.7 and below to give an immunologically detectable, low-molecular-weight component ($s_{20,w}$ approx. 3 S). The digestion process also results in the liberation of a considerable amount of peptide material.

Digestion appears to proceed by a series of components of intermediate starch-gel electrophoretic mobility, the final component moving in the post-albumin position.

Gel diffusion studies reveal that the product is deficient in primate specific antigenic groupings, but carries the antigenic determinants responsible for its cross reactivity with the α_2-macroglobulin homologues of other mammalian species.

INTRODUCTION

The ease with which the 19-S γ-globulin (I_{gM}) component of human serum can be reductively dissociated into sub-units immunologically distinguishable from the parent molecule, is well established[1,2]. However, with the other major 19-S component of human serum, the α_2-macroglobulin, few detailed dissociation studies have been reported. SCHONENBERGER *et al.*[3] were able to dissociate this protein with 5 M urea into two components with $s_{20,w}$ values of 11 and 15.7 S. Reaggregation to a single component occurred on the removal of the urea. Moreover, reduction of the 19-S α_2-macroglobulin to a 6-S component was obtained by ISLIKER[4] using either cysteine, cysteamine or thioglycollate.

The results of these preliminary investigations of α_2-macroglobulin dissociation were substantiated by the work of POULIK[5], who used a urea–starch-gel electrophoretic technique to study the products of reductive cleavage of human α_2-macroglobulin. From these investigations Poulik concluded that α_2-macroglobulin contained sub-units linked, at least in part, by disulphide bonds. However, he stressed that in the light of the observations of SCHONENBERGER *et al.*[3] other forms of linkage must not be excluded.

In the investigations to be described here, an enzymological procedure has been used to cleave the molecule and some of the factors influencing this process have been studied by electrophoretic, immunological and ultracentrifugal techniques. As a result of these studies, procedures have been developed which enabled the complete degradation of the α_2-macroglobulin to a low-molecular-weight (3-S) component which has been characterized by the above techniques. In addition the immunological cross-

reactivity of this product, with the α_2-macroglobulins of other species, has been investigated to determine if there were any structural similarities between these proteins. This investigation is a detailed account of previously reported results[6].

MATERIALS AND METHODS

The human α_2-macroglobulin* was prepared by zone-centrifugation, in a sucrose density gradient, of Cohn's Fraction III-0 (ref. 7). This sample was shown by quantitative gel-diffusion and ultracentrifugal methods to contain less than 2 % contaminating protein (mainly 7-S γ-globulin). A small amount of aggregated α_2-macroglobulin (4 %), detected by ultracentrifugation, was also revealed by "trailing" in starch gel.

The α_2-macroglobulin antiserum used was raised in a rabbit, using the laboratory-prepared protein as antigen. The immunization course was similar to that described by SOOTHILL[9], the antiserum being rendered specific by absorption with Cohn II-1,2 (ref. 10) which contains 7-S γ-globulin.

Immunoelectrophoresis was performed by a technique essentially similar to that described by GRABAR AND WILLIAMS[11] using 1 % (w/v) barbitone-buffered agar (pH 8.6, $I = 0.05$). Gel-diffusion precipitin analyses were also performed in the above agar.

Electrophoresis in starch-gel was performed using the discontinuous buffer system described by POULIK[12]. Analysis in urea–starch-gel (8 M urea in formate buffer, final pH 3.6–3.8) was also carried out using a technique previously described by this author[5].

Analytical ultracentrifugation was performed in 12-mm cells in a Spinco Model E ultracentrifuge at 59780 rev./min and 20°. The samples were dissolved in 0.06 M phosphate buffer containing 0.15 M NaCl, the pH varying according to the experiment. The relative compositions of the protein solutions were determined by measuring the areas under the peaks, by the method of counting squares, after the "fitting" of the appropriate solvent base line.

Enzyme digestion procedures

General procedure: All the enzyme digestions described were performed on 1.5–2.0 g % (w/v) solutions of α_2-macroglobulin in phosphate-buffered saline (pH 6.9, 0.06 M containing 0.15 M NaCl) using 1/50 of the substrate weight of twice crystallized, undialyzed papain (EC 3.4.4.10) (Light and Co. Ltd., Colnbrook (Great Britain)). The solutions also contained EDTA (2 mM) and varying concentrations of cysteine hydrochloride (see later). Following incubation at 37° for 24 h the reactions were terminated by the addition of 0.005 M *p*-chloromercuribenzoate in 0.1 M glycine. (Final concentration of *p*-chloromercuribenzoate, 0.001 M.)

Procedure used in the preparation of the low-molecular-weight component: In previously reported experiments[6] the 3-S component, produced on the partial degradation of α_2-macroglobulin with papain, was resolved from the accompanying high-molecular-weight protein (intact α_2-macroglobulin) by gel-filtration on Sephadex G-200. However, by the use of the modified digestion procedures described below

* In the preliminary report[6] the α_2-macroglobulin investigated was obtained from Behringwerke A.G., Marburg/Lahn.

the complete breakdown of the α_2-macroglobulin to a 3-S component has been achieved; thus no further fractionation procedures (other than dialysis) were required.

Two procedures have been successfully employed; they were developed from the data obtained in experiments studying the effect of pH and cysteine concentration on the papain digestion of α_2-macroglobulin. The first method involves the papain digestion of α_2-macroglobulin at pH 4.3, and 37° for 48 h in the presence of 0.01 M cysteine hydrochloride. The second, alternative procedure, involves the papain digestion of α_2-macroglobulin at pH 5.4 and 37° for 24 h followed by the addition of more papain and a further digestion of 24 h. This reaction was performed in the presence of 0.01 M cysteine hydrochloride. In both procedures the α_2-macroglobulin was dissolved in phosphate-buffered saline (pH 6.9, 0.06 M containing 0.15 M NaCl) containing EDTA (2 mM) to give 1.5–2.0 g % (w/v) solutions which were adjusted to the required pH (4.3 or 5.4) with 0.1 N HCl. Following pH adjustment the papain (1/50th of the substrate weight) was added and the samples incubated as described above.

RESULTS

The effect of pH

In this series of experiments the cysteine hydrochloride concentration was 0.02 M. The protein samples were adjusted to pH values between 5.3 and 8.0 with 0.1 N NaOH or 0.1 N HCl prior to digestion. Immunoelectrophoretic and gel diffusion analyses (illustrated in Figs. 1 and 2) indicate that the α_2-macroglobulin was degraded between pH 5.3 and 5.7 into two immunologically reacting components (see Figs. 1 and 2, Samples B, C and D).

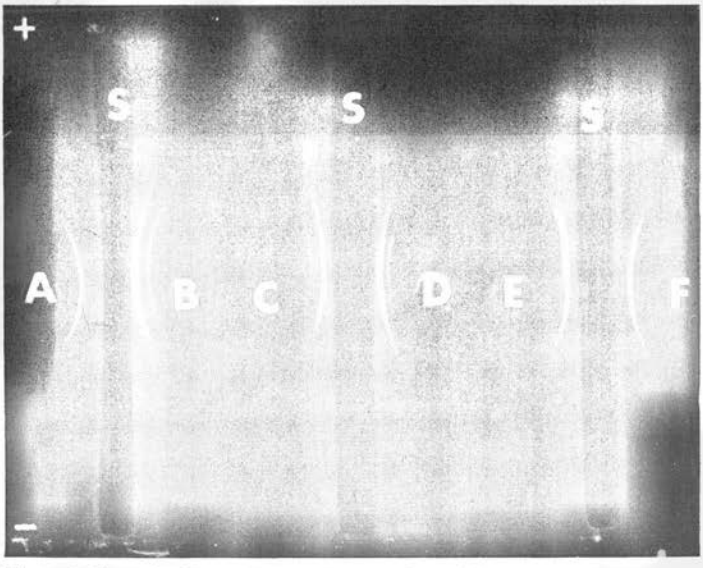

Fig. 1. Effect of pH on the papain digestion of human serum α_2-macroglobulin: Immunoelectrophoretic analyses. A, Intact human α_2-macroglobulin; B, α_2-macroglobulin digested at pH 5.3; C, pH 5.6; D, pH 5.8; E, pH 6.3; F, pH 8.0. All samples contained cysteine (0.02 M). Antiserum S = rabbit anti-human α_2-macroglobulin. Electrophoresis conducted at 20 V/cm for 4 h in barbitone buffer (pH 8.6, I = 0.05).

At higher pH values (pH 6.3, Figs. 1 and 2, Sample E) a certain amount of degradation was observed, whereas at pH 8.0 no visible change appeared to have occurred (Figs. 1 and 2, Sample F).

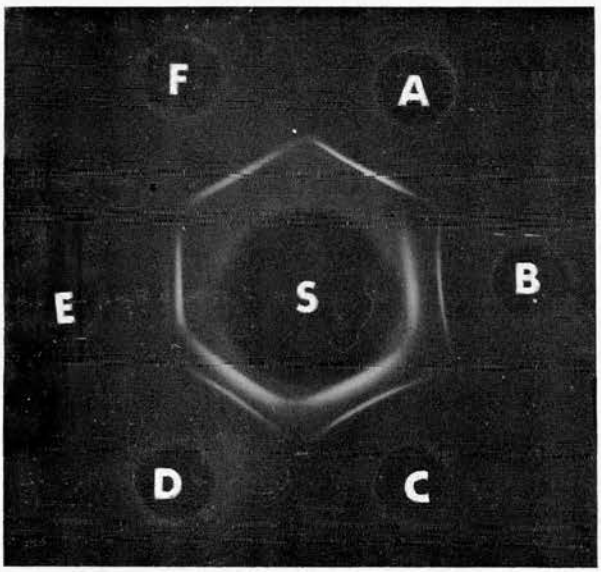

Fig. 2. Effect of pH on the papain digestion of human serum α_2-macroglobulin: gel-diffusion-precipitin analyses. A, Intact human α_2-macroglobulin; B, α_2-macroglobulin digested at pH 5.3; C, pH 5.6; D, pH 5.8; E, pH 6.3; F, pH 8.0. All samples contained cysteine (0.02 M). Antiserum S = rabbit anti-human α_2-macroglobulin. Diffusion in 1.0% agar in barbitone buffer (pH 8.6; I = 0.05).

In samples where degradation was most marked (Samples B and C, Figs. 1 and 2), two components were also observed on starch-gel electrophoresis (Samples B and C, Fig. 3) the new component moving as a post albumin (see also Figs. 7 and 8 later). However, both starch-gel and urea–starch-gel indicated that a certain amount of degradation had occurred in all the samples (see Figs. 3 and 4), and suggested that the degradation may proceed through a series of components of intermediate electrophoretic mobility.

Undegraded α_2-macroglobulin could be detected in all samples by immunological and starch-gel procedures.

The effect of cysteine

Cysteine is generally used in the papain digestion of 7-S γ-globulin[13] and has been shown to increase the yields of low-molecular-weight products during this digestion process[14]. Therefore, experiments were performed with varying concentrations of cysteine hydrochloride (0.005–0.03 M) to study its effect on the papain digestion of the α_2-macroglobulin. The digestions were performed at pH 5.4, as α_2-macroglobulin was readily degraded at this pH. Controls included α_2-macroglobulin samples incubated with papain alone, cysteine alone (0.01 and 0.03 M) and without either of these reagents.

The various analytical procedures used indicated that a proportion of the

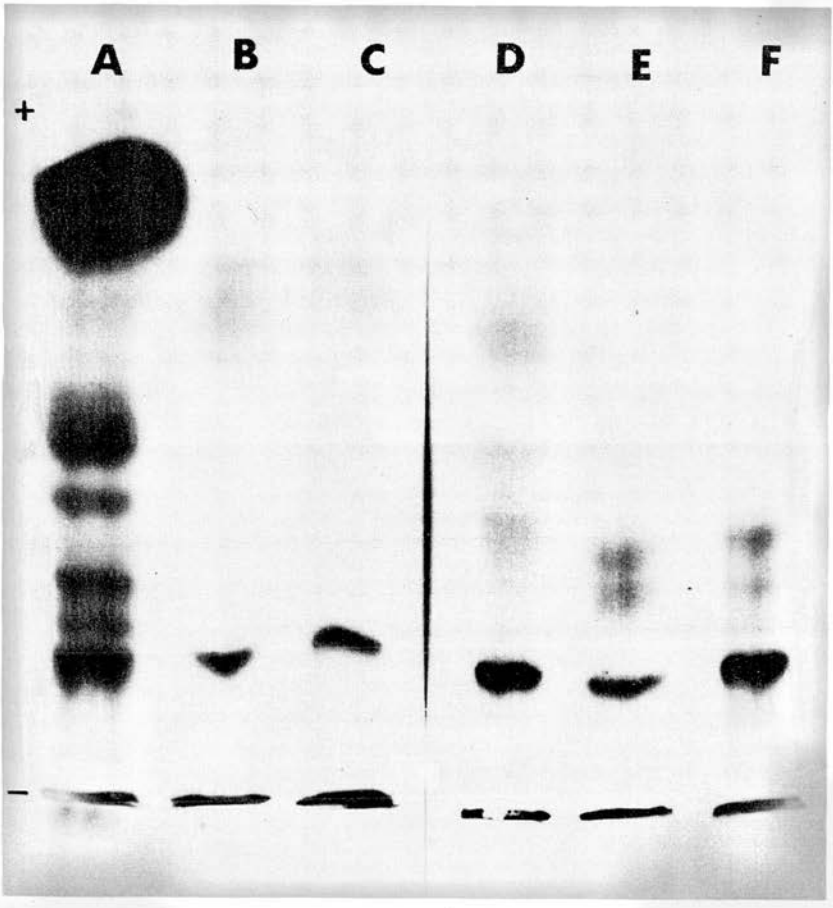

Fig. 3. Effect of pH on the papain digestion of human serum α_2-macroglobulin: Starch-gel analyses. A, Whole serum; B, human α_2-macroglobulin digested at pH 5.3; C, pH 5.6; D, pH 5.8; E, pH 6.3; F, pH 8.0. Electrophoresis conducted at 20 V/cm for 6 h.

α_2-macroglobulin molecules could be enzymically degraded to the immunologically detectable post albumin component in the absence of cysteine at pH 5.4 (see Figs. 5 H and 6 I and Table I).

However, ultracentrifugal analysis indicated that the degradation was not as complete as that observed in the presence of cysteine (compare Samples A and C, Table I) and this observation was confirmed by the results of urea–starch-gel electrophoresis. In the absence of papain no significant changes were observed.

Protein determinations on the samples analyzed in the ultracentrifuge indicated that approx. 40–50 % of the starting material was dialyzable following papain digestion.

The characterization of the low-molecular-weight component

The digestion procedures used in the complete breakdown of the original α_2-macroglobulin molecule also resulted in the production of a considerable amount

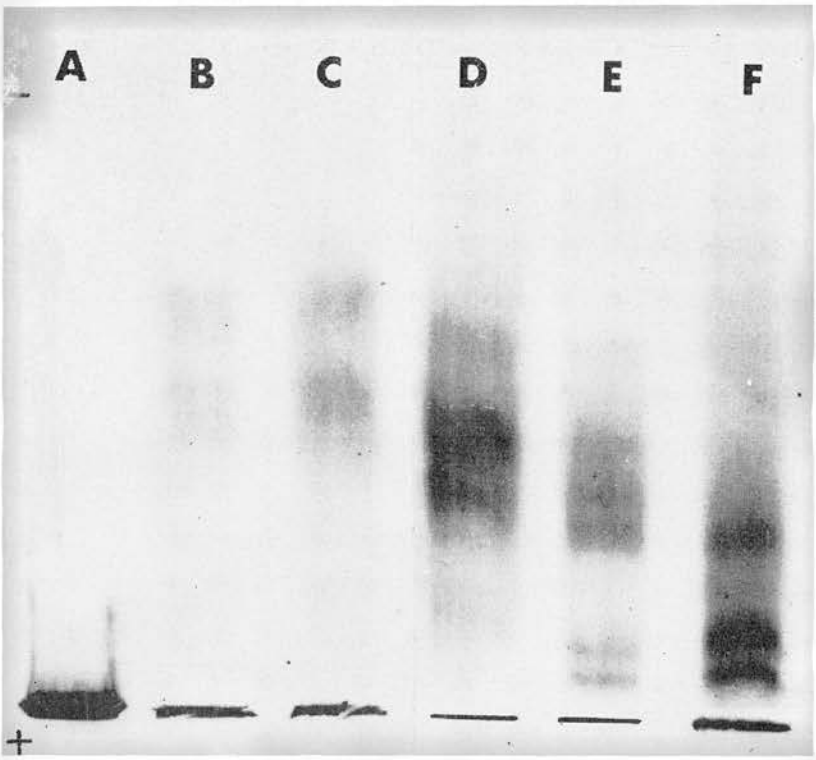

Fig. 4. Effect of pH on the papain digestion of human serum α_2-macroglobulin: Urea–starch-gel-analyses. A, Intact human α_2-macroglobulin; B, human α_2-macroglobulin digested at pH 5.3; C, pH 5.6; D, pH 5.8; E, pH 6.3; F, pH 8.0. Electrophoresis conducted at 20 V/cm for 6 h.

of dialyzable material (approx. 50 % by weight). The product obtained was shown by starch-gel electrophoresis to correspond to the post albumin component observed in partially digested preparations (see Fig. 7).

Furthermore, immunological procedures indicated that this 3-S component with the post albumin mobility was also responsible for the immunological cross reactivity observed in partially digested samples (see Fig. 8).

A comparison by gel diffusion precipitin techniques with the α_2-macroglobulins of a variety of species using a rabbit anti-human α_2-macroglobulin serum indicated that this product was antigenically deficient in comparison to the intact human and rhesus-monkey α_2-macroglobulins. However, the low-molecular-weight material showed very similar cross reactivity to the α_2-macroglobulin homologues of goat, sheep, donkey, horse, cat and dog (see Fig. 9).

These observations were confirmed by serial absorptions of the rabbit anti-human α_2-macroglobulin serum with the digest, when reactivity with human and monkey serum alone was retained (see Fig. 10).

However, it must be added that on absorbing the antiserum with large amounts of the low-molecular-weight product (20 mg per ml of antiserum, that is five times that used in the experiments illustrated in Fig. 10) only weak precipitin lines were obtained with the human and monkey α_2-macroglobulin.

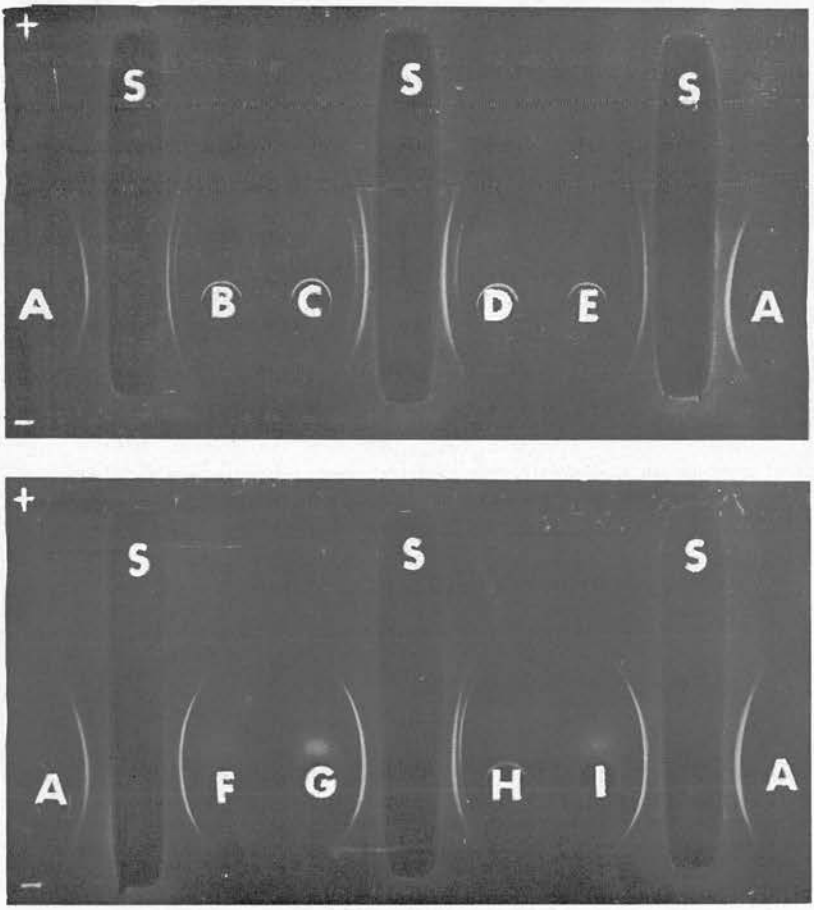

Fig. 5. The effect of cysteine on the papain digestion of human serum α_2-macroglobulin: Immunoelectrophoretic analyses. A, Intact human α_2-macroglobulin. B, C, D and E, α_2-macroglobulin samples containing papain and following concentrations of cysteine: B, 0.005 M; C, 0.01 M; D, 0.02 M; E, 0.03 M. F and G, α_2-macroglobulin samples containing no papain but the following concentrations of cysteine: F, 0.01 M; G, 0.03 M. H, α_2-macroglobulin sample with papain alone. I, α_2-macroglobulin sample with no additions. The pH of Samples B–I was adjusted to 5.4 prior to incubation Electrophoresis conducted at 20 V/cm for 6 h. Antiserum S = rabbit anti-human α_2-macroglobulin.

It should perhaps be stressed that all the immunological changes reported in this study were also observed with a number of specific rabbit anti-human α_2-macroglobulin sera and with rabbit antisera to whole human serum.

DISCUSSION

The results indicate that human serum α_2-macroglobulin is most readily degraded by papain when incubated at pH values below 6. They fail, however, to conclusively establish the role of cysteine during the papain digestion of this serum protein. Thus, although the enzyme appeared to be active in the absence of papain, it is conceivable that the undialyzed preparation used contained trace amounts

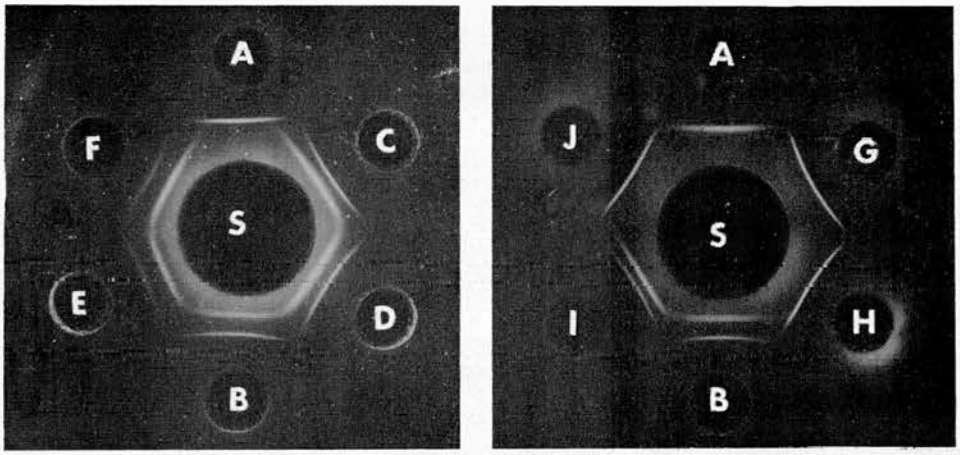

Fig. 6. The effect of cysteine on the papain digestion of human serum α_2-macroglobulin: Gel-diffusion precipitin analyses. A, Intact human α_2-macroglobulin. B, a papain digested α_2-macroglobulin sample as a reference. C, D, E and F, α_2-macroglobulin samples containing papain and the following concentrations of cysteine: C, 0.005 M; D, 0.01 M; E, 0.02 M; F, 0.03 M. G and H, α_2-macroglobulin samples containing no papain but the following concentrations of cysteine: G, 0.01 M; H, 0.03 M. I, α_2-macroglobulin sample with papain alone. J, α_2-macroglobulin sample with no additions. The pH of Samples C–J was adjusted to 5.4 prior to incubation. Antiserum S = rabbit anti-human α_2-macroglobulin. Diffusion in 1.0% agar in barbitone buffer (pH 8.6; $I = 0.05$).

TABLE I

THE EFFECT OF CYSTEINE AND PAPAIN ON HUMAN α_2-MACROGLOBULIN

Relative composition (% of total) refers to the material detectable on ultracentrifugal analysis. For immunological analysis of the samples see Fig. 6, Samples D, G, I and J, respectively. All the samples were equilibrated to pH 5.4.

Sample	*α_2-Macroglobulin solution containing*	*Ultracentrifugal components*	$s_{20,w}$	*Relative composition (% of total)*
A	Papain and cysteine (0.01 M)	1	3.1	50
		2	16.8	50
B	Cysteine (0.01 M)	1	6.7	3
		2	17.7	94
		3	25.6	3
C	Papain	1	2.9	28
		2	16.9	72
D	No additions	1	6.5	2
		2	17.4	94
		3	24.4	4

of cysteine which were sufficient to catalyze the reaction. The increased yields of low-molecular-weight products (compare Samples A and C, Table I) obtained in the presence of cysteine suggest that the role of this reducing agent during the papain digestion of α_2-macroglobulin may be analogous to that it exhibits during the papain digestion of 7-S γ-globulin[14]*.

* *Note added in proof.* Recent digestion experiments performed using iodoacetamide (0.02 M) to block exposed sulphydryl groups, indicate that the papain digestion of α_2-macroglobulin can only be achieved in the presence of reducing agent (Received March 22nd, 1965).

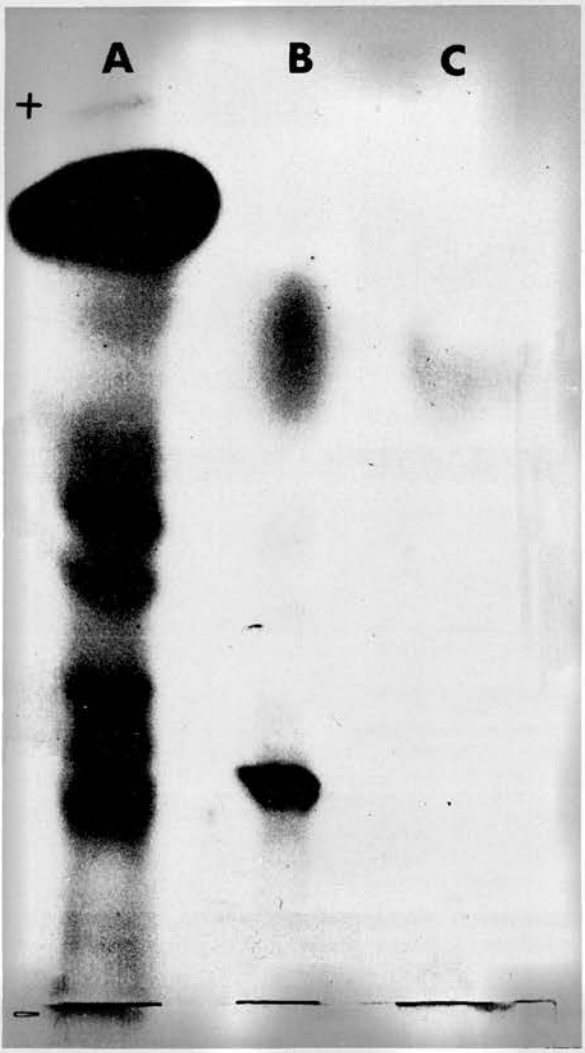

Fig. 7. Starch-gel electrophoresis of the low-molecular-weight component produced by the papain digestion of human serum α_2-macroglobulin. A, Normal human serum; B, partially degraded human α_2-macroglobulin; C, low-molecular-weight component. Electrophoresis conducted at 20 V/cm for 6 h.

The results in Table I also indicate that 0.01 M cysteine had alone no appreciable effect upon α_2-macroglobulin, but greater concentrations of this reducing agent (0.1 M) are able to degrade the molecule[4,15]. However, the products obtained by reduction do not exhibit multiple precipitin lines on immunological analysis[15]. Thus, as previously concluded[5], it would appear that α_2-macroglobulin does contain sub-units linked at least in part by disulphide bonds.

Perhaps the most significant finding resulting from this investigation is that by enzymological procedures one can obtain protein sub-units smaller in size than those produced by chemical methods[3,4]. Furthermore, these products still possess

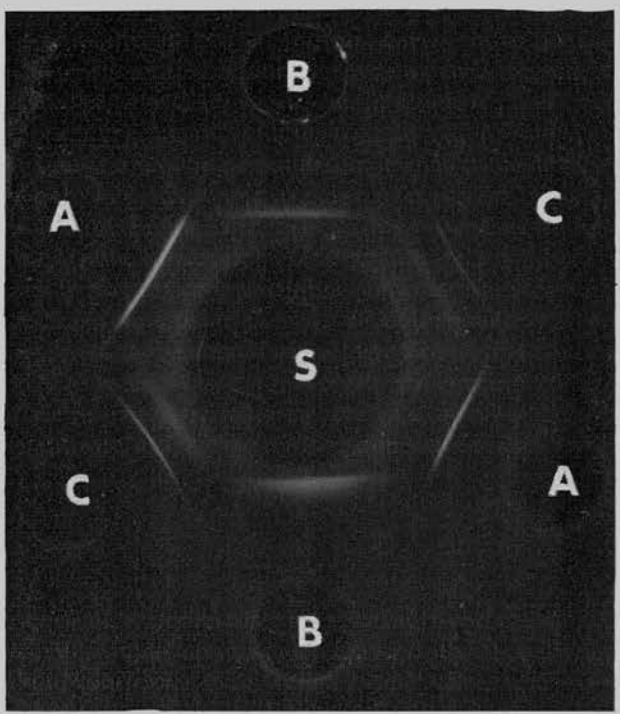

Fig. 8. Gel-diffusion analysis of the low-molecular-weight component produced by the papain digestion of human serum α_2-macroglobulin. A, Intact human serum α_2-macroglobulin; B, low-molecular-weight component of digest; C, partially digested human serum α_2-macroglobulin. Antiserum S = rabbit anti-human α_2-maçroglobulin. Diffusion in 1.0% agar in barbitone buffer (pH 8.6; $I = 0.05$).

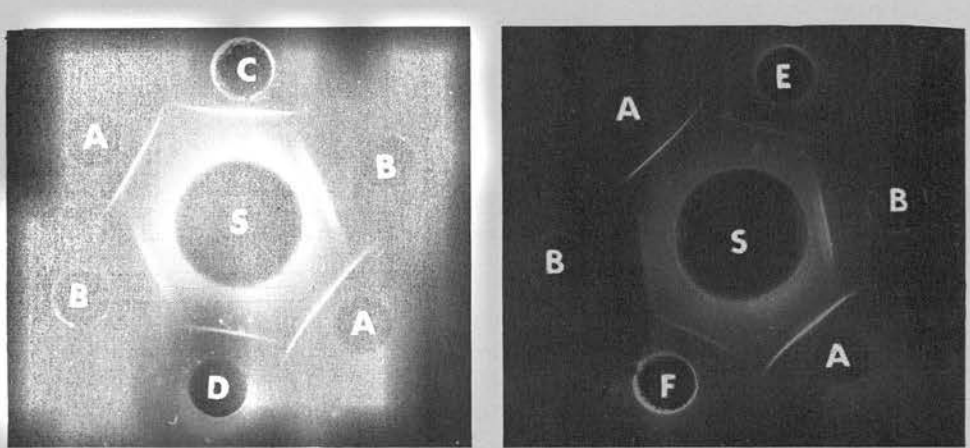

Fig. 9. An immunological comparison of the low-molecular-weight component produced by the papain digestion of human serum α_2-macroglobulin and the α_2-macroglobulin homologues of various species. A, Intact human serum α_2-macroglobulin; B, low-molecular-weight component of digest; C, monkey serum; D, ox serum; E, goat serum; F, sheep serum. Antiserum S = rabbit anti-human serum α_2-macroglobulin. Diffusion in 1.0% agar in barbitone buffer (pH 8.6; $I = 0.05$).

immunological activity and appear to bear a closer antigenic relationship to the α_2-macroglobulins of lower species than did the parent molecule.

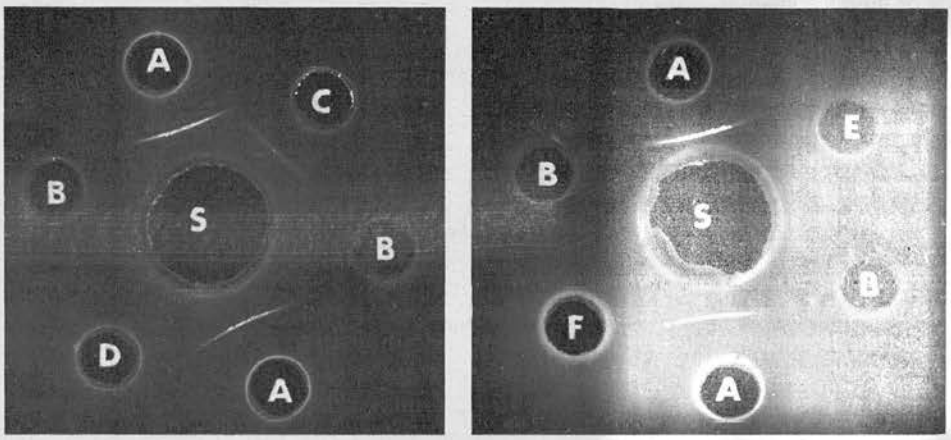

Fig. 10. The immunological cross reactivity of rabbit anti-human α_2-macroglobulin serum after absorption with the low-molecular-weight product of papain digestion of human serum α_2-macroglobulin. A, Intact-human serum α_2-macroglobulin; B, low-molecular-weight component of digest; C, monkey serum; D, ox serum; E, goat serum; F, sheep serum. Antiserum S = absorbed antiserum. Diffusion in 1.0% agar in barbitone buffer (pH 8.6; $I = 0.05$). Compare Fig. 9 with unabsorbed antiserum.

The initial experiments suggested that papain probably selectively cleaved off from the original molecule a low-molecular-weight sub-unit which possessed immunological cross reactivity[6]. It was supposed that this moiety was attached to the parent molecule by a particularly labile bond. However, the more detailed studies reported here suggest that the action of papain is to degrade the entire molecule to this post-albumin component with the liberation of a considerable amount of low-molecular-weight peptide material. Furthermore, the products obtained in this study did not possess the high carbohydrate content initially observed[6]. As a result of the digestion process, the major portion, if not all, of the primate-specific antigenic groupings of the original α_2-macroglobulin molecule were destroyed. However, considering the results of absorption of the α_2-macroglobulin antiserum with excess of the digest product another possible explanation exists, namely that all the original antigenic determinants are present in the fragments but in different relative proportions. Experiments have also to be performed to see if in the digestion process new antigenic groupings have been exposed.

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THE QUANTITATIVE ESTIMATION OF α_2-MACROGLOBULIN IN NORMAL, PATHOLOGICAL AND CORD SERA*

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(Received December 2nd, 1965)

SUMMARY

The α_2-macroglobulin content of normal, pathological and cord sera was estimated by an antibody-agar radial diffusion procedure (employing α_2-macroglobulin standards) which gives highly reproducible results (100 $\pm$ 2.2%).

A significant difference ($P < 0.05$) was observed between the serum α_2-macroglobulin levels of normal Caucasian men (265 $\pm$ 55 mg/100 ml) and women (335 $\pm$ 57 mg/100 ml). In men, the serum concentration of α_2-macroglobulin was greatly increased in ataxia telangiectasia, atopic dermatitis and mongolism and was significantly elevated in pulmonary disorders, diabetes mellitus and agammaglobulinemia. Values were significantly decreased in multiple myeloma and slightly decreased in rheumatoid arthritis. In women, serum concentrations were markedly increased in ataxia telangiectasia, atopic dermatitis and mongolism and were significantly decreased in severe rheumatoid arthritis, macroglobulinemia and multiple myeloma. No significant differences were observed in the limited number of sera from women with pulmonary disorders or agammaglobulinemia.

The α_2-macroglobulin levels of cord sera were elevated (387 $\pm$ 58 mg/100 ml), whereas samples of maternal sera obtained at parturition had normal levels (335 $\pm$ 57 mg/100 ml).

Although α_2-macroglobulin is one of the major components of the complex group of proteins classified as the α-globulins, until relatively recently little was known about its function. Recent *in vitro* studies, however, have indicated that in addition to its probable role as a transport protein[1] for molecules such as human growth hormone[2], and possibly insulin[3], the protein may be involved in a variety of enzyme mechanisms. Such interactions include inhibition of the proteolytic activity

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of trypsin[4] and plasmin[5] and protection of the esterase activity of these enzymes from inhibition by known tryptic inhibitors, *e.g.*, soybean[4,6]. These interactions may be of importance *in vivo*.

The present study was designed to gain further information on the role of α_2-macroglobulin in normal and pathological states. For this purpose we determined the α_2-macroglobulin levels of the sera of normal subjects and patients with chronic diseases. The determinations were performed by a radial diffusion technique recently developed by Mancini *et al.*[7], which has been used successfully for the quantitative measurement of the immune globulins[8]. Several immunological procedures have been used previously to estimate serum α_2-macroglobulin levels, but the studies have not been extensive. These procedures include the turbidimetric assay of antigen–antibody precipitates obtained with a specific antiserum[9,10] and procedures involving diffusion of test sample and specific antiserum in agar plates[11,12]. The radial diffusion technique of Mancini *et al.*[7] makes use of agar plates with specific antibody incorporated into the agar. The test sample is placed in a small well cut in the agar and allowed to diffuse for a predetermined period. The diameter of the antigen–antibody precipitate ring that forms is measured, and the antigen concentration is determined by comparison with the diameter of the rings obtained with known amounts of a purified standard. The technique is simple to perform, gives highly reproducible results, and is readily applicable to the analysis of a large number of samples of small volume.

MATERIALS AND METHODS

Serum samples from 55 normal adult Caucasian subjects (33 men and 22 women) and from a total of 180 male and female patients with a variety of chronic diseases were analysed. Determinations were also performed on cord sera, on paired samples of cord and maternal sera and in 4 cases on samples of sera from infant and both parents. When not tested immediately after collection, the sera were stored at −20° until used.

The α_2-macroglobulin standard used in the studies was prepared by repeated zone centrifugation of Cohn fraction III-0 in sucrose density gradient, followed by starch block electrophoresis to remove trace amounts of γ-globulin contaminants. The final product gave a single line on immunoelectrophoretic and gel diffusion analysis when tested with an antiserum to whole human serum. The antigen was standardized both by the micro-Kjeldahl procedure, as described by Kabat and Mayer[13], and the Folin phenol procedure[14]. From these values the $E_{280\ m\mu}$ was found to be 12.8 for a 1% solution of α_2-macroglobulin.

Antisera to human α_2-macroglobulin were produced in rabbits by injection of the product obtained by repeated centrifugation of Cohn fraction III-0. The antisera were made specific for α_2-macroglobulin by adsorption with Cohn fraction II-1, 2 or with the low molecular weight fraction obtained by zone centrifugation of Cohn fraction III-0. Sodium azide (final concentration 0.1%, w/v) was added as a preservative and the antisera were stored at −20°.

The diffusion plates were prepared as follows. A 2.5% solution (w/v) of Noble agar (Difco, Detroit) in borate–saline buffer (18.6 g boric acid, 2.4 g sodium hydroxide and 9.0 g sodium chloride per liter), pH 8.2, was prepared and transferred to a 56° water bath. An antiserum, which had been diluted with the borate buffer and

equilibrated to 56°, was added to give a final agar concentration of 2%. The antibody–agar mixture was then pipetted between two glass slides of a pre-assembled diffusion unit. Each unit consisted of two glass projection slides (8.3 × 10.2 cm), one of which had been siliconized (Siliclad, Clay Adams, New York) and dried. A U-shaped aluminum spacer (0.13 cm thick) was inserted between the siliconized and non-siliconized slides as a separator; the whole assembly was then clamped with binder clips along the three sides bounded by the metal frame. The slides were warmed to 37°, and the antibody–agar mixture was inserted between them with a prewarmed (37°) pipette. (The warming procedure prevented the formation of air bubbles in the agar.) After the agar had hardened, the clips, siliconized slide and metal frame were removed. Rows of small wells were cut in the agar plate with a tubular cutter (2 mm in diameter) (Fig. 1). Standard antigen, in five different concentrations ranging from 45 to 360 mg/100 ml, and test samples (0.004 ml volume) were placed in the wells with a microsyringe (Hamilton Microliter syringe No. 701, Hamilton Co., Whittier, Calif.). The plate was placed in a petri dish, and the antigen was allowed to diffuse into the agar for about 30 min. The plate was then covered with glycerol to avoid evaporation and incubated at 37° for 2 days. The diameters of the precipitin rings that formed after 2 days (Fig. 1) were measured in two directions at right angles using a Bausch and Lomb eyepiece with incorporated magnifier scale. Sera which gave very large rings

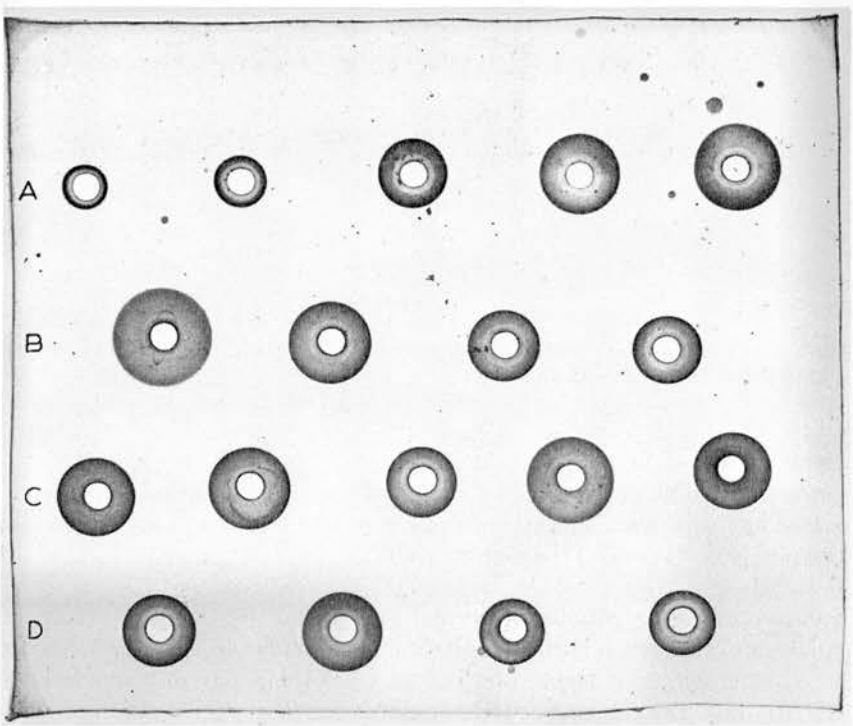

Fig. 1. Antibody–agar plate used in radial diffusion procedure after incubation for 48 h at 37°, showing arrangement of antigen wells and the precipitin rings formed. Wells in row A contain standard antigen in concentrations ranging from 45 to 360 mg %; each well in rows B, C, and D contains a different test serum.

(indicating α_2-macroglobulin concentration in excess of 400 mg/100 ml) were diluted with borate saline buffer and re-examined.

The Student t test was used for statistical evaluation of the data. Values of $P<0.05$ were taken as significant.

RESULTS

Preliminary experiments were performed to determine the optimal conditions for the diffusion procedure. Standard curves were obtained with antigen concentrations ranging from 45 to 360 mg of α_2-macroglobulin/100 ml and with 10- and 20-fold dilutions of antisera. With two antisera tested, larger and better-defined precipitin rings were obtained with the 20-fold dilutions (Fig. 2). The effect of incubation time on the development of the precipitin rings is illustrated in Fig. 3. As shown, no ap preciable change occurred over a 1- to 8-day period.

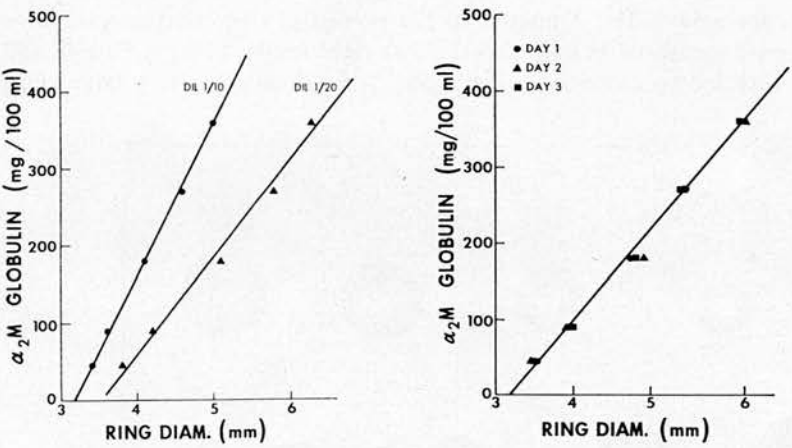

Fig. 2. Effect of antiserum dilution on diameter of precipitin rings in radial diffusion procedure. Plates read after incubation for 96 h at 37°.

Fig. 3. Effect of time of incubation at 37° on diameter of precipitin rings in radial diffusion procedure.

Therefore, a 2-day incubation period was used routinely in subsequent experiments. Repeated analysis of a single serum using a 20-fold dilution of antiserum and a 2-day incubation period revealed the high reproducibility of the procedure (standard deviation $\pm$ 2.2%).

The values obtained in studies of normal and pathological sera are recorded in Table I. Analysis of the sera of normal adult Caucasians showed a marked difference between the α_2-macroglobulin levels of men (265 $\pm$ 55 mg/100 ml) and women (335 $\pm$ 57 mg/100 ml). On the basis of this observation the values obtained with pathological sera were compared with the values obtained with sera from normal subjects of the same sex. Corresponding changes were not always noted in both sexes in patients with the same disease. This discrepancy, however, may only reflect the small number of sera in some groups (*e.g.*, women with pulmonary emphysema, dia-

betes and multiple myeloma), and a detailed analysis of a larger number of samples might reveal a different picture.

Nevertheless, marked increases in the serum α_2-macroglobulin content were observed in men with ataxia telangiectasia, atopic dermatitis and mongolism, and significant but less marked elevations were found in pulmonary emphysema and other pulmonary disorders (mainly asthma), diabetes mellitus and agammaglobulinemia. The levels of this protein were slightly decreased in men with rheumatoid arthritis and significantly decreased in multiple myeloma, but were normal in male patients with Waldenström's macroglobulinemia.

TABLE I

α_2-MACROGLOBULIN LEVELS IN NORMAL AND PATHOLOGICAL SERA

Group	*Male*			*Female*		
	No. of subjects	*α_2-Macroglobulin (mg/100 ml)*		*No. of subjects*	*α_2-Macroglobulin (mg/100 ml)*	
		*Concentration**	*Range*		*Concentration**	*Range*
Normal	33	265 ± 55	150–370	22	335 ± 57	265–460
Pulmonary emphysema	13	294 ± 56	185–390	3	325 ± 15	310–340
Other pulmonary disorders	8	286 ± 69	220–430	11	335 ± 75	190–420
Diabetes mellitus	18	305 ± 88	165–500	2	377 ± 103	305–450
Atopic dermatitis	13	430 ± 103	240–500	20	400 ± 66	285–500
Ataxia telangiectasia	7	420 ± 110	250–510	7	490 ± 26	435–520
Mongolism	8	438 ± 55	345–495	7	414 ± 72	280–485
Rheumatoid arthritis	15	240 ± 51	160–310	12	260 ± 54	215–400
Agammaglobulinemia	6	337 ± 81	260–430	4	336 ± 64	258–395
Macroglobulinemia	8	266 ± 79	165–390	6	268 ± 83	165–385
Multiple myeloma	10	195 ± 54	115–280	2	225 ± 85	165–285

* Results expressed as mean of values obtained by a single estimation on each sample in group ± the standard deviation of the mean.

TABLE II

α_2-MACROGLOBULIN LEVELS IN CORD, MATERNAL AND PATERNAL SERA

Source of sera	*No. of subjects*	*α_2-Macroglobulin (mg/100 ml)*	
		*Concentration**	*Range*
Normal men	33	265 ± 55	150–370
Normal women	22	335 ± 57	265–460
Cord	65	387 ± 58	270–510
Infant–mother	27 each		
Cord		375 ± 55	270–500
Maternal		330 ± 58	240–430
Infant–parent	4 each		
Cord		395 ± 71	350–500
Maternal		325 ± 84	240–405
Paternal		306 ± 52	240–350
Infants	6		
At birth		384 ± 64	305–440
5 days after birth, breast-fed		392 ± 60	305–445

* Results expressed as mean of values obtained by a single estimation on each sample in group ± standard deviation of the mean.

Increased α_2-macroglobulin levels were observed less frequently in the smaller number of sera from the female patients, although the higher initial concentration of this protein in the sera of normal women must be taken into account. As in the case of the male groups, however, the most pronounced increases were found in ataxia telangiectasia, atopic dermatitis and mongolism, whereas decreases were observed in rheumatoid arthritis and multiple myeloma. In contrast to the male subjects, decreased α_2-macroblobulin levels were also observed in macroglobulinemia, whereas no significant changes were found in pulmonary disorders or agammaglobulinemia.

The results of analyses on cord, maternal and paternal sera are recorded in Table II. The levels of α_2-macroglobulin were significantly greater in cord sera than in the sera of either parent or of normal men and women. The α_2-macroglobulin content of the maternal serum exceeded that of the corresponding cord sample in only 8 of the 27 infant–mother pairs studied. The α_2-macroglobulin levels of the maternal samples (obtained at parturition) were similar to the serum levels of normal women. No increases in this protein were observed in sera obtained from 6 infants after 5 days of breast feeding.

DISCUSSION

The mean variation and range of α_2-macroglobulin levels in the normal sera studied were similar to those reported by previous workers[9,11]. The reproducibility was of the same order as that obtained by the turbidimetric procedure of Schultze and Schwick[9] and that determined with other antigens in the radial diffusion procedure[7,8].

Addition of the α_2-macroglobulin levels in normal men obtained in this study and the γ_M-globulin values obtained by Fahey and McKelvey[8] in normal adult males indicates that the total 19 S macroglobulin (α_{2M}- plus γ_M-globulin) content of serum is of the order of 385 mg/100 ml (approximately 5% of the total protein). This value is appreciably higher than those obtained by the analytical ultracentrifugal procedure (approximately 2 to 3% of the total protein). This observation indicates a considerable underestimation by the ultracentrifugal procedure, which probably results from the inherent difficulties in accurately determining the relative composition of serum (especially the minor 19 S component) by measurement of the areas under the peaks after fitting the appropriate solvent base line. The higher values obtained with the radial diffusion method cannot be explained by the presence in normal serum of low molecular weight proteins with similar antigenic determinants to α_2-macroglobulin, since immunological investigation of G-200 Sephadex fractions of whole serum failed to detect any such proteins.

One can only speculate on the cause of the observed differences in the serum α_2-macroglobulin content in men and women. It may be a reflection of basic differences in metabolism, for the α_2-macroglobulin molecule is most probably involved in a number of enzyme processes[4,6]. In addition, the protein may play an important role in the transport of hormones such as insulin[3], human growth hormone[2], and possibly of the steroid hormones, since another α-globulin reversibly binds cortisol and other steroid hormones[15,16].

Elevated α_2-macroglobulin levels have been observed previously in maternal sera (before parturition) and cord sera[1,10], and might also be related to basic differ-

ences in enzyme and hormonal (especially adrenal) activity during pregnancy and fetal development. In this respect the reversible binding of human growth hormone, and possibly of steroid hormones, may be of importance, for significant changes in cortisol-binding α-globulin (not α_2-macroglobulin) have been noted during pregnancy[15]. Despite the increased concentrations of this protein in maternal blood, the concentration in the fetal circulation is relatively low. Such a differential in the levels of the binding protein probably results in the rapid transfer of cortisol from the amniotic fluid, and presumably from the fetus, to the mother[16]. Thus, should α_2-macroglobulin specifically and reversibly absorb hormones and other biologically important molecules, increased levels of this protein in the fetus might assist in the transfer of such molecules from the maternal and placental circulation. Furthermore, α_2-macroglobulin may have growth-promoting properties similar to those of fetuin, the high molecular weight α-globulin found in high concentrations in fetal calf serum[17].

The origin of fetal α_2-macroglobulin is also of theoretical interest in relation to the phenomenon of placental transfer. Franklin and Kunkel[18], in ultracentrifugal studies, detected α_2-macroglobulin in fetal serum, although 19 S (γ_M) macroglobulin was almost entirely absent. The absence of γ_M-globulin has been attributed to the failure of the fetus to synthesize significant amounts of this protein and to the lack of transfer of γ_M-globulin across the placental barrier from the maternal circulation. Thus, it would appear that either (*a*) the fetus is able to synthesize α_2-macroglobulin, (*b*) considerable quantities are synthesized by the placenta, or (*c*) the fetus is able selectively to concentrate α_2-macroglobulin of maternal or placental origin.

The significant increase in the α_2-macroglobulin content of the sera of agammaglobulinemic men has been noted previously[9]. The decreased serum content generally found in the hypergammaglobulinemias (macroglobulinemia and multiple myeloma) may result from the gross imbalance in protein synthesis in these conditions.

Previous reports have indicated that the α_2-macroglobulin levels[12] are usually increased in rheumatoid arthritis. The present studies, however, suggest that α_2-macroglobulin levels are reduced in this condition. In an earlier study on a limited number of arthritic sera, the levels were found to be essentially normal[19], but the present study was restricted to patients with rheumatoid arthritis whose sera contained high levels of rheumatoid factor (latex titers >10000) and of γ_M-globulin.

The clinical and biochemical significance of the increase in α_2-macroglobulin (and other α-globulins) in diabetes mellitus is not known. The increased levels may be a manifestation of insulin-binding properties, which have been attributed to this protein[3]. Nevertheless, should α_2-macroglobulin bind insulin, it could, as in the case of protein-binding of steroids[15,16], (*a*) influence the amount of hormone available for metabolic processes, (*b*) affect the amount available for destruction and hence half-life, and (*c*) modify the activity of the hormone, for in the case of cortisol the protein-bound molecule is inactive[15]. Such interactions may be of extreme importance both in the etiology and therapy of diabetes mellitus.

The significance of the increases in the serum α_2-macroglobulin content in pulmonary emphysema is obscure. This increase may merely represent a nonspecific rise in α_2-macroglobulin due to the chronic infections associated with this disease state similar to the rises in haptoglobin and orosomucoid levels characteristic of chronic disease. The markedly increased values in mongolism (trisomy syndrome) are of interest. This finding might suggest the possible direct involvement of chromo-

some 21 in the regulation of α_2-macroglobulin levels, but such a conclusion is not warranted on the basis of the present data.

Since the fundamental defect responsible for ataxia telangiectasia is unknown, the significance of the increase in serum α_2-macroglobulin content in patients with this disorder cannot be evaluated at present. Whether a relationship exists between the high α_2-macroglobulin concentrations and the low γ_A-globulin levels[20], the defects in delayed hypersensitivity and the telangiectasic and neurologic symptoms in these patients is only conjectural but clearly merits further investigation.

Although only a limited number of pathological sera have been investigated to date, the results indicate considerable variation in the α_2-macroglobulin levels in different pathological conditions, and further investigations should provide data of diagnostic and therapeutic value.

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TRYPSIN STABILIZERS IN HUMAN SERUM. THE ROLE OF α_2-MACROGLOBULIN*

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SUMMARY

The protein in normal serum that combines with trypsin and protects its esterase activity, has been investigated by gel filtration, agar electrophoresis, and immunological procedures, and has been confirmed to be α_2-macroglobulin.

Comparative studies indicated that the isolated α_2-macroglobulin molecule will combine with trypsin in a similar ratio (mole/mole) as the α_2-macroglobulin in whole serum. However, the isolated protein enzyme complex is much more readily inhibited by soybean trypsin inhibitor.

INTRODUCTION

Although α_2-macroglobulin is present in normal human serum in considerable concentrations (200–300 mg/100 ml), little is known about the function of this protein. However, recent investigations indicate that α_2-macroglobulin may be involved in many enzyme mechanisms. For example, this protein can combine with trypsin thus protecting the esterase activity of the enzyme from complete inactivation by trypsin inhibitors (α_1-antitrypsin) in serum and soybean trypsin inhibitor[1–3]; the α_2-macroglobulin can also inhibit both trypsin[1–4] and plasmin[3,4]. Because of its plasmin-inhibitory properties, the α_2-macroglobulin may be important in the complex fibrinolytic mechanism.

Recent reports of hereditary deficiency of serum trypsin inhibitors[5,6], and the increased incidence of certain forms of pulmonary emphysema in such subjects, coupled with the observation of other trypsin inhibitors in serum[7,8] stressed the need for the further characterization of both of the proteins involved and of the nature of the complexes.

The present studies were designed to confirm the presence of macroglobulin trypsin-protectors in whole serum and to demonstrate by sensitive immunological procedures that the protein involved was indeed α_2-macroglobulin[2,3]. Furthermore,

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we have demonstrated that isolated α_2-macroglobulin (although it did bind trypsin) failed to protect the esterolytic activity of trypsin[2,3] as efficiently as the cruder fractions obtained by G 200-Sephadex fractionation of fresh serum.

MATERIALS AND METHODS

Enzymological procedures

The tryptic (esterase) activity of samples was determined by the method of Hummel[9] in which the rate of hydrolysis of *p*-toluenesulfanyl-L-arginine methylester (TAME) is measured by the increase in absorbancy at 247 mμ. One-tenth ml of test solution, 0.3 ml of substrate (0.01 *M* TAME in distilled water), and 2.6 ml of buffer (0.05 *M* Tris buffer containing 0.01 *M* calcium chloride) were pipetted into a cuvette. The appropriate blank contained an additional 0.1 ml of buffer instead of the test sample. The contents of each cuvette were mixed and the absorbancy change recorded for 3 min. Test samples were diluted to give a linear relationship between change in absorbancy and time, so observing zero order kinetics. One unit of tryptic activity was defined as equal to the hydrolysis of one μmole of TAME per minute at 25° and pH 8.1 in the presence of 0.01 *M* calcium ion and was calculated as follows.

$$\text{Units} = \Delta \frac{\text{Absorbance 247 m}\mu\text{/min}}{0.18}$$

where 0.18 equals the increase in absorbancy at 247 mμ resulting from the hydrolysis of one micromolecule of TAME*.

Antitryptic activity was determined by incubating the test solution (30 min at 37°) with a known amount of twice-crystallized trypsin (Worthington Biochemicals) and measuring the residual activity as above.

The soybean trypsin inhibitor used (SBTI) was a three-times crystallized preparation obtained from Worthington Biochemicals.

Fractionation procedures

Sephadex gel filtration was performed at 0° as described by Flodin and Killander[10], using G 200-Sephadex columns (80 × 4.5 cm) equilibrated with 0.05 *M* Tris buffer, pH 8.1, containing 0.01 *M* calcium chloride; this procedure provided an efficient means of separating the two serum α-globulins that are known to interact with trypsin, namely α_2-macroglobulin (molecular weight 820 000, ref. 11) and α-antitrypsin (molecular weight 54 000 ref. 4).

Human α_2-macroglobulin was prepared by repeated zone centrifugation of Cohn fraction III-0 (ref. 12) in a buffered (phosphate pH 6.9, 0.06 *M* containing 0.15 *M* sodium chloride) sucrose density gradient. The final product exhibited only one band on immunoelectrophoresis using an antiserum to whole human serum.

Electrophoretic procedures

Agar-gel electrophoresis was carried out by the method of Wieme[13]; micro-immunoelectrophoresis was performed by the method of Scheidegger[14]. The relative

* The enzyme technique referred to is basically that presented in the catalogue of Worthington Biochemical Corporation, Freehold, N.J.

distribution of tryptic activity in electrophoretic fractions was determined by eluting segments of the gel with a saturated aqueous solution of benzoyl-L-arginine-paranitroanilide (BAPNA)[1,7] for 20 h at 25° with frequent agitation. The extent of hydrolysis of the BAPNA was determined by measuring the increase in absorbance at 383 mμ.

Concentration of proteins

Samples were concentrated by ultrafiltration through 8/32 inch dialysis tubing (at 0°). However, the protein which trailed behind the third peak on G 200-Sephadex filtration (Fig. 3B) was lyophilized.

Immunological studies

The identity of the protein in serum that reacted with trypsin to form a stable complex was further investigated by incubating aliquots of serum containing trypsin, or G 200-fractions of the same, with rabbit antisera specific for one or another human serum proteins. Specific antisera to each of the three immunoglobulins (γG, γA and γM, ref. 16), to lipoproteins (α_1, α_2 and β), and to α_2-macroglobulin were used because all of these proteins may be present in the first peak obtained on the G 200-Sephadex fractionation of serum. The mixtures were incubated at 37° for 4 h, then stored overnight at 0°. The supernatants were examined by the gel diffusion precipitin procedure[17] to determine if the immunoprecipitation of the antigen tested was complete. The tryptic activity of each supernatant was then determined and compared with that obtained in control mixtures containing Tris buffer or normal rabbit serum rather than antiserum.

With certain of the antisera, antigen-antibody precipitin curves were obtained (see Fig. 5). The protein in the precipitate was quantitated by a modified Folin–phenol procedure[15] after washing the precipitate three times in cold physiological saline (0°).

Storage of samples

The G 200-Sephadex fractionations were performed on serum samples drawn just before fractionation and usually took 2 days. The G 200-Sephadex fractions were stored from 1 to 2 days at 0° before determining enzyme activity; the concentrated fractions were refrigerated at −20° for up to one week before analysis. The concentrated α_2-macroglobulin preparations were stored at −20°. In the other studies, the serum (or plasma) samples used had been stored at −20° for 1–4 weeks without any measurable change in enzyme activity.

RESULTS

The effect of adding increased amounts of trypsin to serum or plasma—the demonstration of trypsin stabilizers in whole serum

The enzyme activity of trypsin and whole serum or plasma after incubation at 37° for 30 min is recorded in Fig. 1. The tryptic activity of the samples increased sharply when 100 μg/ml of trypsin was added, remained fairly constant on increasing the trypsin content to approx. 1 mg/ml, and decreased sharply when more trypsin was added. Thus it would appear that normal human serum and plasma (presumably

serum α_2-macroglobulin) possess the capacity to protect about 6 units (50 μg) of tryptic activity from serum trypsin inhibitor and that this property is destroyed if excessive amounts of trypsin are added to the samples.

The addition of trypsin to the samples caused alterations in the Wieme electrophoretic pattern. The observed changes illustrated in Fig. 2 included a frequent splitting in the α_2-band on the addition of small amounts of trypsin (as low as 200

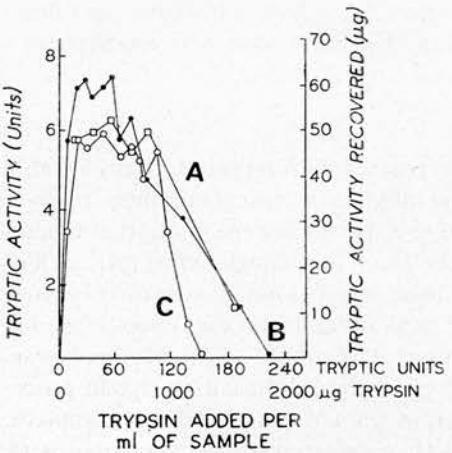

Fig. 1. The tryptic activity of serum and plasma following the addition of increasing amounts of trypsin. A: serum; B and C: plasma (1-ml aliquots). The detection of tryptic activity in samples containing less than 1000 μg trypsin/ml serum demonstrates the presence of trypsin protectors.

μg/ml serum) and the disappearance of this band when larger amounts of the enzyme were added. Other changes noted included an increased intensity of the α_1-band and the appearance of two pre-albumin components. Immunoelectrophoresis using specific antisera showed the α_2-macroglobulin component to be still present but with a slightly increased electrophoretic mobility. Trypsin in concentrations greater than 1 mg/ml decreased the number and intensity of the immunoelectrophoretic precipitin bands.

Distribution of trypsin–protein complexes and trypsin inhibitors in whole serum

The G 200-Sephadex elution patterns obtained by fractionating 5-ml samples of normal serum, with and without trypsin (1 mg/ml), are illustrated in Figs. 3A and B. The antitryptic (anti-esterase) activity was localized in the third (4–5 S) peak in the sample which did not contain trypsin (Fig. 3A). When trypsin was added, enzyme activity was discovered in the first (19 S) peak and antitryptic activity could be detected only in concentrated fractions from the third peak. There was no free trypsin (molecular weight 24 500)[18] in the low molecular weight region (Fig. 3B). Thus the addition of trypsin to serum appeared to decrease the tryptic inhibitory capacity of the serum and produce an enzymatically active, stable, high molecular weight protein–trypsin complex[2]. Of the tryptic units applied to the column, 9% were recovered, all in this high molecular weight fraction. In an experiment with another serum sample, 5% of the applied activity was recovered, a value comparable to that obtained in experiments in which trypsin was added to serum or plasma. This esterase activity of the protein–trypsin complex was not inhibited by SBTI (Table I) nor was it affected

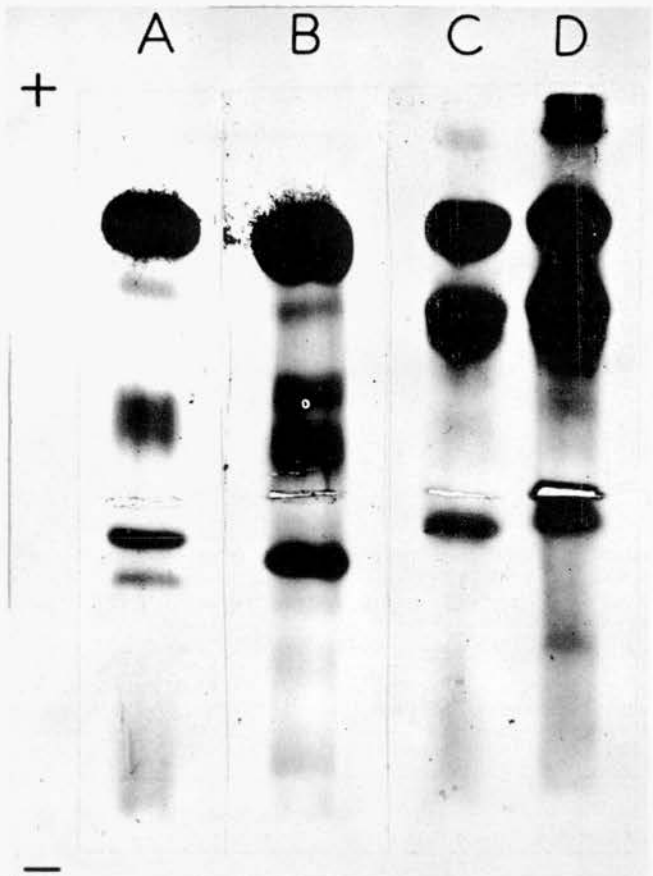

Fig. 2. Wieme electrophoretic analyses of serum samples containing trypsin. A, 0; B, 1000; C 2000; and D, 5000 μg trypsin/ml serum.

by normal serum or the G 200-Sephadex fractions of normal serum (see Fig. 3A) containing antitrypsin in concentrations sufficient to inhibit the equivalent amount of "free" enzyme activity[1,3].

Wieme electrophoresis of whole serum containing trypsin and the active complex obtained on G 200-Sephadex fractionation also confirmed the observations of previous workers, who used the paper electrophoretic technique[1] and found that the active complex had an α_2-globulin mobility. Free trypsin, as originally observed, migrated towards the cathode (Fig. 4).

Immunological characterization of the complexing protein

The results of our studies support the conclusion of previous investigators[2] that the protecting protein in the complex was a high molecular weight α_2-globulin, namely α_2-macroglobulin. However, these studies do not exclude the participation of other serum proteins (possibly trace contaminants) which are eluted with α_2-macroglobulin on G 200-Sephadex fractionation or which move with the α_2-globulins on electrophoresis. Nevertheless, of the specific precipitating antisera used in this study (see

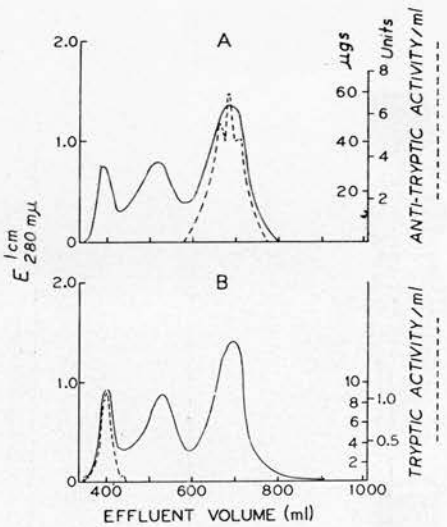

Fig. 3. The G 200-Sephadex distribution of inhibitors and protectors of tryptic esterase activity in whole serum. A: 5 ml fresh serum; B: 5 ml fresh serum containing 5 mg trypsin. Samples fractionated on a column 80 × 4.5 cm. The recovery of tryptic and antitryptic activity expressed as tryptic units and μg trypsin/ml eluate.

TABLE I

RELATIVE STABILITY OF α_2-MACROGLOBULIN–TRYPSIN COMPLEXES TO SOYBEAN TRYPSIN INHIBITOR

Source of trypsin–protein complex	*SBTI added (μg)*	*Tryptic activity (tryptic units)*	*Inhibition (%)*
Whole serum	0	0.155	0
	1.0	0.161	0
	2.5	0.158	0
	5.0	0.155	0
	7.5	0.151	0
	10	0.155	0
Isolated α_2-macroglobulin	0	0.1	0
	1	0.02	80
	5	0.017	84.3
	10	0.017	84.3
	15	0.011	89.5
Free trypsin	0	0.38	0
	2	0.04	89.5

page 365) only the antiserum to α_2-macroglobulin precipitated the tryptic activity from serum and from G 200-Sephadex fractions containing the complex. Fig. 5 shows the results with antisera specific to α_2-macroglobulin, γM and γG-globulins, and normal rabbit serum. The removal of tryptic activity closely parallels the precipitation of α_2-macroglobulin (Fig. 5A), but not of other antigens; the addition of normal rabbit serum had no effect (Figs. 5B, C, D).

The complexing of isolated α_2-macroglobulin with trypsin and its role as an enzyme protector

The above data shows that trypsin was complexing with, and being protected by, the α_2-macroglobulin in whole serum and serum fractions; however, preliminary

studies indicated that the isolated protein (8 molecules α_2-macroglobulin to 1 molecule of trypsin) only weakly protected the enzyme from SBTI (1 molecule enzyme to 1 molecule of inhibitor). Mehl *et al.*[2] have previously suggested that certain commercial preparations of α_2-macroglobulin are unsuitable because their capacity to bind trypsin is destroyed by ammonium sulfate used in the fractionation procedure. Steinbuch[3] observed that the physico-chemical and enzymological properties of isolated α_2-macroglobulin changed if it was left frozen for very long, during freezing or thawing, or lyophilized.

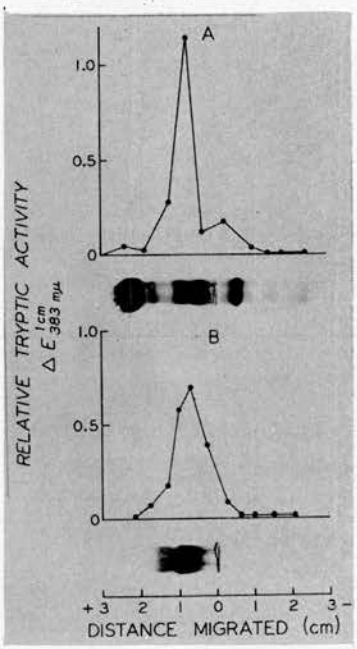

Fig. 4. The Wieme electrophoretic distribution of the serum protectors of tryptic esterase activity. A: whole serum containing trypsin (1 mg/ml); B: esterase-active G 200-Sephadex fraction obtained from above.

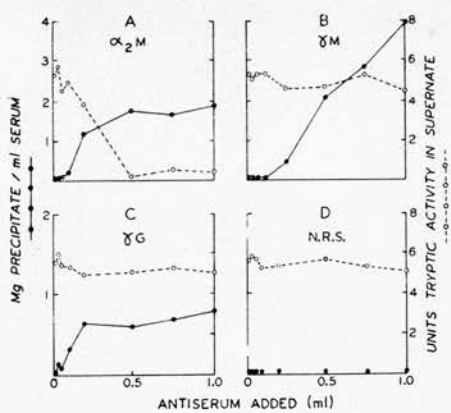

Fig. 5. The effect of specific antisera on the esterase activity of the serum protein–trypsin complex. The rabbit antisera used were to the following human serum proteins: A, α_2-macroglobulin; B, γM-globulin; C-γG globulin. The results in D were obtained with normal rabbit serum.

To establish whether isolated α_2-macroglobulin and trypsin interacted, these two molecules were incubated as previously described (75 mg, α_2-macroglobulin, and 0.5 mg trypsin) and then fractionated on G 200-Sephadex. As in the experiments with whole serum, tryptic activity was eluted with the α_2-macroglobulin (Fig. 6)

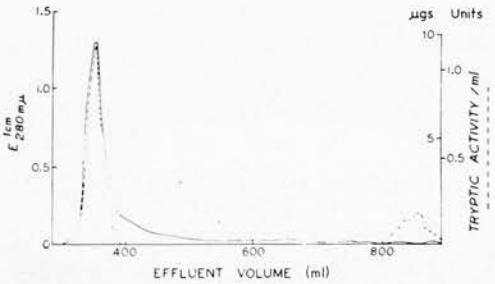

Fig. 6. The interaction of isolated human α_2-macroglobulin and trypsin on G 200-Sephadex. Human α_2-macroglobulin (75 mg) and trypsin (0.5 mg) were fractionated on a column dimensions 82×4.5 cm. Note the recovery of the major part of the tryptic esterase activity accompanies the elution of the α_2-macroglobulin. Recoveries of enzymic activity are expressed as tryptic units and micrograms of trypsin/ml of eluate.

suggesting that the enzyme had complexed with the protein. Furthermore, electrophoresis of the trypsin–α_2-macroglobulin mixture, and the Sephadex peak obtained on the fractionation of the material, revealed that the major part of the trypsin was bound to the α_2-macroglobulin (Fig. 7). Thus, it appears that isolated α_2-macroglobulin is capable of binding trypsin to the same degree as the "native" α_2-macroglobulin present in fresh serum. However, once more the esterase activity of the isolated

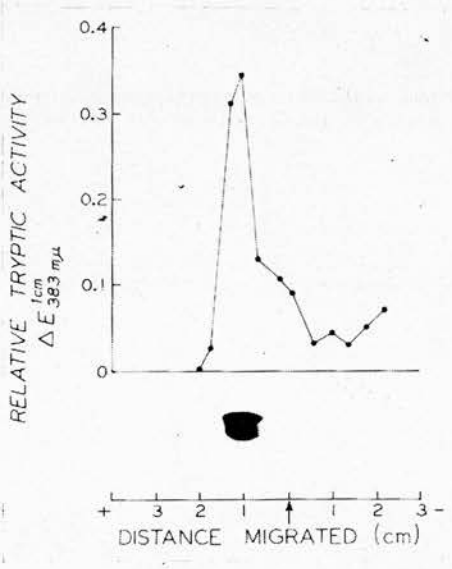

Fig. 7. The demonstration of complex formation between isolated α_2-macroglobulin and trypsin by Wieme electrophoresis. The mixture used was as in Fig. 6. Note esterase activity again accompanies the α_2-macroglobulin peak, the free trypsin migrates to the cathode.

complex was readily inhibited by SBTI (1 mole SBTI/1 mole enzyme), the α_2-macroglobulin offering about 15% of the protection observed in G 200-Sephadex fractions of fresh serum containing approximately half the concentration of α_2-macroglobulin. Furthermore, the latter samples were not inhibited by much greater concentrations of SBTI (40 moles SBTI to 1 mole trypsin) (Table I). Preliminary experiments with the low molecular weight (3 S) fragment produced by the papain digestion of α_2-macroglobulin[19] indicate that this moiety will not bind trypsin.

DISCUSSION

The results obtained on adding increasing amounts of α_2-trypsin to whole serum differed considerably from those of previous investigators[1]; there was no obvious explanation for this apparent discrepancy. Our data presented here suggest that 1 ml of normal serum or plasma can protect the esterase activity of up to 50–60 μg (approx. 6 units) of trypsin from the naturally occurring trypsin inhibitors. However, from 50–60 μg to 1000 μg of trypsin are apparently inhibited by the serum trypsin inhibitors. The loss of enzyme activity when using greater concentrations (over 1000 μg) of trypsin could be attributed to modification of the α_2-macroglobulin protector (which can still be detected by immunological means but possesses an increased electrophoretic mobility), to the production of inhibitory substances on the digestion of other serum proteins, or to auto-digestion of the enzyme.

These experiments, which use specific antisera, confirm by immunological methods the conclusions of previous investigators using other techniques[2,3] namely that the trypsin binding and protecting protein in human serum is α_2-macroglobulin. Although the isolated protein was not as effective a protector as the α_2-macroglobulin in whole serum (Figs. 2 and 3), both appeared to bind trypsin to a similar extent (approx. 1 molecule of "active" trypsin to 3 molecules of α_2-macroglobulin*. The modifications of the structure of the α_2-macroglobulin occurring during the fractionation process may decrease the capacity of the isolated α_2-macroglobulin to protect trypsin by (a) decreasing the affinity (and hence the stability of the complex) between these two molecules; (b) by changing the configuration of the α_2-macroglobulin so that the active site of the enzyme is exposed (rendering it susceptible to inhibition); or (c) by removing additional stabilizers.

Previous investigators[1] using a variety of substrates and procedures including BAPNA[1,5], fibrin–agar in an electrophoretic technique[4], inhibition of clot lysis, and caseinolysis[3], found that approx. 10% of the antitryptic activity in whole serum was in the α_2-globulin region and that α_2-macroglobulin possessed antitryptic activity[1–4]. In investigations not reported here, we have also found that the G 200-Sephadex fractions of whole serum containing the α_2-macroglobulin possess antitryptic activity (anti-proteolytic) when casein and fibrinogen were used, although they failed to inhibit the esterolytic activity of the enzyme as detected by its activity on TAME. Furthermore, the α_2-macroglobulin–trypsin complex possessed only esterolytic activ-

* The calculation was based on the following assumptions: The molecular weight of α_2-macroglobulin is 820 000 (ref. 11), trypsin 24,500 (ref. 18), and normal serum contains approx. 250 mg/l ml of α_2-macroglobulin. The amount of trypsin bound to the protein is calculated from the tryptic activity of the complex and it is assumed that all the trypsin bound to the α_2-macroglobulin possesses the same specific activity as the free trypsin.

ity. Similar properties were also observed by Steinbuch[3]. Thus it would appear that the α_2-macroglobulin molecule can act simultaneously as an inhibitor of the proteolytic activity of trypsin and as a protector of its esterolytic activity. Furthermore, recent observations have shown that most of the esterase activity of the α_2-macroglobulin–trypsin complex is unaffected by concentrations of diisopropylfluorophosphate far in excess of that required to inhibit free trypsin*.

Whether interactions similar to those described above occur *in vivo* remains conjectural, since the *in vivo* trypsin concentration is much lower than used in these experiments. If so, this property of α_2-macroglobulin, as previously stated[1], would provide a mechanism whereby serum might possess tryptic activity in the presence of high concentrations of antitrypsin. Furthermore, such enzyme interaction may be of importance in other enzyme systems for a number of serum enzymes are known to migrate as α-globulins on electrophoresis. Thus, in considering the relationship between enzymes and inhibitors in the pathogenesis of disease, and the therapeutic use of anti-enzymes, consideration must be given to the possible participation of proteins which can protect enzymes, such as α_2-macroglobulin.

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PRELIMINARY NOTES

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The effect of α_2-macroglobulin in human serum on trypsin, plasmin, and thrombin activities

Recent investigations have indicated that human α_2-macroglobulin plays a complex role in a variety of enzymological processes[1–5]. Because of the considerable physiological importance of some of these observations, we have examined further the effect of human serum α_2-macroglobulin on the proteolytic and esterolytic activities of trypsin, plasmin and thrombin.

In order to avoid the losses of activity frequently observed on the isolation of human α_2-macroglobulin[4,5], these investigations have been performed with a partially purified preparation. This was obtained by filtration on Sephadex G-200 at 0°. Fresh serum samples (5–7 ml) from a single donor were separated on columns (80 cm $\times$ 4.5 cm) equilibrated with 0.05 M Tris buffer, pH 8.1.

The enzyme–α_2-macroglobulin complexes were obtained by incubating these substances for 5 min at 37°. The enzymes used were trypsin (twice crystallized, from Worthington Biochemicals Corp.), thrombin (topical bovine product, from Parke, Davis & Co.), and plasmin (obtained by streptokinase activation of plasminogen). The α_2-macroglobulin content of the reaction mixtures was determined by quantitative gel diffusion analysis[6].

The effect of α_2-macroglobulin on the proteolytic activities of trypsin and plasmin was investigated using both casein[7] and fibrinogen substrates[8]. Experiments were performed in which the α_2-macroglobulin rich fraction was added prior to, and on completion of, the assay. The ability of the fraction to protect these enzymes from soybean trypsin inhibitor (SBTI) (3 times crystallized, from Worthington Biochemicals Corp.) was determined by adding this compound to the α_2-macroglobulin enzyme complexes prior to addition of substrate.

The effects of SBTI and diisopropylfluorophosphate (DFP) on the esterolytic activity of the α_2-macroglobulin enzyme complexes were determined by the procedure of ROBERTS[9] using the synthetic substrate, *p*-toluenesulfonyl-L-arginine methyl ester (TAME).

Further details of the quantities of material used are indicated in Tables I and II.

The results obtained when α_2-macroglobulin was added to the enzyme system prior to assay suggest that this protein inhibits the proteolytic activity of trypsin and plasmin (Table I). However, those obtained when α_2-macroglobulin was added on completion of the assay indicate that this protein binds the soluble peptides produced by the action of trypsin and plasmin, thus simulating inhibition.

Furthermore, the same protein also prevented the SBTI and DFP inhibition of plasmin trypsin esterase activity (Table II). The small losses of activity observed

Abbreviations: SBTI, soybean trypsin inhibitor; DFP, diisopropylfluorophosphate; TAME, *p*-toluenesulfonyl-L-arginine methyl ester.

TABLE I

THE EFFECT OF α_2-MACROGLOBULIN ON TRYPSIN AND PLASMIN HYDROLYSIS OF CASEIN AND FIBRINOGEN AND ON SBTI INHIBITION OF THIS HYDROLYSIS

Each assay with α_2-macroglobulin (α_2M) contained 0.43 mg of this protein. SBTI inhibition studies were performed with 0.3 mg of inhibitor.

Substrate	*Enzyme*	*Enzyme Control*	*Enzyme with α_2M added prior to assay*	*Enzyme with α_2M added on completion of assay*	*Enzyme with SBTI*	*Enzyme with α_2M and SBTI*
Casein (40 mg)	Trypsin (20 μg)	25.0*	18.0	18.0	5.0	18.2
	Plasmin (100 μg)	5.0	4.0	3.8	1.0	4.1
Fibrinogen (1 mg)	Trypsin (1 μg)	18.1**	13.0	13.8	6.0	12.0
	Plasmin (17 μg)	0.4	0.30	0.32	0.1	0.31

* μg of casein per min per μg of enzyme.
** μg of fibrinogen hydrolyzed per min per μg of enzyme.

TABLE II

THE EFFECT OF α_2-MACROGLOBULIN ON THE SBTI AND DFP INHIBITION OF TRYPSIN, PLASMIN AND THROMBIN HYDROLYSIS OF TAME

Each assay with α_2-macroglobulin (α_2M) contained 0.43 mg of this protein.

Enzyme	*Inhibitor*		*Enzyme alone*	*Enzyme with inhibitor*	*Enzyme–α_2M complex and inhibitor*
Trypsin (1 μg)	SBTI	0.3 mg	8.37*	0.0	7.45
Plasmin (10 μg)	SBTI	0.3 mg	0.55	0.0	0.465
Thrombin (0.1 unit)	DFP	$1 \cdot 10^{-2}$ M	25.0	17.3	15.3
Trypsin (1 μg)	DFP	$1 \cdot 10^{-6}$ M	19.5	9.2	19.5
Plasmin (10 μg)	DFP	$1 \cdot 10^{-6}$ M	0.65	0.0	0.42

* Moles TAME hydrolyzed per min per μg of enzyme times 10^{-8} as estimated by the ROBERTS procedure[9].

were of the same order as those detected by previous workers and attributed to a decrease in specific activity resulting from complex formation[1,3]. In the experiments with thrombin, where greater DFP concentrations were used, the α_2-macroglobulin was ineffective.

Immunoprecipitation studies utilizing the γG-globulin from specific anti-human α_2-macroglobulin antisera revealed that the esterase-protecting protein was indeed α_2-macroglobulin, thus confirming previous observations[4].

The physiological questions arising from this preliminary study merit further investigation, especially as to the nature of the complexes formed. It is conceivable that this capacity of α_2-macroglobulin to bind and protect both plasmin and trypsin and their products of lysis might be of prime importance with regard to homeostatic processes. Such interactions could (1) ensure a small reservoir of enzyme free from the effects of circulating inhibitors, (2) influence the amount of enzyme and peptide

available for other metabolic processes, (3) affect the amount of these materials available for destruction, and hence (4) also exert an effect on reaction equilibria.

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A SEMIQUANTITATIVE PROCEDURE FOR ESTIMATING SERUM ANTITRYPSIN LEVELS

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A semiquantitative procedure for estimating serum antitrypsin levels

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The antitrypsin levels of a large number of sera (both normal and pathologic) have been determined by a semiquantitative gelatin film method, and compared with those obtained by the TAME procedure. From the values obtained, a simple procedure has been developed which does not require the facilities necessary for an accurate enzymatic assay and which should enable the screening of large populations for α_1-antitrypsin deficiencies.

Methods currently available for estimating antitrypsin levels in serum include enzymologic procedures involving the inhibition of digestion of protein substrates such as casein,[1, 2] fibrinogen,[3] and hemoglobin,[4] and inhibition of hydrolysis of synthetic substrates such as benzoyl-L-arginine paranitroanilide (BAPNA),[5, 6] and p-toluene sulfonyl-L-arginine methyl ester (TAME).[7] In addition, there are immunologic procedures using specific antisera.[8] However, a rapid, simple procedure is needed for the semiquantitative measurement of antitryptic activity, a procedure which would make possible the screening of large populations for the genetically determined α_1-antitrypsin deficiency that appears to be associated with an unusual familial variety of pulmonary emphysema.[9, 10]

Individuals homozygous for the genetic defect possess approximately 10 per cent of the normal level of serum α_1-antitrypsin; heterozygous individuals possess 50 to 60 per cent of the normal concentration of this protein. Since 90 per cent of the antitryptic activity of normal serum is attributable to the α_1-antitrypsin,[11] estimations on whole serum should reflect any major changes in this protein.

We have adapted the gelatin film test used to diagnose trypsin activity in

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stools[12] to provide (1) an inexpensive semiquantitative procedure for determining serum antitrypsin levels, and (2) a simple test (based on 1) permitting the detection of the homozygous and heterozygous deficiency states which should be readily applicable to screening large populations. Both measure the ability of serum to inhibit the capacity of known trypsin standards to digest the gelatin surface of an exposed and developed (black) or an exposed (green) x-ray film. The results obtained in procedure (1) have been compared with those obtained by the TAME procedure.

Procedures

A. Determination of the normal levels of antitrypsin by the gelatin film test. Standard solutions of twice crystallized trypsin (Worthington Biochemicals) containing from 1.0 to 2.5 mg. per milliliter were prepared in Tris buffer (pH 8.1, 0.05M) containing 0.01M calcium chloride. Two drops of each dilution of enzyme were dispensed into separate test tubes (using disposable pipettes); two drops of fresh undiluted test serum (or plasma) were added to each sample, the tubes were shaken to mix the contents, and then allowed to stand for 10 minutes at room temperature. One drop of each mixture was then placed on the gelatin surface of an exposed and developed x-ray film (Fig. 1). The gelatin surface on the lower surface of the film was scraped off after the surface was wet with tap water. The x-ray film was allowed to stand in a Petri dish for 2 hours at room temperature. Finally the digested gelatin was washed off with cold running tap water, leaving a clear area of lysis (Figs. 1 and 2). If the trypsin had been inactivated by the serum antitrypsin, the film was not cleared. The inhibitory capacity of the test serum (milligrams of trypsin per milliliter) was equivalent to the concentration of trypsin (milligrams per milliliter) in the test samples intermediate between the last negative and the first positive result. For example, if the serum sample in-

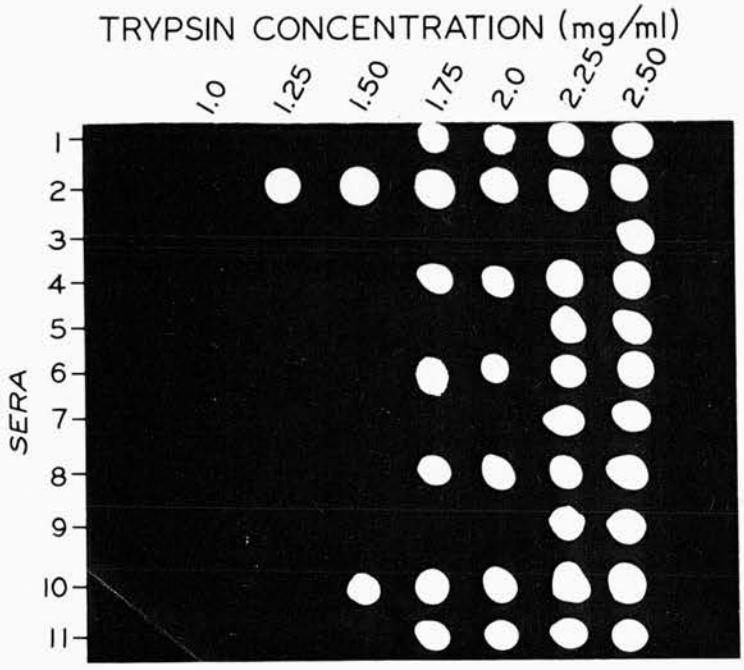

Fig. 1. The estimation of antitrypsin levels in 11 normal sera by the gelatin film procedure.

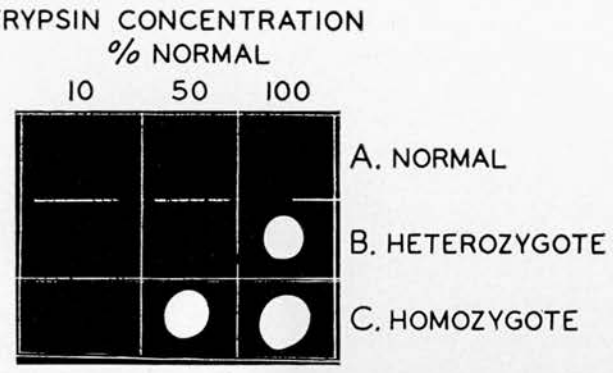

Fig. 2. The detection of the heterozygous and homozygous deficiency states by the rapid screening procedure. *A*, normal; *B*, heterozygous; and *C*, homozygous for α_1-antitrypsin deficiency.

hibited (negative lysis) a 1.5 mg. per milliliter solution of trypsin but failed to inhibit a 1.75 mg. per milliliter solution (positive lysis), then the antitryptic activity of the test serum was calculated at 1.62(5) mg. per milliliter.

B. Rapid screening procedure for the detection of the heterozygous and homozygous traits. The procedure just described indicated that normal sera can inhibit 1.69(5) ± 0.3(8) mg. per milliliter of trypsin. Therefore, standard solutions were prepared containing 10 per cent (0.17 mg.), 50 per cent (0.85 mg.), and 100 per cent (1.7 mg.) of the trypsin usually inhibited by normal serum. The test sera were mixed as before and these three standard solutions applied to the x-ray plate. Sera homozygous for the gene deficiency should inhibit at the most only the weaker sample of trypsin; heterozygous sera should fail to inhibit the 1.7 mg. per milliliter standard. The test was performed with normal sera and with sera homozygous and heterozygous for the α_1-antitrypsin deficiency (see Fig. 2). This technique may also be applied to whole blood. Under these circumstances 4 drops of blood are used for each 2 drops of enzyme standard (see experimental procedure), the increase in volume of the test sample being based on an average packed cell volume of approximately 50 per cent.

TAME procedure. The method used, based upon that described by Hummel[7] involves adding a known volume (0.1 ml.) of a 1 in 100 dilution of serum (in 0.05M Tris, pH 8.6, containing 0.01M calcium chloride) to a standard amount of trypsin (2γ in 0.1 ml.) and determining the ability of this mixture to hydrolyse a 0.01M solution of TAME. The results are compared with those observed in systems containing enzyme and no inhibitor.

Test samples. Because serum antitrypsins are unstable, estimations should be performed on fresh samples wherever possible, or on samples stored at −20° C. without freezing and thawing.

Results

General procedure for semiquantitative estimation of α_1-antitrypsin levels. Analysis of 69 normal sera indicated that 1 ml. of serum was capable of inhibiting 1.69(5) ± 0.3(8) mg. of trypsin; there was no significant difference between males and females (see Table I). Repeated investigations on a single serum indicated that the results were reproducible. However, values obtained by the gelatin film procedure were higher and varied more than those obtained by the TAME technique (Table I).

Rapid screening procedure. Samples of serum from the subjects homozygous for α_1-antitrypsin deficiency were recognized readily by their failure to inhibit the lysis of the gelatin film by the trypsin standards (see Fig. 2). For example,

Table I. Comparison of the estimation of antitrypsin levels in serum by the gelatin film and TAME procedures

Samples	*No. of tests*	*Mean values ± ($6\bar{\chi}$)*	
		TAME (mg./ml.)	*Gelatin film (mg./ml.)*
All estimations*	173	1.48 ± 0.20	1.68(5) ± 0.45
Normal subjects	69	1.51 ± 0.13(4)	1.69(5) ± 0.38
Male	40	1.52 ± 0.09(4)	1.60 ± 0.34
Female	29	1.5 ± 0.19	1.85 ± 0.41
All pulmonary patients	37	1.52 ± 0.14(7)	1.75(5) ± 0.37
Pulmonary emphysema patients	16	1.51 ± 0.11	1.65(5) ± 0.3
Repeated analysis on a single normal serum	10		1.62(5) ± 0.12

*A number of these estimations were performed on aged serum and on pathologic sera not included below. All TAME estimations were performed in duplicate.

the normal serum (*A*) inhibited all three trypsin samples; the heterozygous sample (*B*) inhibited all but the strongest standard (1.7 mg. per milliliter), and the homozygous sample (*C*) inhibited only the weakest standard (0.17 mg. per milliliter). Although the heterozygous samples were normally detected by this procedure, occasionally a "false" normal value would be obtained.

Discussion

Considering the inherent errors in the gelatin-plate procedure resulting from the use of set standards, there is a reasonable correlation between the results obtained by this and the TAME procedure. However, a more detailed analysis of the results revealed that if serum antitryptic levels were more than 1.5 mg. per milliliter (as estimated by the TAME procedure), values from the gelatin-plate procedure were usually too high, and were too low with concentrations lower than this. These discrepancies did not affect the validity of the procedure as a semiquantitative technique or the rapid method for screening populations for low values, but did indicate the errors which could result from the use of this technique as a quantitative procedure. Nevertheless, it should be emphasized that whenever possible the homozygous and heterozygous samples detected by this procedure should be rechecked by more sensitive quantitative techniques, such as that using TAME.

Although too few normal subjects were investigated to determine the incidence of the homozygous and heterozygous traits, two of the 69 normal sera investigated (mixed Caucasian, Negroid, and Asian) had the 50 to 60 per cent levels of inhibitor; an incidence similar to that observed in a much larger Caucasian population.[9] These sera were detected by both the gelatin-film and the TAME procedure. The homozygous and heterozygous traits were not detected in our small number of patients with typical well-characterized emphysema (Table I).

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homozygous for the α_1-antitrypsin deficiency. Keith James wishes to acknowledge the receipt of a Wellcome Foundation travel grant.

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SECTION E - OTHER PUBLICATIONS

The effect of an α-globulin preparation and of polyribonuclease complexes on humoral antibody formation

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THE EFFECT OF AN α-GLOBULIN PREPARATION AND OF POLYRIBONUCLEASE COMPLEXES ON HUMORAL ANTIBODY FORMATION

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SUMMARY

The effects of bovine α-globulin (BAG), bovine-ribonuclease serum albumin (BSA-RNase) and polyribonuclease (poly-RNase) complexes on the primary response of rodents to sheep erythrocytes have been investigated. These preparations frequently failed to cause marked suppression of humoral antibody formation.

INTRODUCTION

Although the reports of Kamrin (1959, 1966) and Mowbray (1963a, b) indicated that α-globulin-rich protein preparations from sera were able to inhibit both cellular and humoral immune responses, other workers have failed to confirm these observations (Spiegelberg & Weigle, 1964; Davis & Boxer, 1965). However, recently Mowbray & Hargrave (1966) claim that a number of factors critically affect the immunosuppressive properties of such preparations when injected into mice and could possibly explain previous failures. It appears that in order to be effective in mice the material has to be injected intravenously 10–20 hr prior to the injection of the antigen and that previously inactive preparations can be rendered active by storage in the frozen form or at 4°C. In addition the latest reports (Mowbray & Hargrave, 1966; Mowbray & Scholand, 1966) indicate that effective α-globulin preparations possess considerable ribonuclease activity and that similar immunosuppression can be achieved using chemically derived ribonuclease complexes. Because of the obvious practical and theoretical importance of these more recent observations we have studied the effect of a BAG preparation and several ribonuclease complexes on the primary humoral antibody response in rodents taking into consideration the factors believed to influence the activity of these materials. In the preliminary results reported in this paper we have failed to achieve routinely a marked suppression of the primary immune response using an α-globulin preparation or ribonuclease complexes.

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MATERIALS AND METHODS

The BAG and the poly-RNase complexes were prepared according to established procedures. The crude BAG (fraction C) was prepared by the method of Mowbray (1963a, b) using bovine serum which had been stored for 2 months at −20°C. Polyacrylamide gel electrophoresis revealed that this product was similar in composition to that originally described by Mowbray (1963a, b). The preparation was stored at −20°C for 1 month prior to use in experiments 1 and 6 but had been stored for 18 months prior to use in experiment 5 (17 months at −20°C, 1 month at +4°C).

The BSA–RNase complexes were prepared according to the method of Mowbray & Scholand (1966). The bisdiazobenzidene coupling mixture contained 138 mg of crystalline bovine serum albumin (BSA) (Armour Pharmaceutical Company) and 25·4 mg of four times crystallized bovine pancreas ribonuclease (Koch–Light Laboratories). The reaction mixture thus contained approximately 2 μmoles of protein and 2 μmoles of enzyme. BSA–RNase preparations 1, 3 and 4 were fractionated at 4°C on columns of G-100 Sephadex (size 52 × 3 cm) equilibrated with 0·1 M-NaCl. The excluded protein peak was concentrated by ultrafiltration at 4°C. Preparation 2 and the poly-RNase complex, prepared according to the method of Mowbray & Scholand (1966), were not subjected to gel filtration.

The protein content of the BAG preparation was determined by the Folin Phenol procedure (Lowry *et al.*, 1951) whilst those of the BSA–RNase complexes were calculated from the extinction coefficient ($E_{1cm\ 1g/100ml\ 280\ m\mu}$ of BSA = 6·0).The ribonuclease activity of the various preparations was determined as previously described (Anfinsen *et al.*, 1954) except that the ribonucleic acid substrate (ex-yeast soluble ribonucleic acid, Koch–Light Laboratories) was prepared as an 0·8% solution in 0·1 M-acetate buffer, pH 5, and was not dialyzed. One unit of ribonuclease activity was that which increased the optical density of the incubation mixture at 260 mμ in a 1 cm cell by 0·01 (Tuve & Anfinsen, 1960).

The immunosuppressive properties of the various preparations were assessed by determining their effect on the primary response of rodents to sheep erythrocytes. The mice used in experiment 5 were TO strain mice obtained from A. Tuck & Son, Rayleigh, Essex. All the other animals were from inbred strains maintained in our own department. Further details of the strains of mice and rats used together with their pre-treatment are recorded in Table 1. In the mice experiments all the materials under test were injected intravenously 17–20 hr prior to the intravenous injection of sheep erythrocytes and the animals were bled 6 days later. The rats on the other hand were injected intravenously 6 hr prior to the intravenous injection of sheep erythrocytes and the animals were bled 5 days later (the time of peak primary response in untreated animals). The erythrocyte agglutinin antibody content of the sera was determined as previously described (James & Anderson, 1967). The Student *t*-test was used for statistical evaluation of the data, the Bessel correction for small samples being applied. Values of $P < 0{\cdot}05$ were taken as significant.

RESULTS

Although the present experiments were performed in a number of strains of mice and in a single strain of rats using a variety of preparations, regular and reliable immunosuppression was not achieved.

In only one experiment (No. 5) out of three did the BAG exhibit a significant immuno-

TABLE 1. The effect of α-globulin and ribonuclease complexes on the primary response of rodents to sheep erythrocytes

Experiment No.	Animal	No.	Pre-treatment				Haemagglutinin response $-\log_2$ titre			Standard error of mean	Difference between means†	Significance of difference between means
			Material	Amount protein (mg)	Amount of enzyme		Range	Mean	Standard deviation			
					μg	units*						
1	C57 Black	10	—	—	—	—	4·32– 7·32	5·92	0·841	0·26		
	strain female	10	BSA	12·5	—	—	3·32– 5·32	4·42	0·755	0·24	1·5	$P<0·001$ ($n=18$; $t=3·9$)
	mice	12	BAG	12·5	Not determined		3·32– 7·32	5·15	1·27	0·37	0·77	$P>0·1$ ($n=20$; $t=1·57$)
2	CBA strain	7	Saline	—	—	—	7·32– 9·32	8·46	0·693	0·26		
	male mice	4	BSA–RNase (1)	0·88	Not determined		8·32–10·32	9·07	0·678	0·34		
		7	BSA–RNase (2)	0·88	Not determined		5·32– 8·32	7·32	1·15	0·44	1·14	$P>0·05$ ($n=12$; $t=2·08$)
3	A strain	12	—	—	—	—	0– 6·32	3·68	2·12	0·61		
	male mice	11	BSA–RNase (3)	0·6	0·8	7·5	0– 6·32	3·87	2·57	0·77		
4	C_3H strain mice											
	Female	12	—	—	—	—	5·32– 8·32	6·99	1·07	0·31		
	Male	12	BSA–RNase (4)	1·5	0·8	6·0	5·32– 8·32	6·74	1·24	0·36	0·25	$P>0·6$ ($n=22$; $t=0·505$)
5	TO strain	10	—	—	—	—	6·32– 9·32	8·22	0·878	0·28		
	male mice	9	BSA	4·0	—	—	4·32–10·32	7·96	1·85	0·58	0·26	$P>0·7$ ($n=17$; $t=0·376$)
		12	BAG	4·0	0·85	12·0	4·32– 9·32	6·90	1·45	0·42	1·32	$P>0·025$ ($n=20$; $t=2·4$)
		11	BSA–RNase (4)	4·0	2·0	16·0	4·32– 8·32	6·05	1·42	0·43	2·17	$P<0·001$ ($n=19$; $t=4·05$)
		12	Poly-RNase	—	150	480	5·32–11·32	7·74	1·62	0·47	0·48	$P>0·4$ ($n=20$; $t=0·801$)
6	Hooded strain	6	—	—	—	—	5·32– 7·32	6·65	0·82	0·33		
	female rats	6	BAG	50	Not determined		3·32– 8·32	6·15	1·72	0·70	0·50	$P>0·5$ ($n=10$; $t=0·587$)

* One unit of activity increases the extinction coefficient at 260 mμ in a 1 cm cell by 0·01.
† The difference between pretreated group and untreated or saline-treated controls.

suppressive effect, while in experiment 1, BSA was observed to cause a more profound effect.

Of the BSA–RNase preparations used Nos. 2 and 4 alone demonstrated any significant effect (experiments 2 and 5 in CBA strain males and TO strain males, respectively). In contrast, preparation 4 failed to suppress the primary immune response of C_3H strain male mice and preparations 1 and 3 were ineffective in CBA strain male and A strain male mice, respectively (experiments 2 and 3). Finally the poly-RNase preparation, which possessed considerable ribonuclease activity, had no significant immunosuppressive effect.

DISCUSSION

These preliminary results once more emphasize the difficulty in obtaining reproducible and reliable suppression of humoral antibody formation using α-globulin-rich serum fractions or ribonuclease complexes (Sims & Freeman, 1966). Throughout the present experiments we have used materials possessing strong ribonuclease activity in amounts roughly equivalent to those previously used. These have been administered at the times and by the routes recommended to achieve maximum immunosuppression and have on occasions been stored for long periods of time, yet in most experiments they failed to cause a marked effect. However, it is still feasible that some of our preparations possessed a low 'specific activity' and that higher doses and repeated administration might have proved effective (Sims & Freeman, 1966). It should also be stressed that in the experiments where suppression was achieved the effect could have been partially attributed to antigenic competition and other non-specific effects (Pokorna & Vojtiskova, 1966; Jennings & Oates, 1967).

It would thus appear that further research will be necessary in order to enable the preparation of reliable and effective products of this type. Once this has been achieved it will then be possible to elucidate the mode of action of these potentially important immunosuppressants.

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SYNOVIAL CELLS

A STUDY OF THE MORPHOLOGY AND AN EXAMINATION OF PROTEIN SYNTHESIS OF SYNOVIAL CELLS

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Because of the infrequency with which fluid is withdrawn from normal joints, most descriptions of the morphology of normal synovial fluid cells are based upon examination of the small quantity of fluid obtained either at autopsy (Ropes and Bauer, 1953) or from patients with non-articular disease following prolonged rest in bed (Coggeshall, Warren, and Bauer, 1940). The cells present have been reported to consist predominantly of monocytes and "clasmatocytes" with small numbers of lymphocytes and only occasional polymorphonuclear leucocytes. Certain other cells, regarded as the actual synovial cells and originating from the joint lining, were extremely few in number (Davies, 1964). Ropes and Bauer (1953) reported an average differential count for normal synovial fluid as:

monocytes 47·9 per cent.
clasmatocytes 10·1 per cent.
lymphocytes 24·6 per cent.
polymorphonuclear leucocytes 6·5 per cent.
unclassified phagocytes 4·9 per cent.
synovial cells 4·3 per cent.

A study of normal synovial membrane (Hamerman and Blum, 1959; Hamerman, Stephens, and Barland, 1961), using the tetrazolium technique for the demonstration of lactic dehydrogenase and DPNH diaphorase activities, not only demonstrated the distribution of the oxidative enzymes, but also revealed the morphological detail of the lining cells more clearly than had previously been seen with routine staining methods.

An extension of this study using the electron microscope to examine the fine structure of normal synovial membrane (Barland, Novikoff, and Hamerman, 1962) revealed the presence of two cell types in the lining membranes:

(i) Numerous type A cells containing many vacuoles, a prominent Golgi apparatus, and finger-like extensions of the cell membrane (filopodia).

(ii) Less frequent type B cells containing abundant ergastoplasm, less extensive Golgi apparatus, few vacuoles, and few filopodia.

It seems reasonable to expect that both these cell types are present in normal synovial fluid.

It is believed that the synovial cells produce the small protein fragment attached to hyaluronic acid (Sandson and Hamerman, 1962), but the synthesis of other specific proteins by the lining cells has not been reported.

Fraser and Catt (1961) and Fraser and McCall (1965) described a method of stripping off some of the lining cells from the synovia of knee joints at autopsy by the use of trypsin. It is possible to compare the properties of cells freed from the synovial membrane in this way with the cells occurring in normal and pathological synovial fluids.

In this and future communications we shall consider the morphological and biochemical features of lining cells obtained from knee joints and make comparisons with normal and pathological synovial fluids and synovial membrane. This paper will be concerned with the types of cells obtained, their superficial morphology, and changes occurring in culture. Evidence for the production of an α-globulin by these cells in culture will also be presented.

Material and Methods

Synovial Cell Suspensions obtained from Autopsy Material.—Synovial fluid was removed through a needle inserted beneath the patella. The joint was washed out with 100 ml. warm phosphate-buffered saline, and about 50 ml. warm 0·25 per cent. trypsin in phosphate-buffered saline introduced and left *in situ* for 30 min. during which time the distended synovial pouches were massaged and the solution frequently withdrawn into the syringe and returned to the joint. The opalescent solution was then withdrawn as completely as possible and transferred to a sterile bottle. The technique, which is essentially that of Fraser and Catt (1961) and Fraser and McCall (1965),

was not always successful. On many occasions the introduced fluid escaped from the joint space and could not be recovered by aspiration. Leakage occurred more frequently from joints of older persons and as the length of time *post mortem* increased. It could not be prevented by "cuffing" above and below the knee. Because of this "leakage" factor it was decided not to force fluid into the joint beyond the point where slight back-pressure was felt in the syringe. On some occasions a solution containing 0·1 per cent. collagenase as well as 0·25 per cent. trypsin was used; on these occasions a slightly better yield of cells was obtained. The cell suspensions, which frequently contained small pieces of tissue and fat globules, were centrifuged at 1,500 r.p.m. for 5 min. as soon as possible after harvesting, and then suspended in Eagle's medium containing 15 per cent. human or calf serum.

Preparation and Examination of Smears.—Smears were prepared directly from the cell deposits. Smears of normal synovial fluid cells obtained before trypsinization were prepared directly from the fluid or after centrifugation when the quantity of fluid obtained was sufficient. Smears of cells from pathological synovial fluids were prepared from the spun deposit. The majority of the smears obtained were air-dried, fixed in methanol, and stained with Jenner–Giemsa, periodic acid-Schiff, methyl green-pyronin, or toluidine blue. Other smears were left unfixed for the detection of lactic dehydrogenase using the method of Nachlas, Walker, and Seligman (1958). Ribonuclease was obtained from Koch Light Laboratories Ltd.

Cell Culture.—Suspensions of the cells in Eagle's medium containing 15 per cent. human or calf serum were transferred to "medical flat" bottles or petri dishes, gassed with 5 per cent. CO_2 in air and incubated at 37° C. Short-term cultures for histochemical and other tests were prepared in 1 cm. polystyrene petri dishes (Oxoid Ltd.) in the bottom of which had been placed a circular cover slip (1 cm. in diameter) specially cleaned for tissue culture. To remove the cover slip after culture without disturbing the cells the supernatant fluid was sucked off, a hole punched in the base of the petri dish with a red-hot needle and the cover-slip pushed up. On some occasions 5 per cent. chick embryo extract was incorporated into the medium. One of the cell lines which grew rapidly was transferred to the revolving Winchester apparatus designed by Wildy (1964). Cells were removed from the glass with 0·1 per cent. trypsin in 0·02 per cent. EDTA in M/180 phosphate in saline.

Examination of Cell Proteins.—Synovial cell cultures for protein investigation were grown in Eagle's medium containing 15 per cent. calf serum from the time of isolation. After removal of culture medium, the monolayer cultures were rinsed with phosphate buffered saline and the cells removed with EDTA-trypsin (20 min. at 37° C.). The cell suspension was centrifuged and the supernate discarded. A soluble extract of the pellet was prepared by suspending the cells in phosphate buffered saline (*p*H 6·9, 0·06M containing 0·15M NaCl) and repeatedly freezing and thawing the suspension. The extract obtained was concentrated by ultra-filtration through $\frac{8}{32}$ in. dialysis tubing (Grant, Rowe, and Stanworth, 1958) to give 0·5 ml. of a product with a protein concentration of approximately 2 g./100 ml. Horizontal starch-gel electrophoresis of the protein was performed using the discontinuous buffer system described by Poulik (1957). The immuno-electrophoretic technique was similar to that of Grabar and Williams (1953) using 1 per cent. (w/v) barbitone buffered agar (*p*H 8·6, I= 0·05). Gel diffusion precipitin analysis was also performed in the above agar. The antisera used were prepared in rabbits using as antigens whole human serum, isolated α_2-macroglobulin, and IgM globulin. Antisera to the purified individual antigens were rendered monospecific by absorption (James, 1965a; Rowe 1962). Hela cell cultures were subjected to a similar procedure and the extract obtained was used as a control.

^{14}C-lysine Incorporation Technique.—Synovial cells freshly obtained from joints at autopsy were mixed with 1/5th volume of human serum in order to neutralize the effect of the trypsin. The cells were then spun down and suspended in lysine-free Eagle's medium containing 0·5 μc. ^{14}C-lysine (Radiochemicals, Amersham). After 24 hours the medium and the suspended cells were removed. Cells attached to the glass were removed with trypsin and added to the cell suspensions after removal of trypsin. The cells were disintegrated by freeze-thawing and homogenization and the homogenate centrifuged at 10,000 r.p.m. for 30 min. The supernate was concentrated by dialysis against Carbowax and subjected to immuno-electrophoresis after the addition of carrier human serum. Two antisera were used. One was from an animal immunized with whole human serum and the other was an antiserum to the three immunoglobulins (IgG, IgA, and IgM). It was prepared by immunizing three separate rabbits with Cohn fraction II human γ-globulin, IgA separated from the serum of a case of IgA myeloma, and IgM from the serum of a case of macroglobulinaemia. The three antisera were then mixed to produce a composite antiserum. The washed and dried plate was clamped to Kodirex *x*-ray film, exposed for one week in the dark and developed with Kodak D19B for 6 min.

Synovial Membrane Specimens.—These were obtained by needle biopsy (Williamson and Holt, 1966) of *post mortem* joints. Frozen sections were prepared using the Frigistor. Paraffin sections were prepared from material fixed in cold ethanol as described by Sainte-Marie (1962). Sections were examined by the methods described for isolated cells.

Results

Synovial Cell Smears

A study of the smears obtained from knee joints at autopsy after the injection of trypsin revealed a cell population of predominantly two types (Type 1

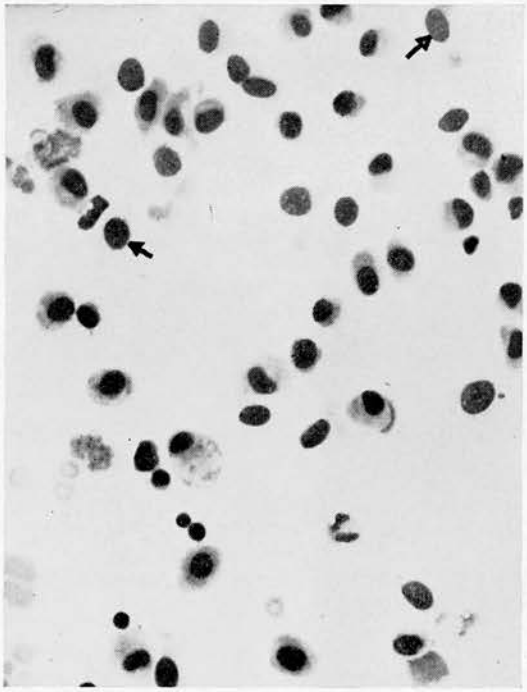

Fig. 1.—Synovial cells (trypsin technique, *post mortem* joint). Type 1 cells predominate. Occasional Type 2 cells (arrows) are also seen. Stain: Jenner–Giemsa. × 380.

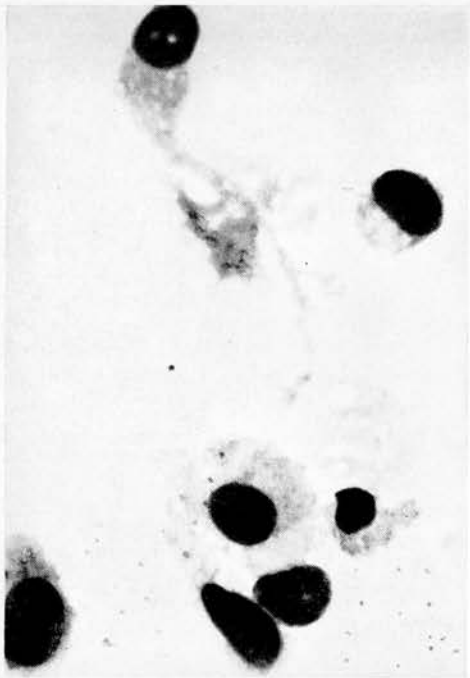

Fig. 2.—High-power view of synovial cells. Azurophilic granules are seen in the Type 1 cells. The single Type 2 cell is easily distinguished by its long cytoplasmic process. Stain: Jenner–Giemsa. × 1,050.

and Type 2). Of these types, Type 1 was far the most commonly seen cell (Fig. 1). When stained with Jenner–Giemsa stain the Type 1 cells were seen to be large mononuclear cells with pale blue-grey cytoplasm containing numerous azurophilic granules (Figs 2 and 3). These granules were usually arranged in a perinuclear pattern, although in some cells they appeared to be scattered throughout the cytoplasm. The oval shaped nucleus was found mainly in a central position and contained one or two prominent nucleoli. Occasionally, multinucleate forms were seen (Fig. 4, opposite). Much variation in shape and size of these cells was seen, and in many the irregular cytoplasmic membrane appeared to be concentrated to give a beaded appearance around the edge of the cell (Fig. 5, opposite). In some cells the nucleus was displaced to the edge of the cell and the very granular cytoplasm was distended with numerous vacuoles (Fig. 6, opposite).

The second type of cell (Type 2) had a large, eccentric, darkly-staining nucleus and deeply basophilic cytoplasm (Figs 2 and 3). One or two nucleoli were occasionally seen but these were not very prominent. The cytoplasm was often elongated to form a short process and the basophilia tended to fade near the tip.

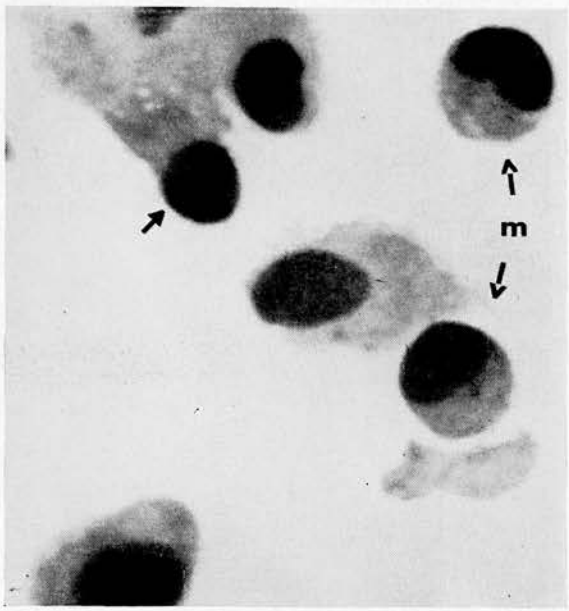

Fig. 3.—A further group of synovial cells. This shows one Type 2 cell (arrow), two cells with the characteristics typical of monocytes (M→), and three Type 1 cells. Stain: Jenner–Giemsa. × 1,440.

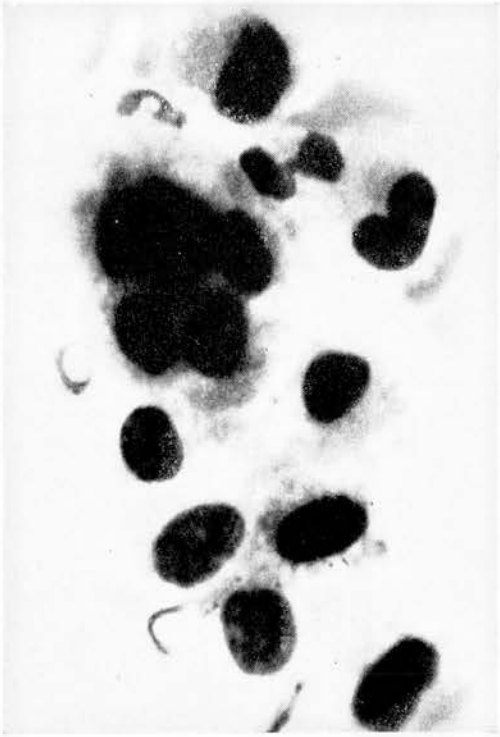

Fig. 4.—Type 1 cells. A multinucleate form is present. Stain: Jenner–Giemsa. × 945.

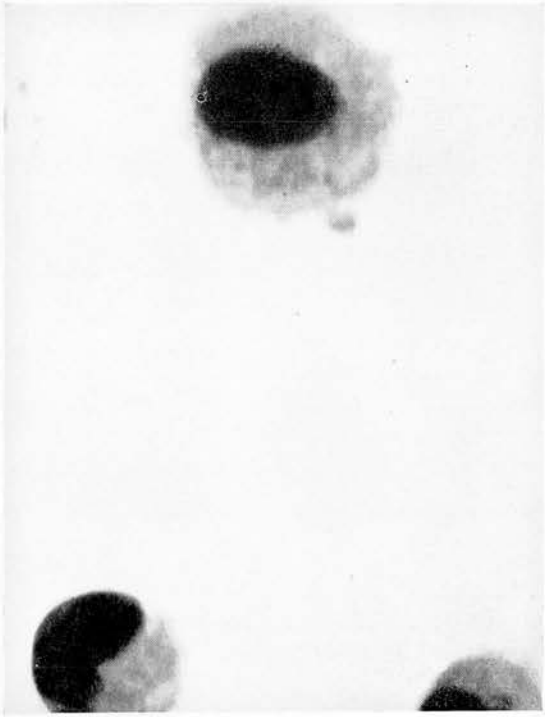

Fig. 5.—Type 1 cell, showing cytoplasmic extensions, which often give a beaded appearance to the cell membrane. Stain: Jenner–Giemsa. × 2,000.

Also seen was another mononuclear cell differing from the Type 1 cell in that it was smaller with an eccentric often indented nucleus, a smooth cell outline, and fewer cytoplasmic particles (Fig. 3). It had the appearance and characteristics of a monocyte. Occasional lymphocytes, polymorphonuclear leucocytes, and mast cells were also observed.

The average differential count in smears from five normal *post mortem* joints was as follows:

Type 1 cells 73 per cent., Type 2 cells 14 per cent., lymphocytes 6 per cent., monocytes 3 per cent., polymorphonuclear leucocytes 3 per cent., mast cells 1 per cent.

On staining with periodic acid-Schiff (PAS) stain, the cytoplasm of Type 1 cells was usually weakly positive. It was granular and appeared to be arranged in a similar way to the azurophilic granules described earlier. Type 2 cells were unstained by this technique (Table, overleaf).

Pyroninophilic staining, indicating the presence of ribonucleic acid (RNA), was observed in both cell types after staining with methyl green-pyronin. Type 1 cells showed a granular staining scattered

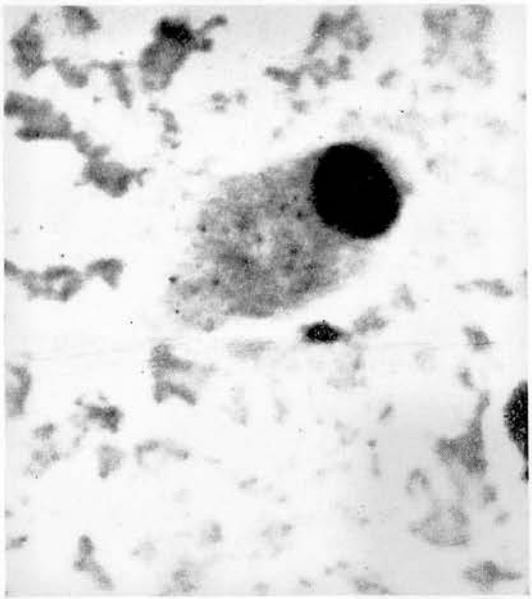

Fig. 6.—Type 1 variant, showing an eccentric nucleus and cytoplasm filled with granules. Stain: Jenner–Giemsa. × 1,200.

TABLE
COMPARISON OF THE STAINING REACTIONS OF TYPE 1 AND TYPE 2 SYNOVIAL CELLS

Stain	Type 1 cell	Type 2 cell
1. Jenner–Giemsa	Cytoplasm: Grey-blue Numerous azurophilic granules Nucleus: Scattered chromatin Prominent nucleoli	Cytoplasm: Deeply basophilic fading towards tip of process No granules Nucleus: Darkly stained Nucleoli not usually seen
2. Methyl green-pyronin	Cytoplasm: Faint granular pyroninophilia Nucleus: Two or more prominent nucleoli	Cytoplasm: Deep pyroninophilic staining fading near tip Nucleus: Nucleoli difficult to distinguish
3. Periodic acid-Schiff	Granular cytoplasmic staining	Unstained
4. Toluidine blue	Occasional metachromatic granules	Unstained
5. For lactic dehydrogenase	Moderate staining Several short irregular cytoplasmic processes	Heavily-stained cytoplasm A single long cytoplasmic process

throughout the cytoplasm. Nucleolar staining was also marked in these cells. Type 2 cells gave intense, general cytoplasmic staining, strongest around the nucleus and fading towards the tip of the cytoplasmic process. Nucleoli were not so easy to distinguish. All pyroninophilic staining was abolished by pre-treatment with ribonuclease.

The majority of cells failed to stain metachromatically with toluidine blue. Occasional Type 1 cells did, however, appear to contain faintly metachromatic granules. The mast cells present gave strong metachromatic staining.

In unfixed smears stained to demonstrate lactic dehydrogenase, long cytoplasmic processes were seen in both Type 1 and Type 2 (Fig. 7). However, considerable variation occurred, some cells having numerous, long branching processes and others very short processes or none at all. This staining technique gave better morphological detail than was seen in fixed smears stained by Jenner–Giemsa stain. Comparable results were obtained when fresh frozen sections of normal synovium were stained to demonstrate lactic dehydrogenase. Similar long cytoplasmic processes were seen (Fig. 8, opposite) and when sections were cut tangentially across the surface of the membrane it was possible to observe the orderly arrangement of these cytoplasmic processes (Fig. 9, opposite).

This arrangement of the cells was not very easily seen in paraffin sections stained routinely. Paraffin sections of synovium stained by the methyl green-pyronin technique demonstrated pyroninophilic staining in most of the lining cells. The majority of the cells showed a fine granular staining while occasionally other cells gave a deep red staining of their cytoplasm.

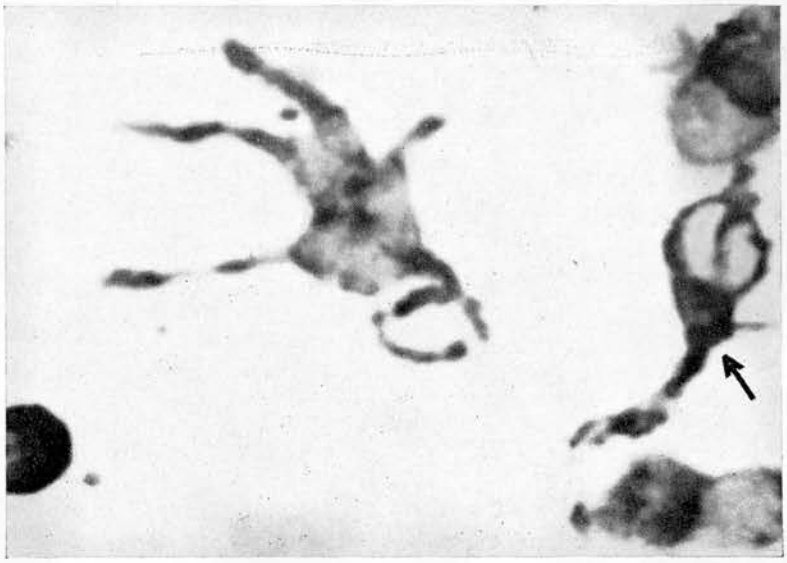

Fig. 7.—Type 1 cell with cytoplasmic processes and a typical Type 2 cell (arrow). Stain: for lactic dehydrogenase. × 1,134.

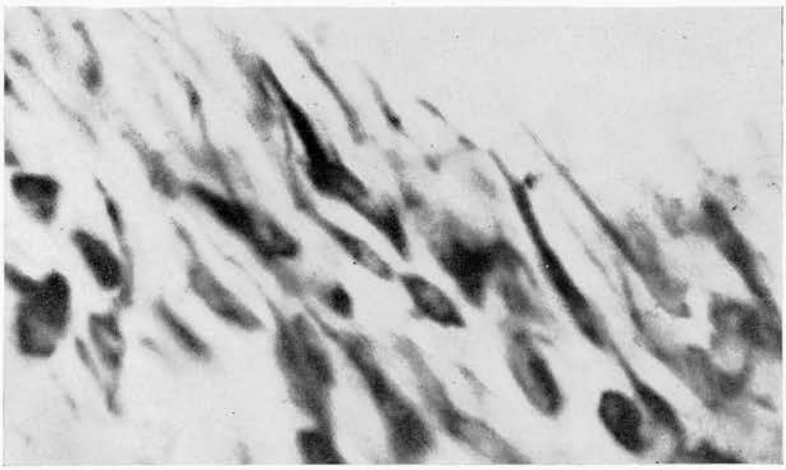

Fig. 8.—Section of normal synovium, showing long cytoplasmic processes of lining cells. Stain: for lactic dehydrogenase. × 756.

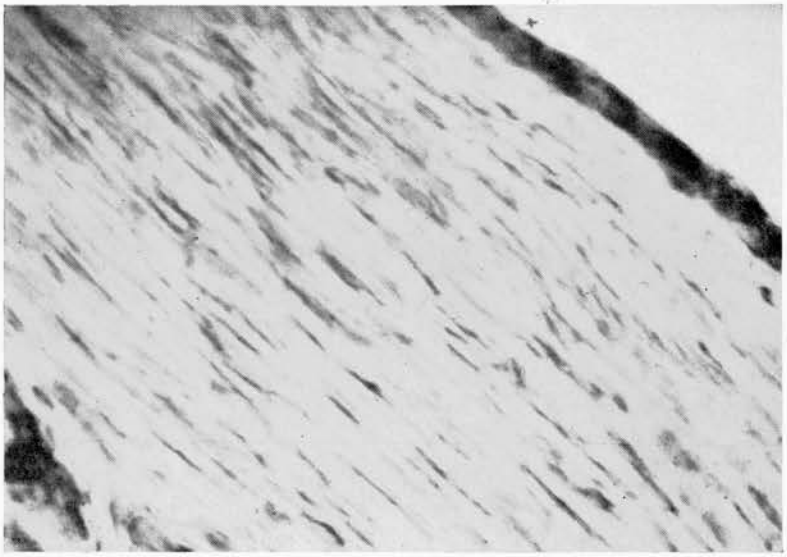

Fig. 9.—Section of synovium. This section was cut tangentially across the surface of the membrane. Note orderly appearance of cytoplasmic processes. Stain: for lactic dehydrogenase. × 300.

Examination of smears of normal synovial fluid cells taken before the introduction of trypsin demonstrated a cell population which was virtually identical with that seen after the addition of trypsin. Far fewer cells were available for examination in these preparations so that differential counts were not very reliable, but the impression gained was that the distribution of cells was about the same as in smears made after trypsin treatment except for a slight increase in lymphocytes and the absence of mast cells.

Over a hundred smears of synovial fluid cells from several different pathological conditions were examined. Considerable differences were found in these smears, but in almost all it was possible to demonstrate cells similar in structure to the Type 1 and Type 2 cells seen in the normal fluid smears.

Synovial Cell Culture

Variable proportions of the cells obtained by trypsinization of joints were viable, as judged by the numbers spread out on the glass after overnight incubation. The proportion depended mainly upon the interval after death which elapsed before the cells were obtained and also apparently upon the age of the patient. Leaving the cells for as short a time as possible in the trypsin also improved the yield of viable cells. The best results were obtained when cells were harvested within 6 hours of death, although a considerable number of viable cells were still present as long as 12 hours after death.

The morphology of cells stained after some days in culture was similar to that of the cells described by Fraser and co-workers (1961, 1965). Variations in

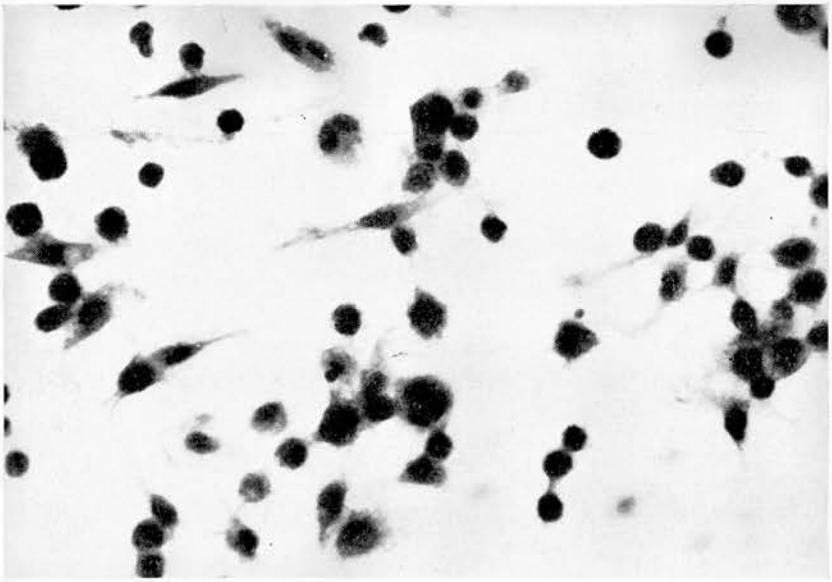

Fig. 10.—Synovial cells (after 48 hrs' culture), showing marked variation in morphology. Stain: Jenner–Giemsa. × 380.

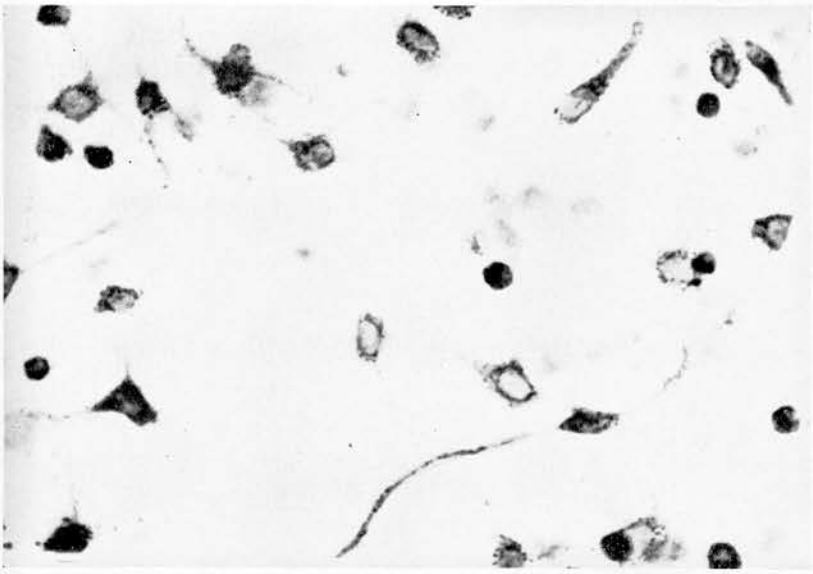

Fig. 11.—Synovial cells (after 48 hrs' culture), stained to demonstrate lactic dehydrogenase. Numerous delicate processes are seen. Stain: for lactic dehydrogenase. × 480.

shape and size of cells were noted: some showed numerous cytoplasmic processes; others were rounded with large eccentric nuclei, prominent nucleoli, and basophilic cytoplasm; still others were spindle-shaped (Figs 10 and 11). It was impossible to say whether one original cell type or more was responsible for these appearances.

Protein Synthesized by the Cultured Cells

Although there was usually no difficulty in keeping the cells alive and spread over the glass for periods of a week or longer, it was more difficult to produce an established culture of dividing cells. On some occasions a contaminant appeared after about one week but usually the cultures remained sterile. Even in sterile cultures the cells did not proliferate, or did so only for a short period. The large numbers of cells required for protein analysis were obtained from only one cell preparation. These cells were cultured for the first few weeks in medium containing 5 per cent. chick embyro extract and 15 per cent. calf serum and subsequently in the same medium without embryo extract. The cells survived for about 4 months.

On starch-gel electrophoresis one of the principal components of the extract was a protein band with an electrophoretic mobility similar to that of α_2-macroglobulin (Fig. 12). The extract also contained traces of another protein with an electrophoretic mobility approximately the same as that of albumin. At least three antigenically distinct components were present on immuno-electrophoretograms using a rabbit antiserum to whole human serum (Fig. 13). The protein extracts were also examined by gel diffusion, using a specific rabbit antiserum to human serum α_2-macroglobulin which had been absorbed with the calf serum in which these cells had been cultured. The absorption step eliminated cross-reactions which might have occurred with calf serum proteins adsorbed on the cells (James, 1965b), but revealed serum proteins manufactured by the cells themselves (Fig. 14, overleaf). The antiserum to human IgM was not absorbed with calf serum as it did not cross-react with calf serum globulins (see Fig. 14). The analysis revealed that the synovial cell extract contained at least three immunologically distinct components, one of which appeared to be identical with human serum α_2-macroglobulin while another reacted with an antibody in the antiserum to human IgM (see Fig. 14). The failure to detect more than one major component on starch-gel analysis is probably due to the failure of large molecular weight

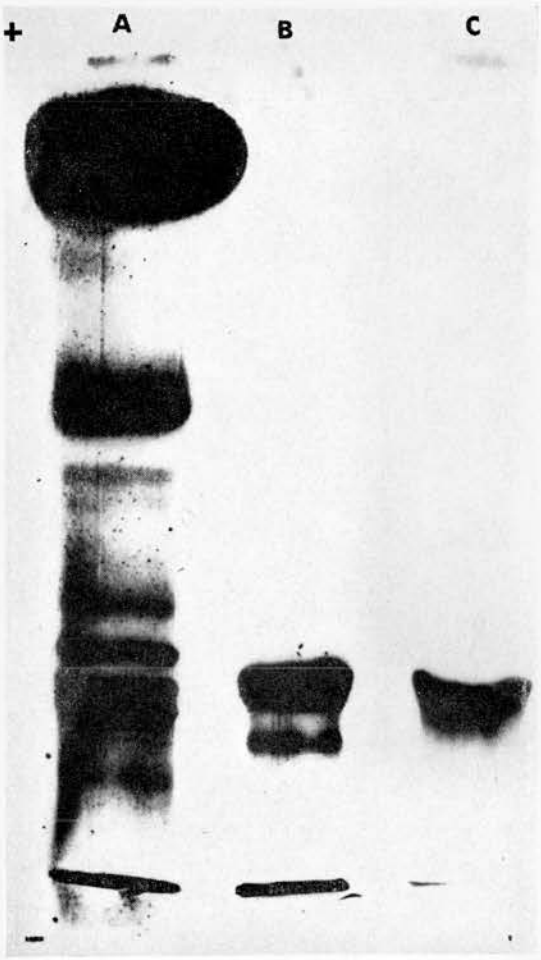

Fig. 12.—Starch-gel electrophoresis of synovial cell extract. Normal human serum (A); human serum α_2-macroglobulin (B); synovial cell extract (C). Electrophoresis conducted at 20 volts/cm. for 6 hrs.

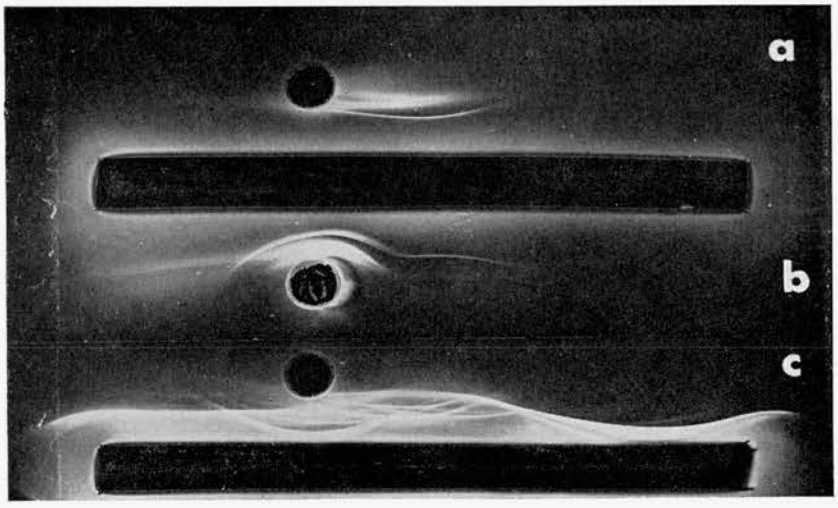

Fig. 13.—Immuno-electrophoretic analysis of synovial cell extract.

A = human serum α_2-macroglobulin

B = synovial cell extract

C = normal human serum

Antiserum = rabbit anti-human whole serum.

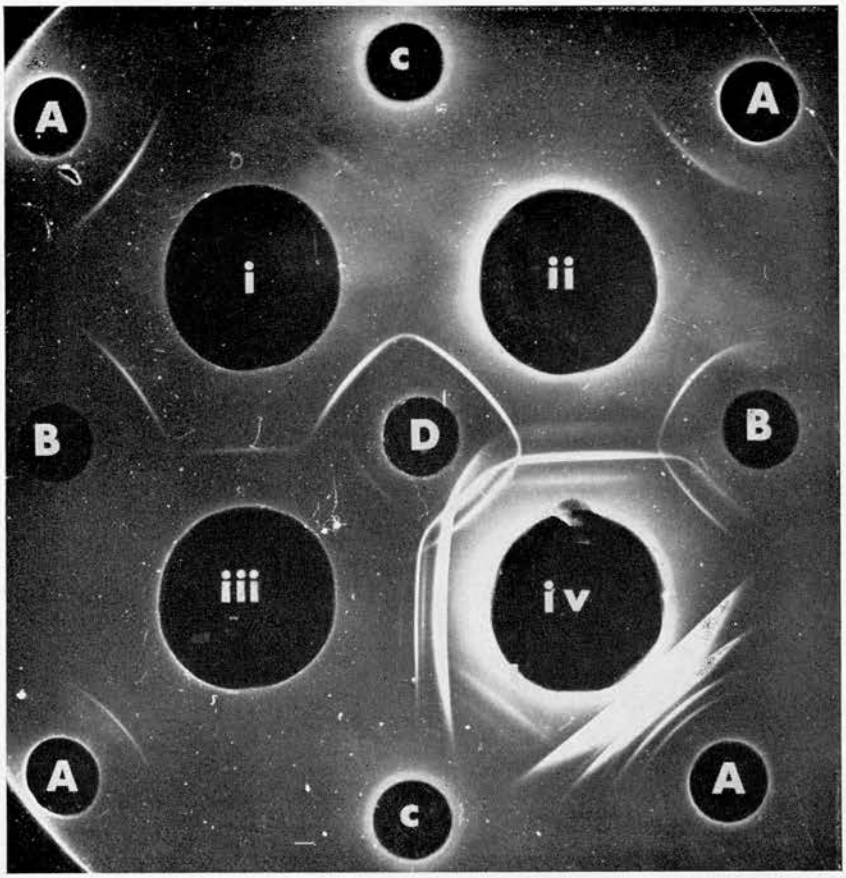

Fig. 14.—Gel diffusion precipitin analysis of synovial cell extract.
Antigens: A = normal human serum
B = human serum α_2-macroglobulin
C = calf serum
D = synovial cell extract
Rabbit antisera to: (i) human α_2-macroglobulin
(ii) human α_2-macroglobulin absorbed with calf serum
(iii) human IgM (19S γ) globulin
(iv) whole human serum

proteins (*e.g.* IgM globulin) to penetrate the gel. The component moving with the same mobility as albumin did not apparently react with the polyvalent antiserum used. The control extract of Hela cells did not show these proteins when subjected to an identical procedure.

Labelling of Synovial Proteins *in vitro* with ^{14}C Lysine

Because of the difficulty in establishing synovial cell lines and the cell selection involved, it was decided to examine the proteins synthesized by freshly-isolated synovial cells. It was not possible to do this by simple extraction of the cells, partly because of the small quantity of cells obtained from each joint and also because of the possibility that human serum proteins might be present on or in the cells. To avoid this source of error, the radioactive amino acid incorporation technique was used. On radio-immuno-electrophoresis of the extracts, a labelled α-globulin line was obtained. It corresponded to a faint precipitin line on the immuno-electrophoretogram which did not appear to be α_2-macroglobulin (Fig. 15, opposite). Moreover, it appeared with only one of the antisera employed. The line did not appear with the polyvalent antiserum. It appeared with only one of the two preparations of joint cells tested.

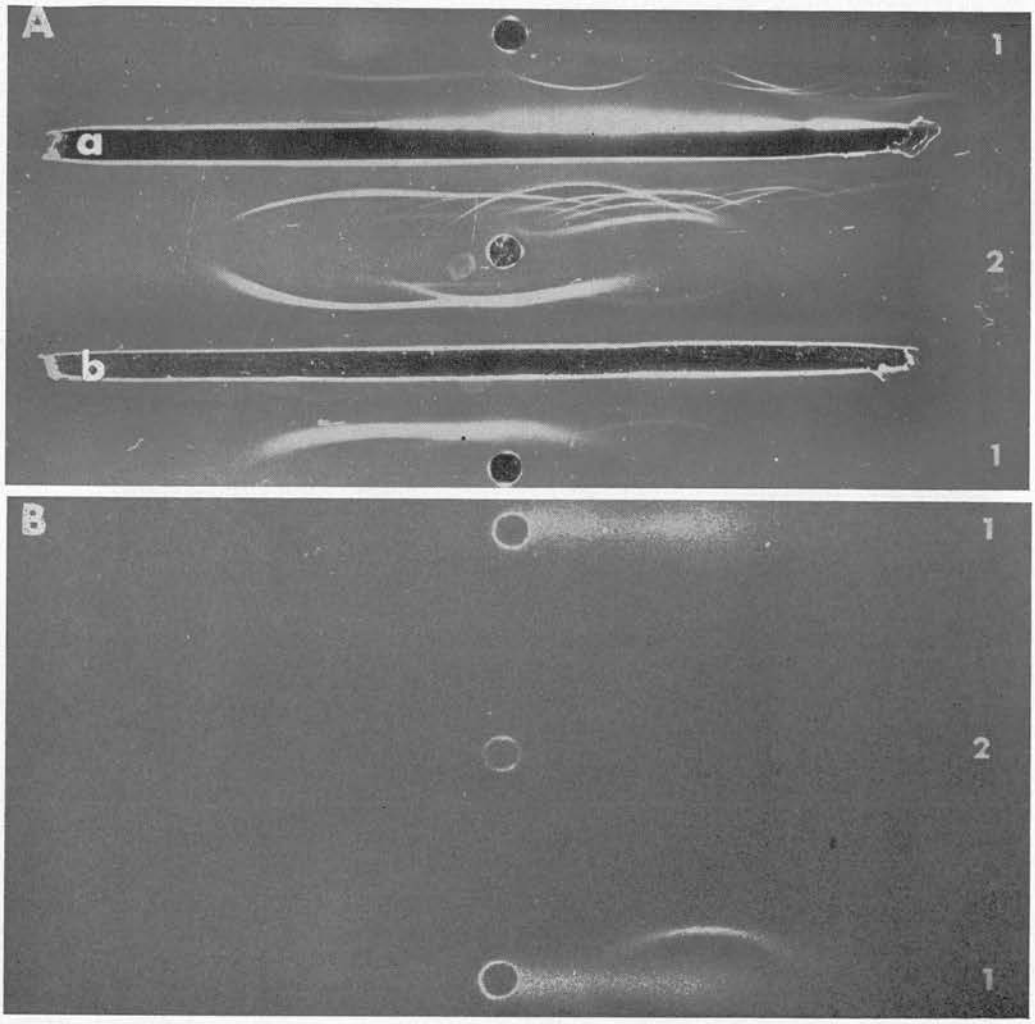

Fig. 15.—Radio-immuno-electrophoresis of synovial cells grown in ^{14}C lysine.

A = immuno-electrophoresis of two synovial extracts (1 and 2) asantigens, together with human serum acting as a carrier

Antisera = (a) rabbit anti-human whole serum
(b) rabbit anti-human gamma globulin

B = autoradiographic picture of immuno-electrophoresis shown in A.

Note marked line in α region of synovial extract I when run against anti-human gamma globulin serum.

Discussion

Morphology

Fraser and Catt (1961) and Fraser and McCall (1965) described a method of trypsinization of knee joints at autopsy as a means of obtaining synovial cells for tissue culture. They concerned themselves mainly with the appearance of cells after culture and gave little account of the morphology of the cells immediately after isolation. That these cells were in fact synovial cells was implied by their cultural characteristics. Further evidence was offered by histological examination of the synovial membrane after the cells had been harvested (Fraser and McCall, 1965). This method gives an excellent means of observing single cells and an opportunity for closer morphological study of synovial cells.

Several studies of normal human and other mammalian synovia using the electron microscope have been made during recent years (*e.g.* Lever and Ford, 1958; Barland and others, 1962, 1964). The work of Barland and others (1962) was of considerable interest in that they distinguished for the first

time two cell types (Types A and B) in normal human synovium. These have since been described in other mammalian synovia (Chapman, Muirden, Ball, and Hyde, 1962; Wyllie, More, and Haust, 1964).

In our study of preparations obtained from knee joints at autopsy it was possible to distinguish two cell types (Types 1 and 2). If it is presumed that these cells have originated from the synovial membrane, then it is probable that the predominant Type 1 cell corresponds to the predominant Type A cell of Barland, and similarly the Type 2 cell corresponds to the Type B cell.

The two cell types were morphologically distinct and it was not possible to distinguish an intermediate cell type as suggested by Chapman and others (1962) and Muirden (1964). However, variants of the Type 1 cell were frequently observed and it is probable that these represent the intermediate types, but in our preparation these cells bore little relationship to the Type 2 cells. Smears made from cultures of synovial cells demonstrated numerous variants and it was not possible to distinguish any specific cell types. Castor and Muirden (1964) described the ultrastructure of synovial cells in culture and they were unable to distinguish different cell types, for these cultured cells had features common to both Type A and B cells.

The staining characteristics of the Type 1 and 2 cells seen in our preparations were consistent with the ultrastructure of Type A and B cells as described by Barland and others (1962). Cytoplasmic basophilia with Jenner–Giemsa stain and deep pyroninophilia with methyl green-pryonin stain in the Type 2 cell suggest the presence of abundant ergastoplasm, whereas the less marked staining in the Type 1 cell is consistent with the sparse ergastoplasm described in Type A cells.

Comparable staining was obtained with sections of synovial membrane. This finding is contrary to that of Shaw and Martin (1962). Much confusion exists in the literature regarding the staining reactions of normal synovial membrane, so the use of smears may enable a clearer concept of the reaction of the two types of cell. The earlier work on the production of mucin and staining reactions of synovial membrane has been reviewed by Bauer, Ropes, and Waine (1940), Davies (1943), Ropes and Bauer (1953), and more recently by Hamerman and Schubert (1962) and Davies (1964).

In our preparations staining with toluidine blue gave faintly positive metachromasia in a small proportion of the Type 1 cells but we feel this to have little significance as it probably represents phagocytosed material.

Staining with periodic acid-Schiff (PAS) reagent gave faint but definite granular staining in the cytoplasm of almost all the Type 1 cells but negative staining in the Type 2 cells. It seems possible that the positive PAS staining which we observed was due to the action of this stain on the lipoprotein membrane of the lysosomes (DeDuve, 1963).

Having established the morphological and staining characteristics of synovial cells in our preparations, an attempt was made to recognize these cells in smears from normal and pathological synovial fluids. Two cell types were distinguishable. The cells simply designated as "synovial cells" by earlier workers (Coggeshall and others, 1940; Davies, 1943; Ropes and Bauer, 1953) apparently correspond to the Type 2 cells of our system. However the Type 1 cell was the predominant type of cell in normal synovial fluid and we presume that such cells correspond to the clasmatocyte and perhaps to some of the monocytes described by these earlier workers.

Staining for lactic dehydrogenase emphasised morphological detail of the cytoplasmic processes characteristic of synovial cells. However, there are still many mononuclear cells without processes.

Function

Since Vaubel (1933) gave the original description of the culture of explants of synovial cells many authors (*e.g.* Bartfeld, 1965; Stanfield and Stephens, 1963) have cultured synovia by a similar technique. No substantial difference in the behaviour of normal and rheumatoid synovia in culture has appeared. Bartfeld observed an increase in giant cell forms in rheumatoid synovia but Stanfield and Stephens found giant cells in about equal proportions in both normal and rheumatoid synovia. We have frequently seen multinucleate cells in smears from joints of normal people.

Fraser and Catt (1961) reported that the addition of serum from rheumatoid patients had a cytotoxic effect in culture on the cells obtained from knee joints at autopsy. We have not been able to confirm this. Myhre (1963) found that certain DEAE cellulose fractions of rheumatoid sera were toxic to human lines and synovial cells as well as being cytolytic to human leucocytes. In spite of our failure to find any specific cytotoxic effects of rheumatoid sera in our cultures, immunospecific phenomena may occur in this disease.

A vast quantity of literature has accumulated describing synovial cell structure and function, and variations encountered in disease states, and there has been much speculation on the origin of synovial fluid and the production of the mucin-like substance, hyaluronic acid. The secretion of this mucosubstance in synovial cell cultures has been recorded by several authors (*e.g.* Vaubel, 1933a, b; Kling, Levine, and Weiss, 1955; Castor and Fries, 1961).

So far it has not been definitely established which of the synovial cell types is responsible for hyaluronate production. One view (that of Barland and others, 1962) is that the Type A cell has a dual function being both secretory and phagocytic.

The Type B cell is structurally adapted for a synthetic role. Coulter (1962) noted the frequent alignment of cysternae or elongated endoplasmic reticulum parallel to the plasma membrane in these cells, and in the opinion of Kemp (1957) this arrangement is characteristic of a protein-producing cell. The nature of the protein secreted is a matter of some interest for it has been presumed for some time that the proteins in synovial fluid all originate from the plasma and that synovial cells play no role in their formation (Decker, McKenzie, McGuckin, and Slocumb, 1959; Hamerman and Schubert, 1962). However, further study (Sandson and Hamerman, 1962) demonstrated the presence in normal synovial fluid of a protein bound to a small percentage of the hyaluronate, and it was thought probable that this protein might participate in the synthesis of hyaluronate in the synovial membrane cells, or that the protein might be added to the hyaluronate at the cell wall and play a role in the passage of hyaluronate into synovial fluid. Hamerman and Sandson (1963) described the formation of a much higher proportion of unusual hyaluronate protein complex in pathological fluids and they considered it possible that cells in the pathological synovial membrane synthesized this unusual compound of hyaluronate and protein. Sandson and Hamerman (1964) demonstrated that an alpha globulin was bound to this hyaluronate protein of pathological synovial fluids. Tests with specific antisera showed that this protein was not ceruloplasmin haptoglobin, or α_2-macroglobulin. An antiserum to hyaluronate protein has been employed by Blau, Janis, Hamerman, and Sandson (1965) to demonstrate by the immunofluorescence technique the presence of this protein in normal synovial lining cells, and it was presumed that this protein was synthesized by these cells.

Recently Kitlowski, Mooney, Rodnan, and Mankin (1965), using radioactive amino acid incorporation techniques with articular cartilage and synovium of rabbits *in vitro*, found a rapid incorporation of ^{3}H-tryptophan, suggesting a considerable synthesis of some protein or proteins other than collagen.

Our results suggest that a protein of α-globulin-like mobility which is antigenically related to serum α_2-macroglobulin is synthesized by cultured synovial cells and that other proteins antigenically related to serum proteins are also synthesized by these cells. It should be borne in mind, however, that polyvalent antisera often contain antibodies to cellular (*e.g.* erythrocyte and leucocyte) proteins as well as to serum proteins, and some cross-reaction of this sort cannot be completely excluded. However, similar findings were not observed with cultures of Hela cells used as controls.

The function of the synthesized α-glycoprotein still remains obscure, but it is of interest that Marr, Owen, and Wilson (1962) demonstrated the presence of an α-macroglobulin in foetal calf serum, and that this protein was one of two glycoproteins having a growth promoting effect in tissue culture. These workers felt that a possible function of these proteins was as carriers of smaller biologically active molecules. Recently James, Johnson, and Fudenberg (in preparation) demonstrated that α-globulin, especially α_2-macroglobulin, appeared to interact with a large number of enzymes and hormones and this may be of extreme importance in cellular development. In synovial fluid this may assist in the nutrition of articular cartilage. Any changes in the level of synthesis of this α-macroglobulin may have a profound effect on the viability of the cartilage and further investigation of this subject is required in pathological conditions, especially in degenerative joint disease.

Summary

Two types of synovial cells (Type 1 and 2) were distinguished in smears of cells obtained from knee joints at autopsy after trypsinization of the joint.

The morphology and staining characteristics are described and these are compared with normal synovial membrane.

Comparisons are also made with the ultrastructure of synovial membrane as reported by other workers.

Similar types of cells were observed in both normal and pathological synovial fluid smears.

Synovial cells in culture were examined.

Evidence is presented for the production by synovial cells of a protein which is antigenically related to serum α_2-macroglobulin. A possible role of this glycoprotein as a carrier protein to assist nutrition of articular cartilage is considered.

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Les cellules synoviales
Étude de la morphologie et de la synthèse protidique

RÉSUMÉ

On distingua deux types de cellules synoviales (Type 1 et type 2) dans des frottis obtenus à partit de genoux lors d'autopsies après trypsinisation de l'articulation.

On décrit leur morphologie et leurs caractéristiques de coloration et on les compare avec ce qu'on observe dans une membrane synoviale normale.

On fait également des comparaisons avec l'ultrastructure de la membrane synoviale observée par d'autres auteurs.

Des types identiques de cellules ont été notés sur des frottis provenant de liquides synoviaux normaux aussi bien que pathologiques.

On examina des cultures de cellules synoviales.

On fournit des preuves de la production par les cellules synoviales d'une protéine antigéniquement apparentée à la macroglobuline-a_2. On prend en considération le rôle possible de cette glycoprotéine comme support protéique facilitant la nutrition du cartilage articulaire.

Las células sinoviales
Un estudio de la morfología y un examen de la síntesis protéica de las células sinoviales

SUMARIO

Se distinguieron dos tipos de células sinoviales (tipos 1 y 2) en tomas de rodillas obtenidas durante autopsias después de triptinización de la articulación.

Se describe la morfología y características tincionales y se comparan con lo que se observa en membrana sinovial normal.

Comparaciones se hacen también con la ultraestructura de la membrana sinovial según descrita por otros autores.

Tipos semejantes de células se observaron en tomas de líquidos sinoviales tanto normales como patológicos.

Se examinaron cultivos de células sinoviales.

Se ofrecen datos de que las células sinoviales producen una proteina antígenicamente relacionada con la macroglobulina-a_2. Se considera el posible papel de esta glicoproteina como un portador protéico para asistir la nutrición del cartílago articular.

THE ACTIVATION OF HUMAN PERIPHERAL LYMPHOCYTES BY PRODUCTS OF STAPHYLOCOCCI

BY

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The Activation of Human Peripheral Lymphocytes by Products of Staphylococci

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Human lymphocyte populations are activated to enter the mitotic cycle *in vitro* to varying degrees by a number of macromolecular substances. Active preparations include phytohaemagglutinin (Nowell, 1960), staphylococcal filtrate (Ling and Husband, 1964), TAB, tetanus toxoid, diptheria toxoid and smallpox vaccine (Elves, Roath and Israëls, 1963), tuberculin (Pearmain, Lycette and Fitzgerald, 1963; Schrek and Rabinowitz, 1963), leucocyte antibodies (Grasbeck, Nordman and de la Chapelle, 1964) and leucocytes from an unrelated individual (Bain, Vas and Lowenstein, 1964; Bach and Hirschhorn, 1964). The fact that lymphocytes may be activated by products of bacteria commonly encountered, and by tissue products and isoantigens, possibly in an immunospecific manner, as well as by phytohaemagglutinin, has promoted the activation phenomenon to a position of extreme importance in immunology. In addition, the different degrees of response of lymphocytes to the stimuli and the wide range of cellular synthetic activity exposed by the stress of stimulation suggest new fields of cytological investigation. In the present paper the effects produced on lymphocytes by two agents of outstanding potency, phytohaemagglutinin and an extracellular product of staphylococci, are compared.

METHODS AND MATERIALS

Preparation of Leucocyte Cultures

Blood was collected aseptically in preservative-free heparin (20 units per ml. blood) in a screw-capped bottle. After addition of one-tenth volume of Benger's 6 per cent dextran in saline, surface air bubbles were exploded with a hot glass rod and a fresh sterile cap was placed loosely on the bottle. The blood was allowed to sediment at 37° C. for 1 hour. The plasma layer containing most of the WBC and about the same number of RBC was sampled for counting and aliquots (usually 0.5 ml.) containing $1–2 \times 10^6$ WBC distributed into 5 ml. bijou bottles containing the substance under test. Culture medium (2.4 ml.) was added and the tightly-capped bottles were incubated at 37° C. for 4 days with occasional mixing. The cell suspensions were then transferred to $3 \times \frac{1}{2}$ in. tubes and centrifuged lightly for 5 minutes. Most of the supernatant fluid was removed and, after a minute or so to allow fluid to drain down the side of the tube, the remainder of the supernatant fluid was removed as completely as possible and the cell deposit (volume approximately 0.01 ml.) taken up in the tip of a clean, fine well-cut Pasteur pipette. Smears were made, fixed for 10 minutes in methanol and stained with Jenner–Giemsa. Counts of 500–1000 lymphocytes were routinely made. Only grossly enlarged cells with prominent nucleoli and highly basophilic cytoplasm were counted as 'transformed' cells. The culture media used were (a) Trowell's hypotonic lymphocyte medium (Trowell, 1963) and (b) Eagle's medium enriched with one-tenth volume of tryptose broth, prepared in the Department of Virology and Bacteriology, University of Birmingham. Both media contained penicillin (200 units per ml.) and streptomycin (100 units per ml.).

Variants of the Procedure

(a) On some occasions lymphocyte suspensions were prepared from defibrinated blood after the addition of high molecular weight gelatin (The Gelatine and Glue Research Association, batch No. 277) according to the procedure of Coulson and Chalmers (1964).

(b) Smears to be treated with fluorescein-conjugated rabbit anti-human γ-globulin (procedure of Elves, Roath, Taylor and Israëls, 1963) were prepared from cells washed twice with phosphate-buffered saline.

(c) ^{3}H-thymidine was the Amersham product (thymidine-(methyl-T), 500–5000 mc./mM); 0.5 μc. was added to cultures 2 hours before harvesting, smears were fixed, washed and coated with Kodak AR 10 stripping film following the technique of Doniach and Pelc (1950). The autoradiographs were developed for 3 days at 4° C. and stained through the gelatin by a method similar to that of Gude, Upton and Odell (1955).

(d) ^{3}H-uridine (Amersham-uridine-5, 6-T(n), 500–3000 mc./mM) was similarly employed except that it was added to the culture 24 hours before harvesting.

(e) ^{32}P phosphate (5 μc.) was added to cultures containing the usual amount of phosphate at zero time. Triplicate cultures were set up in McConkey bottles and placed, with loosely fitting caps, in an air-tight container and gassed with 5 per cent CO_2 in air as recommended by McIntyre and Ebaugh (1962). Cell pellets were analysed for ^{32}P components by the following modification of the Schmidt–Tannhauser procedure: the small pellet of cells was washed twice in warm saline then treated with 2 × 2 ml. of ice cold 5 per cent trichloroacetic acid, the supernates being discarded. The pellet was extracted successively with 2 ml. volumes of acetone, ethanol–ether (3 : 1) and ether (phospholipid fraction). The dry pellet was suspended in N NaOH (1 ml.) for 90 minutes, acidified with N perchloric acid (2 ml.) and centrifuged. The pellet was washed with 0.5 N perchloric acid (2 ml.) and redissolved in N NaOH (DNA fraction) the supernate and washings comprising the RNA fraction. The fractions were counted in a Geiger-Müller liquid counter. Inorganic phosphate determinations were kindly performed by Mr. T. P. Whitehead.

Staphylococcal Cultures

Untyped staphylococci var. aureus were grown for 5 days at 37° C. in bottles almost full of Parker 199 medium from which antibiotics were excluded. The suspensions were sterilized by filtration through a Millipore filter (0.45 μ). Under these conditions a filtrate of potent lymphocyte-activation property was obtained, with little or no haemolysin activity and of low toxicity. Aerating the medium, increasing the glucose content, or adding agar, decreased the yield of active substance. Filtrates containing tritiated polymeric material were prepared by adding 10 μc. of ^{3}H-glucose (Amersham, D-glucose-6-T, 100–500 mc./mM) to 20 ml. of medium. The radioactive filtrates were treated with three volumes of acetone and the deposit redissolved in medium (20 ml.).

Other Reagents

Staphylococcal α- and β-haemolysins were obtained from Burroughs Wellcome and Co. Haemolytic activity was titrated against 2 per cent rabbit red cells in plastic agglutination trays.

Serum from a rabbit immune to staphylococcal antigens. This was obtained by giving a rabbit six intra-peritoneal injections of 2 ml. of staphylococcal filtrate over a period of 3 months.

Phytohaemagglutinin M was the Difco product.

RESULTS

The Effect of Dosage and Time of Incubation on Lymphocyte Activation

The level of nucleic acid synthesis over a 4-day culture period of lymphocytes stimulated with either phytohaemagglutinin or staphylococcal filtrate was markedly dependent upon the dose of stimulant used (Figs. 1 and 2). With phytohaemagglutinin both RNA and DNA synthesis increased linearly with concentration over the lower dose range with a RNA/DNA

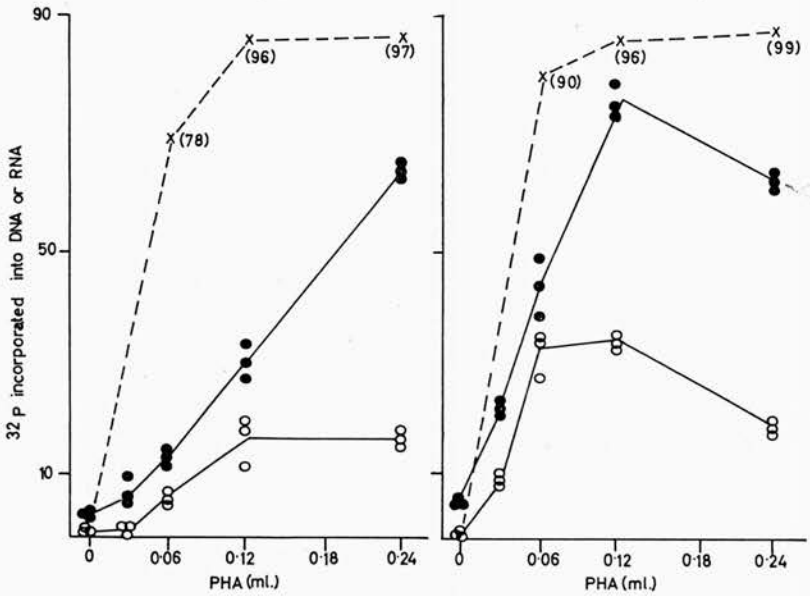

FIG. 1. The incorporation of ^{32}P-phosphate into RNA (●) and DNA (○) (counts/sec. per 10^6 leucocytes) at various concentrations of phytohaemagglutinin. ×, per cent blasts. Two separate batches of cultures are represented.

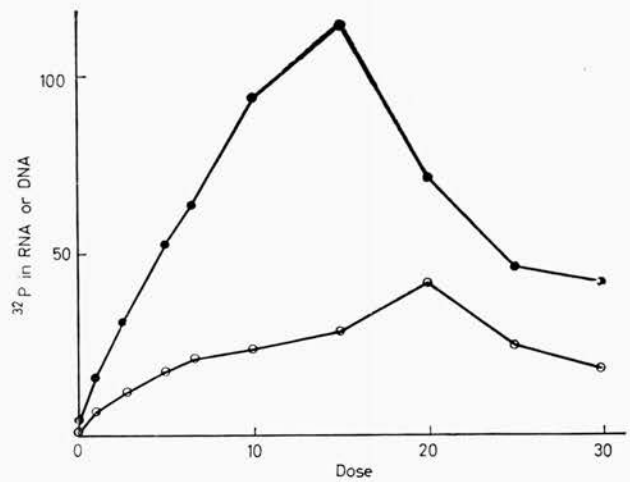

FIG. 2. The incorporation of ^{32}P-phosphate into RNA (●) and DNA (○) (expressed as counts/sec. per 10^6 WBC) at various concentrations of staphylococcal stimulant. A 10× concentrate of active staphylococcal material was prepared as follows: three volumes of acetone were added to one volume of staphylococcal filtrate. The precipitate which formed was separated by centrifugation and dissolved in one-tenth volume of culture medium.

ratio of approximately 1.9. At a dose level where most of the cells were activated a further increase of the concentration of phytohaemagglutinin had little effect on the level of DNA synthesis but still increased the RNA level indicating a direct stimulation of the rate of RNA synthesis per cell. A similar rise in RNA and DNA also occurred with increasing concentrations of staphylococcal stimulant but RNA saturation of response occurred a little before that

TABLE I

COMPARISON OF THE EFFICIENCY OF PHYTOHAEMAGGLUTININ AND STAPHYLOCOCCAL FILTRATE AT THEIR OPTIMAL CONCENTRATIONS IN PROMOTING THE SYNTHESIS OF NUCLEIC ACID

Stimulant/3 ml. culture	^{32}P-*RNA*	^{32}P-*DNA*
Staphylococcal filtrate (1.5 ml.)	24.5	12.0
Phytohaemagglutinin (0.12 ml.)	18.5	9.7
None	3.3	0.2

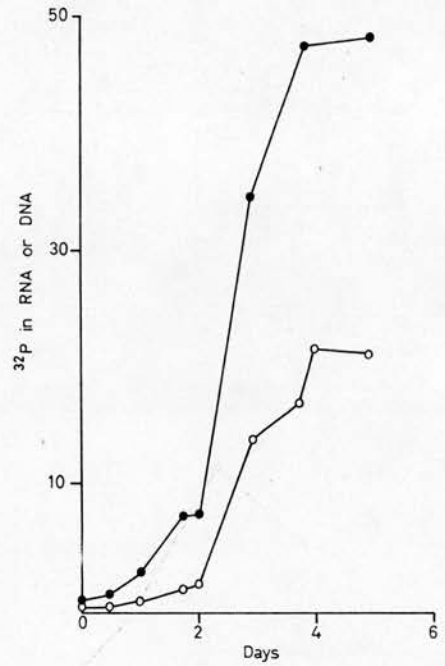

FIG. 3. The incorporation of ^{32}P-phosphate into the RNA (●) and DNA (○) fractions of staphylococcal stimulated cells (expressed as counts/sec. per 10^6 WBC) at various periods of culture. Staphylococcal filtrate: 0.5 ml. per 3 ml. of culture fluid.

of DNA. In both cases a large excess of stimulant (or impurity in it) appeared to be inhibitory. A high dose of stimulant activated most of the cells over a short incubation period: a low dose activated almost the same number over a longer incubation period. When the two stimulants were tested at their respective maximal stimulatory concentrations, the staphylococcal stimulated cells reached higher levels of DNA and RNA synthesis (Table I); the RNA/DNA ratios were similar in the two cases. Little or no DNA synthesis occurred with either stimulant during the first 24 hours of incubation. Maximal nucleic acid synthesis was reached after 4 days with a standard dose of staphylococcal stimulant (Fig. 3).

Effect of Serum Factors on Nucleic Acid Synthesis of Lymphocytes Stimulated with Staphylococcal Filtrate

Cultures routinely contained 14 per cent autologous plasma. Lower rates of nucleic acid synthesis were obtained when this plasma was replaced by sera of other species. Unstimulated lymphocytes survived well in Trowell's simple medium and, to some extent, preferentially, but stimulated lymphocytes did not reach maximal synthetic activity in it. Replacement of 1 ml. of Trowell's medium by 1 ml. of fresh human serum increased the nucleic acid synthesized over the same culture period to varying degrees depending upon the serum used. The biggest increase was obtained with autologous serum.

TABLE II

THE EFFECT OF SERUM COMPONENTS ON THE NUCLEIC ACID SYNTHESIS OF HUMAN LYMPHOCYTES STIMULATED WITH STAPHYLOCOCCAL FILTRATE

Composition of culture				*RNA synthesis* (^{32}P) *(counts/sec./ 10^6 leucocytes)*	*DNA synthesis* (^{32}P) *(counts/sec./ 10^6 leucocytes)*
Leucocyte-rich plasma (ml.)	*Staph. filtrate (ml.)*	*Culture medium (ml.)*	*Other addition (ml.)*		
0.5	0	Trowell (2.5)	—	3.3	0.2
0.5	0.5	Trowell (2)	—	15.0	6.0
0.5	0.5	Trowell (1)	Autologous plasma (1)	49.4	23.7
0.5	0.5	Trowell (1)	Pool serum (1)	13.5	7.6
0.5	0.5	—	Serum dialysate (2)	50.6	25.3
0.5	0.5	—	Dialysed★ serum (2)	13.1	4.3
0.5	0.5	—	Pooled serum dialysate (2)	51.0	24.4
0.5	0.5	—	Dialysed★ serum (2)	14.5	3.9
0.5†	0.5	—	Serum dialysate (2.5)	35.1	18.2
0.5	0.5	Eagle (2)	—	36.0	19.9
0.5	0.5	—	Eagle dialysed against serum (2)	52.6	27.2

★ Dialysed against 2 volumes of Trowell's medium.
† Plasma removed.

The beneficial effect of serum was contained in the dialysate, whereas dialysed serum was frequently inhibitory (Table II). Maximum growth rates appeared to require growth factors present in Eagle's medium and others present only in serum. Additives of thyroxine, triiodothyronine, insulin and yeast extract were ineffective.

Using the Trowell medium–serum dialysate medium, additives of a variety of human sera either had little or no effect on the levels of nucleic acid synthesis or were inhibitory. The inhibitory effect of sera was unrelated to ABO group (Table III). One of the inhibitory sera was from a patient recovering from a staphylococcal infection. Although the inhibitory substance in this serum was shown to be a macromolecule, possibly an antibody, no detectable

inhibition of transformation occurred in a culture containing inactivated serum from the rabbit immune to staphylococcal products. The serum effects in the phytohaemagglutinin stimulated cultures (Table IV) were less clear-cut. When the lymphocytes from two normal individuals were cultured in parallel with isologous or autologous plasma supplements, slightly higher levels of nucleic acid synthesis were obtained with the isologous combinations. The percentages of lymphocytes transformed at various levels of stimulant were the same in the two media, suggesting that deprivation of certain growth factors did not influence the stimulation process but only the subsequent rate of synthetic activity.

TABLE III

THE NUCLEIC ACID SYNTHESIS OF HUMAN LYMPHOCYTES STIMULATED WITH STAPHYLOCOCCAL FILTRATE AND GROWN IN THE PRESENCE OF TROWELL'S MEDIUM WHICH HAD BEEN DIALYSED AGAINST POOLED HUMAN SERUM (2 VOLUMES: 1 VOLUME)

One ml. of this medium was replaced by 1 ml. of serum where indicated.

Medium (ml.)	*Serum (ml.)*	*ABO group*	*RNA-^{32}P*	*DNA-^{32}P*
2	0	—	24.5	12.0
1	Pooled (1)	—	5.0	3.5
1	Auto (1)	A	23.8	11.8
1	Mr. D (1)	B	22.2	6.2
1	Mr. S. (1)	O	18.4	10.2
1	Miss G. (1)	A	12.2	6.2
1	Miss M. (1)	O	24.4	12.2

The figures given are counts/sec. per 10^6 leucocytes.

TABLE IV

THE EFFECT OF SERUM ON THE NUCLEIC ACID SYNTHESIS OF CELLS STIMULATED WITH PHYTOHAEMAGGLUTININ

Eagle's medium (ml.)	*Serum (ml.)*	*RNA synthesis (^{32}P)*	*DNA synthesis (^{32}P)*
2.4	—	16.5	9.5
1.4	Pool 1 (1)	8.5	4.8
1.4	Pool 2 (1)	14.0	8.2
1.4	Serum dialysate (1)	17.4	10.2

Each culture contained leucocyte-rich plasma (0.5 ml.) and PHA-M (0.1 ml.).

The figures given are counts/sec. per 10^6 leucocytes.

Nature of the Activation Process

The large blast-like cells, with prominent nucleoli and basophilic cytoplasm, formed after stimulation with staphylococcal extract were morphologically indistinguishable from those formed after stimulation with phytohaemagglutinin but tended to have more cytoplasm. Activation of the cells was not immediate (Table V). Lymphocytes from whole blood stored overnight at 4° C. were sensitive to staphylococcal stimulation as were lymphocytes cultured for several days at 37° C. before addition of stimulant. Purified lymphocyte suspensions prepared by gradient centrifugation using bovine albumin solutions were also sensitive. Serum proteins were probably not involved in the activation (Table II). Cells grown for 2 days in the presence of tritiated staphylococcal polymeric material contained radioactive

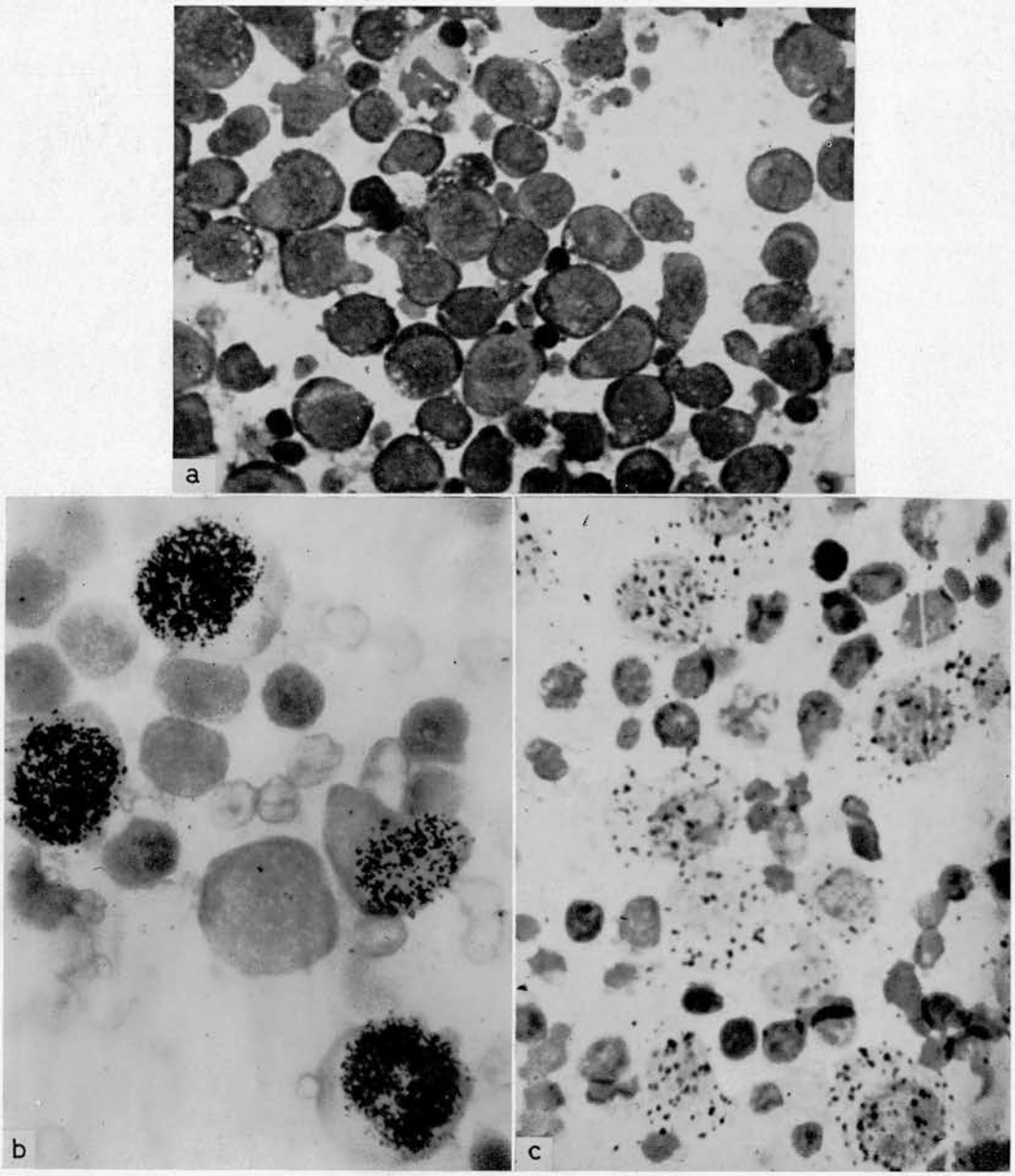

FIG. 4. Morphology and autoradiography of lymphocytes exposed to staphylococcal filtrate. (a) Smear stained with Jenner–Giesma (×630). Culture period, 4 days. (b) Autoradiograph showing heterogeneity in the uptake of ^{3}H-thymidine (0.5 μc., 2 hours) by the transformed cells (×900). Culture period, 3 days. (c) Autoradiograph showing the uptake of ^{3}H-uridine (0.5 μc., 24 hours) into the nucleus and cytoplasm of transformed cells (×630). Culture period, 3 days.

(*Facing p.* 426)

material over the lymphocytes and over other leucocytes present but not over the red cells. After 3 days culture label was no longer present over the activated cells, which had now undergone a blast change, but was still present over the dead granulocytes.

Transformed cells occurred in clumps with the phytohaemagglutinin stimulant but were quite discrete in smears from staphylococcal stimulated cultures (Fig. 4a). Groups of two or three leucocytes were rather more common in stimulated than control cultures as shown by

TABLE V

REACTIVITY OF STAPHYLOCOCCAL FILTRATE WITH LYMPHOCYTES IN RELATION TO TIME OF EXPOSURE OF STIMULANT AND THE EFFECT OF ABSORPTION WITH LEUCOCYTES

Staph. filtrate		*Time cells exposed*	*Blasts (%)*
Volume	*Treatment*		
0	—	—	0.2
0.5	None	15 minutes	0.4
0.5	None	24 hours	68
0.5	None	48 hours	74
0.5	None	4 days	80
0.5	Absorbed with 0.5 ml. packed rabbit spleen cells	4 days	24
0.5	Diluted with 0.5 ml. saline	4 days	34

The total culture time was 4 days.

direct sampling of cultures on days 1–3, but most of the lymphocytes occurred singly. After absorption of staphylococcal filtrate for 24 hours (4 hours at 37° C. and 20 hours at 4° C.) with packed rabbit spleen cells, substantial activity was still present in the supernatant fluid (Table V). The transformed cells did not fluoresce after exposure to fluorescein-conjugated rabbit anti-human γ-globulin.

Heterogeneity of the Cell Response

A marked variation in ^{3}H-thymidine uptake by typical blast cells was found varying from completely negative activity to markedly positive (Fig. 4b). Some of the stimulated cells had presumably entered a post-synthetic period before the isotope was added whereas others had spent the whole of the 2 hours in DNA synthesis. Although grain counts were not undertaken an impression of several steps of activity, rather than a continuous gradation, was obtained. Activated cells took up ^{3}H-uridine into the cytoplasmic RNA as well as into the nucleus on the second day of culture (Fig. 4c). On day 4, by which time many stimulated cells had reached the mitotic stage, a higher proportion of blast cells not in DNA synthesis was found. Mitoses were found on days 3, 4 and 5, again indicating a wide heterogeneity of cell response.

Properties of the Active Material

Most of the active material produced by staphylococci was present in the medium although some activation was produced by extracts of the cocci. Active material survived exhaustive dialysis against saline but staphylococcal filtrate dialysed against an equal volume of culture medium also consistently contained potent activating material in the dialysate. Activity of filtrates was reduced by autoclaving but was little affected by heating in a 100° C. water bath

for 15 minutes. The active material was soluble in 25 per cent (w./v.) trichloracetic acid and saturated ammonium sulphate but was precipitated by 80 per cent (w./v.) acetone and could be redissolved in water with little loss of activity. Incubation with the proteolytic enzyme pronase markedly reduced the activity.

Transforming activity was demonstrable in commercial α- and β-haemolysin preparations (Table VI).

TABLE VI

TRANSFORMATION OF LYMPHOCYTES BY STAPHYLOCOCCAL PRODUCTS

Staph. product	0.1 *ml. of* 1 *in:*				
	1	10	100	500	2000
α-Haemolysin	Toxic	Toxic	0	++	(+)
β-Haemolysin	Toxic	++	++	++	(+)
199 filtrate	+++	(+)	0	0	0

Culture volume = 3 ml. in each case.

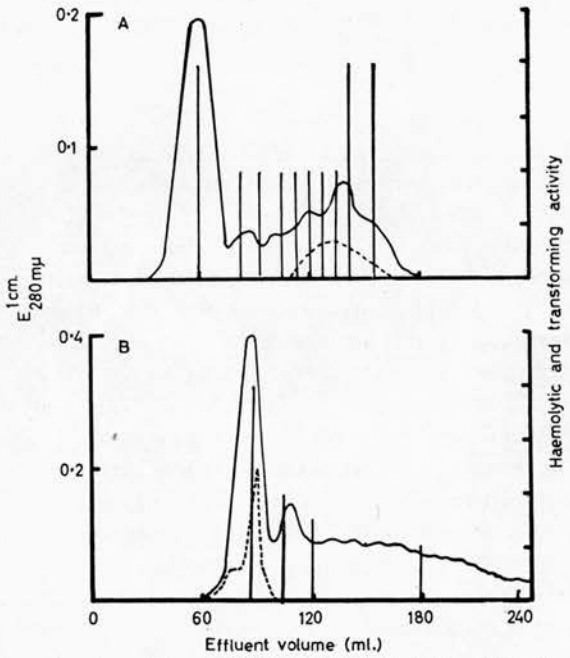

FIG. 5. A. The fractionation of crude staphylococcal filtrate on G-50 Sephadex. B. The fractionation of a commercial α-haemolysin preparation on G-75 Sephadex. Both fractionations were performed at room temperature in phosphate buffer pH 6.9, 0.06 M, containing 0.15 M NaCl. Haemolytic activity (dashed line) and transforming activity (vertical bars) are expressed in arbitrary units. The scale of haemolytic activity is 100 times greater for B than for A.

Partial resolution of the transforming and haemolytic activity of staphylococcal filtrate was achieved on Sephadex G-50 (Fig. 5). In the case of the α-haemolysin the major part of the transforming activity was accompanied by haemolytic activity on DEAE cellulose, gel filtration or G-75 Sephadex and agar gel electrophoresis. The gel filtration distribution of transforming activity in the effluent of the Sephadex column suggested that this activity was

associated with a number of molecules of varying sizes. Only one fractionation of the β-haemolysin was attempted. In this the transforming and haemolytic activities were well resolved, the latter component exhibiting marked affinity for the DEAE cellulose the major part being recovered with the final buffer (0.06 M phosphate, pH 6.9, containing 0.15 M NaCl).

Individual Variations of Response

The lymphocytes of 32 patients of various ages tested with a standard dose of staphylococcal filtrate showed a marked response. One normal individual recently recovered from a staphylococcal infection and one patient with an acute haemolytic crisis following arsenic poisoning were hyporesponsive.

DISCUSSION

Nordman, de la Chapelle and Gräsbeck (1964) have shown that the mitotic stimulant activity of extracts of red kidney beans is closely correlated with their leucoagglutinin content. In support of the view that stimulation of lymphocytes is brought about by attachment of large molecules to their surface these authors showed that rabbit antisera to human leucocytes contained stimulatory activity which could be removed by absorption with leucocytes (Gräsbeck *et al.*, 1964). Vassur and Culling (1964) demonstrated an alteration in the surface charge of lymphocytes exposed to phytohaemagglutinin. Using fluorescein-labelled phytohaemagglutinin Michalowski, Jasinska, Brzosko and Nowosławski (1964) found labelled material over the nucleus whereas using fluorescein-labelled PPD, Witton, Wang and Killian (1963) found fluorescent material in the cytoplasm of lymphocytes from tuberculin-positive persons. Labelled staphylococcal polymer which becomes attached to lymphocytes in culture probably to their surface, may or may not have been the essential activating agent. Most of the surface material disappeared after the transformation process had proceeded, suggesting an active breakdown. The fact that there was little activation of cells exposed to the staphylococcal stimulant for 15 minutes was not compatible with the concept of an agglutinin-like activator with a high affinity for the cell surface. Moreover, a good deal of activity remained after absorption with packed leucocytes. Bacteria are known to produce haemagglutinins and leucoagglutinins and a range of substances chiefly of a lipopolysaccharide nature (Neter, 1956) which attach themselves to sites on the surface of red cells. Substances of this type also have an affinity for the surfaces of other cells, including lymphocytes, and may activate them in the process.

The marked heterogeneity of the cell population responding to staphylococcal stimulant is evident, both in the varied levels of stimulant to which different cells are responsive in a given series of cultures, and in relation to the time of the period of DNA synthesis and cell division at a constant level of stimulant. A similar heterogeneity was noted by Cowling, Quaglino and Davidson (1963) for cells stimulated with tuberculin, and by Michalowski (1963) for phytohaemagglutinin using a 6 hour sampling technique. Phytohaemagglutinin appears to activate all normal human lymphocytes (Schrek and Rabinowitz, 1963; Quaglino and Cowling, 1964; Bernard, Geraldes and Boiron, 1964). Staphylococcal material likewise appears to be a general and non-specific stimulant and shows the same increase in cell response with dose. It differs from tuberculin, which has been shown (Pearmain *et al.*, 1963; Cowling, Quaglino and Barrett, 1964) only to stimulate the lymphocytes of tuberculin positive persons. Cowling *et al.* (1963) found that at least 20 per cent of the lymphocytes of a tuberculin

positive person could be activated over a 7 day culture period. This suggests that even in immunospecific responses a substantial part, and possibly the whole of the lymphocyte population is potentially responsive. Specificity must be expressed in some manner other than clonal; it may be that some, or all, of the lymphocytes of a sensitized individual are responsive to lower doses of the antigen encountered, the level of antigen capable of evoking a response decreasing with each encounter. It might be argued that the almost universal response of human lymphocytes to staphylococcal products simply reflects the likelihood of exposure to this group of antigens.

The low hexose content of active staphylococcal material and its inactivation by pronase would suggest that the active factor may contain protein or polypeptide. Börjeson, Bouveng, Gardell, Norden and Thunell (1964) were able to show that the lymphocyte stimulating factor of phytohaemagglutinin preparations was of protein or polypeptide nature and contained less than 6 per cent carbohydrate. However the products obtained by Punnett and Punnett (1963) contained about 50 per cent polysaccharide. Both the phytohaemagglutinin and staphylococcal activators are of the antigen class but it is doubtful if transforming potency bears any relation to immunogenicity. Staphylococcal substances of varying size appeared to be active.

Maximal levels of DNA and RNA synthesis by cultured cells required the provision of growth materials not present in a simple medium. Serum dialysates were always beneficial whereas whole sera were sometimes inhibitory suggesting that isoantibodies or other large molecules may depress the synthetic activity of the cells. Terasaki, Esail, Cannon and Longmire (1961) have shown that sera from other species are frequently toxic to lymphocytes and that some human sera are toxic to human lymphocytes.

SUMMARY

1. Human lymphocytes are strongly activated by an extracellular product of staphylococci. The stimulated cells have exacting growth requirements.

2. The activation resembles that produced by phytohaemagglutinin in that the lymphocytes of most individuals are affected, the degree of activation increasing with dose of stimulant.

3. The active material is precipitable by acetone, does not contain strong anionic groupings and is destroyed by pronase. The lymphocytes are not grossly agglutinated by active filtrates and the active material is not readily absorbed with rabbit leucocytes.

4. The cell response to stimulation is heterogeneous with respect to time of DNA synthesis and mitosis.

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Studies with Anti-Lymphocytic Antibody and Antibody Fragments

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Introduction

Following the demonstration that IgG preparations from a number of anti-lymphocytic sera exhibited immunosuppressive activity [see reviews JAMES 1967a, 1968] it seemed desirable to investigate the immunological properties of anti-lymphocytic IgG fragments, for it was anticipated that such studies would provide valuable data on the mode of action of this material and might also result in therapeutically superior products (i.e. less antigenic). With these aims in view we have therefore during the course of the last 2–3 years investigated the effect of both divalent $F(ab')_2$ and univalent (Fab') antibody fragments of a number of species specific anti-lymphocytic IgG preparations on a variety of immunological phenomena. These include skin allograft rejection [1] and humoral antibody formation in rats [10, 12] the graft versus host [GVH) response in mice [19] and the *in vitro* transformation of human lymphocytes [24]. In the present communication the results previously reported from the authors department have been summarized and their significance discussed. It should however be noted that studies of a similar nature have also been described by other investigators [3, 16, 21, 22].

Materials

Details of the production and properties of the normal and antilymphocytic IgG preparations used in these studies have been reported elsewhere [12, 19, 24]. The divalent antibody derivatives $F(ab')_2$ of the various IgG preparations were obtained by digestion with pepsin at 37° C for 48 h in 0.1 M acetate buffer (pH 4.0), using 2 mg enzyme for each 100 mg of protein [20]. The univalent products (Fab') were obtained by the subsequent reduction and alkylation of the divalent derivative [5]. The degradation of the IgG preparations to the respective $F(ab')_2$ and Fab' derivatives was shown to be achieved by subsequent immunodiffusion analysis and by measuring the lymphocytotoxic and lymphoagglutinating activity of the products. The structure and basic properties of these preparations is illustrated in figure 1.

Experimental

1. Skin allograft survival studies in rats. The ability of the anti-lymphocytic antibody preparations to delay the cell mediated rejection of skin allografts was investigated in hooded strain rats. These animals received daily intraperitoneal injections (2 ml of 1 g%) of the various preparations for 4 days prior to grafting with albino strain skin. Daily treatment with reduced doses of these preparations (1 ml of 1 g %) was continued for 4 days post grafting. It will be observed from figure 2 that treatment with intact anti-lymphocytic IgG significantly delayed the rejection of skin allografts across this marked histocompatibility barrier. In contrast however, the non cytotoxic univalent and divalent fragments failed to exert a measurable effect [1].

2. Studies on the GVH reaction in mice. In addition to suppressing the cell mediated graft rejection process, anti-lymphocytic antibody has also been shown to diminish the ability of lymphoid cells to produce graft versus host reactions [see review JAMES, 11]. These responses result from the immunological interaction between grafted lymphoid cells and the histocompatibility antigens of the host and one such reaction occurs when parental spleen cells are injected into adult F1 hybrid mice. The occurrence and severity of this reaction can be assessed by measuring the resultant increase in size of the spleen and liver of the F1 hybrid recipient. We have therefore compared the ability of spleen cells from C57Bl mice pretreated with the various preparations to produce a graft versus host reaction upon subsequent injection into normal (C57Bl $\times$ CBA)F1 hybrid recipients.

In these studies the donor animals received daily intraperitoneal injections with 0.25 ml of a 1 g% protein solution of the appropriate preparation on the four days prior to cell transfer. The F1 hybrid recipients were then injected intravenously with 1×10^8 viable spleen cells obtained from the antibody treated parental donors. Nine days after cell transfer the recipients were sacrificed and their spleen and liver weights determined.

The results obtained were similar to those observed in the skin allograft studies, the divalent antibody fragment failing to inhibit the splenomegaly (fig. 3) and hepatomegaly characteristic of the GVH response whilst the intact antibody IgG was most effective in suppressing this response [19].

3. Studies on the primary humoral response in rats. In these studies hooded strain rats received daily intraperitoneal injections for three successive days with the appropriate antibody or normal IgG preparation (2 ml of 1 g% solution). The day following the last injection the animals were challenged by either an intraperitoneal injection of 5 mg of alum precipitated bovine serum albumin or by the intravenous injection of 1×10^9 sheep erythrocytes. The animals were bled at weekly intervals and the circulating antibodies formed in response to the challenge with the test antigens were assessed by standard immunological procedures [12, 13].

These studies revealed that anti-lymphocytic antibody fragments were also relatively ineffective at suppressing the primary humoral response of hooded

strain rats to alum precipitated bovine serum albumin or sheep erythrocytes [10, 12]. The relative ineffectiveness of the divalent antibody derivative is illustrated in figure 4 where it will be observed that rats pretreated with this material readily responded to a subsequent challenge with alum precipitated bovine serum albumin.

4. Studies on human lymphocytes in vitro. Prompted by the suggestion that the immunosuppressive properties of anti-lymphocytic antibody might be due to the transformation and consequent sterile inactivation of lymphocytes [15], and that a measurement of this activity *in vitro* might afford a reliable means of assessing the immunosuppressive potential of anti-lymphocytic antibody, studies were undertaken to determine the transforming activity of intact anti-lymphocytic antibody and antibody fragments. Ideally these studies should have been performed using rodent lymphocytes and their appropriate antibody preparations for this would have allowed an accurate correlation of transforming and immunosuppressive activity. However, due to the difficulties experienced in obtaining measurable and reproducible transformation of rodent lymphocytes *in vitro* the simpler human system was used. This involved adding the appropriate antibody preparations (2 mg) to human peripheral blood lymphocytes (1×10^7 viable cells) in a culture medium consisting of 2 ml of medium 199 and 0.5 ml of heat inactivated autologous plasma. The transformation of the cells into the so called blast forms was assessed indirectly by determining the incorporation of tritiated uridine and thymidine into the nuclear RNA and DNA of the lymphocytes. The tritiated uridine (1.67 μc/ml) was added to the culture at time zero and the cells processed after 18 h incubation at 37° C, while the tritiated thymidine (1.67 μc/ml) was added after 72 h incubation and the cells processed for liquid scintillation counting after a further 24 h at 37° C [24].

These studies revealed that both intact anti-lymphocytic IgG and the divalent antibody fragment obtained on pepsin digestion were both capable of transforming human lymphocytes. In contrast however the univalent (nonagglutinating) antibody fragment was ineffective (fig. 5) (24].

Discussion

From these studies and those of other investigators (summarized in table I) it is apparent that anti-lymphocytic antibody fragments are relatively ineffective at suppressing cellular and humoral immune responses. This suggests that the immunosuppressive effect of anti-lymphocytic IgG is dependent upon the presence of an intact Fc moiety and detailed studies have shown that this region of the molecule is required for complement fixation, and hence lysis of the lymphocyte, and that it may enhance phagocytosis [see review: 16, 23]. On the basis of these observations and our current knowlegde of the structural and biological characteristics of the IgG molecule, the most satisfactory theory of the mode of

action of anti-lymphocytic antibody (see table II) is the destruction of lymphocytes [see reviews: 9, 11] Furthermore the recent observations of MARTIN and MILLER [17] and DENMAN *et al.* [4] indicate, as previously suggested [12, 13, 14, 18] that this need not involve the gross depletion of the lymphocyte population but the preferential destruction (inactivated) of the so called 'antigen reactive' long lived small lymphocytes.

It should perhaps be stressed that these results do not entirely exclude other mechanisms, for the failure of fragments to suppress immune responses may be a reflection of their short half life [7, 16, 22] or their reduced antigenicity [see JAMES, 11]. However recent observations with acid treated antilymphocytic IgG suggest that antigenic competition does not play a major role [2].

Although studies with anti-lymphocytic antibody fragments have, as anticipated, contributed greatly to our knowledge of the mode of action of antilymphocytic antibody they have not led to the development of products possessing distinct therapeutic advantages. Nevertheless it is hoped that other forms of treatment of anti-lymphocytic IgG may reduce some of its more troublesome side effects.

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Summary

The *in vivo* and *in vitro* effects of antilymphocytic antibody and antibody derivatives obtained by the chemical and enzymological treatment of this molecule have been investigated in an attempt to correlate the structure and function of the antilymphocytic IgG molecule, to determine the mode of action of this material and to develop less toxic products.

The results indicate that non-cytotoxic derivatives obtained by acid treatment or enzyme digestion of antilymphocytic IgG [the $F(ab')_2$ and Fab' moieties] all failed to suppress cell mediated immune responses such as skin allograft rejection and the GVH reaction or inhibit the primary humoral response to bovine serum albumin. However the divalent $F(ab')_2$ product obtained from anti-human lymphocyte IgG transforms human lymphocytes *in vitro*.

The significance of these results in relation to the mode of action and heterogenity of anti-lymphocytic antibodies is discussed.

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Fig. 1. This diagram illustrates the basic structure and anti-lymphocytic properties of the antibody and antibody fragments used in the current studies.

Fig. 2. The effect of horse anti-rat lymphocyte IgG and IgG fragments on skin allograft survival (albino strain graft to hooded recipient). Note that the non cytotoxic fragments failed to prolong skin allograft survival indicating that the Fc portion of the molecule is required for effective immunosuppression.

Fig. 3. The ability of spleen cells from C57Bl mice treated with rabbit anti-mouse lymphocyte IgG or its $F(ab')_2$ derivative, to induce a graft versus host response in (C57Bl × CBA)F1 hybrid recipients. The relative spleen weights indicated are mean values obtained from at least 5 animals and the standard deviation was small. Note that treatment of the spleen cell donors with anti-lymphocytic $F(ab')_2$ fails to prevent splenomegaly. Similar observations were also made regarding hepatomegaly.

Fig. 4. The effect of horse anti-rat lymphocyte IgG and its $F(ab')_2$ derivative on the primary immune response of hooded strain rats to alum precipitated bovine serum albumin. Note that only 1 rat treated with intact anti-lymphocytic IgG exhibited a marked and sustained humoral response to the test antigen whereas all the animals receiving the divalent fragment responded well.

Fig. 5. The relative lymphocyte transforming activity of horse anti-human lymphocyte IgG and its fragments. These results were obtained in a number of experiments using lymphocytes from several donors. It will be observed that the intact antibody molecule and its $F(ab')_2$ derivative consistantly stimulate the incorporation of tritiated uridine into nuclear RNA. Similar results were obtained in the thymidine incorporation studies.

Table I. A summary of the properties of anti-lymphocytic antibody and antibody fragments

	Property	Intact IgG	$F(ab')_2$	Fab′	References
A	Lymphoagglutinating	Yes	Yes	No	JAMES and ANDERSON [12];
	Lymphocytotoxic	Yes	No	No	GUTTMAN *et al.* [6]; RIETHMÜLLER *et al.* [22]; MANDEL and ASOFSKY [16]; CHARD [3]
	Lymphocyte transforming	Yes	Yes	No	WOODRUFF *et al.* [24]; RIETHMÜLLER *et al.* [22]
B	Delays rejection of 1st set skin allografts	Yes	No	No	ANDERSON *et al.* [1]; RIETHMÜLLER *et al.* [22]; MANDEL and ASOFSKY [16]
	Suppress graft versus host reaction	Yes	No[1]	No[1]	NAYSMITH and JAMES [19]; MANDEL and ASOFSKY [16]
	Suppress primary humoral response or development of antibody forming spleen cells	Yes	No	No	JAMES and ANDERSON [12]; JAMES [10]; RIETHMÜLLER *et al.* [22]
	Suppress secondary humoral response of 'transferred' sensitized lymphoid cells	Yes	No[2]	No	HARRIS and HARRIS [8]; OGBURN, HARRIS and HARRIS [21]
	Delay renal allograft rejection	Yes	Yes[3]	Yes[3]	GUTTMAN *et al.* [6]

[1] In these experiments the spleen cell donors were treated with the antibody fragments. The *in vitro* treatment of the cells with antibody fragments prior to transfer might give different results for it may interfere with the seeding of the injected cells.

[2] In these studies fragments obtained by papain digestion were used.

[3] In these studies large doses of fragments were used and the effect was less dramatic than that achieved with the intact antibody.

Table II. Theories of the mode of action of anti-lymphocytic antibody

1. The general or specific destruction of lymphocytes.
2. Antigenic competition by lymphocyte bound antigen.
3. Blindfolding of lymphocytes preventing antigenic recognition.
4. Blindfolding of homograft preventing lymphocyte sensitization and/or contact.
5. Sterile inactivation of lymphocytes.
6. Neutralization of thymus humoral factor.
7. Antagonization of lymph node permeability factor.

For further details of these theories see LEVEY and MEDAWAR [14, 15] and JAMES [10, 11].

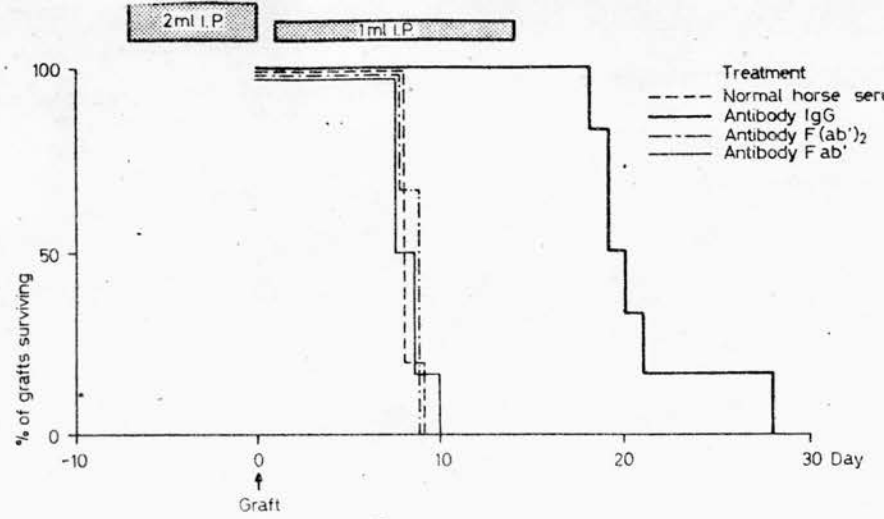

Fig 2

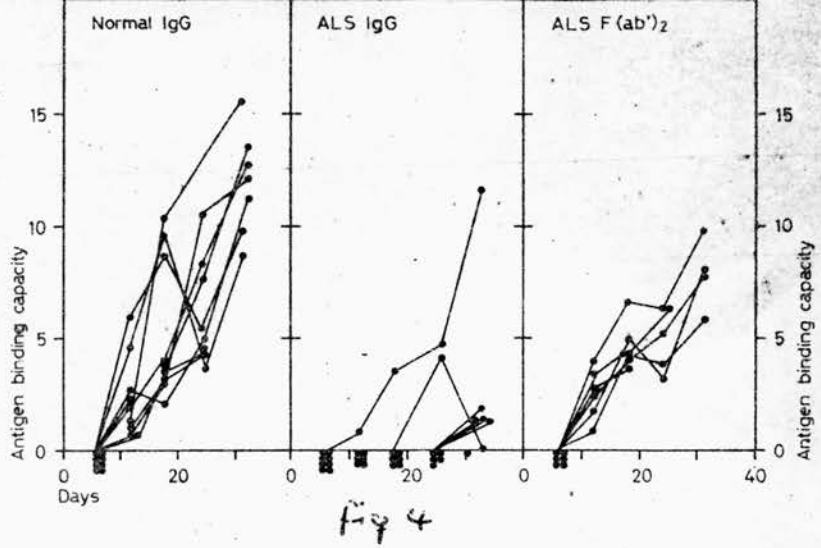

Fig 4

Treatment	Relative ^{14}C-uridine incorporation
Anti-lymphocytic IgG	
Anti-lymphocytic F(ab')$_2$	
Anti-lymphocytic Fab'	
Normal serum	
Normal Fab'	

Fig 5

Preparation	Structure	Properties: Agglutinating	Properties: Cytotoxic	
IgG	L, H, Fab, Fd, Fc, S-S, Papain, Pepsin	+	+	
F(ab')$_2$	L, H, S-S	+	−	Divalent
Fab'	L, H, S-S	+	−	Univalent

Fig 1

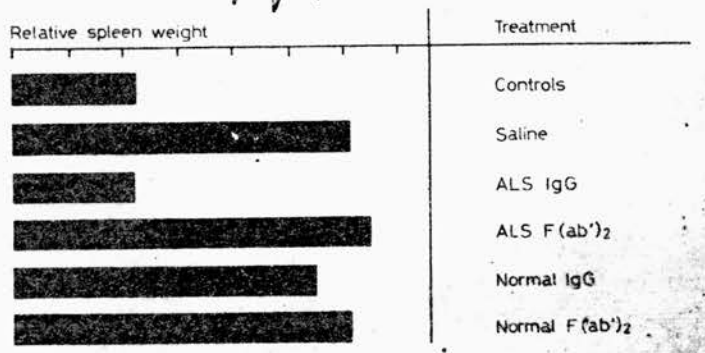

Fig 3

The Characterization of Anti-lymphocytic Antibodies.*

By **Keith James**, Department of Surgical Science, University of Edinburgh. (With Five Text-figures.)

(MS. received May 12, 1969)

Introduction

During recent years the heterogeneity of equine antibodies to a variety of antigens has been convincingly demonstrated (Rockey, Klinman and Karush 1964; Raynaud, Iseaki and Mangalo 1965; Allen, Sirisina and Vaughan 1965; Klinman *et al.* 1966; Klinman and Karush 1967; Johnson and Allen 1968). For example, extensive studies on equine anti-hapten antibodies have shown that the IgG fraction of horse serum contains at least four different proteins, namely the IgGa, IgGb, IgGc and 10S IgG globulins in addition to the IgG(T) and IgM proteins (Rockey *et al.* 1964; Klinman *et al.* 1966). These antibodies differ widely in their ability to precipitate the protein-bound hapten and to fix complement, and their concentrations and binding affinities vary considerably during a course of immunization (Klinman *et al.* 1966).

Because of the current interests in anti-lymphocytic antibodies it seemed important to determine if equine antibodies to human lymphoid tissue exhibited a similar heterogeneity, for it was felt that such studies might provide data of importance in relation to the production, mode of action and purification of anti-lymphocytic antibodies. We have therefore examined in considerable detail the distribution of antibody activity in an equine antiserum obtained by a prolonged course of immunization with human spleen, the activity in the various fractions being assessed by standard *in vitro* procedures. In the present communication the results of these studies are summarized and their significance discussed.

General Materials and Methods

The equine antiserum used in these studies was obtained following a 15-month course of immunization with washed human spleen cell suspensions. During this period the horse received six subcutaneous injections each containing between $11{\cdot}8 \times 10^9$ to $43{\cdot}6 \times 10^9$ lymphocytes of

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varying degrees of viability. This antiserum was incubated at 56° C. for 30 min. to inactivate complement and precipitate the fibrinogen.

An anti-lymphocytic globulin fraction was prepared from an aliquot of the above antiserum by a combined salt precipitation and batch chromatographic technique. This involved the initial precipitation of a crude globulin fraction by the slow addition of 28 per cent (w/v) sodium sulphate, the final concentration being 16 per cent (w/v). Following a suitable period of equilibration against 0·02M phosphate buffer pH 6·5, the crude globulin was batched on diethylaminoethyl cellulose which had also been equilibrated with 0·02M phosphate buffer pH 6·5 (Whatman DE11). The product obtained was concentrated by lyophilization and dialyzed against phosphate buffered saline (pH 7·2 0·06M containing 0·15M sodium chloride) and was shown by immunoelectrophoresis to contain several IgG components.

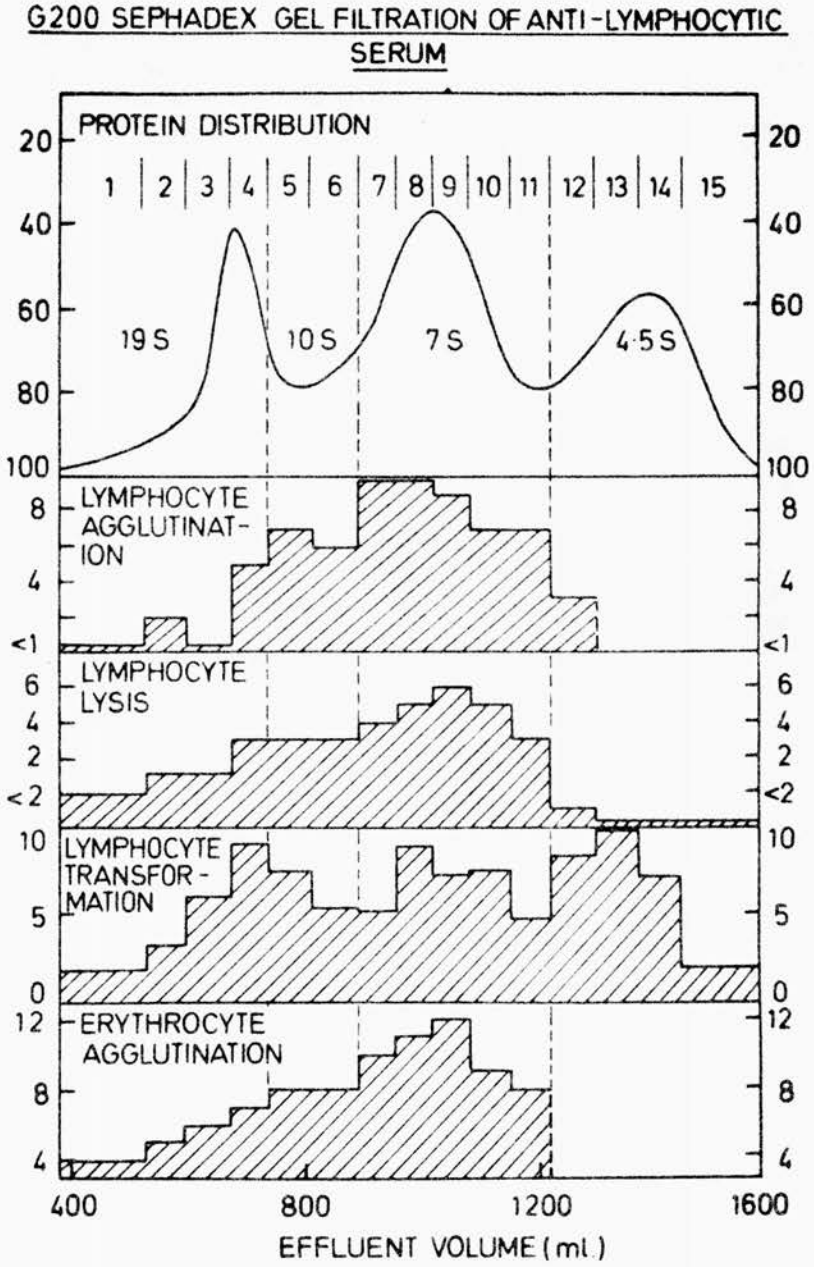
The original antiserum and the globulin fraction derived therefrom were then fractionated by a variety of physicochemical procedures (see later). The various fractions obtained from each experiment were concentrated to the same volume by ultrafiltration, their protein content determined by the Folin Phenol procedure (Lowry *et al.* 1951) and their composition assessed by immunoelectrophoretic analysis using a rabbit anti-equine serum and a goat antiserum to equine H chains.

The lymphocyte agglutinating and cytotoxic activities were determined by standard procedures and the mitogenic properties were assessed by determining the capacity of the various fractions to stimulate the incorporation of tritiated uridine into lymphocyte RNA. In the latter studies all the samples from a particular fractionation were examined simultaneously using the same batch of cells. In addition to the above assays the erythrocyte agglutinin content of the fractions was also determined. The various agglutinin and cytotoxic titres are recorded on the figures as $\log^2$ of the reciprocal titre while the transforming activity has been expressed as a multiple of the value observed in control cultures containing cells and autologous plasma alone. Throughout the studies, control assays were performed using normal equine serum and normal equine IgG. Further details of the above immunization, fractionation and assay procedures have been recorded elsewhere (James *et al.* 1969).

Results

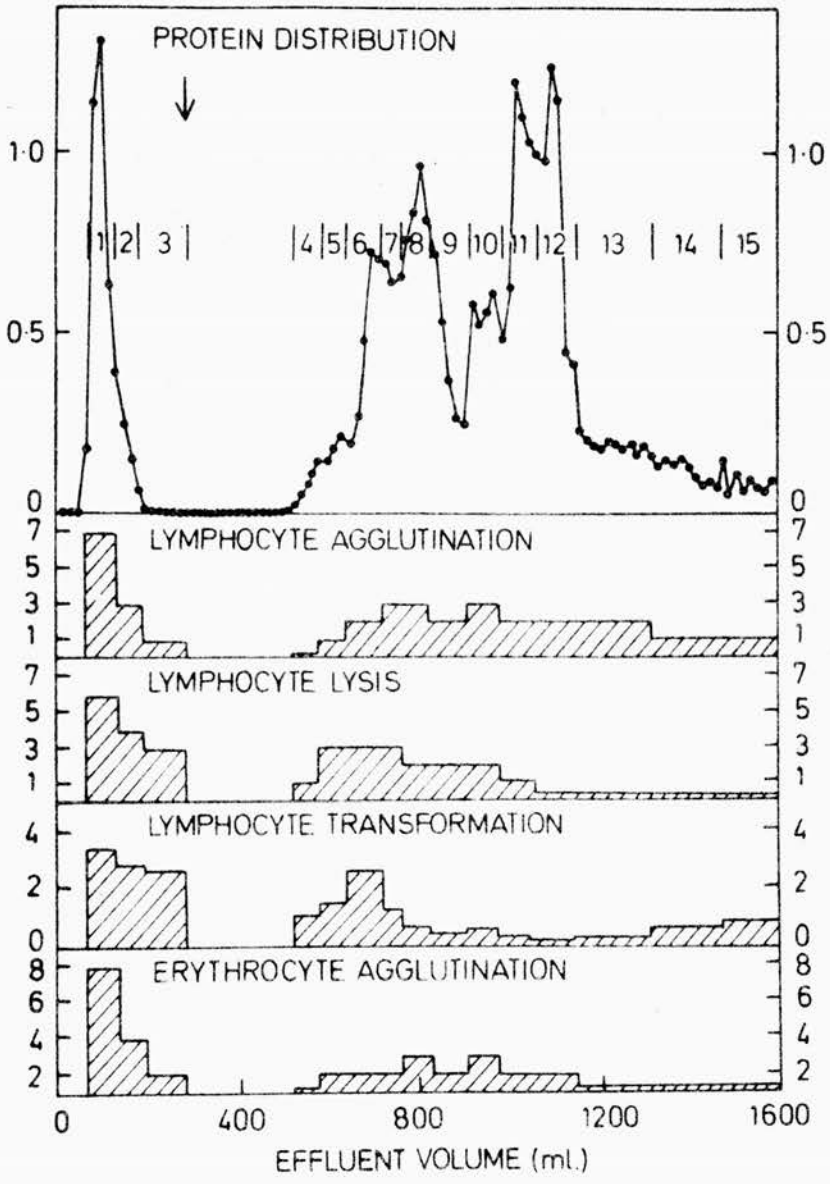
The Distribution of Anti-Lymphocytic Activity in Whole Antiserum

Gel filtration of anti-lymphocytic serum on G200 sephadex under the conditions outlined in text-fig. 1 revealed that the major protion of the



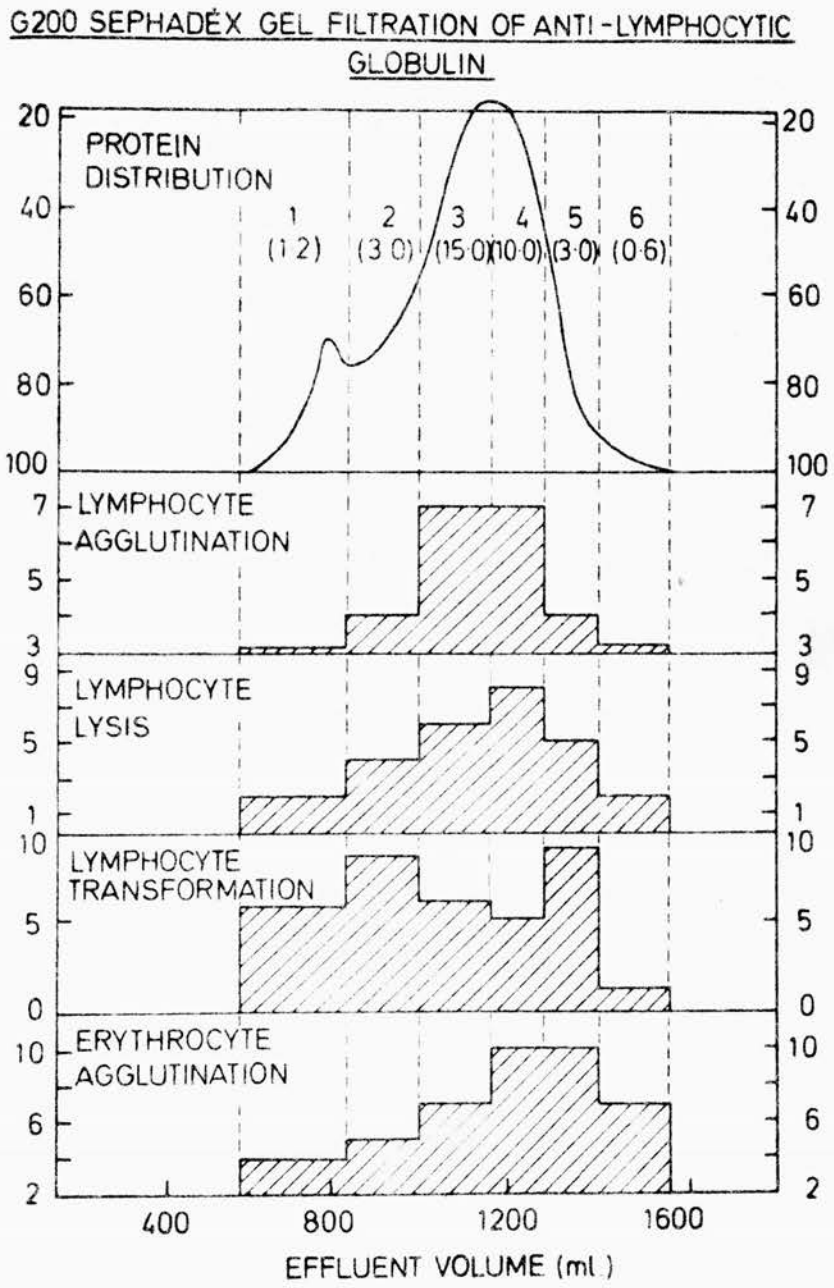
TEXT-FIG. 1.—The Distribution of *in vitro* Activities in Fractions obtained by the G200 sephadex gel filtration of anti-lymphocytic serum.

40 ml. of equine antiserum to human spleen was fractionated on a G200 sephadex column (dimensions 100 × 5·7 cm.). The effluent was pooled as shown in the diagram and concentrated to give 15 fractions of 2 ml. volume. Note that the bulk of the lympho-agglutinating, lymphocytotoxic and erythrocyte agglutinating activities were associated with the 7S fraction, whilst the transforming activity was fairly evenly distributed between the various molecular weight classes.



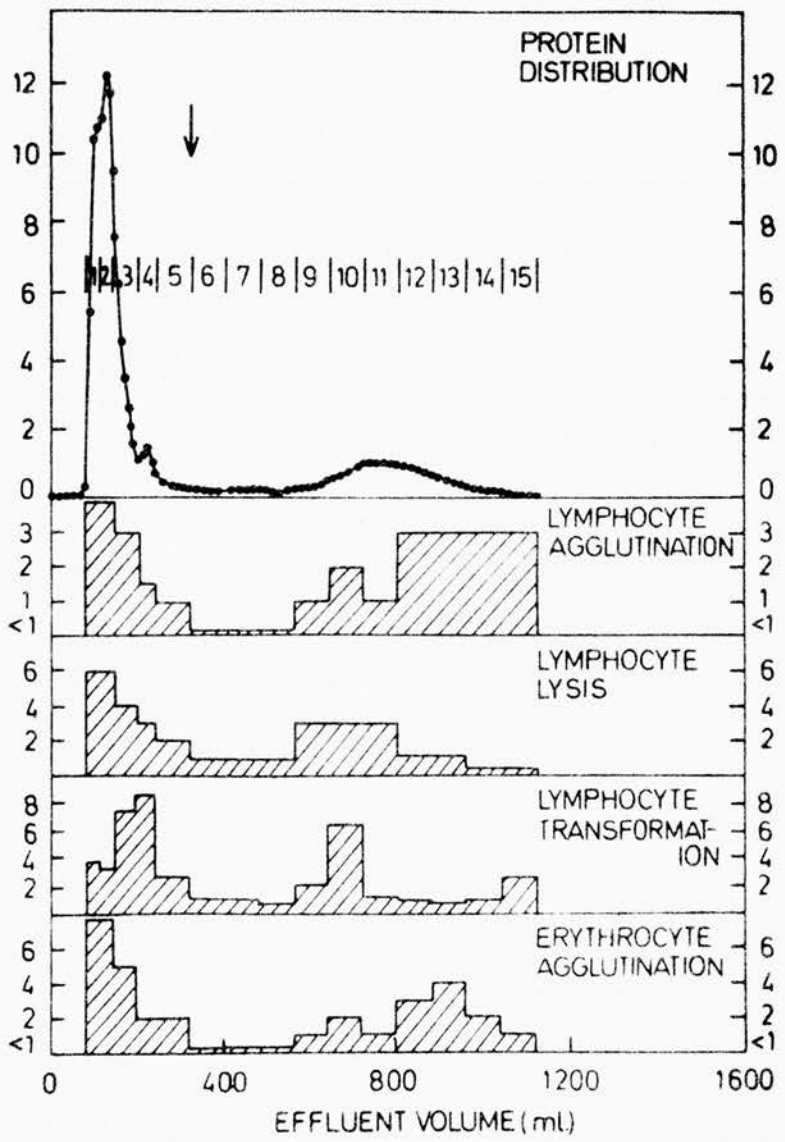
TEXT-FIG. 2.– The distribution of *in vitro* activities in fractions obtained by DEAE cellulose column chromatography of anti-lymphocytic serum.

15 ml. of equine antiserum to human spleen was fractionated on a column of Whatman DE52 cellulose ion exchanger containing 50 g. dry weight of exchanger with an ion exchange capacity of 1 milliequivalent per gram. The serum and exchanger were equilibrated against 0·01 M phosphate buffer pH 8·0 prior to fractionation and the initial elution was performed with this buffer. Gradient elution was commenced at the point indicated by the arrow, the final eluent being 0·3 M NaH^2PO^2. The effluent was pooled as shown in the diagram and concentrated to give 15 fractions of 5 ml. volume. Note that fractions 2, 3 and 6 exhibit transforming activity comparable to that shown by the more strongly agglutinating and cytotoxic fraction 1. Observe also the broad distribution of the various activities indicative of a wide spectrum of antibodies.



TEXT-FIG. 3.—The distribution of *in vitro* activities in fractions obtained by G200 sephadex gel filtration of anti-lymphocytic globulin.

2 g. of anti-lymphocytic globulin prepared as described in the text was fractionated on a G200 sephadex column (dimensions 100 × 5·7 cm.). Note that the bulk of the lympho-agglutinating, lymphocytotoxic and erythrocyte agglutinating activities was located in the 7S peak (fractions 3 and 4). In contrast the lymphocyte transforming activity was more evenly distributed, considerable activity being observed in fractions excluded in the void volume (F1 and 2) which were relatively deficient in other anti-lymphocytic activities.



TEXT-FIG. 4.—The distribution of *in vitro* activities in fractions obtained by the DEAE cellulose column chromatography of anti lymphocytic globulin.

1 g. of anti-lymphocytic globulin was fractionated on a column of DE52 cellulose under the conditions outlined in text-fig. 2. Note that as in text-fig. 2 some fractions possessing relatively low lymphoagglutinating and lymphocytotoxic titres exhibited marked transforming activity (see Fractions 3, 4 and 10 and compare with Fractions 1 and 2). Observe also the distribution of the various activities in proteins with widely varying affinities for the DEAE cellulose.

lymphocyte and erythrocyte agglutinins and the lymphocytotoxins were associated with the 7S fraction of the antiserum which contains the IgG antibodies. In contrast the mitogenic activity was present in high concentrations in all of the fractions studied including those located in the 19S and 4·5S regions.

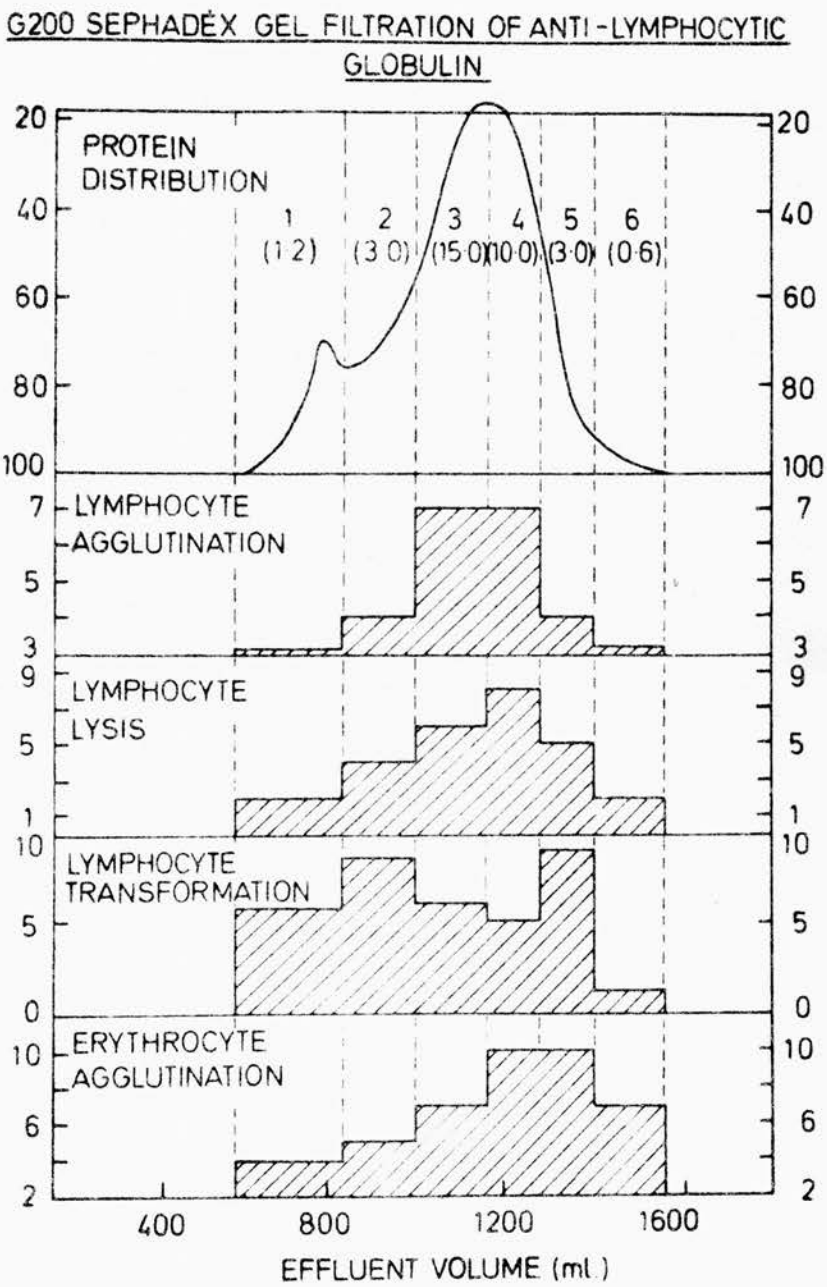
Notable differences in the distribution of the various activities were also observed following column chromatography on diethylaminoethyl cellulose, considerable transforming activity being found in fractions relatively deficient in lymphocyte agglutinating and lytic activity (see text-fig. 2, compare fraction 1 with fractions 2, 3 and 6).

Additional fractionation studies on whole serum were also performed using the gradient ultracentrifuge and Porath column electrophoresis techniques and in general the data obtained, confirmed the above observations (see James *et al.* 1969).

The Distribution of Anti-Lymphocytic Activity in Anti-Lymphocytic Globulin

The additional fractionation studies performed with the anti-lymphocytic globulin preparation confirmed and extended the observations noted with whole serum. Following G200 sephadex gel filtration two protein peaks were observed, a major 7S peak preceded by a minor peak (see text-fig. 3). The major part of the lymphocyte agglutinating and cytotoxic activity was associated with the 7S peak. However, the transforming activity was again more evenly distributed throughout the protein fractions, considerable activity being associated with the weakly cytotoxic fraction 1. Studies performed with mixtures of Blue Dextran 2000 and the anti-lymphocytic globulin preparation revealed that the protein in F1 was excluded in the void volume and subsequent analytical centrifugation indicated that this fraction contained appreciable amounts of 10S protein and other high molecular weight components (10S to 19S). This molecular heterogeneity is characteristic of that frequently observed in human IgA preparations. Immunoelectrophoretic analysis revealed that this fraction contained a gamma 2 globulin component and a faster component with immunoelectrophoretic properties similar to that previously observed in an equine 10S-IgG preparation Rockey *et al.* 1964).

The DEAE cellulose chromatographic studies with the anti-lymphocytic globulin confirmed that the distribution of lymphocyte transforming activity was not directly related to the other anti-lymphocytic activities (see fraction 3 and 4, text-fig. 4). Analytical ultracentrifugation again



TEXT-FIG. 3.— The distribution of *in vitro* activities in fractions obtained by G200 sephadex gel filtration of anti-lymphocytic globulin.

2 g. of anti-lymphocytic globulin prepared as described in the text was fractionated on a G200 sephadex column (dimensions 100 × 5·7 cm.). Note that the bulk of the lympho-agglutinating, lymphocytotoxic and erythrocyte agglutinating activities was located in the 7S peak (fractions 3 and 4). In contrast the lymphocyte transforming activity was more evenly distributed, considerable activity being observed in fractions excluded in the void volume (F1 and 2) which were relatively deficient in other anti-lymphocytic activities.

revealed that the most mitogenic fractions contained appreciable amounts of 10S protein and other high molecular weight components. The electrophoretic and antigenic complexity of the various fractions was demonstrated by immunoelectrophoretic analysis. Fractions eluted with the starting buffer (0·01M phosphate, pH 8·0) contained at least three globulins of gamma 2 mobility while the fractions eluted with the gradient exhibited even greater complexity.

Marked differences in the distribution of mitogenic and other activities were again observed in the fractions obtained following the Porath column electrophoresis of anti-lymphocytic globulin (see text-fig. 5). The transforming activity was eluted in a number of distinct peaks, two of these occurring in fractions in which the other anti-lymphocytic activities were relatively weak (see fractions 32 and 35). In contrast fraction 28 which was rich in these activities was associated with a trough in the lymphocyte transforming activity. The molecular heterogeneity of the strongly mitogenic, weakly cytotoxic fraction 32 was again demonstrated in the analytic ultracentrifuge and the immunoelectrophoretic complexity of all the fractions was also noted.

Discussion

Prior to commencement it was recognized that these studies were limited in that they were confined to a single batch of antiserum produced using an extremely complex inoculum containing a variety of cell types. Nevertheless they have shown that equine antibodies to human lymphoid tissue are physicochemically and fractionally complex, characteristics previously observed with equine antibodies to other antigens (Rockey *et al.* 1964; Klinman *et al.* 1966 and 1967; Allen *et al.* 1965; Johnson *et al.* 1968; Raynaud *et al.* 1968).

As was to be expected in hyperimmune sera, the major proportion of the lymphocyte and erythrocyte agglutinins were located in the 7S-IgG fraction. While the lymphoagglutinins appeared to be almost evenly distributed between protein of gamma 1 and gamma 2 mobility, rather more of the erythrocyte agglutinin and lymphocytotoxic activities were associated with the slow gamma 2 fraction. It should be stressed, however, that different distributions of these activities may be observed in globulin samples prepared by other procedures for the sodium sulphate technique used results in the preferential precipitation of proteins of gamma 2 mobility (James *et al.* 1969).

The marked heterogeneity of proteins capable of lymphocyte transformation and the observation that this property was not always associated

with other anti-lymphocytic activities was of particular interest. The strongly mitogenic, weakly cytotoxic fractions were usually rich in proteins with a sedimentation coefficient of 10S or greater. These high molecular weight components may be naturally occurring or could be the result of the denaturation during fractionation of a particularly labile population of molecules. In this respect recent studies in our laboratory with heat inactivated anti-lymphocytic globulin (70° C. for 30 min.) have indicated that this treatment, which causes considerable denaturation of the molecule, destroys its lymphocyte agglutinating and lytic properties, but fails to affect its mitogenic properties.

There are a number of possible, though untested, explanations of the superior transforming activity of these weakly cytotoxic fractions. The simplest hypothesis is that the mitogenic activity of the anti-lymphocytic globulin preparations is completely independent of the other anti-lymphocytic properties However previous studies with antibody fragments have suggested that lymphocyte transformation was associated with lymphocyte agglutination (Woodruff, Reid and James 1967). Alternatively it may be that the inherent lymphocyte transforming potential of the more concentrated, highly cytotoxic fractions has not been manifested for a variety of reasons. A major weakness of the present transformation studies is that the samples investigated have been of widely differing protein concentration. Thus in certain cases the systems may have been oversaturated and feedback or other forms of inhibition may have occurred. Preliminary studies performed in our laboratory suggest that this may be the case, for dilution of the highly cytotoxic, weakly mitogenic fraction 2 (see text-fig. 4) results in marked increase in its transforming activity and further experiments are underway to elucidate this phenomenon.

The marked transforming activity observed in the 4·5S region was unexpected. Initially it was believed that this could be due to the presence of growth-promoting substances or low molecular weight antibody (Rockey *et al.* 1964). However more recent studies in our laboratory suggest that it may have been caused by small amounts of contaminating sephadex.

Although in the present studies we have not succeeded in isolating any of the IgG sub-components and have made no attempt to assess the immunosuppressive properties of the various fractions, the data obtained is of importance in relation to the preparation and clinical use of this material and in the elucidation of its mode of action. The anti-lymphocytic antibody preparations used to date have undoubtedly been physico-chemically and functionally complex, including those obtained by immunoabsorption procedures (Woodruff 1968). Because of this the observed *in vitro* and *in vivo* properties will have been the net effect of a

number of factors, some of which were acting in competition. For example, molecules such as the IgGc and IgG(T) globulins, which exhibit a high avidity for the lymphocyte but fail to fix complement, may inhibit the lytic effect of cytotoxic antibodies of the IgGa and IgGb type (Klinman *et al.* 1967; Johnson and Allen 1968). Furthermore variations in the relative amounts of cytotoxic and non-cytotoxic anti-lymphocytic antibodies may explain the losses in immunosuppressive activity which have been observed following hyperimmunization and fractionation (Levey and Medawar 1966; Clunie *et al.* 1968; Jooste *et al.* 1968).

The observation that marked mitogenic activity may occur in relatively non-cytotoxic fractions lends support to previous observations that this property is not a reliable indication of the immunosuppressive potential of anti-lymphocytic antibody preparations (Riethmüller *et al.* 1968; James 1969).

The present results emphasize the need for further studies on the development, fractionation and immunosuppressive properties of anti-lymphocytic antibody molecules. It is believed that such studies will contribute greatly to our understanding of the mode of action of anti-lymphocytic antibodies and lead to the development of improved products for therapeutic use.

Acknowledgments

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