STUDIES ON MONONUCLEAR PHAGOCYTE RECEPTORS

by

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DECLARATION

I confirm that the work in this thesis was conceived, planned and executed by myself.

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THE UNIVERSITY *of* EDINBURGH

SUMMARY

The presence or absence of various plasma membrane receptors on human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages has been described and compared. The receptors include those for the complement components C3b, C3b´ and C3d (CR_1, CR_3 and CR_2), the Fc portions of IgG and the Fc portions of IgM, and "lectin-like" receptors which recognise bacterial cell wall sugars. A rosette assay using sheep erythrocytes coated with various intermediates as indicator cells was used to visualise all the receptors except "lectin-like" receptors. These were detected by the binding of Staph. albus to macrophages. Complement receptors were demonstrated using purified human complement components, diluted human AB serum or zymosan depleted serum (R3) as sources of complement to coat the sheep red cells sensitised with rabbit anti sheep erythrocyte IgM. Although comparisons between the different cell types must be treated with come caution since heterologous systems were used, human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages all expressed C3b receptors, Fc (IgG) receptors and "lectin-like" receptors. Both Fc(IgG) and "lectin-like" receptors were expressed at both 0°C and 37°C whereas CR_1s were not expressed at 0°C. Mouse peritoneal macrophages had the highest levels of Fc (IgG) and "lectin-like" receptors and guinea pig alveolar macrophages expressed slightly lower levels of these receptors whereas human peripheral blood monocytes (and other leukocytes) showed a comparatively low degree of Staph. albus binding. On the other hand, human monocytes appeared to possess more complement receptors (CR_1) with higher affinity than those on mouse peritoneal macrophages, and guinea/...

guinea pig alveolar macrophages expressed very low levels of complement receptors. Human monocytes also expressed CR_3 but not CR_2 and had cryptic receptors for the Fc portion of IgM. The presence of these latter three receptors on mouse peritoneal and guinea pig alveolar macrophages were not examined. Human neutrophil "lectin-like" receptors appeared to be qualitatively different from "lectin-like" receptors on other phagocytes in that they were only inhibitable by D-galactose and not by D-glucose.

The capacity of various chemoattractants to increase the percentage of leukocytes binding complement-coated red cells has been termed complement receptor enhancement. This phenomenon has been described for human monocytes, neutrophils and eosinophils and for mouse peritoneal macrophages and guinea pig alveolar macrophages. It is thought to represent a general biological process whereby adhesion of cells to appropriately opsonised particles is increased.

Casein and f-Met-Leu-Phe, both recognised chemotactic factors, enhanced the expression of CR_1s on human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages, in a dose-dependent fashion. Concomitantly with this chemoattractant induced increase in complement receptors, "lectin-like" receptors were inhibited whereas Fc (IgG) receptors were unaffected by chemotactic factors. A number of recognised monocyte chemoattractants including lymphokines, C. parvum extract and other formyl-methionyl peptides, and a chemokinetic agent, human serum albumin, increased the percentage of human monocytes which formed rosettes with complement coated erythrocytes. Comparable results were obtained irrespective of whether purified complement components or whole serum was used as a source of complement. Unformylated methionyl peptides/...

peptides had no effect on human monocyte complement receptors. Pharmacological mediators other than chemoattractants had varying effect on human monocyte complement receptors. Histamine either had no effect on these receptors or decreased complement receptor expression on normal human monocytes. On the other hand complement receptors on monocytes from atopic patients responded to histamine by enhanced expression of complement receptors. Prostaglandin $F_{2\alpha}$, serotonin, bradykinin and isoprenaline (at 10^{-5} moles.l^{-1}) which are known to increase intracellular cGMP levels, enhanced complement receptor expression whereas histamine isoprenaline at other concentrations, which increase cAMP levels in cells, either had no effect or decreased complement receptor expression. Prostaglandins E_1 and E_2 which also increase cAMP levels and are also chemotactic, increased the percentage of monocyte complement rosettes. Maximal complement receptor expression was generally achieved with 30 minutes incubation in the presence of chemoattractant but when the chemotactic factor was removed, monocyte complement enhancement was reversible, returning to normal values in approximately 120 minutes. Monocyte complement receptor enhancement and murine macrophage "lectin-like" receptor inhibition did not occur at 0°C. Monocyte and neutrophil complement receptor enhancement had a temperature threshold level at around 23°C below which no complement receptor enhancement was evident.

These data confirm and extend previous findings on leukocyte-receptor enhancement on human eosinophils and indicate that the phenomenon may have potential as a clinical test for macrophage function both in health and disease.

The importance of the various surface receptors studies on macrophages in the inflammatory process and the significance of their modulation by inflammatory agents, is discussed.

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CHAPTER I - INTRODUCTION

1.0 HISTORICAL BACKGROUND

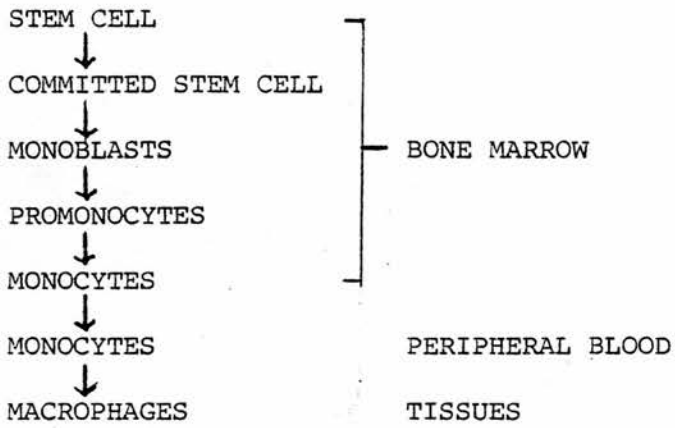
The concept of immunology arose from the general observation that those who survive an infection seldom contact that disease again. Both Edward Jenner and Louis Pasteur had evolved valid methods for immunization but the mechanisms involved in protection remained obscure until the end of the 19th century. In 1890 von Behring and Kitasato at Koch's Institute in Berlin showed that immunity developed against tetanus was due to the production of a specific neutralising substance or antibody in the blood of the immune animal and that such antitoxin immunity could be transferred to another animal. Subsequently it was shown that antibodies (so-called precipitins) were developed against a wide variety of foreign material by the animal into which they were injected. The interest in serotherapy led to further understanding of the immunological properties of serum. In 1893 Buchner described a heat-labile bactericidal constituent (alexine) in normal serum. Two years later Bordet showed that there were two components in immune serum which were bactericidal - one was thermolabile and also present in normal serum and the other was thermostable and specific. Ehrlich (1900) thus considered that the evidence for the presence of antitoxic and antimicrobial activity in serum was sufficient to explain the immunity observed in vivo. Metchnikoff (1905), on the other hand, thought that the serum components merely stimulated the phagocytes which he had observed in blood and the destruction of foreign material such as bacteria by these microphages and macrophages was the main mechanism of protection. The dispute between the proponents of humoral and cellular immunity was partially resolved by Sir Almoth Wright (1903) and others who showed that the

presence/...

presence of the heat labile factor in normal serum and/or the specific serum factors from immune animals prepared bacteria for phagocytosis. This cellular immune response together with a purely humoral immune response involving antibody and complement provided the basis for observed immunity to infections.

Aschoff (1924) first defined the reticuloendothelial system by the ability of cells to take up dyes in vivo. Its function was thought to be involved in the removal of bacteria, other foreign material and effete tissue elements. More recently van Furth et al. (1972) have defined the mononuclear phagocyte system according to various functional criteria. Until relatively recently the scavenging of foreign material was believed to be the only role macrophages played in host defence mechanisms. However it has become clear over the last few years that macrophages play an essential part in the development and regulation of cellular and humoral immunocompetence by their involvement in both the afferent and efferent limbs of the immune response (Unanue, 1972; 1978).

MONONUCLEAR PHAGOCYTE SYSTEM



NORMAL STATE

Connective tissue (histiocyte)
liver (Kupffer cell)
Lung (alveolar macrophage)
lymph nodes (free and fixed macrophages; interdigitating cell?)
spleen (free and fixed macrophage)
bone marrow (fixed macrophage)
serous cavities (pleural and peritoneal macrophages)
bone (osteoclasts)
nervous tissue (microglial cell)
skin (histiocyte; Langerhans cell?)
synovia (type A cell)
other organs (tissue macrophage)

INFLAMMATION

exudate macrophage
exudate - resident macrophage
epithelioid cell
multinucleated giant cell (Langerhans type and foreign-body type)

Cells included in the mononuclear phagocyte system in normal and inflamed tissues.

2.0 DEFINITION

Cells belong to the mononuclear phagocyte system as defined by van Furth et al. (1972), (see table).

Various functional criteria are used to distinguish mononuclear phagocytes from other cells. Cytochemical methods include the demonstration of non specific esterase (Yam et al., 1971), an enzyme located diffusely in the cytoplasm of almost all monocytes and macrophages. Peroxidase is present in granules in monoblasts, promonocytes, monocytes and exudate macrophages (van Furth et al., 1970) but is only demonstrable in resident macrophages using electron microscopy (Daems and van der Rhee, 1980) which detects peroxidatic activity in rough endoplasmic reticulum, nuclear envelope and the Golgi apparatus.

Morphology of macrophages depends on: the stage of maturation, tissue location and methods of elicitation. Generally the size of the cell varies from 10 - 25μ and the shape of the nucleus is oval or kidney shaped. The nuclear/cytoplasm ratio is relatively high in promonocytes in the bone marrow. Monocytes in the bone marrow have an indented nucleus and a lower nuclear/cytoplasm ratio and peripheral blood monocytes have an essentially similar morphology with more granules present in the cytoplasm. Tissue macrophages are generally larger than monocytes and contain more granules (van Furth et al., 1970; 1980). As macrophages mature the plasma membrane becomes more ruffled and surface microvilli increase.

Mononuclear phagocytes endocytose avidly. Soluble molecules are internalised either nonselectively by fluid phase pinocytosis or selectively by attaching to the macrophage membrane surface via receptors/...

receptors. However other cells such as fibroblasts also pinocytose albeit at a lower rate. Phagocytosis of particulate material is another major functional property of macrophages (Silverstein et al., 1977). Ingestion of latex particles is often used to demonstrate the presence of macrophages (Kohl et al., 1977) although the demonstration of Fc receptors by the ingestion of opsonised particles is a more useful criterion (Huber et al., 1969). Promonocytes are not as actively phagocytic as monocytes and a lower percentage of cells have Fc or complement (C3) receptors (56% of human promonocytes have Fc receptors and 31% have C3 receptors compared to 91% human monocytes with Fc receptors and 88% with C3 receptors (van Furth et al., 1980)). Fc and C3 receptors are also present on human skin and peritoneal macrophages. Similar results were found with mononuclear phagocytes from mice in which other tissue macrophages can also be examined. Van Furth et al. (1980) detected Fc receptors on murine macrophages from liver and lung as well as on skin and peritoneal macrophages. C3 receptors were present on peritoneal and skin macrophages but were almost not detectable on alveolar macrophages (2.2%) and only 33% of liver macrophages were C3 receptor positive.

The use of antisera would probably be the best method for detecting mononuclear phagocytes. However there are problems with cross reactivity with other cell types (Springer et al., 1979). Some recent reports have been published on the use of monoclonal antibodies which appear to recognize specific membrane determinants of mononuclear phagocytes and not other cell types (Todd et al., 1981; Rosenberg et al., 1981).

Generally more than one criterion must be examined before a cell can be assigned confidently to the mononuclear phagocyte system especially since the demonstration of particular markers is never 100% positive.

3.0 ORIGIN AND KINETICS

During ontogeny, haematopoietic stem cells, of mesenchymal origin, migrate to foetal liver where immature mononuclear phagocytes develop (Cline and Moore, 1972). Haematopoiesis begins in the bone marrow from the 3rd week of gestation in the mouse and continues throughout adult life. The most immature cell of the mononuclear phagocyte system in the bone marrow is the monoblast and its characteristics have been described by van der Meer *et al.* (1980) in bone marrow cultures. Monoblasts differentiate into promonocytes which are the direct precursors of monocytes. These remain in the bone marrow for a short time before they are released into the blood stream (van Furth *et al.*, 1972; van Furth, 1980). The kinetics of monocytopoiesis and turnover have been studied in several species including man *in vivo* and *in vitro* by measuring the incorporation of tritiated thymidine. Whitelaw (1972) calculated the half life for circulating human monocytes to be 71 hours. *In vitro* incorporation of tritiated thymidine indicated that monoblasts and promonocytes are actively dividing cells unlike monocytes and macrophages (van der Meer *et al.*, 1980). It is generally accepted that all tissue macrophages originate from the bone marrow (van Furth *et al.*, 1972). Although Volkman (1976) suggested from his experiments with tritiated thymidine incorporation that resident macrophages in the tissues undergo a slow rate of division and this is sufficient for self-renewal, the incorporation of label was very low. Daems and van der Rhee (1980) have suggested that resident macrophages are not derived from blood monocytes because they are the only macrophages without peroxidase activity within granules. However Beelen *et al.* (1980) has shown the existence of the "exudate-

resident/...

resident macrophage" which is a transitional cell between monocytes or exudate macrophages and resident macrophages, and Bainton (1980) has shown that monocytes in culture acquire resident macrophage characteristics.

Monocytopoiesis in inflammation is controlled by colony stimulating factor (CSF) and a serum factor (factor inducing monocytosis (FIM)). Macrophages are a potent source of CSF and the production is increased following stimulation with endotoxin (Eaves and Bruce, 1974) or antigen (Metcalf, 1971). Thus macrophages at the site of inflammation, by producing CSF, have a positive feedback mechanism which increases the proliferation of monocytes. Van Waarde et al. (1977) detected a serum factor (FIM) of mice in the early stages of inflammation which induced release of monocytes from the bone marrow.

Little is known about exactly how macrophages migrate to the tissues nor about the ultimate fate of these cells. There appears to be little recirculation via the blood stream. However they do migrate to lymph nodes and may die there (McPherson and Steer, 1980).

Although there is some controversy over the steady-state recruitment of resident macrophages it would appear that macrophages in inflammatory foci are recently derived from peripheral blood monocytes.

4.0 ROLE OF MACROPHAGES IN THE IMMUNE RESPONSE

Studies in vivo and in vitro have shown that macrophages are necessary for the development of a primary immune response. Experiments with live macrophages showed that macrophages briefly exposed to antigen and then injected into histocompatible hosts led to the development of specific immunity and a much greater response than if soluble antigen was injected instead (Spitznagel and Allison, 1970). Since macrophages are mobile cells, Unanue (1972) suggested that by phagocytosing antigen they can transport it to distant sites and by modifying the antigen make it more immunogenic for presentation to T and B cells.

Macrophages or adherent cells are needed in almost all immunogenic reactions in vitro. In the induction of a specific immune response in cultures of spleen cells (containing a mixture of T and B lymphocytes) antibody production was greater with antigen-pulsed macrophages than with soluble antigen alone (Katz and Unanue, 1973). The majority of humoral responses are T cell dependent and the generation of T helper (T_H) cells in response to stimulation by soluble antigens is dependent on the presence of a critical number of macrophages (Erb and Feldman, 1975a) and the macrophages and lymphocytes must share some identity at some portion of the major histocompatibility complex (MHC) for successful detection of the antigenic signal borne by the macrophages (Rosenthal and Shevach, 1973). The subsequent T lymphocyte - B lymphocyte interaction also requires macrophages (Shortman and Parker, 1971; Feldman, 1972). Both intact macrophages and a soluble factor released from macrophages (interleukin 1, IL-1) are required to support the differentiation of human/...

human B cells into immunoglobulin-secreting cells (Rosenberg and Lipsky, 1981). Activation of B lymphocytes by T cell-independent antigens appears to involve antigen presentation to B cells by macrophages (Boswell et al., 1980).

Macrophage-lymphocyte interaction may occur indirectly by the secretion of antigen complexed to soluble products or directly by cell-cell contact. Evidence for the former mechanism exists for the induction of T_H cells in the mouse (Erb and Feldman, 1975b). However soluble macrophage products do not elicit T lymphocyte proliferation in the absence of intact macrophages. Ultrastructural studies (Lipsky and Rosenthal, 1973) have shown that macrophages from several different sources are able to interact with T and B cells and in the presence of antigen the binding is dependent on sharing MHC gene products and leads to the proliferation of bound lymphocytes. The histocompatibility restriction of macrophage-T cell interactions in the presentation of antigen appears to be involved in the genetic control of the immune response which is regulated by the immune response (IR) genes linked to the MHC (McDevitt and Chinitz, 1969). Using two inbred strains of guinea pigs which differed genetically only at the I region of the MHC and had different immune responses in vivo, Rosenthal and Shevach (1973a, b) were able to show that antigen induced proliferation of T cells was dependent on the presence of antigen pulsed macrophages from the responder strain.

The I region of the MHC also codes for protein molecules (Ia antigens) which are expressed on the surface membranes of monocytes, macrophages, B cells and activated T cells (Sachs and Cone, 1973; Cullen et al., 1974). These determinants may be receptors with/...

with broad specificities which focus or orient antigen to T and B cells and allow recognition to occur (Feldman, 1977). For a given macrophage's repertoire of immune response gene products, their function may be to specify what portions of an antigen are seen by the T lymphocytes. The immune response to insulin as an antigen in guinea pigs is under genetic control (MHC linked). Barcinski and Rosenthal (1977) have shown that two different strains of guinea pig recognise distinct antigenic regions of insulin and they suggested that macrophages select discrete regions of the antigenic molecule for recognition by the T cell.

Farr et al (1979a,b) showed that when immune T cells (to Listeria monocytogenes) are cultured with macrophages and dead bacteria, the MHC restriction lay in the I-A subregion. Immune T cells established tenacious contact and bound only to macrophages bearing both antigen and the appropriate Ia molecule (Ziegler and Unanue, 1979). Similar histocompatibility restrictions are also evident in man. The secondary proliferative response of human T lymphocytes to low concentrations of PPD is dependent on the presence of macrophages with identical HLA-D determinants (Bergholtz and Thorsby, 1977).

After this initial essential interaction with T cells, macrophages secrete a number of growth and differentiation products including IL-1 (previously called lymphocyte activating factor (LAF) (Aarden et al., 1979)), which induces some T lymphocytes to proliferate and differentiate, leading to T_H cell production. Once this occurs B cells may be activated into antibody production, other T cell subsets are called into action and lymphokines produced which may lead to macrophage activation (Unanue et al., 1976). Other factors released may decrease lymphocyte/...

lymphocyte proliferation since relatively high concentrations of macrophages in culture systems depress lymphocyte activity (Nelson, 1976). These suppressive factors affect a variety of T and B lymphocyte proliferative (Kirchner, 1978) and non proliferative (Varesio et al., 1979) responses to antigens and mitogens. Prostaglandins may be involved and may act by inducing T suppressor activity (Fischer et al., 1981).

Macrophages are also required for cell mediated immunity both in vivo and in vitro. They are necessary for the generation of cytotoxic T cells in allograft rejection (Wagner et al., 1972) and have proved to be the stimulator cell in contact sensitivity (Thomas, 1977). Macrophages themselves play an effector role in cell mediated immunity. Specifically activated T cells secrete lymphokines which activate macrophages in vitro to become nonspecifically bactericidal, tumoricidal and antiviral (Adams, 1976). These lymphokines produced in vitro include macrophage inhibition factor (MIF) (David, 1966), MAF (Fowles et al., 1973) and a chemotactic factor for monocytes (Ward et al., 1969). Presumably these lymphokines are involved in vivo in attracting macrophages to the site of inflammation, immobilising and activating them. Recently Sher et al. (1980) have demonstrated the presence of a soluble mediator which induces exudates rich in Ia positive macrophages.

Intensive study into the biochemical nature of activated macrophages has demonstrated that these cells secrete many biologically active substances not normally secreted. Most of this knowledge comes from in vitro work and the relative in vivo importance of such factors/...

factors has to be inferred (Cohn, 1978; Karnovsky and Lazdins, 1978; Allison, 1978). The products secreted (see Davies and Bonney (1979) for a review) include the neutral proteases, plasminogen activator, elastase and collagenase. However, the increase in enzyme secretion did not necessarily correlate with cytocidal activity (Karnovsky and Lazdins, 1978). They may function to degrade tissue around the inflammatory site and aid macrophage migration. Collagen degradation products are also chemotactic for macrophages (Chang and Houck, 1970). The conversion of plasminogen to plasmin by plasminogen activator may then lead to the activation of certain cascade systems including the clotting system and complement system. Activation of these systems in vitro induced rapid spreading by nonstimulated macrophages (Bianco et al., 1976) in a manner apparently analogous to that observed with macrophages which have been activated in vivo (North, 1968). The complement factor involved in in vitro spreading of macrophages has been identified as the proteolytic fragment of factor B, Bb, of the alternate pathway of complement (Bianco et al., 1979). They also showed that activated factor B inhibited the migration of normal macrophages. Since macrophages synthesise and secrete the necessary components for the generation of Bb (Brade and Bentley, 1980) it is possible that in vivo macrophages are attracted to the site of inflammation and then immobilised by local production of Bb. Sundsmo and Gotze (1979) have suggested that Bb may exert its action on macrophages by cleaving C5 which is present on the macrophage membrane. Complement receptors could thus enhance cell activation since complement-activating substances are coated with C3b Bb P (C3-C5 convertase of the alternate pathway of complement) and may be bound via the C3b to complement/...

complement receptor bearing macrophages, thereby bringing the convertase into contact with membrane C5 and causing cell activation. A heat-labile plasma factor induced human monocytes to kill xenogeneic red blood cell targets (Muchmore et al., 1979). Evidence suggested that factor B or the late acting complement components were involved.

Activation of the alternate pathway also leads to the production of C3b which has been shown to induce lysosomal enzyme release from macrophages (Schorlemmer et al., 1976). Some of these enzymes cleaved C3 and thus amplification of the system is possible.

Mononuclear phagocytes also secrete α-2-macroglobulin a plasma proteinase inhibitor against a broad spectrum of proteinases (Hovi et al., 1977) which may act to regulate the effects of protease activity in inflammatory reactions.

Stimulated macrophages secrete certain arachidonic acid oxygenation products, prostaglandin E_2 (PGE_2) being the major product of most mononuclear phagocytes studied (Bonney et al., 1980). Prostaglandins are probably important in inflammatory lesions (Bray, 1980). Certain prostaglandins (PGE_1, PGE_2, PGA_2 and PGB_2) have been shown to be chemotactic and/or to inhibit spreading or adhesion of mouse peritoneal macrophages in vitro (Cantarow et al., 1978) and may be involved in modulating the immune response. They can suppress many lymphocyte functions such as lymphocyte proliferation to Con A (Metzgar et al., 1980) although Kelly et al. (1979) found that inhibition of the cyclo-oxygenase pathway reduced lymphocyte transformation in response to PHA. These contradictory results may reflect concentration differences. Very low concentrations of prostaglandin E may enhance mitogenesis whereas high doses may inhibit suggesting that/...

that there may be a regulatory mechanism which decides the outcome of the inflammatory response. Exogenous E-type prostaglandins inhibited MIF production (Gordon et al., 1976) as well as production of mitogenic lymphokines and inflammatory macrophages produce PGE_2 in response to lymphokines. Thus macrophage derived PGE_2 may provide an extracellular mediator of T-cell function (Bray et al., 1978) and by this feedback mechanism inhibit lymphokine-dependent activation of macrophages (Schultz et al., 1978).

Other arachidonic acid metabolites are also synthesised by macrophages in response to inflammatory stimuli. 5-hydroxyeicosatetraenoic acid (5-HETE) and 11-HETE which are chemotactic for both neutrophils and eosinophils (Goetzl et al., 1980) are elaborated by phagocytosing rabbit alveolar macrophages via the lipoxygenase pathway (Valone et al., 1980). Production of these chemotactic factors may serve to amplify the cellular component of the inflammatory reaction. Inhibition of the lipoxygenase pathway (Kelly et al., 1979) has been shown to reduce lymphocyte transformation in vitro. Thus modulation of T-cell function may also be affected by these products of arachidonic acid metabolism.

Oxidative metabolism is also increased in activated macrophages and includes increased glucose oxidation, oxygen consumption and hexose monophosphate shunt activity (Nathan and Root, 1977). Superoxide anions (Johnston et al., 1978) and hydrogen peroxide (Nathan et al., 1979a) are secreted and may play an important role in the killing of tumour cells and parasites (Nathan et al., 1979b and c). They may also be involved in the destruction of connective tissue and inflammatory mediators themselves and hydrogen peroxide formation suppressed lymphocyte proliferation to Con A in vitro (Metzgar et al., 1980).

Interferons/...

Interferons are secreted by activated macrophages and these may be involved in the activation of killer T lymphocytes and natural killer cells (Djeu et al., 1979) and they also inhibit both proliferative and nonproliferative activity of lymphocytes by cell to cell contact (Schwartz et al., 1978).

Other changes in activated macrophages include increased adherence, spreading and ruffling of membranes (Blanden, 1968), increased pinocytic (Edelson et al., 1975) and phagocytic capabilities (Bianco et al., 1975). In the latter study thioglycollate-induced mouse peritoneal macrophages exhibited altered complement receptor function in that complement coated particles were bound and ingested by these macrophages whereas normal mouse peritoneal macrophages appear to bind and not ingest these particles. Recent studies in vitro (Griffin and Griffin, 1980) have suggested that a T-cell factor is involved. Macrophage plasma membrane receptors for the Fc fragment of IgG augment the killing and phagocytosis of infectious agents during a secondary immune response (Zuckerman and Douglas, 1979) and their expression is also altered in activated macrophages. A T-cell factor from immune T cells restimulated with antigen in vitro increased Fc receptor expression on cultured macrophages (Steinman et al., 1980).

Although ideas are changing frequently about the role of macrophages in the immune response, they obviously play an important part in the induction of the immune response where antigen presentation and specific interactions with T cells (and possibly B cells) are regulated by I-region genes, possibly at the level of Ia expression on/...

on macrophages. In cell mediated immunity the principle effector cells are macrophages which have been activated independently of MHC genes by lymphokines produced by activated T lymphocytes. Activated macrophages are involved in chronic, allergic and inflammatory diseases and are able to nonspecifically kill bacteria, parasites, tumour cells and viruses. By interacting with other cells including granulocytes and lymphocytes and with various cascade systems, macrophages are able to regulate the immune response by amplifying or suppressing their effects.

5.0 CHEMOTAXIS

During an inflammatory response, the production of soluble mediators at the site of injury or invasion by micro-organisms attracts phagocytes to leave the circulation and migrate to the focus of injury where the cells either phagocytose foreign material or release various substances including lysosomal enzymes for the digestion of tissues and foreign materials, and other inflammatory mediators such as prostaglandins. One mechanism by which leukocytes accummulate at inflammatory sites may be chemotaxis or directed migration. Although it has not been possible as yet to show that chemotaxis occurs *in vivo*, studies *in vitro* have shown that cells can respond to chemotactic substances in their environment in at least two different ways: by chemotaxis and by chemokinesis or random movement. *In vitro* methods of assessment should distinguish between the two modes of movement (Keller *et al.*, 1977). Microscopic methods are used including time-lapse photography where individual cells are followed (Allan and Wilkinson, 1978; Wilkinson and Allan, 1980) but they are time consuming and laborious. Measurement of cell movement using Boyden chambers or under agarose methods do not differentiate between chemotaxis and chemokinesis, unless a checkerboard assay is carried out (Zigmond and Hirsch, 1973).

5.1 Chemotactic factors

Many chemotactic factors have been described and their structures, chemical and biological properties and origins vary considerably. However their *in vivo* role is not always clear. Probably in any inflamed tissue there are multiple factors of varying potency and different factors may be important at different stages of inflammation.

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5.2 Endogenous chemotactic factors

These include the complement derived proteins, C5a and $C5a_{des\ arg}$; blood coagulation products, kallikrein, plasminogen activator and fibrin split products; factors generated from the proteolytic degradation of collagen, and soluble substances released from granulocytes, macrophages and activated T lymphocytes.

5.3 Complement derived factors

These are especially important due to their potency and the variety of pathways which lead to their formation (Ward et al., 1965). Decreased neutrophil chemotaxis in animals and man deficient in key complement components (Rosenfeld et al., 1974; Cochrane et al., 1970) and animals artificially depleted of complement (Cochrane et al., 1970) suggest that complement components are important in vivo. This is also borne out by the isolation of chemotactically active complement factors from inflamed tissues (Ward and Zvaifler, 1971; Hill and Ward, 1971).

The complement cleavage factor, C5a, is a potent chemoattractant for monocytes and macrophages (Snyderman et al., 1971 and 1972) and other leukocytes (Hugli and Muller-Eberhard, 1978). It had previously been thought that another complement-derived anaphylotoxin C3a was also chemotactic for leukocytes but a report by Fernandez et al. (1978) clearly refutes these earlier findings. Contamination of minute amounts of C5a accounted for observed chemotactic activity observed with C3a and possibly also C567. C5a is normally converted to an anaphylotoxin-inactive form $C5a_{des\ arg}$ by a carboxypeptidase B-like enzyme present in normal serum but $C5a_{des\ arg}$ remains chemotactic (Fernandez et al., 1978; Chenoweth and Hugli, 1980).

C5a is not only generated by the classical and alternate pathways of complement but also through direct activation by C5 cleaving proteases which/...

which have been demonstrated in leukocytes, platelets, myocardium and human epidermis (Weksler and Coupal, 1973; Levine et al., 1976). These proteases are called chemotaxigens i.e. they generate chemotactic activity but are not themselves chemotactic. They may be important in complement-associated infections where the classical or alternate pathways cannot be demonstrated.

5.4 Chemotactic factors generated by other cascade systems

Activation of the kinin-generating and the coagulation/fibrinolytic pathways are frequent accompaniments to inflammation and provide a potential source of chemoattractants. Many stimuli including endotoxin, basement membranes and exposed native collagen activate Hageman factor. This is the first recognised event in the contact phase of the intrinsic system of blood coagulation and chemotactic factors are produced via this cascade system (Ward, 1968; Bianco et al., 1976). Prekallikrein and plasminogen proactivator are converted to their activated forms kallikrein and plasminogen activator by Hageman factor. Both conversion products are chemotactic in vitro (Kaplan and Austen 1975). Plasminogen activator cleaves serum plasminogen, thus generating plasmin, a proteolytic enzyme which acts on fibrin to produce fibrinolytic peptides which are also chemotactic for macrophages (Snyderman and Mergenhangen, 1976).

In vitro chemotactic factors have been produced by the enzymatic degradation of collagen and immunoglobulins (Chung and Houck, 1970; Aoki et al., 1976). Collagen degradation products may be produced in inflammatory reactions in vivo by the action of collagenase, which is produced by activated macrophages in vitro (Wahl et al., 1974), on tissue substrates.

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5.5 Cellular-derived chemotactic factors

Lymphocytes when incubated with mitogens or antigens produce and release soluble mediators of inflammation termed lymphokines (Dumonde et al., 1969). Some of these have been shown to be chemotactic for neutrophils, monocytes, macrophages and basophils (Ward et al., 1969).

Human leukocytes from PPD-sensitive humans released a chemotactic substance of molecular weight, 12,500 daltons when they were incubated with PPD antigen (Altman et al., 1973). A similar substance was also produced when lymphocytes were incubated with mitogen. It was termed lymphocyte-derived chemotactic factor or LDCF (Altman et al., 1974) and is isoelectrically and antigenically distinct from C5a.

Both activated T and B lymphocytes produce LDCF. T lymphocytes required the presence of macrophages for its production but B cells apparently did not (Wahl et al., 1975). In vivo studies have suggested that a chemoattractant indistinguishable from the LDCF produced by antigen-stimulated lymphocytes in vitro was produced in delayed-type hypersensitivity (DTH) reactions in vivo (Postlethwaite and Snyderman, 1975) concomitantly with the appearance of macrophages at the inflammatory site.

Tumour cells have also been reported to produce a chemotactic factor for macrophages (Meltzer et al., 1977) with peak levels produced during log phase. It was distinguishable from LDCF by its molecular weight and behaviour on ion-exchange chromatography. Thus chemotactic factors released by tumours or proliferative cells may provide an endogenous mechanism for macrophage accumulation.

5.6/...

5.6 Lipid-associated chemotactic factors

Prostaglandins have been reported to be chemotactic for leukocytes (Cantarow et al., 1978) although others have not found consistent chemotactic activity (Goetzl et al., 1980). However products of arachidonic acid oxidised via the lipoxygenase pathway were chemokinetic and chemotactic for neutrophils and eosinophils and less so for monocytes (see Goetzl et al., 1980, for a review of the effects of HETES on leukocyte functions). Two HETES, 5-HETE and 11-HETE are synthesised and secreted by rabbit alveolar macrophages in response to the inflammatory stimulus, zymosan (Valone et al., 1980). Most chemotactic agents are chemotactic and chemokinetic at similar concentrations and deactivate cells at very low concentrations whereas HETES were chemokinetic at a lower dose and deactivated at a similar concentration to that which induced chemotaxis. They also did not affect oxidative metabolism or induce lysosomal enzyme release unlike most other chemoattractants studied. However one of the precursors of HETEs augmented the enzyme releasing capacities of some of the other chemotactic factors including C5a.

5.7 Exogenous chemotactic factors

5.8 Bacterial chemotactic factors

Production of chemoattractants by a variety of micro-organisms has been demonstrated. Keller and Sorkin, (1967a) found that culture filtrates of Staph. albus and E. coli contained chemotactic activity for neutrophils and Wilkinson et al. (1974) showed that C. parvum extracts were chemotactic for macrophages. These chemoattractants were produced by bacteria during log phase of growth (Ward, 1974). Some organisms possess molecules which may activate the alternate pathway of complement (Ward et al., 1973; McBride et al., 1975), and they/...

they also produce chemotaxigens capable of directly cleaving C5 to produce the anaphylotoxin and chemotactic factor, C5a (Keller and Sorkin, 1967a).

5.9 Synthetic chemotactic factors

The above bacterial factors could not be attributed to a single common entity. Schiffman et al. (1975) found that N-terminal blocked methionyl di and tripeptides were chemotactic for neutrophils and macrophages. The initiation of protein synthesis in prokaryotes uses a formylated amino acid and this amino acid together with some of the adjacent amino acids are cleaved from the nascent protein. They speculated that the chemotactic response to formylated peptides (the positively charged terminal amino group must be blocked for chemotactic activity to be evident in the peptide) is a simple recognition system by which mammalian leukocytes can detect microbial agents since eukaryotic cells mainly initiate protein synthesis with nonacylated methionine.

This discovery allowed the synthesis of highly purified structurally defined substances, previously unavailable for studying the physiology and biochemistry of chemotactic factor interactions with leukocytes. Maximum chemotactic activity required that the positively charged terminal amino group must be neutralised and N-formylation was preferable to N-acylation (Showell et al., 1976). Methionine was not obligatory for the N-terminal position since other amino acids of similar hydrophobicity could be substituted. In the second position an amino acid with a nonpolar side chain was necessary for maximal chemotactic activity and the most active peptides were those with phenylalanine in the third position. However Wilkinson (1979) has found that the structural/...

structural constraints on N-terminal blocked peptides need not be so specific for chemotactic activity. The most active peptides were those which were amphipathic with a considerable hydrophobic moiety.

Thus there are numerous ways in which chemotactic factors can be generated which provides an efficient mechanism for host defence.

5.10 Regulation

During an inflammatory process, macrophages, having migrated to the inflammatory lesions must be retained in these sites where they become activated and carry out their effector role in inflammation in a restricted local environment. Control mechanisms may be at the level of the chemotactic factor by blocking its production or activity and at the cellular level by rendering the phagocytes unresponsive to all chemotactic factors. Incubation of cells with high concentrations of chemoattractants as would be found at the inflammatory foci, deactivates leukocytes (Ward and Becker, 1968) and thus cells may be confined in the lesion.

Plasmin and activated factor B, Bb may be produced at inflammatory sites and Bianco et al. (1976, 1980) have shown that in vitro, both these factors induced spreading of macrophages and inhibition of migration.

Cell directed inhibitors of chemotaxis are produced by inflammatory effector cells including neutrophils (Goetzl, 1975) and lymphocytes (David, 1966) which produce lymphokines, leukocyte MIF and macrophage MIF. In addition to the cell-derived factors, the serum proteinase inhibitors $\alpha 1$ antitrypsin and $\alpha 2$ macroglobulin inhibited chemotaxis of leukocytes (Goetzl, 1975).

Several/...

Several serum proteins may act directly to inactivate chemotactic factors. C5a is inactivated by at least two serum proteins, anaphylotoxin inactivator and chemotactic factor inactivator. The latter protease(s) also has activity against kallikrein and plasminogen activator, both of which are also inhibited by α2 macroglobulin, and kallikrein is also inhibited by C1 esterase inhibitor (Ward et al., 1977).

A greater knowledge of the influencing factors involved in regulation of chemotaxis may allow better control of inflammatory disease. For instance monocyte chemotaxis in vitro is decreased in patients with influenza (Pike et al., 1977) and also in neoplastic disease (Boetcher and Leonard, 1974; Hausman et al., 1975).

5.11 Mechanism of chemotaxis

Most of the studies on the mechanism of chemotaxis have been carried out on neutrophils. However macrophages have been investigated as well and show similar responses as neutrophils and it is assumed that the majority of results obtained with neutrophils are applicable to macrophages.

Margination into the blood vessels is necessary before phagocytes can migrate through the tissues to the site of inflammation and it has been shown that chemotactic substances caused granulocytes to aggregate in vitro and when they were injected in vivo neutropenia resulted (O'Flaherty et al., 1977). This suggests that chemotactic agents must first make the phagocytes more sticky so that they can adhere to blood vessel walls, possibly by decreasing the negative charge which is present on most biological membranes and therefore minimising charge repulsion between the phagocyte membrane and the capillary wall (Gallin et al., 1975).

Chemotactic/...

Chemotactic factors probably initiate responses by interacting at the cell membrane. The response to chemotaxins is very rapid and the presence of specific receptors for certain chemotactic factors on leukocyte membranes has now been demonstrated. Evidence for the existence of formyl methionyl peptide receptors on rabbit and human neutrophils has been shown by various workers (Aswanikumar et al., 1977; Williams et al., 1977; Vithauskas et al., 1980). Binding was rapid, saturable, specific and reversible and the specificity of binding by the peptides correlated with biological activity. Similar receptors have also been demonstrated on inflammatory and resident peritoneal guinea pig macrophages (Snyderman and Fudman, 1980) and on a human monocyte cell line (Pike et al., 1980). Human neutrophils also have C5a receptors (Chenoweth and Hugli, 1978, 1980) and again the binding was rapid, specific and saturable. $C5a_{des\ arg}$ and $C5a_{1-69}$ (lacking five C-terminal residues which does not have chemotactic activity) both bind to the C5a receptor suggesting that the receptor recognition site is in an internal portion of the molecule but the activation site is represented in the C-terminal end (Chenoweth and Hugli, 1980). The receptors for f-Met-Leu-Phe and C5a appear to be distinct since C5a did not inhibit formyl peptide binding to neutrophils (Aswanikumar et al., 1977; Williams et al., 1977). Neutrophils also have receptors for a chemotactic factor generated in neutrophils by urate crystals (Spilberg and Mehta, 1979). Binding was saturable and specific in that the chemotactic factors f-Met-Leu-Phe, gly-his-gly and C5a did not displace the binding of the cell-derived chemotactic factor.

K_D values for all the receptors were similar to the concentration required to give half maximal response and the number of binding sites was usually about 10^5 per cell. F-Met-Leu-Phe receptors were also present/...

present on isolated cell membranes (Vithauskas et al., 1980).

Membrane-modifying agents perfringolysin, a cholesterol binding agent, and phospholipase C diminished the chemotactic responsiveness of neutrophils to f-Met-Leu-Phe, suggesting that the receptors may be intimately associated with hydrophobic domains in the lipid bilayer and depend on a defined lipid environment for effecting their function (Wilkinson, 1979).

Thus receptors for high potency chemotactic factors do exist on leukocyte surfaces. Other chemoattractants such as denatured proteins may not have specific receptors but may exert their influence by hydrophobic interactions with the membrane (Wilkinson, 1974). Some of these chemotactic factors such as casein are also amphipathic which appeared to be one of the major criterion for chemotactically active formyl peptides (Wilkinson, 1979).

The interaction of formyl peptides and C5a with whole cells was rapid but dissociation was very slow (Vithauskas et al., 1980; Chenoweth and Hugli, 1978). Interaction of the formyl peptide and its receptor led to rapid internalisation possibly by pinocytosis (Vithauskas et al., 1980; Niedel et al., 1979) and loss of binding site activity. More receptors were lost from the membrane than the number of molecules bound suggesting that other mechanisms are also involved apart from endocytosis, possibly the binding sites become inactivated. Deactivation following incubation with formyl peptides was also proportionally greater than the loss of receptor sites suggesting that a post receptor event was at least partially responsibly for deactivation apart from simply receptor loss (down regulation).

5.12/...

5.12 Transduction

Chemotactic factors increased the permeability of leukocytes to the cations Na^+, K^+ and Ca^{2+} (Naccache et al., 1977) which are required externally for optimal responsiveness to chemotactic factors (Showell and Becker, 1976; Gallin and Rosenthal, 1974). The alteration in Na^+ and K^+ fluxes may have been due to activation of the Na^+,K^+ ATPase as chemotactic factors increased the activity of this enzyme in isolated neutrophil plasma membranes (Becker et al., 1978). Gallin and Gallin (1977) measured membrane potential changes following activation with chemotactic factors in macrophages using standard electrochemical techniques and Gallin et al. (1980) measured similar changes in human monocytes using a membrane potential sensitive cyanine dye. They found that chemotactic factors induced a K^+ dependent membrane hyperpolarization. Similar experiments with neutrophils showed that the hyperpolarization triggered by chemoattractants was inhibitable by oubain, suggesting that there may be a direct effect on the Na^+,K^+ ATPase (Seligman et al., 1980).

Ca^{2+} also appeared to contribute to the membrane hyperpolarization triggered by chemotactic agents. Both an increase in permeability to external Ca^{2+} and release of Ca^{2+} from intracellular stores may play a role in chemoattractant induced membrane potential changes (Naccache et al., 1979a; Gallin et al., 1980). Electron microscopic studies have indicated that f-Met-Leu-Phe caused circumferential loss of plasma membrane-bound Ca^{2+} (Hoffstein, 1979) presumeably to the interior of the cell.

Transmethylation reactions mediated by S-adenosyl-L-methionine (SAM) appear to be involved in cell movement. Inhibition of this pathway/...

pathway blocked carboxy-O-methylation of monocyte proteins concomitantly with inhibition of monocyte chemotaxis (Pike et al., 1978). A rapid stimulation of carboxy-O-methyltransferase activity in rabbit neutrophils by f-Met-Leu-Phe has been reported (O'Dea et al., 1978) although Pike et al. (1979) found that chemoattractants had no effect on the methylation of proteins in guinea pig macrophages which would seem to contradict their earlier data (Pike et al., 1978). Activation of the chemotactic factor receptors also stimulated another enzyme, protein methylesterase which hydrolyses the methyl ester groups on the methylated proteins (Venkatasubramanian et al., 1980). They suggested that the increased turnover of protein methylesters may result in oscillations between charged and uncharged forms of the methyl accepting protein. This may contribute to alterations of membrane events which may occur in the earliest stages of chemotaxis.

Chemotaxins inhibited the methylation of phosphatidylethanolamine (PE) in macrophages (Pike et al., 1979) thereby inhibiting the formation of phosphatidylcholine (PC). Increased turnover or degradation of phospholipids was not detected and since PC is the major lipid in the membrane, the ratio of PE/PC would be increased in newly synthesised membrane. A continuous supply of new membrane lipid and cholesterol was required for human monocyte and neutrophil chemotaxis (Pike and Snyderman, 1980). They suggested that when the chemotactic factor bound to the cell, phospholipid methylation was inhibited locally which may lead to changes in the biophysical properties of the membrane such as fluidity. These asymmetric membrane alterations may be necessary to establish polarised cellular orientation.

Although Hirata et al. (1979) also found that chemoattractants inhibited phospholipid methylation, increased degradation of PC and not/...

not decreased synthesis accounted for their observation. The disappearance of methyl groups was associated with release of arachidonic acid which suggested that phospholipase A_2, an enzyme stimulated by Ca^{2+} (Gullis and Rowe, 1975), which removes an unsaturated fatty acid from phospholipids was activated by chemotaxins. Accumulation of phosphatidyl-N-monomethylethanolamine within the membrane increased membrane fluidity (Hirata and Axelrod, 1978). The release of arachidonic acid is potentially important since it is the precursor of prostaglandins and lipoxygenase products, HETEs which play a critical role in inflammation. Phospholipase activation by chemotactic factors may be mediated by an increase in intracellular Ca^{2+}. Inhibition of the lipoxygenase pathway in neutrophils which is the main pathway for arachidonic acid in neutrophils (Borgeat and Samuelsson, 1979) prevented increased membrane permeability to Ca^{2+} following stimulation with chemoattractants. Displacement of Ca^{2+} from intracellular stores was unaffected, suggesting that the two processes of Ca^{2+} metabolism can be separated, one preceeding the other and are related to arachidonic acid metabolism (Naccache et al., 1979b). Ca^{2+} fluxes may also play a role in the transduction of chemotactic factor binding to the receptor and inducing methylation of proteins.

Actin and myosin are probably involved in chemotaxis. Stimulation of neutrophils with a gradient of chemoattractant led to an increase in the concentration of actin filaments in the pseudopod put out by the neutrophil as an initial response to the gradient. The concentration of actin was maintained in the membraneous portion of the cell facing the gradient (Oliver et al., 1978). Microfilaments appear to be involved in both chemotaxis and chemokinesis since both are inhibited by cytochalasin B (Zigmond and Hirsch, 1972).

Microtubule/...

Microtubule involvement is not so clear. It has been suggested that microtubules are not required for spatial detection of or for locomotion towards a chemotactic factor but are important for accurate turning by leukocytes and for maintenance of the polarity of the moving cell (Allan and Wilkinson, 1978).

Ca^{2+} fluxes may be involved in microtubule and microfilament assembly (Gallin and Rosenthal, 1974).

Chemoattractants induced a transient increase in intracellular levels of cAMP (Simchowitz et al., 1980) before other changes were observed. However agents which are known to increase intracellular cAMP decreased locomotion (Hill, 1978). Possibly chemotaxin induced increases in cAMP may be compartmentalised whereas these agents induce an overall increase in cAMP. Chemotactic factors also induced a small and transient increase in cGMP and agents which increase intracellular levels of cGMP enhanced locomotion (Hill, 1978). Probably these agents modulate rather than initiate chemotaxis possibly by affecting microtubule assembly either directly or through stored Ca^{2+} (Zurier et al., 1974). The accumulation of lysophosphatidylcholine mentioned above may play a crucial role in the regulation of the guanyl cyclase (Shier et al., 1976).

5.13 Other leukocyte changes induced by chemotactic factors

Metabolism is dramatically altered but these changes are not necessarily associated with the actual directed migratory response. Both aerobic glycolysis and HMPS activity increased following incubation with chemotactic factors even when the cells had been deactivated and no longer responded chemotactically (Goetzl and Austen, 1974). However these pathways may provide ATP for membrane-associated transport systems,/...

systems, microfilament contractions and possibly microtubule assembly. Lysosomal enzyme release was triggered by chemoattractants. Although the concentration of chemotactic factors was higher than that necessary for migration the structure/activity relationship of some of the chemotaxins such as the formylated peptides was the same (Showell et al., 1976). N-formylated peptides also produced chemiluminescence in leukocytes (Hatch et al., 1978; Lehmeyer et al., 1979; Beswick and Kay, 1981). Chemiluminescence is probably a measurement of superoxide and myeloperoxidase dependent reactions. Structure/activity relationships suggest that these activities are elicited through interaction of the peptides with a common receptor.

Directed migration results from the culmination of a complex series of biochemical events. Initial recognition, at least with some chemotactic factors is through the reversible interaction with specific membrane receptors. This may induce perturbations in the membrane, possibly through conformational changes in the receptor molecule and rearrangement of membrane lipids. These alterations may affect membrane-associated enzymes such as the Na^+,K^+ ATPase which increases the sensitivity of neutrophils to chemoattractants. Local permeability changes for divalent cations may also be affected. The displacement of intracellular membrane Ca^{2+} may increase phospholipase activity and thereby increase the production of arachidonic acid which may be necessary for increased permeability to external Ca^{2+}. Influx of Ca^{2+} leads to elevated levels of intracellular Ca^{2+} and may activate the cell's contractile system. Alterations in membrane lipids may also affect cyclic nucleotide levels and these may also be involved in microtubule assembly. Both methylation of phospholipids and proteins are affected by chemotaxins and may/...

may be involved in the control of membrane-associated enzyme and permeability. They may also induce local changes in membrane fluidity which may be necessary for pseudopod formation and cellular orientation in a chemotactic gradient. Activation of metabolic processes may or may not be necessary for chemotaxis per se and may be more important when the cell has reached the inflammatory site and can effect its inflammatory role.

6.0 MACROPHAGE PLASMA MEMBRANE

The chemical composition of macrophage membranes is similar to that of other mammalian cells and consists of 46% protein, 41% lipid and 8% carbohydrate (Nachman et al., 1971a). SDS gel electrohporesis of solubilised membranes from rabbit alveolar macrophages elicited by B.C.G. yielded seven to nine major proteins with molecular weights between 7×10^4 daltons and 14×10^4 daltons. Pratt and Cook (1979), in a more recent study on resident rabbit alveolar macrophages, isolated five major glycoproteins with molecular weights varying between 2.8×10^4 and 8×10^4 daltons. These discrepancies may reflect differences between resident and stimulated macrophage membranes. Yin et al. (1980) reported that there were quantitative and qualitative differences between plasma membrane polypeptides of resident and activated macrophages. Kaplan et al. (1979) isolated five major polypeptides and seven to eight minor bands from [^{35}S] methionine labelled cells (thioglycollate induced mouse peritoneal macrophages). The major proteins had molecular weights between 4.5×10^4 and 31×10^4.

The major lipids in macrophage plasma membranes consist of phosphatidyl choline, sphingomyelin and phosphatidyl ethanolamine as well as a more unusual lipid, lyso (bis) phosphatidic acid (Mason et al., 1972). The fatty acids of macrophage phospholipids contain an unusually high proportion of arachidonic acid compared to other leukocytes (Stossel et al., 1974).

6.1 Turnover of macrophage plasma membrane constituents

Nachman et al. (1971b) found a seven to eight hour half life of [^{14}C]-leucine labelled proteins in the plasma membrane of rabbit alveolar macrophages. Although these proteins had varying rates of synthesis/...

synthesis, all the labelled proteins were rapidly degraded at the same rate. These cells are known to endocytose at a rapid rate and this may have accounted for the bulk removal of membrane constituents. A more rapid endocytic rate with partial recycling and partial degradation would also fit their data. The turnover of most surface polypeptides of molecular weight greater than 5×10^4 daltons in thioglycollate induced mouse peritoneal macrophages was very slow ($t_{\frac{1}{2}}$ was greater than eighty hours (Kaplan et al., 1979)). Using the lactoperoxidase iodination method for studying cell surface proteins Yin et al. (1980) reported that glycoproteins on mouse peritoneal macrophage membranes were degraded at different rates, suggesting that the membrane is not turned over as a single unit as was previously implied by the results of Nachman (1971b). Again the turnover of most polypeptides was slow ($t_{\frac{1}{2}}$ = 21.7h). It had previously been suggested that pinocytosis was the mechanism for the turnover of plasma membrane proteins (Edelson and Cohn, 1976) in order to explain the decrease in 5′ nucleotidase activity in thioglycollate and endotoxin induced peritoneal macrophages which show an increased pinocytic rate. However the turnover of plasma membrane proteins is much slower than the expected pinocytic rate (Steinman et al., 1976) of two cell surface equivalents per hour in resident peritoneal macrophages. It is possible that membrane is selectively excluded from endocytic vesicles or that it is recycled after endocytosis as suggested by Muller et al. (1980) who radiolabelled phagolysosomal membranes and showed that the label was rapidly (within 30 minutes) returned to the plasma membrane. Similarly with ligand-induced pinocytosis, Fc receptors regenerated within 30 minutes after pinocytosis of a surface load of receptors saturated with small antigen-antibody/...

antibody complexes (McKeever et al., 1976). Rapid recycling of pinocytosis receptors on rat alveolar macrophages for glycoprotein also occurs (Stahl et al., 1980). So at least some membrane constituents are not degraded after internalisation but appear to be recycled. The slow turnover of membrane proteins may also reflect the degradation rate within the lysosome.

Both Kaplan et al. (1979) and Yin et al. (1980) also found that there was a more rapid phase of label loss from membrane proteins apparently due mainly to low molecular weight species. Yin et al. (1980) recovered this portion of the label in the medium suggesting that it was shed directly from the cell surface and not endocytosed. Therefore there may be two different modes of cell surface protein turnover. It is not clear whether individual proteins are specifically removed by one route or the other.

Others have studied the kinetics of regeneration of plasma membrane components after exposure of intact cells to hydrolytic enxymes. Following trypsin treatment a membrane protein involved in cholesterol metabolism required seven hours for full recovery of normal cholesterol exchange rates and required protein synthesis (Werb and Cohn, 1972). Fc receptors on a macrophage cell line were regenerated after removal with trypsin ($t_{\frac{1}{2}} < 12h$) and the restoration was blocked by the protein synthesis inhibitor, cyclohexamide (Unkeless and Eisen, 1975). However it is impossible to conclude from these data whether these rates reflect a normal membrane protein synthetic rate or an altered rate due to a damaged cell surface.

Following phagocytosis of latex particles there was a decrease in plasma membrane constituents in the mouse peritoneal macrophage

(5'/...

(5′ nucleotidase, phospholipids and cholesterol) (Werb and Cohn, 1972). Fc receptors were also decreased following phagocytosis of latex particles from rabbit and human alveolar macrophages (Daughaday and Douglas, 1976) and to a lesser extent by human blood monocytes (Schmidt and Douglas, 1972). Approximately six hours after phagocytosis these plasma membrane constituents started to return to normal levels and the regeneration was dependent on protein synthesis. The amount of new membrane synthesised was proportional to the amount of membrane interiorized around the latex particles suggesting that interiorization of membrane constituents and their subsequent degradation is tightly coupled to the cellular processes involved in membrane biosynthesis. However internalization is not a completely random process. The membrane transport systems for amino acids and nucleotides in neutrophils and macrophages (Tsan and Berlin, 1971) and complement receptors in macrophages (Daughaday and Douglas, 1976) appeared to be excluded from membrane internalisation during phagocytosis of latex. However when neutrophils were treated with colchicine, phagocytosis of latex particles resulted in the internalisation of the transport carriers. Thus the apparent segregation of transport and phagocytic sites appears to be under the control of cytoskeletal elements (Ukena and Berlin, 1972).

Phospholipid synthesis is increased following phagocytosis (Elsbach, 1968). However there is no evidence that phospholipid synthesis is necessary for phagocytosis except when large numbers of particles are ingested (Pike and Snyderman, 1980).

7.0 ENDOCYTOSIS

Utilising their capacity for endocytosis, macrophages fulfill many important roles in host defence against micro-organisms, parasites and possibly invasive neoplasms, in scavenging foreign material and senescent or damaged cells. It provides a mechanism for the acquisition of nutrients from the external environment, the down regulation of cell surface receptors and is possibly involved in plasma membrane turnover. It has important secondary consequences by triggering macrophage secretory functions.

Endocytosis has been studied *in vivo* by measuring the plasma clearance rates of various substances. Liver and spleen macrophages are the main cells involved in plasma clearance. However these studies are complicated by the presence of serum opsonins and other host factors such as hormones. Most of the work on endocytosis has been carried out *in vitro* using readily available mononuclear phagocytes including peripheral blood monocytes, mouse peritoneal macrophages and also alveolar macrophages and macrophage cell lines.

Endocytosis can be divided into two separate categories: phagocytosis which involves the internalization of particles with diameter greater than 1μ and pinocytosis which involves the uptake of soluble substances. Pinocytosis, itself, can be subdivided into micropinocytosis in which membranous vesicles of diameter less than 0.1μ are interiorized and macropinocytosis in which the vesicles have a diameter greater than 0.2μ. Alternatively endocytosis can be divided into two different categories: non-receptor mediated endocytosis which is mainly fluid phase or non-adsorbtive pinocytosis and is more or less a passive process dependent on the external concentration of the substances/...

substances being pinocytosed and the ability of the macrophage membrane; and receptor-mediated pinocytosis which includes phagocytosis and adsorptive pinocytosis (Silverstein et al., 1977).

7.1 Pinocytosis

7.2 Non receptor mediated or fluid phase

Macrophages form smooth or uncoated vesicles which invaginate from the plasma membrane, trapping fluid and solutes from the extracellular environment when the membrane fuses to form the vesicles. Using horse radish peroxidase as a marker for this process it was shown that uptake was directly proportional to the concentration in the medium and was temperature dependent (Steinman et al., 1976).

7.3 Receptor mediated or adsorptive pinocytosis

Another population of vesicles formed by macrophages are the so-called coated vesicles which are involved in the selective uptake of proteins and particles by adsorptive pinocytosis. One of the most detailed in vitro studies is that of Goldstein and Brown (1977) on the uptake of low density lipoprotein (LDL) by human fibroblasts. Cell surface receptors for LDL are located in coated pits which invaginate when the ligand is bound and are internalized. These receptors are specific for LDL and are saturable. Several substances have been shown quantitatively to exhibit features of adsorptive uptake in vitro by macrophages including polycation-polyanion complexes (Seljelid, 1973) lysosomal enzymes (Stahl et al., 1978) and glycoconjugates (Stahl et al., 1978, 1980; Shepherd et al., 1981).

7.4/...

7.4 Phagocytosis

By this process macrophages ingest particulate material which first attaches to the cell membrane by specific or nonspecific receptors or binding sites.

7.5 Energy requirements

Phagocytosis and pinocytosis are temperature dependent events and with phagocytosis there is a minimum threshold level below which particles are not ingested (Kaplan and Mørland, 1978). ATP is thought to provide the chemical energy for the process since phagocytosis is accompanied by increased cyanide-insensitive oxygen consumption, glycolysis and HMPS, and glycolytic inhibitors such as sodium fluoride and iodoacetimide inhibit phagocytosis (reviewed by Karnovsky et al., 1970). However many of these alterations are the result of phagocytosis and are not required for the event to take place (Silverstein et al., 1977). Michl et al. (1976a, b) have shown that alterations in ATP levels did not correlate with the phagocytic capabilities of mouse macrophages and Stossel et al. (1970) found no changes in ATP levels during phagocytosis of latex particles.

However creatine phosphate consumption was increased during phagocytosis (Silverstein and Loike, 1980) suggesting that the ATP content of phagocytosing macrophages is maintained at least in part by rephosphorylation of ADP by the cells' creatine phosphate content which is present in three to five times molar excess of ATP.

7.6 Cyclic nucleotides

Most investigators have not observed changes in cAMP levels in phagocytosing neutrophils (Stossel et al., 1970). Ignarro and George (1974)/...

(1974) found elevated cGMP levels during particle ingestion by neutrophils. However omission of Ca^{2+} from the medium prevented the rise in cGMP and since phagocytosis normally proceeds in the absence of Ca^{2+} it is unlikely that ingestion itself is regulated by these changes.

7.7. Contractile elements

Microfilaments appear to be involved in the endocytic process as suggested by their presence in phagocytic cells, the association of actin with the plasma membrane and the ability of cytochalasins to inhibit phagocytosis (Axline and Reaven, 1974) unlike agents which depolymerize cytoplasmic microtubules. Microfilaments have a diameter of 50 Å, are composed of actin, and are found in abundance beneath the plasma membrane in the cortical cytoplasm (Reaven and Axline, 1973). Macrophages are 10% by weight actin and 1% myosin (Hartwig and Stossel, 1975). When the pseudopod first forms the orientation of microfilaments is random. However adjacent to the particle being ingested microfilaments are arranged in ordered bundles and as the pseudopod advances round the particle these filaments become more parallel to the long axis of the pseudopod (Reaven and Axline, 1973). The filaments also become more densely packed beneath the ingesting particle (Griffin et al., 1976).

Other macrophage proteins interact with actin in vitro to cause viscosity changes and movement. Actin, myosin, actin-binding protein (ABP) and a Ca^{2+}-dependent actin regulator protein (gelsolin) have been purified from rabbit alveolar macrophages (Hartwig and Stossel, 1975; Yin and Stossel, 1980). Actin and ABP form gel lattices in vitro and macrophage myosin in the presence of Mg^{2+}-ATP contracts the actin filaments isometrically (Stossel and Hartwig, 1976). Immunofluorescence studies/...

studies have shown that both myosin and ABP are present in the peripheral cytoplasm or cortex (Stendahl et al., 1980). Gelsolin in the presence of Ca^{2+} inhibited the formation of the actin-ABP lattices, suggesting that this protein may be the physiological regulator of actin gelation (Yin and Stossel, 1980).

7.8 Mechanism of phagocytosis

Electron microscopic studies by various workers have shown that during phagocytosis of certain particles such as IgG coated sheep red blood cells, EA_G, pseudopodia surround the particle until they meet and fuse on the far side of the particle, forming the vacuole (LoBuglio et al., 1967; Griffin et al., 1975a, b). Internalization by this process appears to involve a segmental response to phagocytic stimuli. Red cells were bound to macrophages using ligands which did not promote phagocytosis. The cells were incubated with latex particles or antibody coated bacteria and it was observed that the latter were ingested but the red cells were not, suggesting that the putative molecules which link surface receptors to the underlying cytoskeleton do not diffuse rapidly within the membrane and only couple to the receptors at the site of phagocytosis. A zipper mechanism has been proposed to explain the observed surrounding of the particle by pseudopodia (Griffin et al., 1975b; Shaw and Griffin, 1981). Ingestion only proceeds upon sequential circumferential binding of macrophage receptors to IgG distributed evenly around the target cell. When antibody was not distributed evenly as with lymphocytes coated with anti-immunoglobulin IgG capped at one pole, phagocytosis did not take place. After macrophages had bound EA_G at 4°C, they were treated with anti-macrophage IgG and allowed to warm up to 37°C; no ingestion occured. Initial attachment does not necessarily predestine the particle/...

particle for ingestion. The direction and extent of movement of the macrophage membrane is governed by the distribution of ligands on the surface of particles and by continued availability of macrophage surface receptors to interact with the ligands.

However phagocytosis of other particles including complement coated particles appears to involve a different series of events. Electron microscopic studies revealed the formation of craters or pits in the macrophage membrane into which the particle directly sank. There only appeared to be regions of close contact at the bottom of the pit which does not agree with the "zipper hypothesis" of Griffin et al. (1975b) in which regions of close contact to the walls of the crater would be expected. This type of phagocytosis was less sensitive to cytochalasin B than the zippering mechanism (Kaplan, 1977) suggesting that either microfilaments are not involved or that a different set of microfilaments mediate crater formation. Bundles of filaments at a deeper level may be relatively resistant to cytochalasin B and may exert a downward pull in the case of internalization of complement coated particles.

From these in vivo and in vitro studies Hartwig et al. (1980) have proposed a "rigidity-shear" hypothesis to explain how macrophages form and extend pseudopodia in the zippering mechanism. Two opposing forces are involved - rigidity which comes from the lattice and shearing from the myosin moving actin filaments. They assume that the cortical layer in cells is in an actin-lattice structure which spans the entire cortex of the cell. The lattice is subject to regional control so that the cell can alter its structure in distinct regions. ABP is normally attached to the membrane but when a particle binds to the membrane via its receptors, ABP is released and cross links the actin filaments in this/...

this region which leads to a local increase in rigidity. Myosin, incorporated into the lattice, in the presence of Mg^{2+} and ATP contracts the actin filaments isometrically and increases the tension in the filaments. Myosin can then pull filaments adjacent to the cross-linked filaments from a region of lower rigidity into a region of higher rigidity by an isotonic contraction.

Since some of the actin filaments are attached to the membrane this pulling pinches the membrane up and toward the particle and new attachments can occur between the membrane and the particle. Further ABP dissociates and results in movement of the rigidity gradient into the advancing pseudopod, propagating its movement. A secondary response in regions of particle membrane attachment is an increase in cytoplasmic Ca^{2+} which may be released from plasma membrane stores by the interaction of opsonised zymosan or immune complex precipitates (Hoffstein, 1979). This increase in free Ca^{2+} in the presence of gelsolin leads to the dissolution of the ABP-actin lattice. As an increase in rigidity advances with the pseudopod, a secondary wave of solution follows behind it, maintaining a rigidity gradient, where rigidity is greatest at the tip of the pseudopod.

7.9 Post endocytic events

Endocytic vesicles once formed detach from the plasma membrane and fuse with pre-existing membrane bound vesicles called lysosomes which release their hydrolytic enzyme contents into the fused phago-lysosome (Cohn, 1970) where their ingested substances are digested.

7.10 Metabolic consequences of ingestion

Metabolism in general is increased, particularly cyanide-insensitive oxygen consumption, H_2O_2 production and glucose oxidation via the HMPS/...

HMPS pathway (Karnovsky et al., 1970). The generation of oxygen metabolites, superoxide anion and singlet oxygen (Johnston, 1976, 1978) and release of H_2O_2 (Nathan and Root, 1977; Nathan et al., 1979b) appears to be important in bacterial and parasite killing in macrophages.

Endocytosis stimulates the secretion of neutral proteinases and these enzymes include collagenase, elastase and plasminogen activator (Davies and Bonney, 1979). Lysosomal enzyme secretion is also triggered by phagocytosis and these enzymes include β-glucuronisae and N-acetyl-β-glucosaminidase. Ingestion of zymosan and opsonised erythrocyytes leads to prostaglandin E release from macrophages (Bonney et al., 1980).

5-HETE and 11-HETE, chemotactic factors for neutrophils and eosinophils are released by rabbit alveolar macrophages after ingestion of opsonised particles (Valone et al., 1980). Secretion of these various substances during and following ingestion suggests that phagocytosis plays a significant role in the host inflammatory response (Davies and Allison, 1976).

8.0 THE GENERATION OF CELL BOUND C3b, C3b´ and C3d BY THE CLASSICAL AND/OR ALTERNATE PATHWAYS OF COMPLEMENT

There are cascade systems in the blood of all vertebrates which are activated by the conversion of proteolytic zymogens to active proteases. Three of these systems, the complement system, clotting system and kinin system play an essential part in the defence of the host against injury and infection. Cascade systems allow rapid responses which are localised by the high concentration of proteolytic inhibitors in the blood.

The complement system is the most complex of the cascade systems and has emerged as an integral part of the immune system. It is activated by two independent pathways - the classical and the alternate pathways. The former is activated by immunoglobulins after interaction with antigen (Porter and Reid, 1979; Müller-Eberhard, 1975, 1980). The alternate pathway can also be activated by antibody aggregates but through a different route and may also operate in the absence of specific antibody. It is activated by naturally occuring polysaccharides and lipopolysaccharides and by certain micro-organisms, viruses, parasites and mammalian cells depending on the chemical composition on their cell surfaces (Götze and Müller-Eberhard, 1976; Müller-Eberhard and Schreiber, 1980).

The concentration of complement in the blood is between two and three g/l with the third component, C3, present in the greatest quantities of all the components (100mg/100ml). The complement system comprises of twenty plasma proteins; six constitute the classical pathway of activation: C1q, C1r, C1s, C2, C3 and C4, and six the alternate pathway: C3, B, D, β1H or factor H, C3b inactivator (C3bINA or KAF or factor I) and properdin. Whereas the latter three proteins are considered to be regulatory proteins in the complement system, they are essential

for/...

for the activation of the alternate pathway. Five proteins participate in the membrane attack pathway: C5, C6, C7, C8 and C9. This pathway is regulated primarily by S-protein which inhibits the formation of the membrane attack complex (MAC). The classical pathway regulators are C1 inhibitor (C1INH), C4 binding protein and C3b1NA. The activation peptides of C3, C4 and C5 and their anaphylotoxin activity are controlled by serum carboxypeptidase B.

Complement has five principle biological functions:

(a) it forms ligand molecules such as C3b which can bind to biological particles through its metastable binding site and then link these particles to phagocytic cells via their C3b receptors.

(b) it elaborates inflammatory mediators, C3a, C4a and C5a which have anaphylotoxin and/or chemotactic activities, Bb which inhibits leukocyte migration and induces spreading and C3e which has leukocytosis inducing properties (Ghebrehiwet and Müller-Eberhard, 1979) which may be important in the granulocytosis response to gram positive bacterial infections.

(c) it is bacteriolytic by generating a unique membranolytic multimolecular complex which is capable of weakening and disassembling lipid bilayers and of forming transmembrane channels.

(d) it solubilises immune complexes (Miller and Nussenzweig, 1975; Takahashi et al., 1980). The importance of this function is evidenced by certain patients with complement deficiencies who have systemic lupus erythematosus which is related to immune complex deposition (Einstein et al., 1975). Solubilisation of immune complexes may also be important in the immune response to soluble antigens. Parish and Chilcott (1975) postulated that complement receptors might facilitate T and B lymphocyte interactions by stabilising the union of soluble antigens/...

antigens (which would have to be coated with natural antibody and complement) with antigen specific receptors on complement receptor positive B cells.

(e) it may play a role in antigen focusing and specific responses in relation to intercellular co-operation. Depletion of C3 prevents localisation of antigen in lymphoid follicles and B memory cells are not generated in the germinal centres (Klaus and Humphrey, 1977). They suggested that antigen-antibody complexes bound to dendritic cells via C3b receptors on the dendritic cells and Fc receptors on B cells, provide the necessary environment for generating memory cells. C3 may act by strengthening the bridge between the dendritic cells and B lymphocytes. C3 depletion also depresses T-dependent antibody responses but leaves T-independent responses unimpaired. C3 may promote bridging between antigen-carrying macrophages and/or T and B lymphocytes or may enhance the binding of antigen to B cells (Pepys, 1976). However other workers have reported that C3 does not play a role in the in vitro antibody response (Waldman and Lachman, 1975). Koopman et al., (1976) has suggested that the transient generation of free C3b either by proteolytic enzymes or by the C3 convertase may augment the inflammatory response by stimulating B cells to elaborate chemotactic and other lymphokines.

C3 is the most versatile of the twenty proteins of complement and fulfills many different functions in the system.

C3 has a molecular weight of about 180,000 daltons and is composed of two polypeptide chains linked by disulphide bonds, an α chain of about 105,000 and a β chain of about 75,000 daltons (Bokisch et al., 1975).

Activation/...

Activation of the classical pathway after antibody has bound to antigen which may be on the cell surface or soluble protein or other molecule. The first component of complement which consists of a complex of C1q, C1r, C1s and Ca^{2+}, binds to the C_H2 domain of IgG (Colomb and Porter, 1975) or the C_H4 domain of IgM (Brown and Koshland, 1977) via the C1q subcomponent (Müller-Eberhard et al., 1966a). A catalytic site is then exposed in C1s of the C1 complex (Stoud et al., 1966) which cleaves C4 into C4a and a much larger fragment C4b. The loss of C4a exposes a hydrophobic site in C4b through which it binds to cell membranes. C2 binds to C4 and is split into C2a which remains bound and C2b which dissociates (Nagasawa and Stoud, 1977). C2a contains an enzymatic site which is responsible for the cleavage of C3 (Müller-Eberhard et al., 1966b). C3 is cleaved into C3a, one of the three anaphylotoxins (Dias da Silva et al., 1967) and the major fragment, C3b. Concomitant with this cleavage a metastable binding site is expressed on C3b through which C3b can firmly attach to a large variety of biological particles and cell membranes by a covalent ester linkage (Law, Lichtenberg and Levine, 1979; Sim et al., 1981; Tack et al., 1980). The binding site probably resides in the C3d fragment which is part of the α chain of C3b since C3c was released by the action of C3b1NA and other serum proteins while C3d was retained by the cell (Ruddy and Austen, 1971).

C3b is also generated by the alternate pathway. Activation consists of initiation and amplification. It is believed that there may be a continuous generation of the priming convertase C3Bb at a slow turnover rate by the interaction between C3, B and D (and properdin). Deposition of C3b on cell membranes requires the enzymatic cleavage of fluid phase C3 in order to generate the metastable C3b, capable of binding to biological particles (Schreiber et al., 1978). It is also possible/...

possible that certain chemical groups of the surface of cells may cleave this bond, thereby attaching C3 to cell membranes. C3 thus bound may behave functionally like C3b and serve as the subunit in the initial C3 convertase. The initial convertase would be surface bound C3Bb rather than fluid phase C3Bb.

Spontaneous hydrolysis of the thioester bond in C3 initiates a conformational change which exposes the binding site for factor B (Vogt et al., 1978). The C3B complex is activated by factor D and generates the first C3 convertase of the pathway C3Bb. The proteolytic activity of this enzyme on native C3 subsequently generates the first molecule of metastable C3b which is then deposited onto potential target sites.

Surface bound C3b through either the classical or the alternate pathway is then subjected to two competing processes - amplification and inactivation. Amplification occurs when additional C3b molecules are deposited around the original C3b molecule. Factor B binds to the first C3b molecule and C3bBb is produced by the action of factor D. A possible amplification loop is generated because C3bBb is a C3 convertase and each generated molecule of C3b can repeat this process (Muller-Eberhard and Gotze, 1972) with the subsequent deposition of many more molecules of C3b. Many of the functions of cell bound C3b can be abolished by the action of C3bINA which acts as a proteolytic enzyme (Ruddy and Austen, 1971). This enzyme requires β1H for its action possibly because β1H binds to C3b and the resulting conformational change exposes a site susceptible to cleavage of C3bINA (Pangburn et al., 1977). The α chain of C3b is cleaved into two fragments without affecting the β chain (Law et al., 1979; Gaither et al., 1979). All three polypeptides are held together by disulphide bonds and

therefore/...

therefore the cleaved molecule C3b´ has the same molecular size as intact C3b. C3b´ is covalently bound to the cell surface through the larger α chain fragment. Conversion of C3b to C3b´ is accompanied by loss of immune adherence to human erythrocytes, functional haemolytic activity and the ability to form on the cell surface the factor B, factor D, properdin dependent amplification C3 convertase. However C3b´ like C3b is recognised by specific surface receptors on phagocytes (Ross and Rabellino, 1979) and may induce ingestion (Newman et al., 1980). C3b´ is very susceptible to attack by proteases which cleave a second bond in the α chain to produce two fragments, C3b and C3d. The C3d portion remains covalently linked to membrane and the C3c fragment is released into the medium (Ruddy and Austen, 1971). Further tryptic digestion of C3c produces C3e which is probably derived from the α chain and has leukocytosis inducing activity (Ghebrehiwet and Müller-Eberhard, 1979). In vitro a low concentration of trypsin is required to cleave C3b´ to produce C3c and C3d. However it is not clear which enzymes are involved in vivo. Plasmin may be used to substitute for trypsin but high concentrations are required. Elastin from neutrophils also cleaves C3b´ and this lysosomal enzyme may represent the in vivo component (Carlo et al., 1979).

A control system apparently exists in that C3b binds to foreign particles and not to host cells. It appears capable of distinguishing between activators and non-activators of the alternate pathway (Pangburn et al., 1980) which is reflected in a differential accessibility of bound C3b to β1H (Kazatchkine et al., 1979). The competition between factor B and β1H binding to cell-surface-associated C3b is influenced by the amount of sialic acid on the cell surface. The presence of sialic acid increases the affinity of cell bound C3b for β1H but not for factor B but natural activators of the alternate pathway have low amounts/...

amounts of sialic acid on their surfaces.

Both the classical and the alternate pathways are potentially cytolytic. C5 is cleaved by either C5 convertase, C1423b or C3bBbC3b, into two fragments, C5b and C5a. C5b forms part of the MAC and C5a which is an anaphylotoxin and chemotactic factor, is released. The formation of the C56789 complex or MAC appears to be a self-assembly process (Podack et al., 1980) which exert a direct physical attack on the target membrane leading to target cell destruction (see Boyle and Barson, 1980, for a review).

9.0 MACROPHAGE COMPLEMENT RECEPTORS

9.1 Function

Complement receptors are involved in the selective elimination of invasive pathogens by macrophages. Their biological importance is evidenced by patients with hereditary C3 deficiency or abnormal C3 metabolism. These patients whose serum opsonising capacity is diminished (Alper et al., 1976), have an increased susceptibility to life-threatening recurrent infections which normally can be controlled by phagocytes in normal serum. However other complement-mediated functions are also absent in C3-deficient sera and the lack of C3 opsonising capacity cannot account for all the effects observed. Both complement and Fc receptors are important in bacterial killing by human monocytes (Leijh et al., 1979). Triggering of these receptors after phagocytosis of opsonised micro-organisms appears to be necessary for intracellular killing to take place.

C3b induces lysosomal enzyme release from macrophages presumably by interacting with their C3b receptors (Schorlemmer and Allison, 1976). This may be important in inflammatory reactions where alternate pathway activation occurs. The C3b thus generated may act on macrophages stimulating release of enzymes involved in the inflammatory response. Some of these enzymes can cleave C3 to form more C3b thus amplifying the system.

There is some controversy about whether or not C3b alone is sufficient to stimulate release of superoxide anion (O_2^-) or oxidative metabolism (Newman and Johnston, 1979; Goldstein et al., 1976). However synergy between C3b and Fc binding to their respective receptors caused enhancement of oxidative metabolism and degranulation.

C3b/...

C3b receptors on macrophages may play a role in antigen presentation to B lymphocytes by enhancing the approximation of different cell types involved in co-operation (reviewed by Pepys, 1976). There may be a functional basis for the fact that EA_MC adhere to macrophages but may not be ingested (Mantovani et al., 1972). In a primary immune response, immune complexes would remain on the surface of macrophages and therefore be in a position to stimulate immunocompetent cells.

Antibody-dependent cell cytotoxicity by human monocytes was enhanced by the presence of complement on IgG coated erythrocytes (Kurlander et al., 1978) or IgG coated Raji cells (Kessler and LoBuglio, 1980) although the presence of complement alone on these target cells did not mediate lysis by monocytes.

Generally a rosetting technique with complement coated red cells is used to directly visualise complement receptors (Bianco, 1976) or ^{51}Cr labelled erythrocytes have been used (Atkinson et al., 1977). Lymphocyte complement receptors have been investigated using fluoresceinated anti C3 antiserum and soluble C3 and C3b or radiolabelled C3 and C3b (Theofilopoulos et al., 1974). However neither of these two methods has been used to examine macrophage complement receptors. The production of anti C3 receptor antibodies (Fearon, 1980; Gerdes and Stein, 1980, 1981; Dobson et al., 1981) may be used in the future to detect C3 receptors.

C3 receptors have a widespread tissue distribution and can be detected on the surfaces of primate erythrocytes (Nelson, 1963), many nonprimate platelets (Nelson, 1963, neutrophils (Anwar and Kay, 1977a), macrophages (Mantovani et al., 1972), monocytes (Ehlenberger and Nussenzweig, 1977), a subpopulation of lymphocytes (primarily B lymphocytes) (Ross and Polley, 1975) mast cells (Vranien et al., 1980) eosinophils (Anwar/...

(Anwar and Kay, 1977) and epithelial cells of normal renal glomeruli (Schreiber and Penny, 1979).

9.2 Specificity

Macrophages do not have receptors for complement components C1 or C2 (Wellek et al., 1976). Human monocytes have receptor activity for C4 (Ross and Polley, 1975) as well as for C3 (Ehlenberger and Nussenzweig, 1977) although the affinity of the monocyte receptors for C3 is much greater than it is for C4. Ross and Polley (1975) showed that the C3b receptor and C4b receptor are the same receptor on human lymphocytes and different from the receptor for C3d. Probably the same receptor binds both C3b and C4b on monocytes as well. Macrophages may also have receptors for soluble C3 and C3b as has been shown for lymphocytes (Theofilopoulos et al., 1974). Although this has not been shown formally on intact macrophages, isolated complement receptors from macrophages bound both soluble C3 and C3b (Schneider et al., 1981a).

It had previously been thought that C3bINA converted C3b into C3d but it is now realised that C3b is first degraded to C3b′ and then to C3d by the action of an unidentified protease. Many studies which have investigated the presence of C3d receptors, may have been studying C3b′ receptors especially when C3bINA was used. However many early preparations of C3bINA also probably contained protease activity and therefore C3d may have resulted. The use of heat inactivated serum as a source of C3bINA may have led to the production of C3b′ or C3d depending on the concentration of proteases present and the length of incubation. Therefore interpretation of previous data is difficult.

Ross and Rabellino (1979) have shown that human monocytes (and immature neutrophils) have two distinct complement receptors, one for C3b, CR1, and one for C3b′, CR3, and do not have receptors for C3d, CR2./...

CR2. Lymphocytes have receptors for all three C3 cleavage products although apparently the same receptor, CR2, binds both C3b′ and C3d. However Carlo et al. (1979) found that human monocytes also had all three receptors. The receptors for C3b and C3d have been shown to be antigenically distinct on human tonsil cells (Stein et al., 1981) and CR1 isolated from human erythrocytes which is antigenically similar to CR1 present on human leukocytes, is distinct from CR2 and CR3 (Dobson et al., 1981). In vivo deposition of C3b via the classical or alternate pathways will probably be converted rapidly into C3b′ and therefore the C3b′ receptor may be more important physiologically. Phagocytosis via C3b′ receptors has been observed by activated macrophages (Newman et al., 1980).

The presence of C5b receptors on macrophages has not been investigated although it has been suggested that human neutrophils do possess C5b receptors which are important in phagocytosis (Segerling and Opferkuch, 1977). C5b receptors on lymphoid cells are apparently identical to C3 receptors (Landen and Dierich, 1979).

9.3 Number of receptors/cell

At present the use of complement coated erythrocytes does not allow estimations of the numbers of C3b receptors/cell or the affinity constants. EAC appears to bind to macrophages with fewer regions of close contact than EA_G (Kaplan, 1977) which may be a reflection of fewer receptors. Complement receptors have a greater affinity than Fc receptors since fewer IgG molecules are required per red cell to promote ingestion when complement is also present (Ehlenberger and Nussenzweig, 1977). Using an antibody prepared against solubilised complement receptors, 48,000 binding sites per monocyte were detected (Fearon, 1980).

9.4/...

9.4 Ultrastructure

Complement receptors appeared to be randomly distributed over the surface of endotoxin-induced macrophages with none present at the extreme periphery of the cells (Kaplan, 1977). The binding to complement receptors is temperature dependent. At 4°C very few complement coated erythrocytes bind to macrophages (Lay and Nussenzweig, 1968). The number of red cells bound increases with increasing temperature and at 37°C maximum binding is reached. These differences in binding with temperature changes may be related to the fluidity of the membrane.

In most systems studied no requirement for Ca^{2+} or Mg^{2+} for binding to complement receptors could be demonstrated. However binding of EAC to mouse macrophages attached to a glass surface required Mg^{2+} (Lay and Nussenzweig, 1968).

Cytochalasins inhibited rosette formation on alveolar macrophages with a pattern of reactivity: cytochalasin A > E > D > B, and vinblastine and colchicine also inhibited EAC binding (Atkinson et al., 1977). An intact cytoskeleton consisting of microfilaments and microtubules may be required for effective binding between C3-sensitised particles and macrophage C3 receptors. Michl et al. (1979) found that complement receptors, unlike Fc receptors, did not appear to be mobile in the plane of the membrane. C3b receptors are clustered in the membranes of human monocytes and neutrophils and do not diffuse laterally in the plane of the membrane (Petty et al., 1980).

9.5 Phagocytosis via C3 receptors

The importance of C3b in promoting adherence of sensitised erythrocytes or bacteria to phagocytes is not disputed but there is some controversy in the literature about whether or not complement receptors mediate phagocytosis. Huber et al. (1969) reported that human peripheral blood/...

blood monocytes did phagocytose via their complement receptors whereas Ehlenberger and Nussenzweig (1977) observed that complement coated erythrocytes were bound but not ingested by human monocytes. Van Furth et al. (1979) found that although 98% of human monocytes bound, only 16% ingested EAC. Other particles such as complement coated E. coli were phagocytosed by human monocytes (Horwitz, 1980) and soluble heat aggregated IgM was ingested in significant amounts in the presence of complement by mouse and guinea pig macrophages (Van Snick and Masson, 1978; Kijlsta et al., 1978). Also, both phagocytosis and killing has been observed with agammaglobulinaemic human serum (Williams and Quie, 1971). Resident mouse peritoneal macrophages bound but did not ingest EAC whereas thioglycollate induced macrophages both bound and ingested EAC (Bianco et al., 1975) and cultured human monocytes were also found to ingest C3b and C3b' coated red cells (Newman et al., 1980). However both normal and stimulated mouse peritoneal macrophages will phagocytose EAC if additional C5 deficient mouse serum was added providing extra ligands between macrophages and erythrocytes (Bar-Shavit et al., 1979). Uncleaved C3 deposited on the macrophage surface via complement receptors may be cleaved by C142 convertase on the target cell surface which would provide extra bridging between the cells in a similar manner to that described by Dierich and Landen (1977). Since complement components are expressed on the plasma membranes of different cells and macrophages synthesize certain complement components (Brade and Bentley, 1980) these additional complement bridges may be important in vivo. The presence of extra ligands may thus allow the cell to phagocytose using the zipper mechanism described by Griffin et al. (1975b).

The changes observed with complement receptors on activated macrophages may therefore reflect an increase in numbers or affinity of complement receptors rather than a change in function.

The/...

The opsonisation of erythrocytes coated with complement may be different from opsonisation of bacteria especially because of varying densities of complement on the surface and the relative size of the particles coated. Therefore the results of the phagocytosis experiments should be interpreted according to the functional state of the cell and the type of particles to be ingested. On bacteria and other particles which activate the alternate pathway there are surface molecules which "non specifically" induce phagocytosis and synergism between these binding sites and complement receptors inducing phagocytosis has been observed (Czop et al., 1978).

Complement receptors also play a role in enhancing phagocytosis mediated by Fc receptors (Ehlenberger and Nussenzweig, 1977). Monocytes ingested EA_G more efficiently in the presence of complement with a limiting amount of antibody. It has been suggested that IgG through its Fc fragment directly stimulates particle ingestion but is relatively inefficient at inducing particle ingestion (Mantovani et al., 1972). Although complement receptors were involved in the binding of these particles only Fc receptors mediated phagocytosis as shown by Shaw and Griffin (1981). They allowed erythrocytes coated with IgG and complement to attach to resident mouse peritoneal macrophages at 4°C and then blocked the non-engaged Fc receptors with anti-macrophage serum. When the cells were warmed up to 37°C phagocytosis did not occur. However using another approach Michl et al. (1979) suggested that complement bound to the Fc fragment of IgG in immune complexes rendered them ineffective as ligands for macrophage Fc receptors and the binding occured via complement receptors alone. If this also occurs on particles coated with IgG and complement, then it is difficult to explain how ingestion can occur via Fc receptors in Shaw and Griffin's model.

9.6/...

9.6 Modulation of the expression of complement receptors

As described above, activated or stimulated macrophages phagocytose EAC unlike unstimulated macrophages which mediate attachment only (Bianco et al., 1975; Newman et al., 1980). The mechanism of this change in function may represent an increase in density or affinity or may be due to the synthesis of a new type of complement receptor. The observation that thioglycollate induced macrophages showed changes in complement receptor function suggested that lymphokines may be involved. Lymphokines produced by *in vivo* primed T cells incubated *in vitro* with macrophages and priming antigen had no effect on resident macrophage complement receptors (Griffin and Griffin, 1979). However macrophages which had ingested IgG coated particles triggered the elaboration of a unique lymphokine from T cells. This lymphokine altered complement receptor function on resident macrophages, which had been cultured for 48 hours in the presence of FCS, so that they ingested EAC as well as binding them (Griffin and Griffin, 1980). The lymphokine which itself had no effect on Fc receptor expression had a molecular weight less than 10,000 daltons which is lower than the molecular weight of MIF/MAF, lymphotoxin or interferon.

New protein synthesis was not required and Griffin and Griffin suggested that the lymphokine may exert its effect by increasing the rate of insertion into the membrane or by rearranging existing complement receptors in relation to their cytoskeletal elements involved in phagocytosis. Observations made by Kaplan *et al.* (1978) and Michl *et al.* (1979) tend to suggest the second hypothesis. They showed that although Fc receptors were motile in the plane of the macrophage membrane, complement receptors on resident macrophages did not appear to be so. However thioglycollate/...

thioglycollate induced macrophages were able to redistribute their complement receptors on their plasma membranes suggesting that there may be alterations in the attachment of complement receptors to the cytoskeletal elements.

The effects of lymphokines may be important in host defence mechanisms. Many micro-organisms and possibly tumour cells shed antigens which become complexed to specific immunoglobulin. When macrophages ingest these immune complexes, Fc receptor expression is decreased (Doughaday and Douglas, 1976; Griffin, 1980) and macrophages can no longer phagocytose the micro-organisms. However ingestion of immune complexes by macrophages would trigger T cells to produce a lymphokine which increases complement receptor expression on macrophages which could then ingest micro-organisms via their complement receptors.

Chemotactic factors also modulate the expression of complement receptors. Eosinophil specific chemoattractants enhanced complement receptors on eosinophils without affecting the expression of Fc receptors (Anwar and Kay, 1977b, 1978). Similarly Goetzl et al. (1980) has shown that HETEs increased eosinophil and neutrophil complement receptors without affecting Fc receptors on these cells. Thus as cells migrate to the site of inflammation and chemotactic factor production, the alteration in their complement receptor expression may prepare them for more efficient ingestion of opsonised particles.

9.7 Nature of complement receptors

Trypsin sensitivity of complement receptors suggests that they consist in part of protein (Lay and Nussenzweig, 1968; Doughaday and Douglas, 1976). Biosynthetic labelling with [^{14}C]-glucosamine suggests that/...

that the receptor is a glycoprotein (Schneider et al., 1981). The behaviour of C3 receptors isolated from KBr lysates of the membrane fraction of human tonsil cell homogenates suggested that the receptor is a lipoprotein complex (Gerdes and Stein, 1981).

Landen et al. (1979) have suggested that α_1-antitrypsin may serve as a C3 receptor on certain cells including Raji cells and B lymphocytes though not erythrocytes. However Stein et al. (1981) using antisera to α_1-antitrypsin failed to inhibit EAC agglutination with C3 receptor bearing cells or to stain C3 receptor positive cells.

9.8 Isolation of complement receptors

A 205,000 daltons glycoprotein (gp 205) was solubilised from human erythrocyte membranes with the non-ionic detergent, Nonidet-P40 and purified to homogeneity with various chromatographic techniques including reversible adsorption to Sepharose-C3 (Fearon, 1980). Rabbit anti gp 205 inhibited rosette formation of erythrocytes, monocytes, neutrophils and B lymphocytes, with EAC3b but not EAC3b´ or EAC3d. Immunoprecipitation with anti gp 205 gave a single membrane protein with an apparent molecular weight of 205,000 on SDS-PAGE from solubilised membranes of erythrocytes, monocytes, neutrophils and B lymphocytes. The gp 205 thus appeared to be the C3b receptor of all four cell types. Since red cells only have CR_1, this receptor must be CR_1. Dobson et al. (1981) using essentially the same technique obtained a receptor with a similar molecular weight (195,000 daltons) with or without prior reduction of disulphide bonds. Rabbit antibody inhibited EAC14b and EAC3b rosetting to erythrocytes, peripheral blood and tonsil B lymphocytes, B lymphoblastoid cells, monocytes and neutrophils. However the antibody had no effect on the binding through CR_2 or CR_3.

Using/...

Using a different species, cell type and method of separation Schneider et al. (1981) obtained a complement receptor with a molecular weight of 64,000 daltons. They radio-iodinated rabbit alveolar macrophage membranes and then solubilised the membranes with Nonidet-P40. The C3b receptor was purified on Sepharose-C3b or Sepharose-C3. A single peak was obtained which had ligand binding activity in that it rebound specifically to Sepharose-C3b or Sepharose-C3. Inhibition experiments suggested that the receptor had a higher affinity for C3b than for C3.

Immunoprecipitation of KBr lysates of the membrane fraction of human tonsil homogenates, followed by SDS-PAGE yielded a complement receptor with a molecular weight of 1×10^6 daltons which behaved like a lipoprotein complex. The protein moiety consisted of disulphide bridged polypeptide chains with an apparent molecular weight of 38,000 daltons. CR_1 and CR_2 receptors had similar molecular structures but the recognition units for C3b and C3d were located on separate polypeptide chains (Gerdes and Stein, 1981).

The relationship between these receptors is not known and the differences may partly reflect the method of separation and partly species variations. It would seem likely that the receptor is part of a lipoprotein complex and the various molecular weights obtained for the glycoprotein moiety may reflect polymerization of individual subunits (or may be degradation products).

10.0 MACROPHAGE Fc RECEPTORS

10.1 Function

Macrophage Fc receptors are involved in the selective binding and ingestion of antigens and bacteria (Reynolds, 1974) and effete autologous cells (Kay, 1975). These receptors bind to the Fc portion of immunoglobulins which have been produced against and bind to foreign antigens. Apart from their involvement in phagocytosis, Fc receptors are also important in IgG-dependent extracellular lysis with erythrocytes (Walker, 1977) and tumour cells (Shaw et al., 1978) as targets and possibly in IgM-dependent cell cytotoxicity.

Inflammatory mediators including prostaglandins, particularly PGE_2 are released after ingestion of antibody/antigen complexes or antibody-coated red cells, (Bonney et al., 1980) by resident mouse peritoneal macrophages. Fc fragments of IgG also stimulated release of PGE_2 by human monocytes (Passwell et al., 1980) suggesting that binding of a ligand to Fc receptors, even in the absence of phagocytosis may play an important role in monocyte activation.

Interaction with Fc receptors after ingestion of opsonised micro-organisms by human monocytes, appears to be necessary for intracellular killing to take place (Leijh et al., 1979).

Methods used to visualise Fc receptors include the rosette technique with antibody-coated erythrocytes (Bianco, 1976) or ^{51}Cr labelled erythrocytes can be used to count the receptors directly. Other methods include fluorescein, radioactive, peroxidase or ferritin labelling of antibody-antigen complexes or aggregated immunoglobulin complexes.

Fc receptors are present on many cells apart from macrophages and monocytes (Mantovani et al., 1972; Huber and Fudenberg, 1968) including/...

including lymphoid cells of the B,T and null cell groups (Basten et al., 1972; Anderson and Grey, 1974; Stout and Herzenberg, 1975) neutrophils (Messner and Jelinek, 1970) eosinophils (Anwar and Kay, 1977a), basophils (Segal et al., 1981) and platelets (Pfueller et al., 1977).

10.2 Specificity

Most studies have shown that mononuclear phagocytes have Fc receptors for specific subclasses of IgG. Fc receptors for IgM and IgE have been shown but there does not appear to be receptors for IgA.

Human monocytes have receptors for IgG_1 and IgG_3 but not for IgG_2 or IgG_4. Alexander (1980) found that IgG_3 bound with greater affinity than did IgG_1 whereas an earlier report using a different technique suggested that monocytes had greater affinity for IgG_1 (Abramson et al., 1970). It is not known whether human monocytes have one receptor or two. However mouse macrophages probably possess two distinct receptors (Unkeless, 1980), one for monomeric IgG_{2a} which is trypsin sensitive (FcRI) and a trypsin insensitive receptor for aggregated IgG_{2b} and probably aggregated IgG_1 as well (Diamond and Scharff, 1980) (FcRII). Phospholipase sensitivity suggests that FcRII is lipid dependent whereas FcRI is not (Anderson and Grey, 1978). Varients of macrophage cell lines were established which expressed either one or the other receptor (Unkeless and Eisen, 1975). Other workers have found different results and Grey et al. (1977) has suggested that the discrepancies in the literature may be due to different techniques used to detect Fc receptors.

There may be functional differences between these receptors which may be important in vivo since IgG_{2a} is produced early in the immune response whereas IgG_{2b} is present in hyperimmune serum.

Haskill/...

Haskill and Fett (1976) suggested that antibody-dependent cell cytotoxicity (ADCC) was mediated by IgG_{2a} since macrophage mediated ADCC against a tumour cell line was only blocked by IgG_{2a}. However Walker (1977) obtained contradictory results: IgG_{2b} mediated ADCC and IgG_{2a} mediated phagocytosis. Diamond et al. (1978) on the other hand, reported that macrophages phagocytosed IgG_{2a} and IgG_{2b} coated red cells.

Using a monoclonal antibody, Diamond and Yelton (1981), have identified a third Fc receptor for IgG_3 on mouse peritoneal macrophages and on some macrophage cell lines. This receptor is trypsin resistant, mediates phagocytosis and can also mediate ADCC against shee red blood cells.

Fc receptors possess relatively weak affinity for monomeric IgG and a high affinity for immune complexes (Phillips-Quagliata et al., 1971). The summation of individual receptors indentical to the monomeric receptor may account for the high affinity binding. It has also been suggested that binding proceeds by a co-operative mechanism (Leslie and Alexander, 1980).

Binding to the Fc receptor is probably through the C_H2 domain although this has not been unequivocably demonstrated. Studies with guinea pigs (Alexander et al., 1978a), mice (Unkeless and Eisen, 1975) and man (Alexander et al., 1978a) have suggested that the C_H2 domain is involved. However other reports indicated that the C_H3 domain may be involved (Dissanayake and Hay, 1975; Okafor et al., 1974) and Diamond et al. (1979) showed that IgG_{2b} bound to mouse macrophages via its C_H3 domain and IgG_{2a} through its C_H2 domain.

Binding by resident murine macrophages has also been shown to be IgM dependent (Lay and Nussenzweig, 1969) and glycogen elicited murine macrophages also ingest EAM (Mantovani, 1981). IgM anti erythrocyte antibody/...

antibody was found to be cytophilic for mouse macrophages (Tizard, 1969). These results suggested that there may be Fc receptors for IgM but rosetting techniques failed to detect them (Huber et al., 1968). However Rhodes (1973) reported that although guinea pig splenic macrophages did not bind IgM coated red cells they possessed a Fc receptor for monomeric IgM and not for pentameric IgM. Haegert (1979a) using a direct plaque forming assay detected IgM Fc receptors on human monocytes which recognised non-aggregated IgM. Neuraminidase treatment increased the number of IgM Fc receptors and they became detectable by a rosette assay (Haegert, 1979b). These receptors were trypsin resistant like mouse IgG_{2b} and IgG_3 receptors and human Fc (IgG) receptors. These IgM Fc receptors may be involved in both phagocytosis and IgM dependent cell cytotoxicity.

Fc receptors for IgE have also been described on macrophages (Capron et al., 1975; Joseph et al., 1977). Prior incubation of macrophages with IgE complexes induced macrophages to become cytotoxic when bound to S. mansoni shistosomula via the Fc fragment of IgE.

10.3 Number of receptors/cell

The number of Fc receptors on the surface of macrophages lies in the region of 10^5 to 10^6 per cell depending on the assay used. Alexander et al. (1979) using radiolabelled trimers of rabbit IgG detected 3×10^4 binding sites per human monocyte with an affinity constant, Ka equal to $5 \times 10^7 M^{-1}$, whereas Unkeless and Eisen (1975) detected 10^5 binding sites per mouse peritoneal macrophage for IgG_{2a} ($Ka = 10^8 M^{-1}$) and 5×10^4 binding sites per cell for IgG_{2b} ($Ka = 10^7 M^{-1}$) using mouse monomeric myeloma proteins of the respective subclasses. Phillips-Quagliata et al. (1971) found that there were 2×10^6 sites per rabbit/...

rabbit alveolar macrophage. These differences may reflect technical differences or species variations.

10.4 Ultrastructure

Electron microscopic studies indicated that there is an 8nm distance between a bound immune complex and the macrophage membrane which is smaller than the length of an immunoglobulin molecule suggesting that there may be a compact membrane pocket within the membrane (McKeever et al., 1976).

Fc receptors appeared to be randomly distributed over the entire macrophage surface but are more concentrated in the perinuclear regions, cytoplasmic veils and pseudopodia (Kaplan et al., 1975).

The binding to Fc receptors is not temperature dependent unlike complement receptors (Anwar and Kay, 1977a).

Cytochalasin B not only inhibited phagocytosis of EA_G but also binding (Atkinson and Parker, 1977) suggesting that subplasmalemmal microfilaments are involved in the binding. The Ca^{2+} ionophore, A23187 also blocked the binding of EA_G (Sanners and Spicer, 1979). However other workers have found that binding of EA_G to macrophages is independent of Ca^{2+} (Lay and Nussenzweig, 1969) and Mg^{2+} (Davey and Ascherton, 1967).

10.5 Modulation of Fc receptor expression

A number of studies have shown that activated macrophages possess more IgG receptors (Zuckerman and Douglas, 1979; Bianco et al., 1975) than resident or unstimulated macrophages. Although it has been suggested that the observed increase was due to the greater cell size of activated macrophages (McKeever et al., 1976) lymphokine rich supernatants/...

supernatants from mixed leukocyte cultures increased the number of radiolabelled IgG binding sites per cell on cultured human monocytes without increasing the cell volume (Guyre et al., 1981). Circulating human monocytes from patients with chronic granulomatous diseases, tuberculosis, sarcoidosis and regional enteritis have increased IgG receptor activity as measured by rosetting and phagocytosis of EA_G (Zuckerman and Douglas, 1979). Enhanced Fc receptor expression was also observed in monocytes from patients with malignancies (Rhodes, 1977). Activation must have occured before the monocytes reached the tissues suggesting that there may be feedback mechanisms operating which act on either bone marrow precursors or peripheral blood cells. In contrast to monocytes, macrophages in chronic granulomas eventually show decreased Fc receptor activity (Mariano et al., 1976).

Thioglycollate stimulated mouse peritoneal macrophages showed increased phagocytic capacity for EA_G when cultured in vitro for 48 hours in the presence of serum. However macrophages from LPS hyposensitive C3H/HeJ mice lost the capacity to bind and phagocytose EA_G. A lymphokine rich supernatant from Con A-stimulated spleen cells (Vogel and Rosenstreich, 1979) and cAMP agonists (Vogel et al., 1981) corrected this defect. They also showed that the lymphokines (which they suggested may be MAF) increased cAMP levels in macrophages. The inability of C3H/HeJ mice to differentiate in vitro with respect to Fc receptor expression may be due to an inability to respond to a signal (possibly a lymphokine) which by altering cAMP levels in the macrophage affects the expression of surface markers. CAMP agonists also corrected an Fc-mediated phagocytic defect in a macrophage cell line grown under suboptimal conditions. The effects of cAMP agonists required/...

required five to ten hours to become evident suggesting that changes in cAMP levels were not the immediate signal for phagocytosis but allowed the cell to enter a permissive state (Muschel et al., 1977). Insulin which increased cAMP levels also decreased phagocytosis of EA_G in normal macrophages (Muschel et al., 1977). Rhodes, (1975) found that insulin or a phosphodiesterase inhibitor which increased cAMP levels in macrophages inhibited the observed increase in Fc receptors on cultured macrophages.

Prostaglandins PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and PGA_1 increased the phagocytosis of opsonised particles at physiological concentrations and decreased phagocytosis at higher concentrations (Razin et al., 1978). This may be important in vivo since prostaglandins are produced in inflammatory reactions and may play a role in enhancing the phagocytic capacity of macrophages.

10.6 Nature of the Fc receptor

Human Fc receptors and mouse IgG_{2b} are trypsin resistant (Lay and Nussenzweig, 1968; Unkeless, 1980). However both IgG_{2a} and IgG_{2b} receptors became trypsin sensitive when detergent solubilised (Anderson and Grey, 1978). Trypsin sensitivity, the evidence that [^{14}C]-glucosamine is incorporated into the receptor in vivo (Kulczycki et al., 1980) and neuraminidase or sodium borohydride treatment of isolated Fc receptors (Mellman and Unkeless, 1980) suggest that Fc receptors are glycoproteins.

Sucrose density sedimentation of detergent lysates from macrophages indicated that the soluble Fc receptor was associated with phospholipids (Anderson and Grey, 1977). Since phospholipases inhibited the expression of/...

of Fc receptors (Walker, 1976a) phospholipids may be important in the functional activity of the receptor. More recent evidence suggests that only the IgG_{2b} receptor is lipid dependent (Anderson, 1980).

10.7 Isolation of Fc receptors

Various workers using different methods of isolation have obtained putative Fc receptor molecules with apparent molecular weights varying between 35,000 and 100,000 daltons. The relation between these proteins and measurable Fc receptors on cell surfaces is not clear. Anderson and Grey (1977) detected Fc receptor activity in detergent lysates but attempts to purify by affinity chromatography were not successful. More recently Fc receptors have been purified by rabbit antiglobulin-sepharose affinity chromatography (Kulczycki et al., 1980) and appear to have a molecular weight between 35,000 and 55,000 daltons. Separation by affinity chromatography and inhibition studies indicated that the soluble Fc receptor did not appear to discriminate between IgG_{2a} and IgG_{2b} suggesting that the micro-environment in the membrane confers the specificity on the receptor. Mellman and Unkeless (1980) isolated two polypeptide chains from a mouse macrophage cell line with molecular weights of about 60,000 daltons and 47,000 daltons which also recognised both IgG_{2a} and IgG_{2b}.

However both Lane et al. (1980) and Anderson (1980) were able to separate two distinct Fc receptors, one of which recognised IgG_{2a} and the other recognised IgG_{2b}.

As with complement receptors, the differences found between size and specificity of the various Fc receptors isolated, may partly reflect the methods of separation and partly species variations.

11.0 NON SPECIFIC RECEPTORS OR RECEPTORS FOR FOREIGN SUBSTANCES ON MACROPHAGES

Macrophages ingest a wide variety of particles including polystyrene (latex) particles (Doughaday and Douglas, 1976), extracted yeast cell walls (zymosan) (Czop et al., 1978), senescent red cells (Kay, 1975), chemically modified erythrocytes (Rabinovitch, 1968; Capo et al., 1979) syngeneic tumour cells (Piessens, 1978), xenogeneic cells (Cabilly and Gallily, 1977), bacteria (Weir and Ögmundsdóttir, 1980) and parasites (Capo et al., 1979).

Thus macrophages are able to discriminate between self and alterations to self as in effete or damaged tissue components or aberrant self constituents such as tumour cells. They are also capable of distinguishing between foreign material and self components. The biological importance of this is probably most obvious in the lung where alveolar macrophages clear the alveolar respiratory surfaces of small particles (Heppleston, 1963). The receptors involved may represent a primitive form of recognition.

The mechanisms that underlie these highly important recognition capacities of macrophages is in the main unknown. Nonspecific binding may be dependent on the hydrophobicity of the foreign particle (Wilkinson, 1976) and membrane biomechanical factors may contribute to macrophage binding and ingestion of foreign substances. Positively charged substances such as cationized ferritin bind more avidly than negatively charged substances which are repulsed by the macrophage's own negatively charged surface.

Nonspecific binding may, however, in at least some instances, be mediated by specialised structures on macrophage membranes. Some of these/...

these binding sites may be specific receptors which bind defined substrates and are saturable. Receptors for certain groups of substances differ from receptors for other substances in their susceptibility to digestion by hydrolytic enzymes. Monocyte ingestion of zymosan was more sensitive to trypsin than was phagocytosis of latex particles (Czop et al., 1978). Receptors for glutaraldehyde treated erythrocytes (Rabinovitch, 1968) and bacteria (Ögmundsdóttir et al., 1978) were destroyed by trypsin. Michl et al. (1976a, b) found that 2-deoxyglucose decreased phagocytosis via Fc and complement receptors but did not affect the phagocytosis of nonopsonised zymosan or latex particles. The effects of 2-deoxyglucose appeared to be related to its potential effects on glycosylation and not to ATP levels.

Benoliel et al. (1980) using five different test particles, EA_G, glutaraldehyde treated erythrocytes, leishmania, latex beads and tumour cells, found that there was no cross inhibition of binding although ingestion of some particles inhibited the ingestion of certain others. The pattern of inhibition of binding of these particles by various inhibitors of cellular functions and potential modulators of intermolecular forces was also different for each particle. Electron microscopy indicated that the binding of EA_G and Leishmania to macrophage membranes involved large areas where the interacting membranes were separated by a low density gap of constant width whereas glutaraldehyde-treated erythrocytes and latex interactions with the macrophage membrane were irregular. These workers suggested that there exists several groups of functionally linked binding structures on macrophage membranes which in some cases require active cell participation and may or may not involve hydrophobic interactions. The role of specific receptors for these substances (apart from EA_G) was not clear.

The/...

The involvement of serum proteins in nonspecific binding of target particles ot macrophages is difficult to assess and may involve Fc or complement receptors in some cases. For instance cytophilic antibody may be adsorbed onto the macrophage surface (Choi et al., 1974) and macrophages themselves synthesise certain complement components (Brade and Bentley, 1980) and express them on their cell membranes. Aging erythrocytes accumulate autoantibodies which are very difficult to remove (Kay, 1975). However for at least some nonspecific receptors evidence suggests that Fc and complement receptors are not involved (Czop et al., 1978; Michl et al., 1976a; b).

12.0 RECEPTORS FOR GLYCOPROTEINS AND POLYSACCHARIDES

Macrophages possess specific receptors for glycoproteins and glycoconjugates (Stahl et al., 1978, 1980; Shepherd et al., 1981); for desialated erythrocytes (Kolb and Kolb-Bachofen, 1978; Nagamura and Kolb, 1980; Czop et al., 1978); for bacterial cell wall carbohydrates (Ögmunsdóttir and Weir, 1976; Ögmunsdóttir et al., 1978; Freimer et al., 1978) and for tumour cells (Weir et al., 1979). These specific lectin-like receptor activities may explain some of the so-called non-specific recognition by macrophages. Certain foreign substances have terminal sugars expressed on their surfaces e.g. zymosan consists of polyglucose and polymannose in association with protein and lipid (Northcote and Horne, 1952); specific types of pneumococci and certain other bacteria contain glucose residues, either terminal or easily cleaved to the terminal position, in their outer cell wall (Larm and Lindberg, 1976). N-acetylglucosamine residues comprise the backbone and terminal positions in cell walls of bacteria including Staph. aureus (Strominger et al., 1976).

12.1 Function

These receptors may be important in cleaving micro-organisms from the lung and other exposed tissues before humoral immunity is generated. They may be involved in macrophage recognition of altered self including transformed cells (Weir et al., 1979) and effete autologous enzymes (Stahl et al., 1978) and effete red cells (Kolb and Kolb-Bachofen, 1978). Spontaneous cytotoxicity for certain xenogeneic target cells (red blood cells) by human monocytes was inhibited by simple carbohydrates (Muchmore and Blaese, 1979) suggesting that lectin-like receptors on macrophages may be involved in NK activity.

Lectin-/...

Lectin-like receptors may also function as self-self recognition units as well as being involved in self-nonself recognition. Antigen-specific lymphocyte proliferation which is dependent on the presence of macrophages was inhibited by simple sugars (Muchmore and Blaese, 1979) suggesting that the sugar specific receptor on human monocytes may also be involved in T cell recognition.

Clearance of lysosomal enzymes by Kupffer cells or other macrophages also appears to be mediated by a lectin-like receptor (Schlesinger et al., 1978; Stahl et al., 1978). These receptors may play a role in enzyme salvage by Kupffer cells since lysosomal enzymes are secreted continuously. Pinocytosis via these receptors on macrophages may regulate the lysosomal enzyme concentration in the immediate environment and therefore be important in the inflammatory response where lysosomal enzyme secretion is increased. They may also be involved in the intracellular movement of lysosomal enzymes and in the actual assembly of the lysosome.

IgM antibody-antigen complexes are pinocytosed by rat liver non-parenchymal cells via a similar receptor. It appears to recognise mannose oligosaccharides which become accessible on IgM after it has complexed with antigen (Day et al., 1980).

12.2 Specificity

Mouse peritoneal macrophages possess "lectin-like" receptors which recognise unopsonised Gram positive and Gram negative bacteria (Ögmundsdóttir and Weir, 1976; Freimer et al., 1978). The ability of various monosaccharides to inhibit binding was found to correlate with their presence in the bacterial cell wall. D-glucose, D-galactose,

N-/...

N-acetyl-D-glucosamine and N-acetyl-D-galactosamine all of which are present in C. parvum cell walls inhibited the binding of C. parvum to mouse peritoneal macrophages to approximately the same extent. However fucose and rhamnose which were not detected in the C. parvum cell wall were also inhibitory. Binding of mutant strains of S. typhimurium deficient in certain sugars, were not inhibitable by these particular monosaccharides. Attachment of tumour cells and mouse embryo fibroblasts in growth phase to mouse peritoneal macrophages was also inhibited by various monosaccharides (Weir et al., 1979).

Both rat Kupffer cells and alveolar macrophages specifically recognised glycoproteins or glycoconjugates with terminal mannose, N-acetyl-glucosamine or fucose on their oligosaccharide chains (Schlesinger et al., 1978; Stahl et al., 1978; Shepherd et al., 1981). The uptake was specific and saturable. Inhibition studies indicated the following order of potency for sugar recognition by this system: L-fucose = D-mannose > N-acetyl-D-glucosamine ≈ D-glucose > D-xylose ⋙ D-galactose = L-arabinose = D-fucose (Shepherd et al., 1981). These results suggested that orientation about the fourth carbon (C-4) was critical. C-6 appeared to be less important but still essential because removal of C-6 (xylose) showed decreased binding. The orientation about C-2 may also play a role since glucose and mannose (C-2 epimers) varied in inhibitory potency by an order of magnitude.

Neuraminidase treated erythrocytes are taken up by Kupffer cells in vivo (Janik et al., 1978). Both rat Kupffer cells and rat peritoneal macrophages bound desialated red cells via a D-galactose specific receptor in vitro (Kolb and Kolb-Bachofen, 1978; Nagamura and Kolb, 1980). Inhibition studies with monosaccharides, oligosaccharides and/...

and glycoproteins indicated that the receptor recognised N-acetyl-D-galactosamine, D-galactose, L-fucose and D-fucose. D-glucose was also inhibitory but only when present on glycoproteins and not as a monosaccharide. However after phosphorylation at position 6, D-glucose was as inhibitory as D-galactose (Schlepper-Schaffer et al., 1980). Desialated orosomucoid was bound with thirty to forty times greater affinity than the most effective mono- and oligosaccharide in this system suggesting that the receptor may recognise a specific sequence of saccharides.

12.3 Number of receptors/cell

With particulate material such as erythrocytes and bacteria it was not possible to estimate the number of receptors per macrophage. However rat alveolar macrophages expressed about 75,000 binding sites per cell for mannose-BSA (Stahl et al., 1980).

12.4 Ultrastructure

Ultrastructural analysis of the binding between desialated erythrocytes and Kupffer cells by electron microscopy showed broad contact zones between the two surfaces (Schlepper-Schaffer et al., 1980). Extension of pseudopodia around the red cells suggested that phagocytosis would occur according to the zipper hypothesis of Griffin et al. (1975b).

Attachment of bacteria, red cells or glycoproteins is not temperature dependent (Ögmundsdóttir and Weir, 1976; Kolb and Kolb-Bachofen, 1978; Stahl et al., 1978).

The presence of external Ca^{2+} was required for the binding of bacteria, desialated erythrocytes and glycoproteins (Ögmundsdóttir and/...

and Weir, 1976; Kolb and Kolb-Bachofen, 1978; Stahl et al., 1978; Shepherd et al., 1981) and Mg^{2+} was also required for bacterial binding (Ögmundsdóttir and Weir, 1976).

Binding of desialated erythrocytes to rat peritoneal macrophages or Kupffer cells was not inhibited by cytochalasin B or colchicine suggesting that neither microfilaments nor microtubules are involved (Nagamura and Kolb, 1980: Schlepper-Schaffer et al., 1980). The effects of these agents on the attachment of bacteria or glycoproteins has not as yet been tested.

12.5 Modulation of the expression of "lectin-like" receptors

Alterations in the expression of "lectin-like" receptors on mouse peritoneal macrophages which recognise bacterial cell wall sugars is described in this thesis. Modulation of other "lectin-like" receptors has not been reported.

12.6 Nature of "lectin-like" receptors

The glycoprotein receptor, and the receptors recognising bacteria and tumour cells were trypsin sensitive (Stahl et al., 1978; Ögmundsdóttir et al., 1978; Weir et al., 1979). Pronase inhibited the binding of C. parvum and tumour cells to mouse peritoneal macrophages (Ögmundsdóttir et al., 1978; Weir et al., 1979) and the binding of desialated erythrocytes to rat Kupffer cells (Schlepper-Schaffer et al., 1980). ß-galactosidase, phospholipases C + D and periodate treatment decreased the binding of C. parvum or tumour cells to mouse peritoneal macrophages (Ögmundsdóttir et al., 1978; Weir, et al., 1979). These enzyme studies suggest that the receptors are protein in nature and the sensitivity of the receptors recognising bacteria or tumour cells indicates/...

indicates that it is a glycoprotein. The effects of phospholipases on these latter receptors suggest that phospholipids may be important in the functional activity of the receptor either directly or to provide the correct microenvironment.

12.7 Isolation of "lectin-like" receptors

Townsend and Stahl (1981) have isolated a mannose-binding protein from rat liver by affinity chromatography which has the same binding characteristics as that observed *in vivo* with plasma clearance of lysosomal enzymes and *in vitro* with rat Kupffer cells and alveolar macrophages. SDS-PAGE under reducing conditions revealed a single band with a molecular weight of about 35,000 daltons.

Isolation of the other "lectin-like" receptors has not been reported as yet.

CHAPTER II - AIMS OF THE PRESENT STUDY

Mononuclear phagocytes are involved in inflammation and the clearance of particulate agents and play an important role in both the afferent and efferent limbs of the immune response (Unanue, 1972, 1978) and probably also in the control of neoplasia (James et al., 1977). They synthesise and secrete a wide variety of products and are involved in anabolic and catabolic metabolism. Cell-cell contact and membrane stimulation are important features of many macrophage functions such as phagocytosis, pinocytosis, cell recognition and antigen presentation and thus many biological activities of macrophages are dependent on their surface receptors. The aims of the present study were to establish more precisely the presence and properties of membrane receptors for IgM, IgG, C3b, C3b´ , C3d, C4, and "lectin-like" receptors on mononuclear phagocytes, and to develop a greater understanding of environmental influences which may modulate the expression of these receptors and therefore the functional capacities of macrophages. Human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages comprise populations of mononuclear phagocytes from different sources, different maturation states and have different biochemical characteristics and physiological roles. The functions of these cells may depend on the level of expression of their surface receptors and accordingly this was investigated and comparisons made between different cell types. Heterogeneity of macrophage populations was first described by Walker (1976) who showed that macrophages from a single tissue could be separated into subclasses of cells that are functionally distinct. Therefore in these studies possible surface receptor heterogeneity within the populations was also examined.

It had previously been reported that complement receptors on human peripheral blood eosinophils were enhanced following incubation with/...

with various pharmacological mediators to which they migrate in chemotaxis and/or chemokinesis. A number of eosinophilotactic agents, including the eosinophil chemotactic factors of anaphylaxis (ECF-A) (the tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu), histamine and one of its major catabolites imidazoleacetic acid selectively enhanced receptors for complement (C3b and C4b) (CR_1) on human eosinophils in a dose- and time-dependent fashion (Anwar and Kay, 1977, 1978) with no apparent effect on Fc (IgG) receptors or C3b' receptors (CR_3). The phenomenon of complement receptor enhancement may be an important feature common to all phagocytic cells. It is proposed that in vivo chemotactic factors both attract phagocytes to the site of inflammation and via complement receptor enhancement increase their capacity for attachment to C3b opsonised particles, thereby increasing phagocytosis and killing. Enhanced killing of complement coated Schistosomula by eosinophils pretreated with ECF-As or by neutrophils preincubated with a synthetic chemotactic agent, f-Met-Leu-Phe indicates the possible significance of complement receptor enhancement in vivo (Anwar et al., 1980). It seemed important to investigate chemoattractant induced complement receptor enhancement by other phagocytes and accordingly various mononuclear phagocytes were examined. Three different macrophage populations were studied - human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages - because they exhibit regional differences in their biochemistry and function. They can modify their own and other cells' mechanism of complement receptor enhancement and its possible consequences in vivo, the effects of various chemoattractants and other pharmacological mediators on surface receptors of functionally distinct macrophages were examined.

CHAPTER III - MATERIALS AND METHODS

SECTION I -

BUFFERS AND REAGENTS

1.0 BUFFERS

All chemicals were analytical grade, obtained from BDH Chemicals Ltd., Poole, Dorset, unless otherwise stated.

1.1 Dulbecco's phosphate buffered saline (modified) D.PBS

This was prepared by dissolving 8.0g NaCl, 0.2g KCl, 1.08g Na_2HPO_4, 0.2g KH_2PO_4, 0.089g $CaCl_2 2H_2O$ and 0.203g $MgCl_2 6H_2O$ up to 1 litre with distilled water.

1.2 Phosphate buffer (Sorensen's)

A. M/15 sodium phosphate

9.465g Na_2HPO_4 was dissolved in distilled water and made up to 1 litre.

B. M/15 potassium acid phosphate

9.08g KH_2PO_4 was dissolved in distilled water and made up to 1 litre.

Various amounts of the above 2 mixtures were mixed to give the required pH.

1.3 Phosphate buffered saline (PBS)

PBS was prepared by mixing 8.5g NaCl, 1.07g Na_2HPO_4 and 0.39g NaH_2PO_4 and adjusting the volume to 1 litre with distilled water.

1.4 Hepes buffer

Hepes buffer was prepared by dissolving 23.83g Hepes buffer (Flow Laboratories, Irvine, Scotland, U.K.) in approximately 90ml distilled water. Sufficient 5N NaOH (about 5ml) was added to adjust the pH to 7.6 - 7.7 and the final solution adjusted to 100ml with distilled water. This gives a one molar solution.

1.5/...

1.5 Medium 199

This was prepared by mixing 10ml of 10 times concentrated medium 199 (Flow Laboratories, Irvine, Scotland, U.K.) with 3ml of 1M Hepes buffer and adjusting the volume to 100ml with distilled water.

1.6 Eagle's Minimum Essential Medium (MEM)

Eagle's MEM was prepared by mixing 10ml of 10 times concentrated Eagle's MEM (Flow Laboratories, Irvine, Scotland, U.K.), 3ml 1M Hepes buffer, 1ml of 0.2M glutamine and 0.1ml of penicillin and streptomycin (200,000 $U.ml^{-1}$) and adjusting the volume to 1 litre with distilled water.

1.7 Formol-saline

10% formol-saline was prepared by making 10ml formalin up to 100ml with PBS. This stock solution was diluted to 1% with medium 199 as necessary.

1.8 0.001M Acetate buffer

A. 0.001M Sodium acetate

0.136g $NaCH_3COOH.3H_2O$ was dissolved in 1 litre of distilled water.

B. 0.001M Acetic acid

57µl of 17.5M stock solution of acetic acid were diluted to 1 litre with distilled water.

Approximately 1 litre of A and 3 litres of 3 were mixed to give 0.001M acetate buffer, pH 5.0.

1.9 Five times Veronal (5 X Veronal)

This was prepared by mixing 42.5g NaCl, 1.875g sodium barbitone and 2.875g barbitone and adjusting the volume to 1 litre with distilled water.

1.10/...

1.10 10% Gelatin

10g gelatin was dissolved in 100ml hot distilled water and 10ml volumes dispensed into universals and autoclaved.

1.11 0.03M $CaCl_2$

4.41g $CaCl_2 2H_2O$ was dissolved in 1 litre of distilled water.

1.12 0.1M $MgCl_2$

20.33g $MgCl_2 6H_2O$ was dissolved in 1 litre of distilled water.

1.13 Gelatin-Veronal buffer (GVB^{++})

This was prepared by mixing 200ml 5X Veronal, 10ml 10% gelatin, 5ml 0.03M $CaCl_2$ and 5ml 0.1M $MgCl_2$ and adjusting the volume to 1 litre with distilled water.

1.14 Gelatin Veronal buffer (GVB^{--})

This was prepared according to the above buffer recipe without the addition of 0.03M $CaCl_2$ and 0.1M $MgCl_2$.

1.15 5% Dextrose ($D5W^{++}$)

50g dextrose, 5ml 0.03M $CaCl_2$ and 5ml 0.1M $MgCl_2$ were dissolved in 1 litre of distilled water.

1.16 Dextrose-gelatin-Veronal buffer ($DGVB^{++}$)

Equal volumes of GVB^{++} and $D5W^{++}$ were mixed together.

1.17 Isotonic EDTA

89.33g disodium EDTA was made up to 1 litre with warm distilled water. 780ml 0.3M NaOH was added to adjust the pH to 7.4. The final molarity of EDTA is 0.086M.

1.18/...

1.18 0.01M EDTA GVB^{--}

100ml of 0.086M EDTA was adjusted to 860ml with GVB^{--}.

1.19 Lysis buffer

This was prepared by dissolving 8.2g NH_4Cl, 1.0g $KHCO_3$ and 0.037g Na_2EDTA in 1 litre of distilled water.

1.20 Alsevers

21g dextrose, 8g trisodium citrate. $2H_2O$, 4.2g NaCl and 0.4g anhydrous citric acid were dissolved in 1 litre of distilled water. This solution was aliquoted and autoclaved.

2.0 STAINS

2.1 White cell diluting fluid

A stock solution was prepared by adding 0.1g gentian violet to 100ml of 1% acetic acid. To use the stock was diluted 1:10 with 1% acetic acid and 1 part cell suspension was added to 9 parts stain.

2.2 Trypan blue

1% aqueous trypan blue was added 1:1 with the cell suspension.

2.3 Eosinophil stain

A. 0.1% methylene blue

0.1g methylene blue was added to 100ml propan-1-2-diol.

B. 0.1% phloxine

0.1g phloxine was added to 100ml propan-1-2-diol.

1ml of each of the above 2 stains was mixed with 2ml distilled water and 1 part cell suspension added to 9 parts stain.

2.4 Non specific esterase stain

This method is modified from that of Yam, Li and Crosby (1971). Cells of the mononuclear phagocyte series are stained diffusely brown.

A. Hexazotised pararosaniline

Solution A 1g pararosaniline (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was dissolved in 25ml 2N HCl by heating gently. The solution was cooled, filtered and stored at 4°C.

Solution B 4g sodium nitrate was dissolved in 100ml distilled water.

Immediately before use, equal parts of A and B were mixed.

B./...

B. α napthyl acetate substrate

45mg α napthyl acetate (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was mixed with 2.5ml N, N dimethylformamide, 45ml Sorensen's phosphate buffer, pH 7.2, and 1.8ml freshly hexazotised pararosaniline. This solution was filtered before use.

Cell smears were incubated in the above mixture for 45 minutes at room temperature or for 20 minutes at 37°C.

2.5 May Grunwald/Giemsa stain

May Grunwald stain was diluted 1:1 with PBS.

Giemsa stain was diluted 1:10 with PBS.

Cell smears were stained for 2 minutes in each stain in the above order, followed by 5 minutes destaining in PBS.

2.6 Eosinophil stain

A. Chromotrope

1g of chromotrope was dissolved in 1% phenol.

B. Tartrazine

0.1g tartrazine was dissolved in 1% acetic acid.

Cell smears were incubated in the chromotrope stain for 15 - 20 minutes, washed and counterstained for 30 seconds with tartrazine.

SECTION II -

PURIFICATION OF CELLS

1.0 HUMAN MONOCYTES AND NEUTROPHILS

Monocytes and neutrophils were separated according to the method of Boyum (1968). Blood from healthy donors was drawn into plastic tubes containing 10 U of heparin.ml^{-1}, and then diluted 1:4 with normal saline. The diluted blood was layered onto Ficoll-Hypaque cushions (Pharmacia, Uppsala, Sweden) (s.g. 1.078) and centrifuged for 30 minutes at 400g. After the interface cells (monocytes and lymphocytes) were collected, the pellet containing granulocytes and red blood cells were resuspended in 0.82% ammonium chloride to lyse the erythrocytes as described by Boyle (1968). The separated cells were finally washed twice in D.PBS and resuspended in medium 199.

2.0 Human eosinophils

Blood was obtained from atopic eczema patients or healthy donors with eosinophilia. Leucocyte rich plasma was prepared by mixing 1 part 6% Dextran T70 (Pharmacia, Uppsala, Sweden) and 4 parts heparinised blood and incubating for 30 minutes at 37°C. The plasma was layered over cushions of sodium metrizoate (Nyegaard and Company, As, Oslo, Norway) (s.g. 1.140) and centrifuged for 30 minutes at 400g. The pellet containing neutrophils, eosinophils and red blood cells was treated as for neutrophils above.

3.0 ANIMALS

Dunkin-Hartley guinea pigs (250 - 400g) and CF1 mice (age 6 - 10 weeks) were from the Bacteriology Department breeding colony.

4.0 MOUSE PERITONEAL MACROPHAGES

These were obtained by peritoneal lavage with D.PBS and heparin (10 $U.ml^{-1}$).

5.0 GUINEA PIG ALVEOLAR MACROPHAGES

These were collected by tracheo-bronchial lavage (Myrvik, Lenke and Farris, 1971) using D.PBS and heparin (10 $U.ml^{-1}$) from animals anaesthetised with intra-peritoneal sodium phenobarbital.

6.0 LYMPHOCYTE SUPERNATANTS

A cell suspension containing up to 90% lymphocytes was obtained from defibrinated blood as described by Boyum (1976). The separated cells were resuspended to a concentration of 3×10^6 lymphocytes ml^{-1} in medium 199. Under sterile conditions and with added penicillin and streptomycin, 9µl of phytohaemagglutinin (PHA-P, Wellcome Reagents Ltd., Beckenham, Kent) were added to 3ml of the mononuclear cell suspension. The cells were incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO_2. After incubation the supernatants were harvested by centrifugation, aliquoted and stored at -70°C until use. The unstimulated lymphocyte supernatants were prepared in an identical fashion with the exception that PHA-P was added at the end of the 3 day incubation period.

SECTION III -

PREPARATION OF INDICATOR CELLS

1.0 PREPARATION OF RABBIT ANTISHEEP ANTIBODY

1.1 Preparation of sheep stroma

250ml of whole sheep's blood was diluted 1:1 with Alsevers and washed once with normal saline. The cells were slowly added to ice cold 4.4×10^{-4}M acetic acid, mixing continuously for a few minutes. The stroma was allowed to settle overnight at 4°C. After removal of most of the supernatant fluid, the sediment was centrifuged at 800g for 20 minutes at 4°C. The resultant pellet was washed repeatedly in cold 0.001M acetate buffer, pH 5.0 until most of the haemoglobin was removed. The packed stroma was washed 3 times with 0.15M NaCl, resuspended in 45ml 0.15M NaCl and boiled for 1 hour. When cool the stroma was ultrasonicated to a smooth suspension and stored at -70°C.

1.2 Rabbit antisheep stroma

To raise antiserum to the stroma, adult rabbits were injected intravenously in a series of 11 injections spread over 12 days. The amount injected was gradually increased from 0.1 to 2ml. The rabbits were test bled on the 4th day after the last injection and bled out if the titre was high enough. The resultant antiserum was heated at 56°C for 1 hour to destroy complement, and stored at -20°C.

1.3 Fractionation of IgM and IgG antibodies from whole serum

A. Ammonium sulphate precipitation

This was carried out according to Heide and Schwick (1978). 20ml of whole heat inactivated serum was precipitated with 16.4ml of a saturated ammonium sulphate solution (45% ammonium sulphate) which was added dropwise into the serum over a period of 20 to 30 minutes, stirring/...

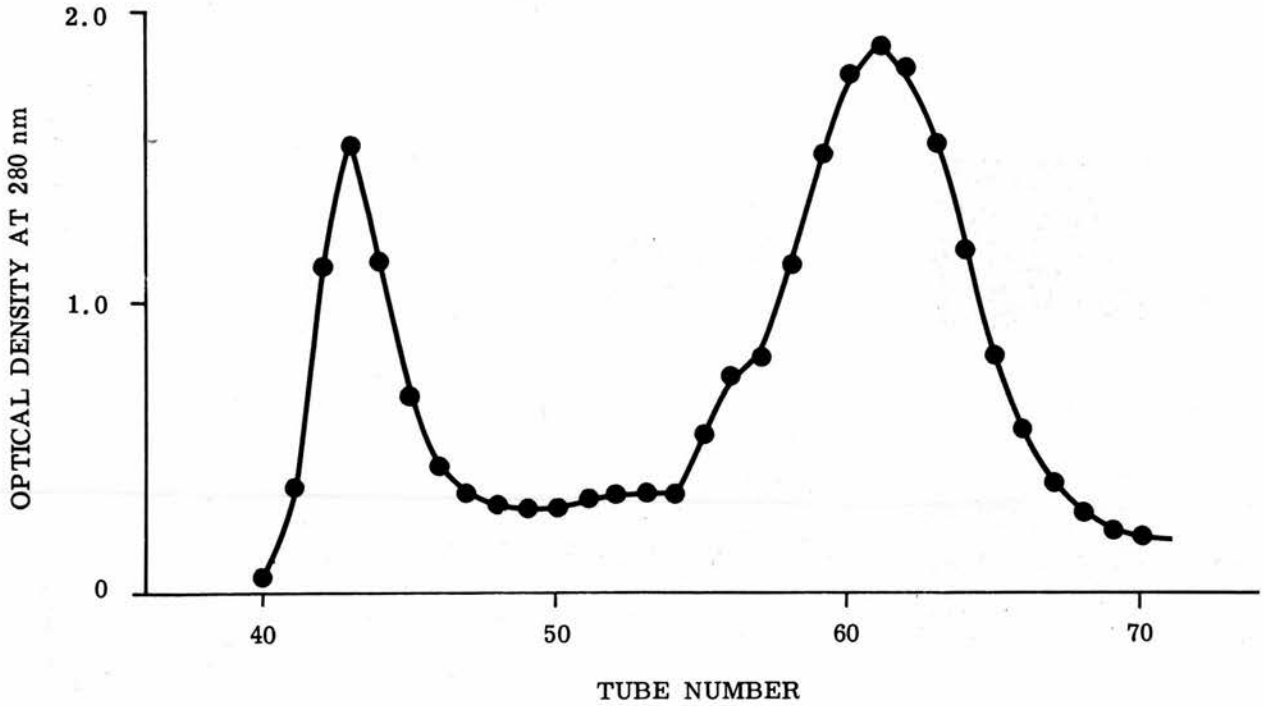


Fig. 1

Sephadex G-200 fractionation of rabbit anti sheep erythrocyte sera.

stirring continuously. The treated serum was centrifuged at 10,000g for 20 minutes at 4°C and the precipitate dissolved in 5ml distilled water. This was dialysed against 5 litres of distilled water for 12 to 18 hours at 4°C and then against 5 litres of PBS for 6 to 12 hours. The dialysed solution was stored at -20°C.

B. Fractionation with Sephadex G-200

This was carried out according to Shevach et al.(1972). 2.5ml of the above ammonium sulphate fractionated serum was applied to a Sephadex G-200 column (bed volume = 750ml) at 4°C and eluted with PBS using a flow rate of $14ml.hr^{-1}$. 5ml fractions were collected. The optical density at 280nm for each fraction was measured (Fig. 1). The ascending half of the first peak (IgM fraction) was pooled, and the upper three-quarters of the second peak (IgG fraction) was pooled. Each pool was dialysed against 5 litres of distilled water for 18 hours at 4°C, freeze-dried and resuspended to one-tenth their original volume.

The IgM and IgG pools were further purified according to Fahey and Terry (1978).

C. Further purification of IgM. Fractionation with Protein A-Sepharose CL-4B

0.5g of Protein A (Staph. aureus) covalently bound to Sepharose CL-4B (Pharmacia, Uppsala, Sweden) were swollen for 3 hours in 4ml PBS at 4°C, poured into sterile 10 cc syringe containing a thin layer of glass wool. The gel was allowed to settle to a packed bed volume of approximately 1.7ml before washing with 25ml PBS.

The entire IgM sample from the above Sephadex G-200 fractionation was applied and eluted with PBS, 1ml fractions being collected. All fractions with OD_{280} greater than zero were pooled, dialysed against distilled/...

distilled water, freeze-dried and reconstituted in PBS to 1mg protein. ml^{-1}.

D. Further purification of IgG. Fractionation with DEAE-52

Approximately 3.5ml of pre-swollen DEAE-52 (Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) were mixed with 0.01M Tris: 0.02M NaCl (pH 7.5) and poured into a 60ml syringe containing a thin layer of glass wool.

The entire IgG sample from the above Sephadex G-200 fractionation was applied to the DEAE-52 column and eluted with 50ml Tris NaCl buffer. 1ml fractions were collected and the OD_{280} readings for each were recorded. All fractions with an OD_{280} reading greater than zero were treated as above.

Samples of IgM, IgG and whole serum were tested in an immunodiffusion assay (carried out by the South-East Scotland Regional Blood Transfusion Service, Royal Infirmary, Edinburgh) and the IgM and IgG fractions determined to be uncontaminated by other immunoglobulins.

2.0 DETERMINATION OF IgM AND IgG TITRES BY AGGLUTINATION OF SHEEP RED CELLS

Sheep red cells were washed twice in normal saline and resuspended to 1 x 10^8 cells.ml^{-1} in $DGVB^{++}$. 50µl of red cells and 50µl of doubling dilutions of antibody were incubated together in V-shaped well microtitre plates for 1 hour at 37°C. From the settling pattern of the sheep cells the agglutination titre could be ascertained.

3.0 PREPARATION OF ANTIBODY COATED SHEEP RED CELLS

3.1 Preparation of rabbit antisheep IgM coated erythrocytes (EA_M^{rab})

Sheep blood was drawn asceptically into sterile Alsevers solution and stored at 4°C. The red cells were washed twice in cold saline and once in 0.01M $EDTAGVB^{--}$. The cell concentration was adjusted to 1×10^9 cells.ml^{-1} and IgM antisheep red blood cells was added, the amount depending on the titre calculated above. The mixture was incubated for 30 minutes at 37°C, followed by 30 minutes at 4°C with frequent shaking. The cells were washed once in 0.01M $EDTAGVB^{--}$ and twice in $DGVB^{++}$ and stored at 4°C. The cells were kept for not longer than 5 days and were washed daily with $DGVB^{++}$.

3.2 Preparation of rabbit antisheep IgG coated erythrocytes (EA_G^{rab})

The method for preparing EA_G^{rab} was exactly the same as that for EA_M^{rab} except that IgG was used to sensitise the red cells instead of IgM.

4.0 PREPARATION OF COMPLEMENT COATED SHEEP RED CELLS

4.1 Preparation of yeast

This method was modified from U. Hadding (1967).

250g of fresh baker's yeast was suspended in 1 litre of PBS and autoclaved for 30 minutes at 120°C, washed with PBS until the supernatant was clear and resuspended in 250ml PBS containing 0.1M mercaptoethandl. The mixture was incubated at 37°C for 2 hours with frequent stirring and washed once with saline to remove most of the mercaptoethanol. The yeast was stirred at room temperature for 2 hours in 250ml of normal saline containing 20% w/v 0.2M phosphate buffer (pH 7.2) and 20mM iodoacetimide. The pH was checked periodically. After washing 3 times with PBS, the yeast was resuspended in 1 litre of the same buffer and re-autoclaved for 30 minutes at 120°C and washed until the supernatant was clear. Finally the yeast was suspended in 500ml of GVB^{++} containing 0.01% azide and stored at 4°C.

4.2 Titration of yeast

After washing twice with $DGVB^{++}$, various dilutions of yeast were incubated with an equal volume of serum for 30 minutes at 37°C. The suspension was centrifuged at 400g for 10 minutes and the supernatants collected.

0.1ml of the various supernatants was added to an equal volume of EA_M^{rab} (10^8 cells.ml^{-1}) and incubated for 30 minutes at 37°C. The dilution which was just below that which gave detectable lysis was $^1/5$ and this was the dilution used (see next section).

4.3/...

4.3 Preparation of EAC

Two different methods of preparing EAC were used.

A. Human AB serum as a source complement

Fresh human AB serum was obtained from a suitable donor and checked for the presence of Forssmann's antibody by incubating neat serum with sheep red cells. If lysis occurred the serum was adsorbed with sheep red cells at 4°C with frequent shaking for several hours. The serum was centrifuged to remove the red cells and then aliquoted and stored at -70°C for not longer than 6 months. For each experiment a fresh ampoule was used and not refrozen.

Equal volumes of EA_M^{rab} at a concentration of 1×10^8 cells.ml^{-1}, and human AB serum diluted with $DGVB^{++}$ to a suitable concentration, were mixed and incubated for 30 minutes at 37°C. The cells were washed and resuspended in the same buffer to give a final concentration of 1×10^8 cells.ml^{-1}.

B. Zymosan depleted serum as a source of complement

With human neutrophils and eosinophils and mouse peritoneal and guinea pig alveolar macrophages the concentration of serum required to give an optimal number of rosettes would have lysed the red cells. So, for experiments involving these cells, a different method of preparing EAC was used.

Equal volumes of yeast prepared as described above in part 4 and AB serum were mixed and incubated for 30 minutes at 37°C. The yeast was centrifuged and the remaining supernatant, termed R3, was used as the source of complement. The alternate pathway of complement was activated and the terminal components of complement - C5 to C9 were used up. Although a considerable proportion of C3 is also turned/...

turned over, it is still possible to coat the sheep cells with more C3 than if the highest possible concentration of serum was used.

Equal volumes of R3 diluted with $DGVB^{++}$ and EA_M^{rab} at a concentration of 1×10^8 cells. ml^{-1} were mixed and incubated for 30 minutes at 37°C. The cells were washed and resuspended in $DGVB^{++}$ to give a final concentration of 1×10^8 cells. ml^{-1}.

5.0 PREPARATION OF EAC USING PURIFIED HUMAN COMPLEMENT COMPONENTS

Purified human complement components were obtained from Cordis Laboratories, Miami, U.S.A.

5.1 Preparation of EA14

EA_M^{rab} was mixed with 400 effective molecules of human C1 per red cell, incubated for 15 minutes at 37°C and washed twice with $DGVB^{++}$. EA14 was prepared by the addition of 6,000 molecules of C4 and incubated for 30 minutes at 37°C, washed twice and resuspended in $DGVB^{++}$ at a concentration of 1×10^8 cells.ml^{-1}.

5.2 Preparation of EA1423b

EA1 was prepared as described above. 400 effective molecules of C4 were added and the mixture incubated at 37°C for 30 minutes. After 2 washes with $DGVB^{++}$, a further 400 molecules per cell of C1 were added to EA14 and incubated for 15 minutes at 37°C. The cells were washed twice and incubated with 50 effective molecules of C2 per cell for 10 minutes at 30°C. Without washing varying amounts of C3 per cell were added to EAC142 and incubated at 37°C for 30 minutes. After washing twice with $DGVB^{++}$, the EAC1423b was diluted to 10^8 cells.ml^{-1}.

5.3 Preparation of EAC1423b´

Two methods of preparing EAC1423b´ were used.

A. C3b inactivator

EAC1423b cells were prepared as described above in part 5.2, usually with 500 molecules of C3 per cell. The cells were incubated with purified C3b inactivated (KAF) (Cordis Laboratories, Miami, U.S.A.) at/...

at a concentration which reduced immune adherence (see below) by 95%, for 1 hour at 37°C. The cells were washed twice with $DGVB^{++}$ and resuspended to 1 x 10^8 cells.ml^{-1}.

B. EDTA-chelated AB serum

EAC1423b were incubated with neat AB serum chelated with 10mM EDTA for 15 minutes at 37°C, washed twice and resuspended to a concentration of 1 x 10^8 cells.ml^{-1}.

5.4 Preparation of EA1423d

EA1423d was prepared by incubating EAC1423b' (made by either of the above two methods) with 0.25mg trypsin per 10^8 cells for 2.5 minutes at 37°C. The reaction was stopped by flooding the cells with ice-cold $DGVB^{++}$ and the cells washed twice with $DGVB^{++}$ and resuspended to a concentration of 1 x 10^8 cells.ml^{-1}.

6.0 IMMUNE ADHERENCE

Human red cells have receptors for C3b and not for C3b' or C3d. In order to demonstrate the presence of C3b an immune adherence assay was set up in V-shaped microtitre plates. In each well 50µl of human O red cells (10^8 cells.ml^{-1}) and 50µl of test sheep red cells (10^8 cells.ml^{-1}) were mixed and incubated at 37°C for 2 hours. From the settling pattern it was possible to determine which complement coated sheep red cells had C3b deposited on their surfaces. If the cells were spread out over the bottom of the well immune adherence had occurred whereas if the cells had pelleted the test cells did not have C3b on their cell membranes. Using this assay it was possible to demonstrate that as expected EA1423b cells were immune adherence positive as were EAC prepared using AB serum as a source of complement. EA1423b' and EA1423d were immune adherence negative.

7.0 PREPARATION OF BACTERIA

7.1 Preparation of Staphylococcus albus (Staph. albus)

Staph. albus maintained by aerobic subculture on horse blood agar was obtained from the Bacteriology Department teaching collection.

A suspension of bacteria was prepared by growing an innoculum aerobically in 10-15ml of horse digest broth plus 3% glucose for 24 hours at 37°C and then transferred into 100ml of the same medium and incubated for a further 19 hours at 37°C in a shaking incubator. The cultures were harvested in log phase and centrifuged for 20 minutes at 2,000g at 4°C, washed once in normal saline and killed by 24 hour exposure to 0.5% formalin at 4°C. The killed bacteria were washed 3 times in saline, resuspended in about 10ml saline and stored at 4°C.

Since an optical density at 540nm corresponds to 2×10^7 Staph. albus. ml^{-1}, the concentration of bacteria could be easily determined.

SECTION IV -

ROSETTE FORMATION

1.0 DETECTION OF Fc AND COMPLEMENT RECEPTORS ON CELLS IN SUSPENSION

A portion (0.1ml) of sheep red cells coated with immunoglobulin and/or complement (10^8 cells.ml^{-1}) was added to 0.1ml of leukocytes (2×10^6 cells.ml^{-1} or in the case of monocytes or macrophages 1×10^6 monocytes or macrophages.ml^{-1}). The mixtures were centrifuged at 100g for 10 minutes at 4°C and the pellets incubated at 37°C for 20 minutes for EA_M^{rab} or EAC and for 30 minutes at 0°C for EA_G^{rab}. The pellet was gently resuspended in medium 199 containing 1% formal saline and smears prepared on clean glass slides in duplicate. These were dried quickly in air, fixed in methanol and monocytes, macrophages and neutrophils were stained with May Grunwald/Giemsa and eosinophils stained with Chromotrope and tartrazine. In some experiments the monocytes were fixed in 2.5% glutaraldehyde and stained using α-napthyl acetate histochemistry. Leukocytes with 3 or more adherent red cells were termed rosettes. In each slide 200 leukocytes were counted and the percentage rosetted cells calculated.

2.0 DETECTION OF Fc AND COMPLEMENT RECEPTORS ON CELLS IN MONOLAYERS

2.1 Preparation of monolayers

Eosinophils and neutrophils were suspended in Eagle's MEM to give a final total cell count of 2×10^5 cells.ml^{-1}. Monocytes and macrophages were suspended to give a final concentration 2×10^5 monocytes or macrophages.ml^{-1}. One ml of the above cell preparations was layered onto 13mm diameter (No 1) glass coverslips in 16mm diameter tissue culture plates (Costar, Cambridge, Mass., U.S.A.) and incubated for 1 hour at 37°C. Non adherent cells were removed by washing with D.PBS.

2.2 Fc and complement rosette formation

0.5ml of EA_G^{rab} or EAC (10^8 cells.ml^{-1}) was added to each monolayer, centrifuged for 5 minutes at 100g and incubated for 40 minutes at 37°C. Non adherent red cells were removed by very gentle washing with D.PBS.

Coverslips were air dried, fixed in methanol and stained with May Grunwald/Giemsa. Positive binding was scored as those leukocytes with two or more red cells attached. In each coverslip 200 leukocytes were counted and the results expressed as the percentage of cells binding organisms.

3.0 DETECTION OF "LECTIN-LIKE" RECEPTORS ON CELL MONOLAYERS

3.1 Staph. albus binding to cell monolayers

Monolayers were prepared as described above in part 2.1. The coverslips were overlaid with 1ml Staph. albus in D.PBS and incubated for 2 hours at 4°C. Non attached organisms were removed by repeated washing with D.PBS.

3.2 Effect of sugars on Staph. albus binding

5.58g of glucose or galactose were dissolved in cold distilled water to give an isotonic solution of 310mM, filtered and stored at 4°C. They were diluted as necessary with D.PBS.

Cell monolayers were incubated with 0.5ml of solutions of glucose and galactose for 20 minutes at 4°C. The cells were then washed and the Staph. albus binding assay performed as described previously.

SECTION V -

EFFECT OF CHEMOATTRACTANTS, PHARMACOLOGICAL MEDIATORS AND NEURAMINIDASE ON LEUKOCYTE MEMBRANE RECEPTORS

1.0 REAGENTS

F-Met-Leu-Phe was a gift from Dr. Derek Hudson (Royal Postgraduate Medical School, London), f-Met-Leu-Phe, Met-Leu-Phe and Met-Met-Phe were gifts from Miles Laboratories (Stoke Poges, England). F-Met-Phe was obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Other materials were obtained as follows. Histamine acid phosphate and casein (BDH Chemicals Ltd., Poole, Dorset, U.K.); C. parvum 10390 supernatant was a gift from Dr. W. McBride (Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, U.K.); human serum albumin (Sigma Chemical Company, St. Louis, Missouri, U.S.A.); prostaglandins E_1, E_2 and $F_{2\alpha}$ were a gift from Dr. J. Pike (Upjohn Company, Kalamazoo, U.S.A.); bradykinin triacetate, 5-hydroxytryptamine creatine sulphate complex and isoprenaline (Sigma Chemical Company, St. Louis, Missouri, U.S.A.); neuraminidase from Vibrio cholerae, E.C. 3.2.1.18., 500 U ml^{-1} (BDH Chemicals Ltd., Poole, Dorset, U.K.).

2.0 PREPARATION OF REAGENTS

2.1 F-Met peptides

The f-Met peptides were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 10^{-2} moles.l^{-1} and further diluted in medium 199 to the required concentrations. At the highest dose used (10^{-5} moles.l^{-1}) the concentration of DMSO was about 10^{-2} moles.l^{-1} and at this concentration had no effect on cell viability as determined by trypan blue dye exclusion and locomotion *in vitro* through micropore filters (Kay, Glass and Salter, 1979).

2.2 Casein

Casein was dissolved in distilled water and the pH raised to 11 with 1N NaOH. The pH was then adjusted to 7.4 with 1N HCl. 9 parts of the casein solution were then added to 1 part of 10 times concentrated medium 199 with Hepes buffer, pH 7.4.

2.3 C. parvum 10390 supernatant

The *C. parvum* supernatant was prepared from formaldehyde-killed *C. parvum* organisms which had been washed twice in 0.9% NaCl, lyophilised and reconstituted in distilled water. The reconstituted material had a concentration of 7mg.ml^{-1} dry weight which represented 3×10^{11} organisms.

2.4 Prostaglandins

The prostaglandins E_1, E_2 and $F_{2\alpha}$ were dissolved in alcohol at a concentration of 10^{-2} moles.l^{-1} and further diluted in medium 199. At the highest dose used (10^{-4} moles.l^{-1}) the concentration of alcohol had no effect on cell viability.

All other compounds were dissolved in medium 199.

3.0 EFFECTS OF CHEMOATTRACTANTS AND OTHER MEDIATORS ON LEUKOCYTE RECEPTORS

Equal volumes of leukocytes (2×10^6 cells.ml^{-1}) and various concentrations of the compounds under study or medium alone as a control were mixed and incubated in a shaking water bath usually for 30 minutes at 37°C. The cells were then washed twice in medium 199 and the numbers adjusted to their original concentration in the same medium. The cells were then rosetted as described previously.

In some experiments cell monolayers were incubated with 0.5ml of solutions of the chemoattractants for 30 minutes at 37°C. After gentle washing the rosetting assays were performed as described previously.

4.0 NEURAMINIDASE TREATMENT OF HUMAN PERIPHERAL BLOOD MONOCYTES

5×10^6 total mononuclear cells were incubated with 5 U of neuraminidase per 1×10^6 total cells in the presence of 2% bovine serum albumin (BSA) for 30 minutes at 37°C. Control cells were incubated in the same way without the presence of neuraminidase. Each set of cells were washed twice with 0.2% BSA and resuspended to their original volume of 1 ml. Rosette assays were performed at this stage. The remaining mononuclear cells were then incubated with fluid phase IgM (0.004ml of 1/40 titre per 10^6 total cells) in 0.2% BSA for 30 minutes at 4°C, washed twice with 0.2% BSA and rosette assays performed as before.

STATISTICS

Measurements from duplicate incubations from at least three separate experiments were pooled, the mean and standard errors calculated and the observations compared using the student's t-test. Such pooling was considered to be statistically valid since in the present study the variation within each experiment was similar to the variations between individual experiments.

CHAPTER IV - RESULTS

SECTION 1 -

DETECTION OF RECEPTORS FOR IMMUNOGLOBULIN AND COMPLEMENT, AND LECTIN-LIKE RECEPTORS ON HUMAN MONOCYTES, MOUSE PERITONEAL MACROPHAGES AND GUINEA PIG ALVEOLAR MACROPHAGES

1.0 INTRODUCTION

Membrane receptors for the Fc fragment of immunoglobulin and complement components have been demonstrated on a number of phagocytic cells. Although most studies have been on lymphocytes, other cells such as mononuclear phagocytes, granulocytes, mast cells and basophils have also been shown to possess these receptors. The presence of complement and Fc receptors on lymphocytes is believed to be involved in antigen presentation and on phagocytic cells they function as recognition sites in mediating the binding of particles ranging in size from antigen-antibody complexes to whole cells coated with antibody and complement. Apart from being involved in phagocytosis of these particles, Fc and complement receptors are also important in other functions of leukocytes including antibody-dependent cell cytotoxicity.

In the last few years lectin-like receptors have been described on mouse macrophage membranes that recognize bacterial cell wall sugars leading to macrophage bacterial adherence. These receptors are likely to be of importance in relation to the pathogenesis of bacterial infection.

The present studies were concerned with defining and comparing the presence of complement, Fc and lectin-like receptors on a variety of mononuclear phagocytes from different species and environments - human peripheral blood monocytes, mouse peritoneal macrophages and guinea-pig alveolar macrophages. These preliminary experiments were also necessary for determining the optimal conditions for demonstrating the presence of these receptors before studies on the modulation of receptor expression could be undertaken in later experiments.

Visualisation/...

Visualisation of Fc and complement receptors was carried out using the rosette technique with sheep red cells coated with immunoglobulin and/or complement. Attachment of Staph.albus to phagocytic cells in a bacterial adherence assay was used to demonstrate the presence of lectin-like receptors.

SECTION II -

DETECTION OF RECEPTORS FOR IMMUNOGLOBULINS ON HUMAN MONOCYTES, MOUSE PERITONEAL MACROPHAGES AND GUINEA PIG ALVEOLAR MACROPHAGES

1.0 DETECTION OF RECEPTORS FOR IgG ON MONONUCLEAR PHAGOCYTES

1.1 Determination of optimal experimental conditions: Time course and temperature

Binding of rabbit anti-sheep IgG coated sheep red cells (EA_G^{rab}) to human monocytes was both time and temperature dependent (Figs. 2 and 3 and Table 1). At 37°C the percent rosetted cells remained constant (about 35%) for 20 minutes and then decreased to 28% by 60 minutes (Fig. 2). The number of monocytes which had phagocytosed red cells increased from 15% at 5 minutes to 35% at 60 minutes. Maximum binding of EA_G^{rab} to monocytes at 4°C was observed by 30 minutes (Fig. 3) and thereafter remained constant. Table 1 shows that Fc receptor expression is temperature dependent; at 30 minutes with $^1/_{20}$ dilution of antibody, 25% of the monocytes formed rosettes at 0°C whereas at 23°C and 37°C a slight increase in the number of rosetted cells was observed (34% and 32.5% respectively).

In order to investigate effects on binding of EA_G^{rab} to mononuclear phagocytes all subsequent experiments were carried out at 4°C for 30 minutes.

1.2 Effect of increasing the IgG concentration on human monocyte, mouse peritoneal macrophage and guinea pig alveolar macrophage rosette formation with EA_G^{rab}

Dose response curves for human monocytes, mouse peritoneal macrophages and guinea pig macrophages were carried out, varying the amount of IgG antibody coating the sheep red cells (Fig. 4). The percentage of rosette formation by mononuclear phagocytes was directly dependent on the IgG concentration. At $^1/_{10}$ dilution of the antibody (agglutination titre = $\frac{1}{8}$), 63% of guinea pig alveolar macrophages/...

macrophages bound EA_G^{rab} whereas both human monocytes and mouse peritoneal macrophages appeared to express fewer Fc receptors (47% and 49% respectively). However at lower concentrations of antibody mouse macrophages had the highest level of binding and human monocytes the lowest level of binding.

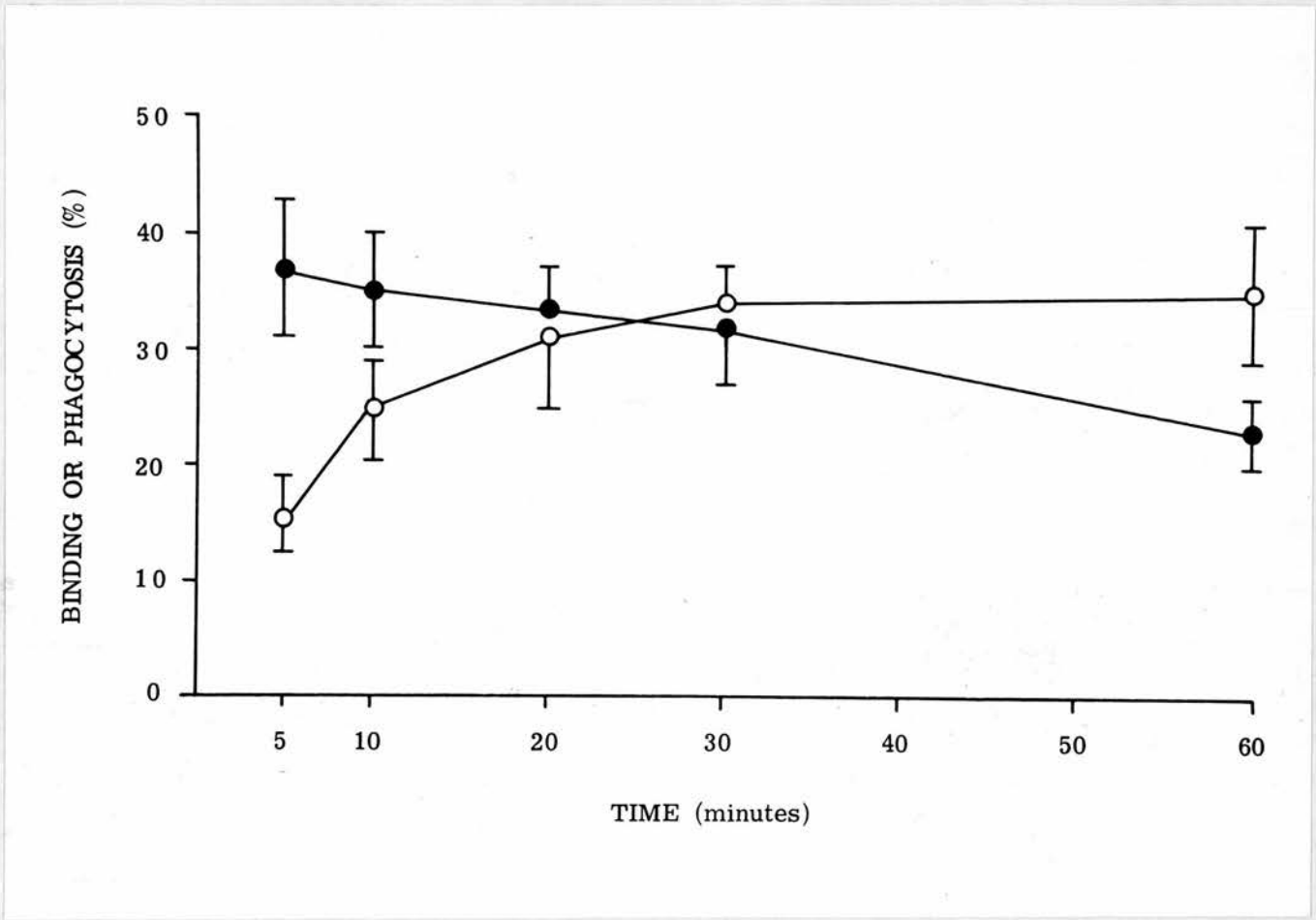


Fig. 2

The time course of the binding (●——●) or phagocytosis (o——o) of EA_G^{rab} by human monocytes at 37°C, using $^1/_{20}$ dilution of antibody. Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

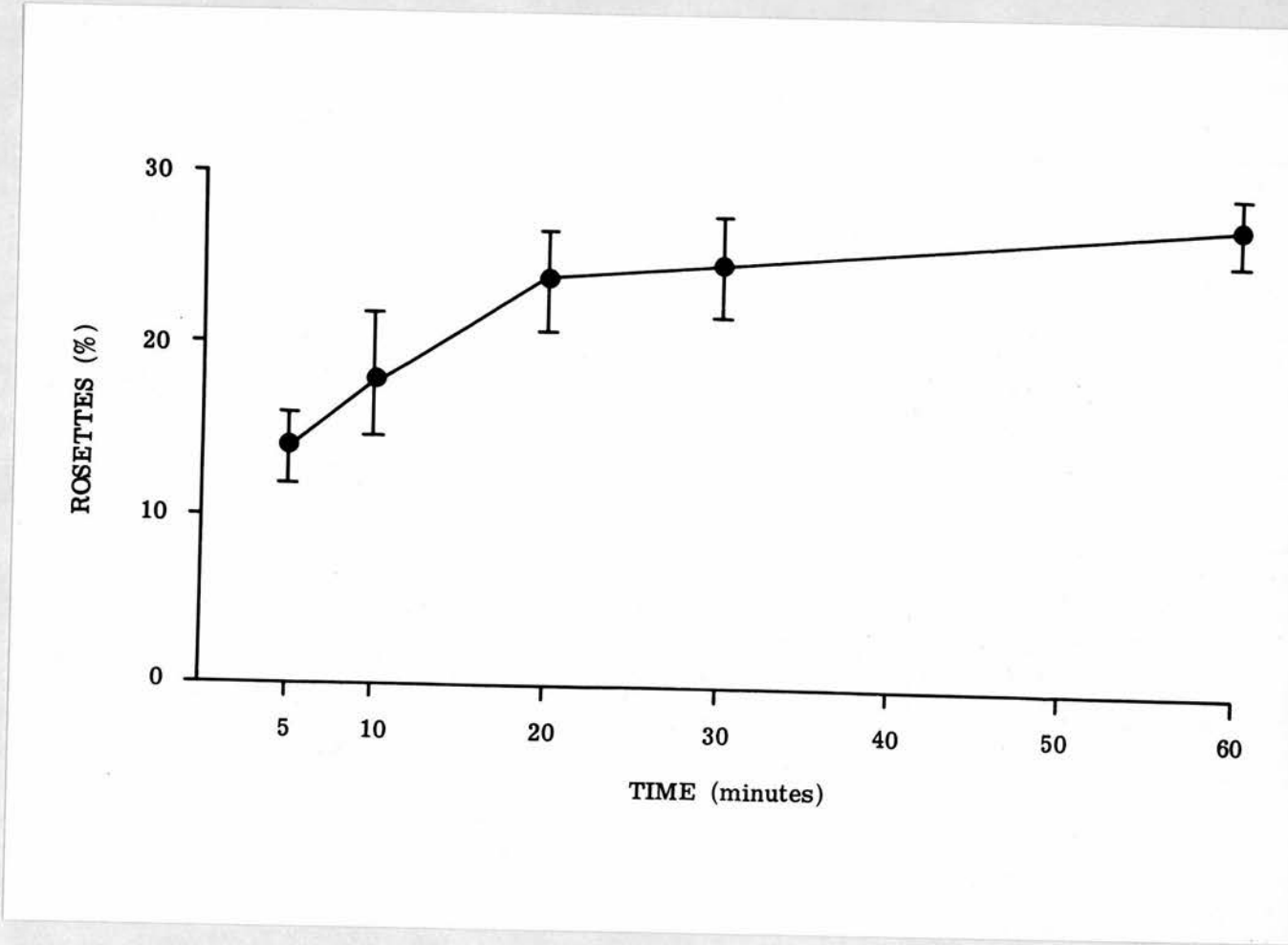


Fig. 3

The time course of the binding of EA_G^{rab} by human monocytes at 0°C using $^1/_{20}$ dilution of antibody. Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

TEMPERATURE °C	BINDING %
0	25 ± 2
23	34 ± 5
37	32.5 ± 8

TABLE 1

The effect of temperature on the binding of EA_G^{rab} to human monocytes. The results represent the mean of 3 experiments ± 1 S.E.M.

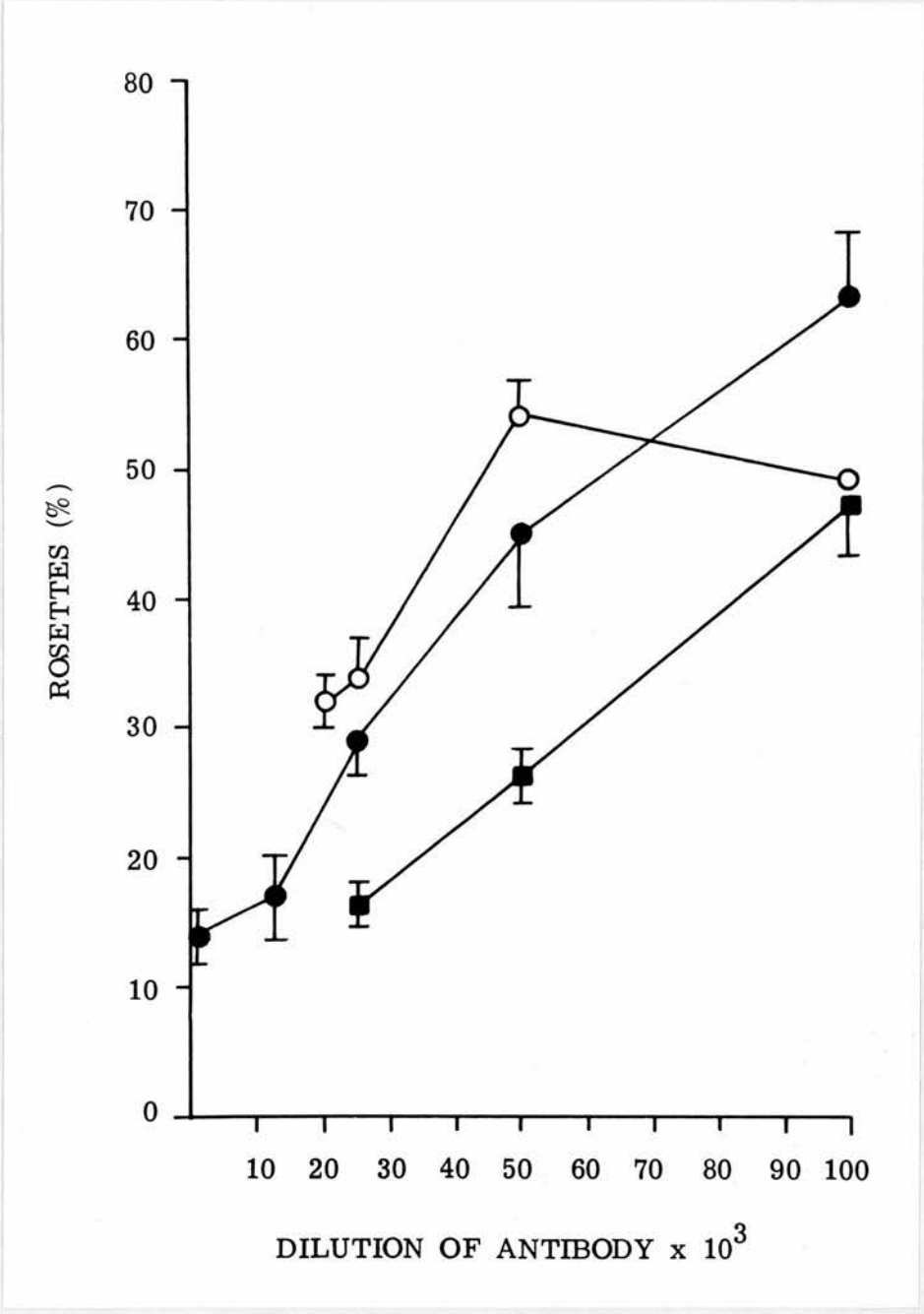


Fig. 4

The effect of increasing the amount of sensitising antibody (IgG) on the binding of EA_G^{rab} by guinea pig alveolar macrophages (●——●), mouse peritoneal macrophages (o——o) and human monocytes (■——■). Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

2.0 DETECTION OF RECEPTORS FOR IgM ON HUMAN MONOCYTES

Sheep red cells sensitised with a subagglutinating titre of rabbit anti-sheep IgM (EA_M^{rab}) were tested for their capacity to form rosettes with human monocytes. The experiments were performed at 37°C for 20 minutes and no rosette formation was observed (Fig. 5). However treatment of the monocytes with neuraminidase led to the formation of rosettes with EA_M^{rab}. These newly expressed receptors for EA_M^{rab} could be blocked by further incubating the monocytes with fluid phase IgM. In contrast Fc and complement receptors were relatively unaffected by neuraminidase action on monocyte membranes; a slight increase in Fc receptors and a slight decrease in complement receptors was observed (results not shown).

Fc receptors for IgM on human monocytes are therefore, not normally expressed and there is no possibility that rosettes formed with sheep red cells sensitised with IgM and coated with complement were due to IgM receptors.

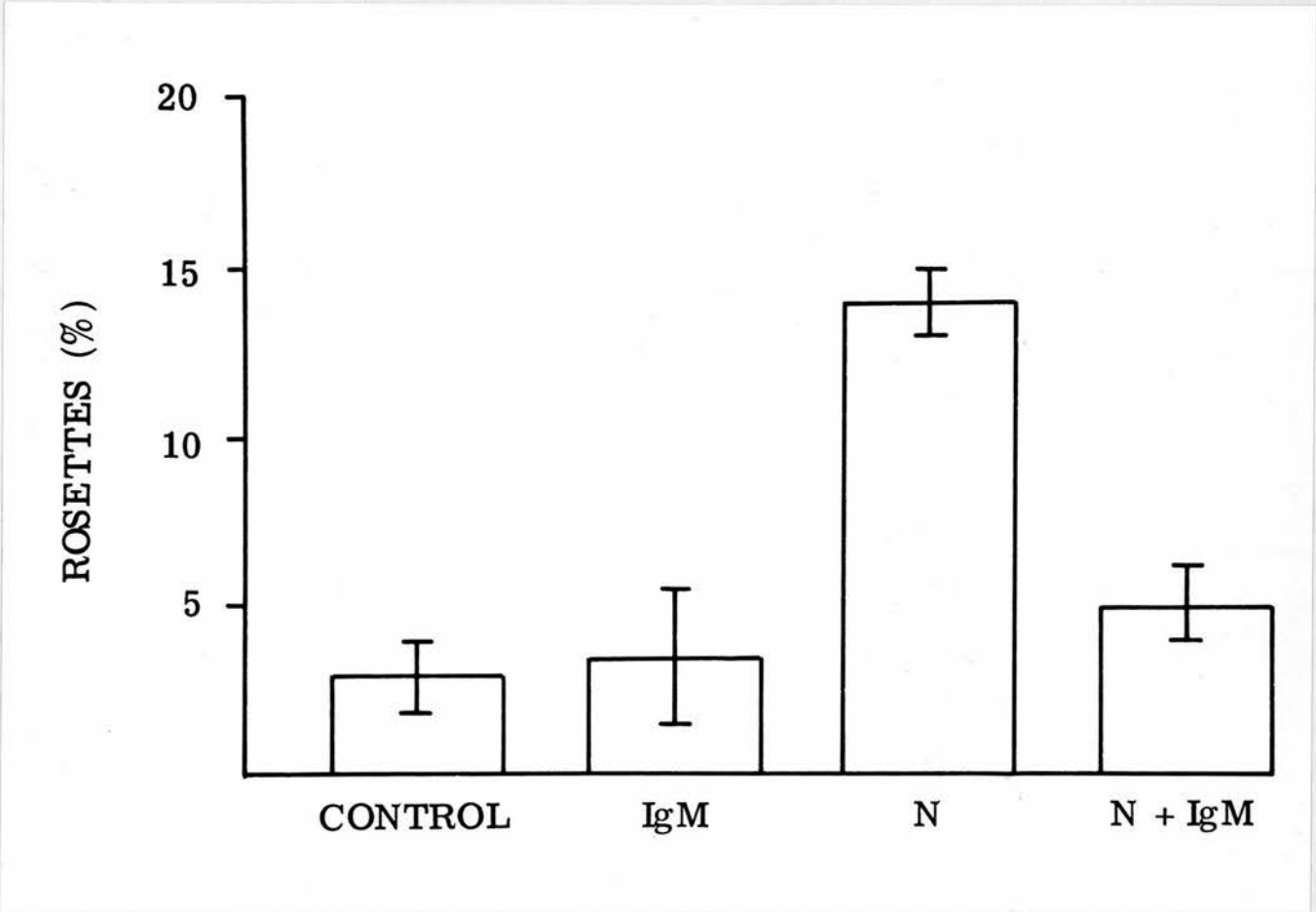


Fig. 5

The effect of neuraminidase on EA_M^{rab} rosette formation by human monocytes. (control) untreated, (N) neuraminidase, (IgM) fluid phase IgM, (N + IgM) neuraminidase and fluid phase IgM. Each column represents the mean $\pm$ 1 S.E.M. of three experiments.

3.0 SUMMARY

Human peripheral blood monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages formed rosettes with EA_G^{rab}. The number of rosetting cells was directly related to the amount of sensitising antibody and was time and temperature dependent. A comparison of the expression of Fc receptors on the three types of cells was made.

Human monocytes only formed rosettes with sheep red cells sensitised with IgM after treatment with neuraminidase.

SECTION III -

DETECTION OF RECEPTORS FOR VARIOUS HUMAN COMPLEMENT COMPONENTS ON HUMAN MONOCYTES, MOUSE PERITONEAL MACROPHAGES AND GUINEA PIG ALVEOLAR MACROPHAGES

1.0 DETERMINATION OF OPTIMAL EXPERIMENTAL CONDITIONS FOR DETECTING C3 RECEPTORS ON HUMAN MONOCYTES

1.1 Time course and temperature

Various different methods for visualising complement receptors were used but all involved sheep red cells sensitised with rabbit anti-sheep IgM (EA_M^{rab}). Most of the experiments which were carried out to detect complement receptors on human monocytes used EA_M^{rab} incubated with human AB serum which activates the classical pathway and coats the red cells with the third component of complement. Binding of these red cells (EAC) to human monocytes was time and temperature dependent (Fig. 6 and Table II). In contrast to Fc receptor expression, virtually no binding of EAC was found at 4°C. The number of monocytes rosetting at 23°C was about half that observed at 37°C (19% and 37% respectively). Fig. 6 shows that there was a time dependent increase in rosette formation at 37°C for the first 20 minutes and thereafter the percentage of rosettes remained relatively constant. Little or no phagocytosis of EAC was observed for the first 20 minutes but with longer incubation times, up to 25% of the monocytes had phagocytosed one or more sheep red cells.

In order to observe complement receptors in this type of assay the optimal conditions were 20 minutes at 37°C.

1.2 Effect of increasing the concentration of AB serum on human monocyte C3 receptors

A dose response curve was obtained by varying the dilution of AB serum used to coat the IgM sensitised erythrocytes (Fig. 7). At the lowest dilution used ($^1/200$) 65% of human monocytes had bound/...

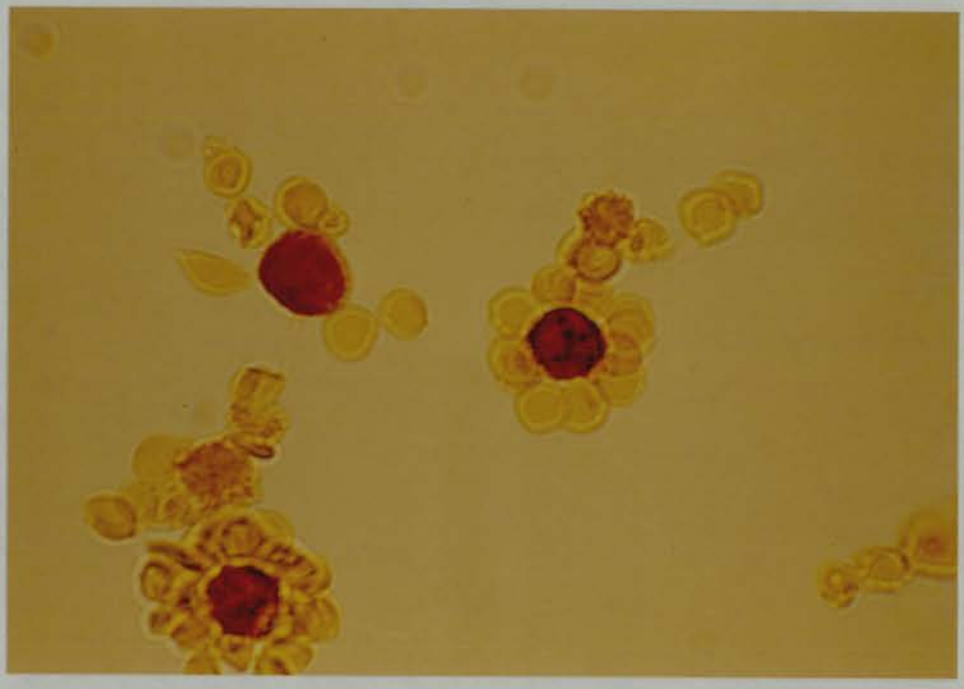


Plate I

Human peripheral blood monocyte rosette formation with EAC (prepared with AB serum as a source of complement). (Non-specific esterase, X 1000 magnification).

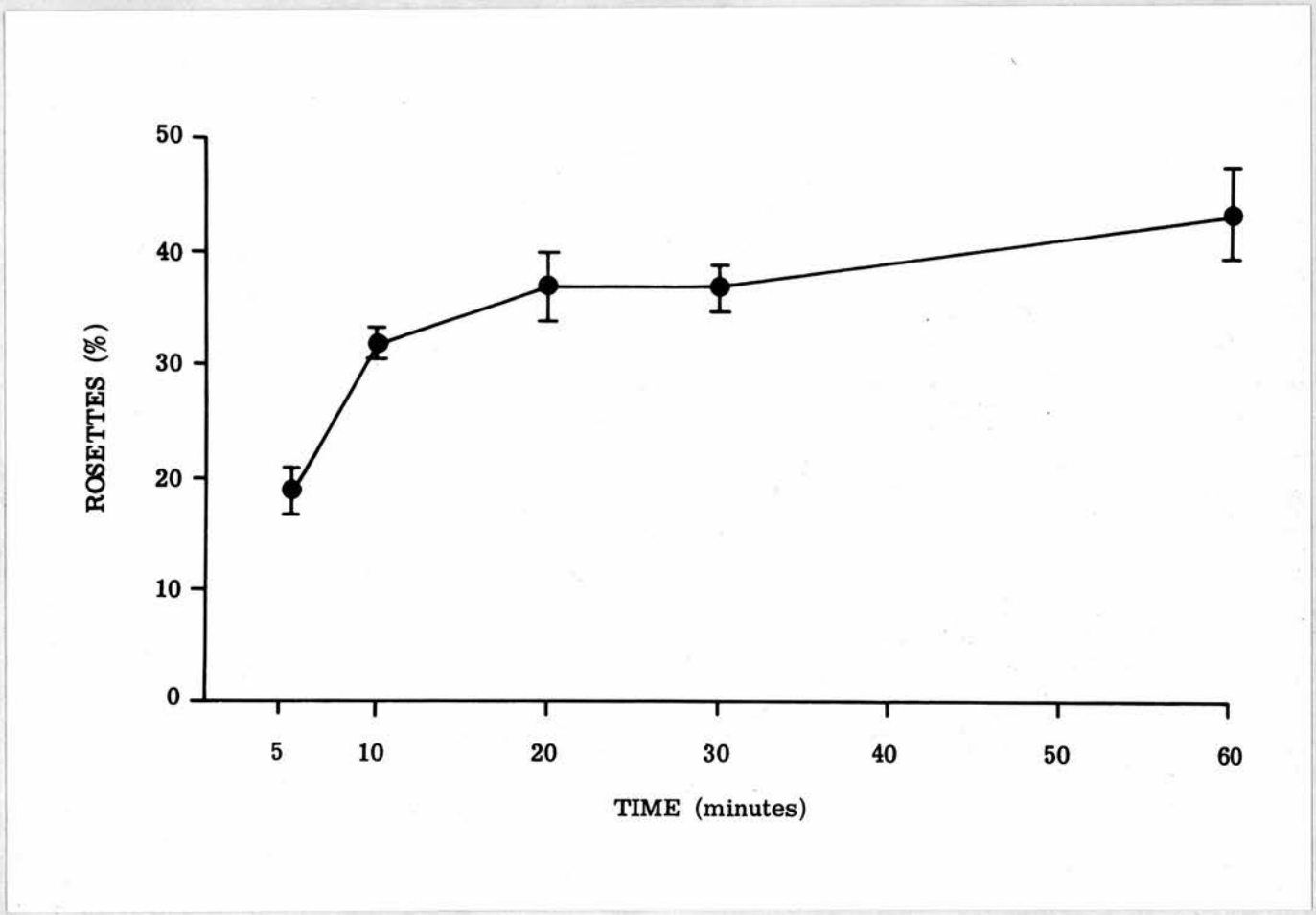


Fig. 6

The time course of EAC rosette formation by human monocytes at 37°C, using $^{1}/_{700}$ dilution of AB serum. Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

TEMPERATURE °C	BINDING %
0	3.5 ± 2
23	19 ± 1.5
37	37 ± 3

TABLE II

The effect of temperature on the binding of EAC to human monocytes. The results represent the mean of 3 experiments ± 1 S.E.M.

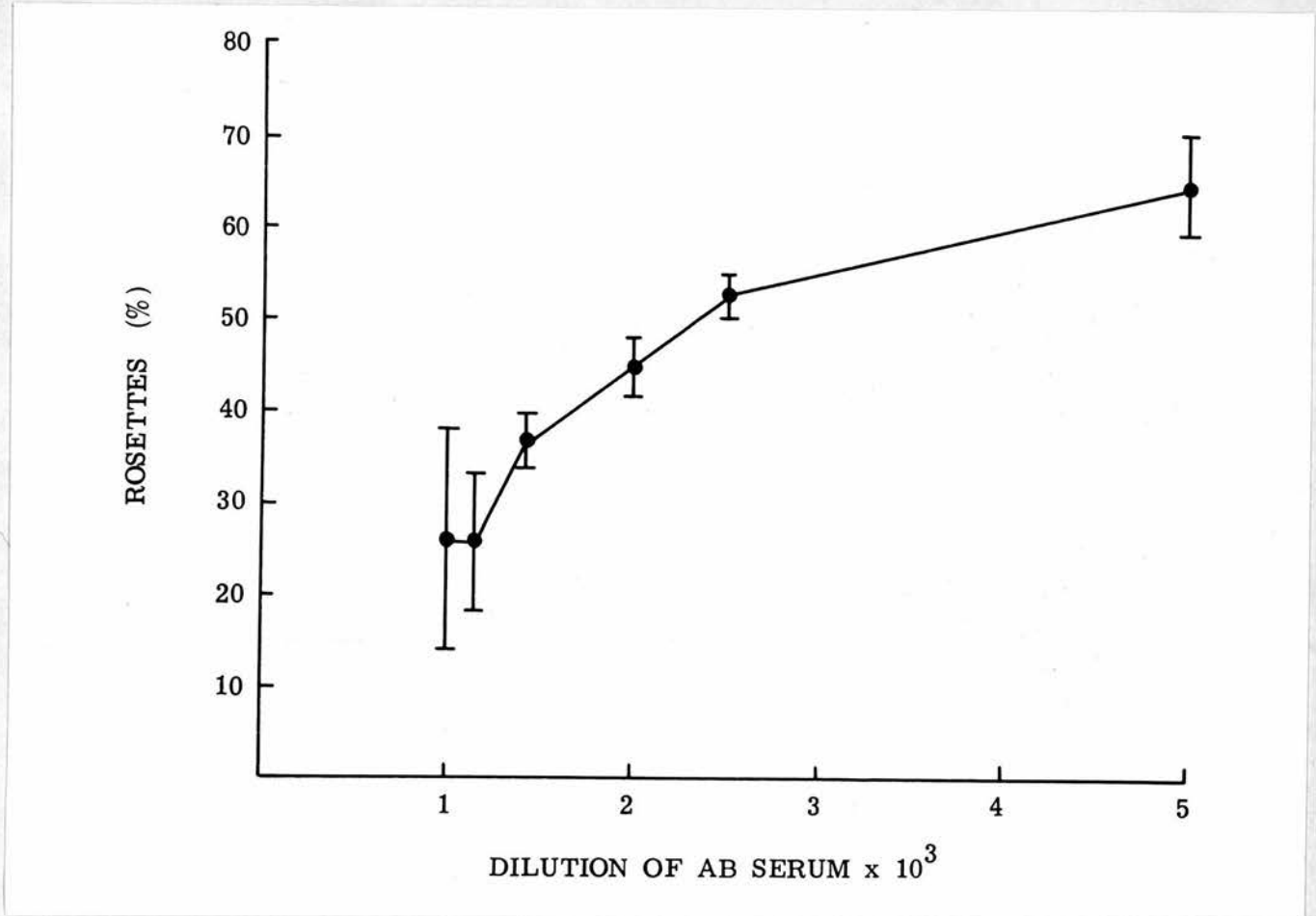


Fig. 7

The effect of increasing the concentration of AB serum on the binding of EAC by human monocytes. Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

bound 3 or more red cells. Lysis of the red cells meant that lower dilutions than $^{1}/_{200}$ could not be used. The percent rosettes observed was directly proportional to the dilution of AB serum.

1.3 Dependence of human monocyte complement receptor expression on the concentration of purified human C3b

Some experiments with human monocytes were carried out using purified human complement components to coat EA_M^{rab} rather than using AB serum. EA_M^{rab} coated with purified human C1 (EAC1) did not form rosettes with human monocytes at 37°C. When purified C4 was added to EAC1 to form the intermediate EAC14 no rosette formation was observed even when the amount of C4 was increased from 400 molecules C4/cell (the optimal number of C4 molecules required for the ultimate deposition of C3) to 6,000 molecules C4/cell. When C2 was added to EAC14 (400 molecules C4/cell) no rosettes were detected. Rosette formation with human monocytes occurred once C3 was added to EAC142 to produce EAC1423b. By varying the number of molecules of C3 added to EAC142 it can be seen in Figure 8 that there is a direct relationship between the percent rosetting monocytes and the amount of C3 present on the red cells. At the highest concentration of C3 used (2,000 molecules/cell) 63% of the monocytes formed rosettes.

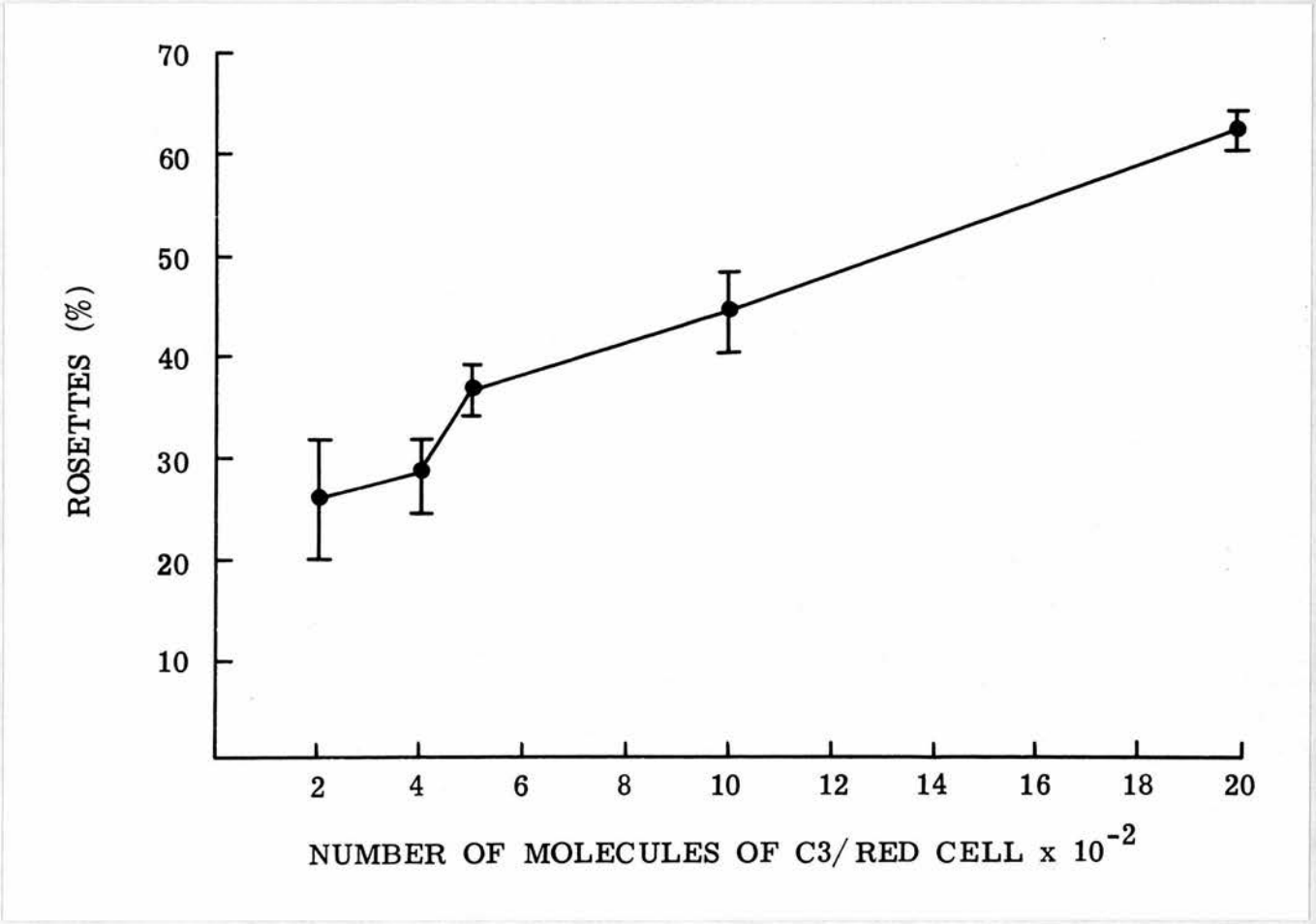


Fig. 8

The effect of increasing the number of C3 molecules on the binding of EAC1423b by human monocytes. Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

2.0 ROSETTE FORMATION BETWEEN HUMAN MONOCYTES AND EAC3b′

Red cells coated with C3b′ were prepared by either treating EAC1423b cells with purified human C3b inactivator (KAF) or with human AB serum chelated with 10mM EDTA (see materials and methods). These cells were no longer immune adherence positive in contrast to untreated EAC1423b or AB serum coated red cells (EAC).

Human monocytes formed rosettes with EAC1423b′ cells prepared by either of the above 2 methods (Fig. 9). When EAC1423b′ cells were prepared from EAC1423b using 1,000 molecules C3/cell, the percent monocyte rosettes was increased from 37% to 46% in the case of EDTA serum treated EAC1423b and 49% with KAF treated EAC1423b. The increase in monocyte rosettes using 500 molecules C3/cell was not so marked (33% to 38% using EDTA serum treated EAC1423b). However using either 500 or 1,000 molecules C3/cell, monocyte rosettes with C3b′ coated red cells had more erythrocytes per cell and appeared to be more tightly bound.

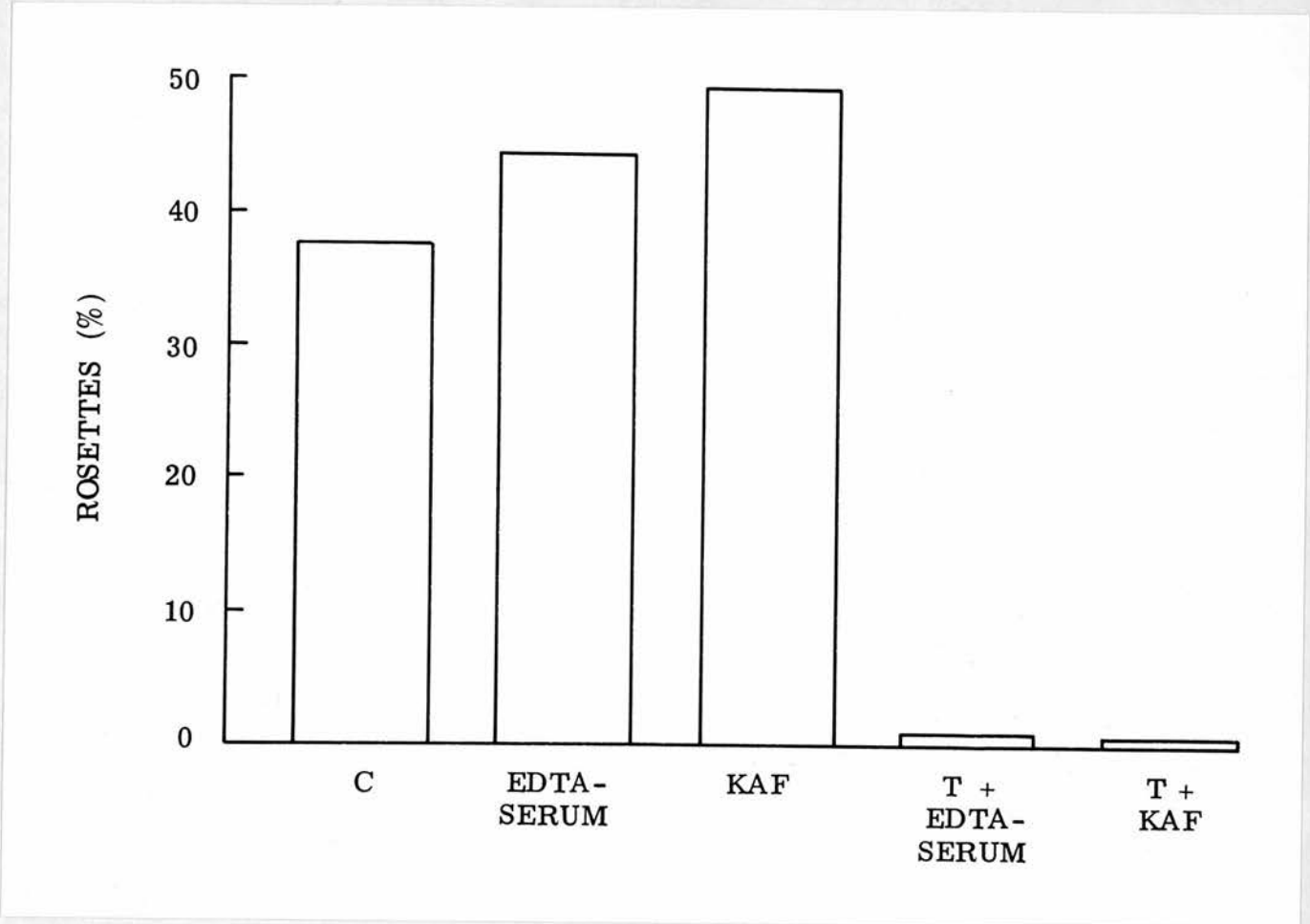


Fig. 9

The effects of C3b inactivator and trypsin on the rosetting of complement-coated sheep red cells (EAC1423b:1000 molecules C3 per red cell) by human monocytes. EAC1423b were incubated with purified C3b inavtivator with or without trypsin (T+KAF and KAF respectively) or were incubated with EDTA-chelated human AB serum (as a crude source of C3b inactivator) with or without trypsin (T+EDTA-serum and EDTA-serum respectively). The percentage rosetting by such treated EAC1423b by human monocytes was compared with untreated controls (C). The results are expressed as the mean of two experiments.

3.0 ROSETTE FORMATION BETWEEN HUMAN MONOCYTES AND EAC3d

EAC1423d cells were prepared by trypsinising EAC1423b' cells (either KAF or EDTA serum treated EAC1423b). Human monocytes did not form rosettes with these cells (Fig. 9).

4.0 DETECTION OF COMPLEMENT RECEPTORS ON MOUSE PERITONEAL MACROPHAGES AND GUINEA PIG ALVEOLAR MACROPHAGES

Mouse peritoneal macrophages and guinea pig alveolar macrophages did not form rosettes with EA_M^{rab} coated with AB serum even at $^1/200$ dilution of serum. However by using zymosan depleted serum (R3) (see materials and methods) which removes the terminal complement components C5 to C9, it was possible to increase the amount of C3 deposited on the red cells without causing lysis. As can be seen in Fig. 10 both mouse and guinea pig macrophages formed rosettes using this method albeit that considerably higher concentrations of R3 were required than for human monocytes ($^1/150$ dilution gave 26 $\pm$ 2% human monocyte rosettes). Guinea pig alveolar macrophages expressed considerably fewer C3 receptors than mouse peritoneal macrophages using this method. Only 17% of the guinea pig cells formed rosettes at $^1/2$ dilution of R3 whereas 55% of mouse peritoneal macrophages formed rosettes at $^1/10$ dilution of R3.

Some experiments were carried out using mouse peritoneal macrophage monolayers rather than using cell suspensions. In order to obtain between 20 to 30% rosettes a slightly lower dilution of R3 was used - $^1/35$ instead of $^1/50$.

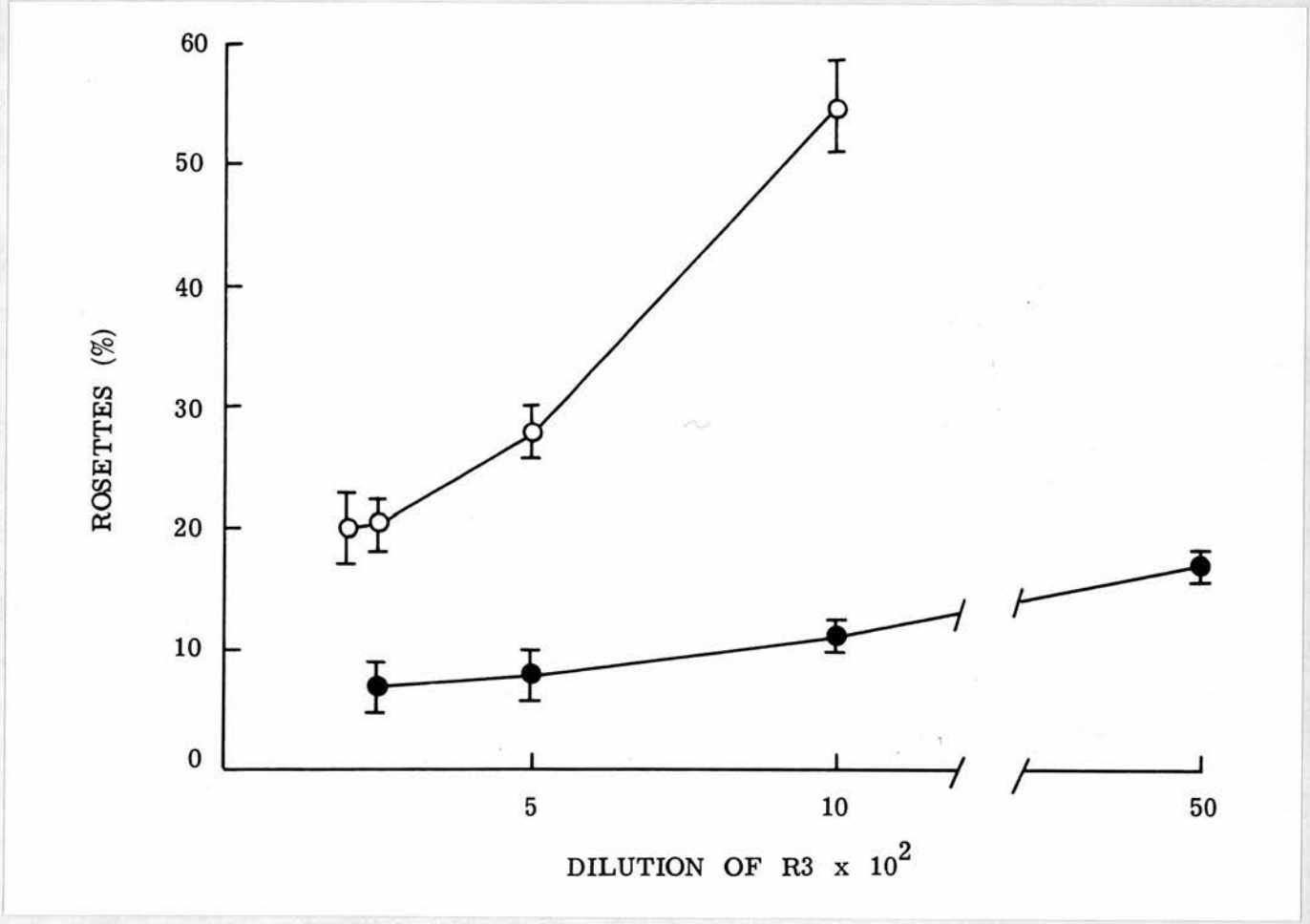


Fig. 10

The effect of increasing the concentration of zymosan-depleted AB serum (R3) on the binding of EAC by mouse peritoneal macrophages (o——o) and guinea pig alveolar macrophages (●——●). Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

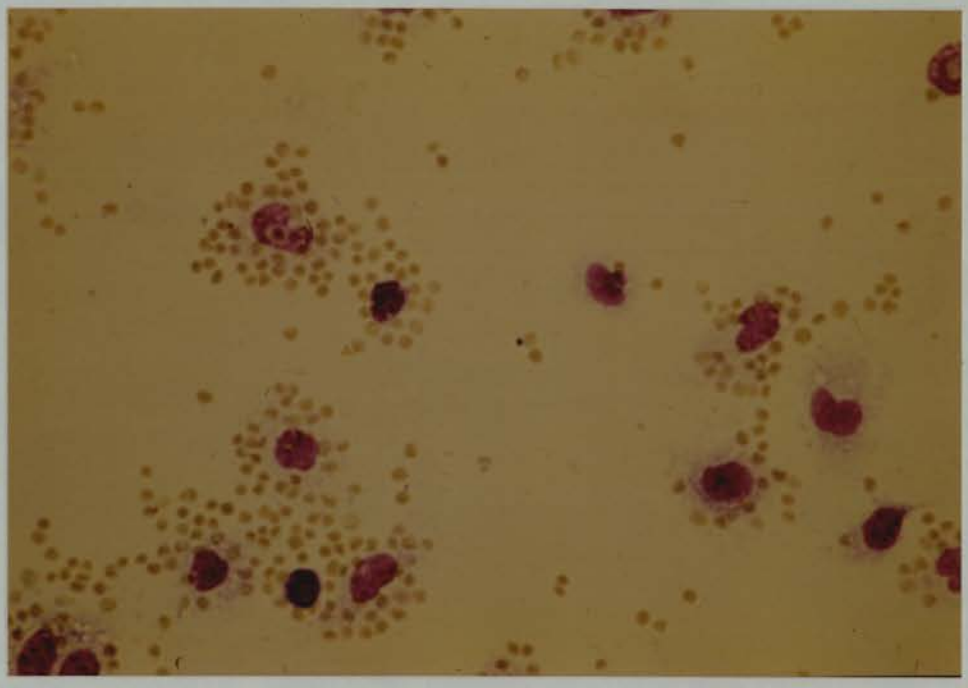


Plate II

Mouse peritoneal macrophage monolayer rosette formation with EAC(R3) (May Grunwald/Giemsa, X1000 magnification).

5.0 DETECTION OF COMPLEMENT RECEPTORS ON HUMAN NEUTROPHILS AND EOSINOPHILS

In a few experiments modulation of complement receptors on human peripheral blood monocytes, neutrophils and eosinophils was studied. Both neutrophils and eosinophils express fewer complement receptors than monocytes and therefore require more complement on the indicator red cells before rosette formation can take place. R3 was used to coat EA_M^{rab} in these experiments since $^1/200$ dilution of AB serum was insufficient to promote binding. For 23 $\pm$ 3% human neutrophils to bind 3 or more red cells a $^1/25$ dilution of R3 was required and for eosinophils a $^1/10$ dilution was required to give 26 $\pm$ 3% rosettes.

6.0 SUMMARY

The capacity of sheep red cells (E) coated with various purified human complement components or human AB serum to form rosettes has been studied with human peripheral blood monocytes, neutrophils and eosinophils, mouse peritoneal macrophages and guinea pig alveolar macrophages.

Untreated sheep red cells (E), E sensitised with IgM, EAC1, EAC14, EAC142 and EAC1423d did not form rosettes with human monocytes. EAC1423b, EAC1423b´ and EAC on the other hand, formed rosettes with human monocytes. The number of rosetting cells was directly related to the amount of complement coating the red cells and was time and temperature dependent.

Human peripheral blood neutrophils and eosinophils, mouse peritoneal macrophages and guinea pig alveolar macrophages appeared to express fewer receptors for human complement than human monocytes and required a higher concentration of complement deposited on the erythrocytes before they formed rosettes.

Together with the results from Section II it was concluded that human monocytes bear membrane receptors for human complement components C3b and C3b´, for Fc portion of IgG and have hidden Fc receptors for IgM. Guinea pig alveolar macrophages and mouse peritoneal macrophages have receptors for C3b and the Fc portion of IgG

SECTION IV -

DETECTION OF "LECTIN-LIKE" RECEPTORS ON HUMAN MONOCYTES, NEUTROPHILS AND EOSINOPHILS, MOUSE PERITONEAL MACROPHAGES AND GUINEA PIG ALVEOLAR MACROPHAGES

1.0 DETERMINATION OF OPTIMAL CONDITIONS FOR BINDING OF STAPH. ALBUS TO MOUSE PERITONEAL AND GUINEA PIG ALVEOLAR MACROPHAGES

Optimal conditions for Staph. albus binding to C3H mouse peritoneal macrophages had already been determined by H. Ogmundsdottir (Ph.D. thesis, 1979) when she showed that binding of bacteria to mouse peritoneal macrophage monolayers took place at 4°C and required 2 hours incubation for maximal binding.

Assuming that this would be true for other cell types dose response curves were carried out under these conditions as shown in Fig. 11. The number of macrophages binding organisms was directly related to the concentration of bacteria used although at concentrations greater than 10^8 bacteria ml^{-1} no further increase in binding was observed. Mouse peritoneal macrophages showed a higher level of binding than did guinea pig alveolar macrophages ($63 \pm 4\%$ and $36 \pm 5\%$ respectively at 10^9 Staph. albus ml^{-1}).

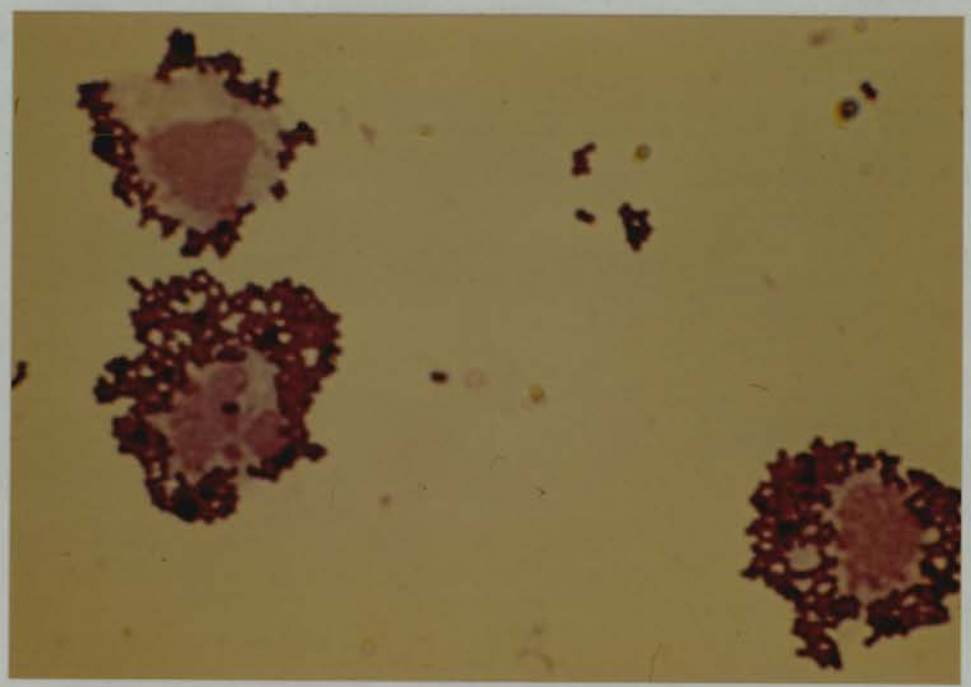


Plate III

Staph. albus binding to mouse peritoneal macrophage monolayer (May Grunwald/Giemsa, X2000 magnification).

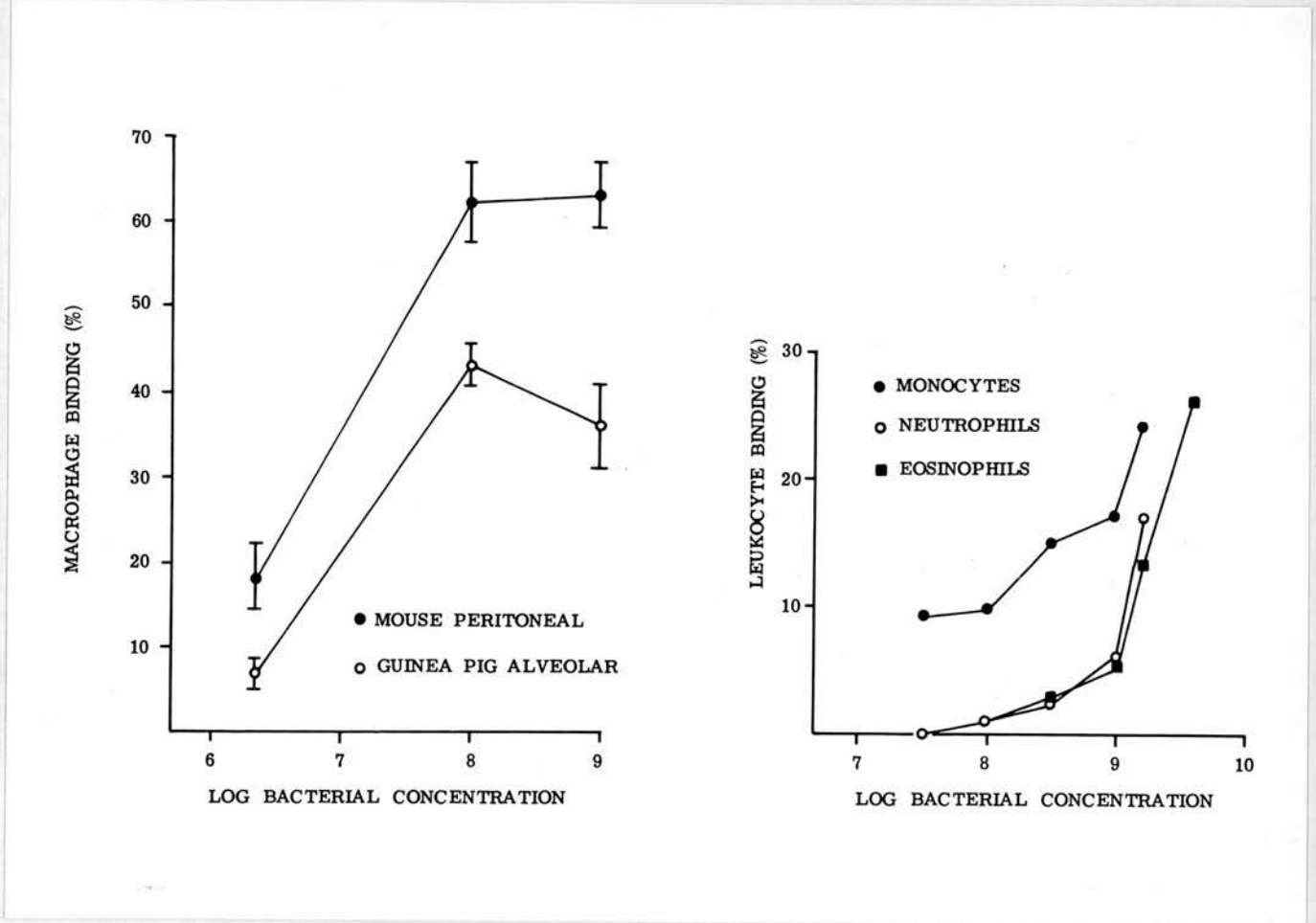


Fig. 11

The effect of increasing concentrations of Staph. albus on the binding by mouse peritoneal and guinea pig alveolar macrophages and human peripheral blood leukocytes. Each point represents the mean $\pm$ 1 S.E.M. of four experiments for mouse peritoneal and guinea pig alveolar macrophages and the mean of two experiments for human leukocytes.

2.0 DETERMINATION OF OPTIMAL CONDITIONS FOR BINDING OF STAPH.ALBUS TO HUMAN PERIPHERAL BLOOD MONOCYTES, NEUTROPHILS AND EOSINOPHILS

Staph. albus binding to human leukocyte monolayers was performed as described above at 4°C for 2 hours. As shown in Fig. 11 the number of leukocytes binding organisms was directly proportional to the concentration of Staph. albus. Human peripheral blood leukocytes showed a considerably lower degree of binding than that observed with mouse and guinea pig macrophages. A concentration of 2×10^9 organisms.ml^{-1} was required before 22% monocytes or 17% neutrophils bound 2 or more organisms and eosinophils required 6×10^9 bacteria.ml^{-1} before 23% cells showed positive binding. Above 10^{10} organisms.ml^{-1} the presence of bacteria in the background made it difficult to estimate binding and therefore detection of leukocytes binding bacteria at these concentrations was not possible.

3.0 INHIBITION OF STAPH. ALBUS BINDING TO MOUSE PERITONEAL MACROPHAGES AND GUINEA PIG ALVEOLAR MACROPHAGES BY D-GLUCOSE AND D-GALACTOSE

Inhibition of binding of Staph. albus to C3H mouse peritoneal macrophages by D-glucose and D-galactose had previously been described by Freimer et al. (1978). Similar inhibition studies were carried out with CF1 mouse peritoneal macrophages and guinea pig alveolar macrophages.

Staph. albus were used at a concentration which gave 20 to 30% binding.

Monolayers were pre-exposed to a range of concentrations of D-glucose and D-galactose (5-20 mM) prior to overlaying the Staph. albus as described in the materials and methods. D-galactose and D-glucose reduced the binding of Staph. albus to both guinea pig alveolar and mouse peritoneal macrophages at 10 and 20 mM concentrations (Fig. 12). Inhibition was directly related to sugar concentration except that 10 mM galactose inhibited bacterial binding to guinea pig alveolar macrophages to a greater extent than did 20 mM galactose ($38 \pm 5\%$ and $24 \pm 19\%$ respectively). With both cell types 20 mM glucose was more inhibitory than 20 mM galactose and binding to mouse macrophages was reduced to a greater extent by 10 mM glucose than by 10 mM galactose ($30 \pm 5\%$ and $23 \pm 9\%$ respectively). ($p = N.S.$)

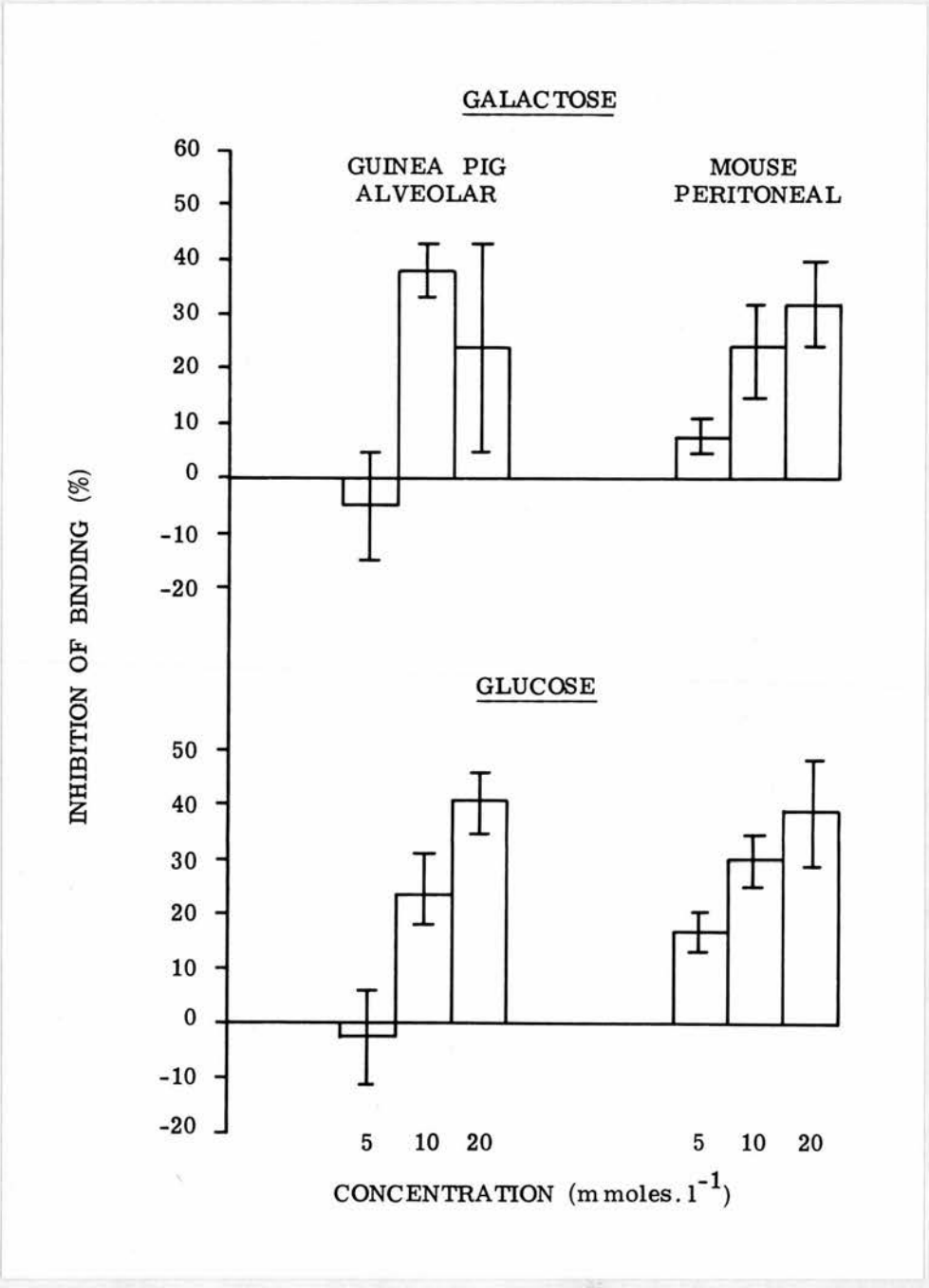


Fig. 12

The effect of D-glucose and D-galactose on Staph.albus binding to mouse peritoneal and guinea pig alveolar macrophages. The percentage inhibition of binding is calculated as the difference between control and test divided by the control X 100. Each column represents the mean $\pm$ 1 S.E.M. of six experiments for mouse macrophages and four experiments for guinea pig macrophages.

4.0 INHIBITION OF STAPH. ALBUS BINDING TO HUMAN PERIPHERAL BLOOD MONOCYTES, NEUTROPHILS AND EOSINOPHILS

The effects of D-glucose and D-galactose on the binding of Staph. albus to human leukocyte monolayers was investigated as described above for mouse and guinea pig macrophages. Staph.albus was used at a concentration of 2×10^9 organisms.ml^{-1} for monocytes and neutrophils which gave mean control values of 22% and 17% respectively; for eosinophils a concentration of 6×10^9 bacteria.ml^{-1} was used which gave a mean control value of 23%. As shown in Fig. 13 D-galactose reduced the binding of Staph. albus to all 3 leukocyte types at 10 and 20 mM concentrations, with 10 mM galactose being slightly more inhibitory especially with regard to the monocytes - 10 mM galactose inhibited binding by $43.5 \pm 2\%$ compared to 20mM galactose which caused $26 \pm 7\%$ inhibition. (p = N.S.)

In contrast to D-galactose, D-glucose inhibited binding of Staph. albus to monocytes and eosinophils but not to neutrophils at any of the concentrations tested. Binding to monocytes and eosinophils was reduced by 10 and 20 mM glucose.

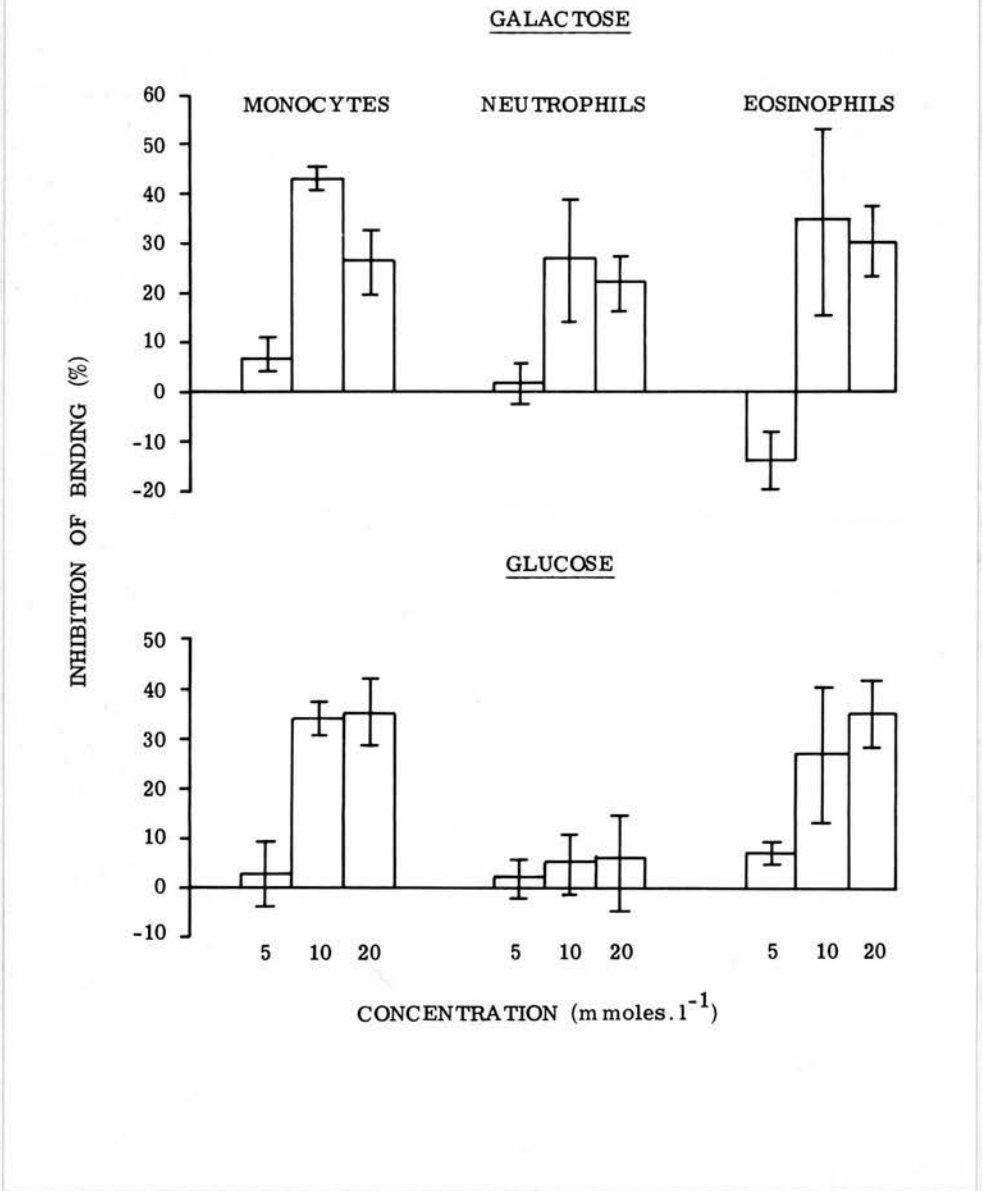


Fig. 13

The effect of D-glucose and D-galactose on Staph. albus binding to human peripheral blood leukocytes. The percentage inhibition of binding is calculated as in Fig. 12. Each column represents the mean $\pm$ 1 S.E.M. of five experiments.

5.0 SUMMARY

Staph. albus bound in a dose dependent fashion to CF1 mouse peritoneal macrophages, guinea pig alveolar macrophages, and human peripheral blood monocytes, neutrophils and eosinophils. Mouse macrophages showed the highest degree of binding and guinea pig macrophages had a slightly lower level of binding. However even at the highest possible concentrations under the assay conditions human peripheral blood leukocytes did not express more than 23% positive binding compared to the highest values observed for mouse and guinea pig macrophages (63% and 43% respectively).

D-glucose and D-galactose inhibited Staph. albus binding to all the cell types investigated except for human neutrophils which were only inhibited by D-galactose.

SECTION V -

EFFECTS OF CHEMOATTRACTANTS AND OTHER PHARMACOLOGICAL MEDIATORS ON MONONUCLEAR PHAGOCYTE RECEPTORS

1.0 INTRODUCTION

Co-workers had previously reported that complement receptors on human eosinophils were enhanced by a number of eosinophil chemotactic factors (including the ECF-A tetrapeptides, histamine and imidazole-acetic acid) in a dose and time dependent fashion (Anwar and Kay, 1977b and 1978). The phenomenon of complement receptor enhancement may be an important feature common to all phagocytic cells. Therefore it seemed important to determine whether other human peripheral blood leukocytes such as monocytes and other phagocytic cells from different species and environments such as mouse peritoneal macrophages and guinea pig alveolar macrophages underwent similar changes. In the earlier sections of this thesis the monocyte cell membrane was shown to bear receptors for C3b and C3b', the Fc portion of IgG and IgM and to have lectin-like receptors. Accordingly these receptors were investigated to determine whether their expression could be altered and thus gain further insight into phagocytic membrane changes in response to their environment and the possible consequences of such changes. Studies were undertaken to examine the effects of substances known to promote their migration *in vitro* including the chemotactic factors casein, formyl methionyl peptides and lymphokines from PHA-stimulated human lymphocytes, on the expression of receptors on mononuclear phagocytes. The exact mechanism by which cells respond to chemoattractants which cause random or directed migration is not clear although participation of "recognition units" on the cell membrane has been suggested particularly with regard to the formyl methionyl peptides (Aswanikumar *et al.*, 1977; Williams *et al.*, 1977; Snyderman and Fudman, 1980) and C5a (Chenoweth and Hugli, 1978).

The/...

The relationship between Fc, complement and lectin-like receptors and those proposed for chemotactic factors is unknown at present.

Membrane responses to pharmacological mediators (including prostaglandins, bradykinin, serotonin and histamine) which have been shown to play important roles in host defence mechanisms and in inflammatory diseases, were also examined.

2.0 EFFECTS OF CHEMOATTRACTANTS ON HUMAN MONOCYTE COMPLEMENT AND Fc RECEPTORS

2.1 Effect of increasing concentrations of casein

In these experiments the monocyte suspensions were incubated for 30 minutes at 37°C with increasing concentrations of casein at 1, 2 and 3 $mg.ml^{-1}$ and the cells were then added to indicator sheep red cells to test their rosette forming capacity (see materials and methods).

Casein enhanced the percentage of monocyte C3b rosettes in a dose dependent fashion (Fig. 14) irrespective of whether EAC or EAC1423b were used as indicator cells. In contrast there was a small but insignificant increase in the percentage of EA_G^{rab} (Fc) rosettes. With the highest dose of casein the mean values for the enhancement of Fc receptors was 13% whereas with EAC and EAC1423b the increase was 92% and 100% respectively. Increasing the concentration of casein to 4, 5 or 6 $mg.ml^{-1}$ had no effect on the expression of Fc receptors (results not shown).

2.2 Time course of monocyte complement receptor enhancement

Enhancement of complement receptors by casein at a concentration of 3 $mg\ ml^{-1}$ was dependent on the time of incubation. Maximal effects were observed at about 30 minutes after which there was no further increase (Fig. 15). Similar results were obtained irrespective of whether EAC or EAC1423b were used as indicator red cells. A small but insignificant increase in Fc receptors was also observed in this time course study and was maximal after approximately 60 minutes. By 120 minutes this small increase had returned to the original value./...

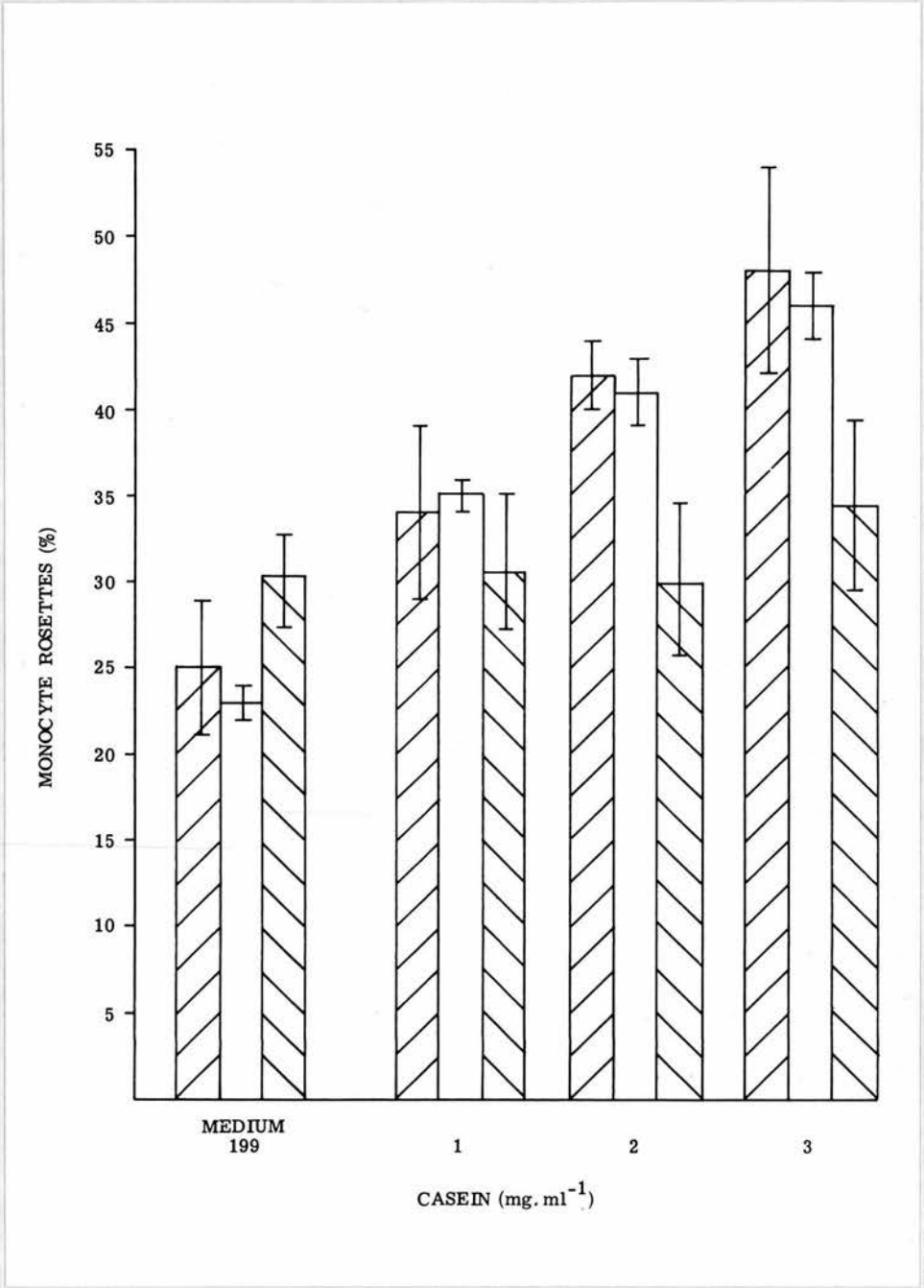


Fig. 14

The effect of casein on monocyte C3b and IgG rosettes ▨ EAC1423b, □ EAC, ▧ EA_G. Each column represents the mean ± 1 S.E.M. of three experiments.

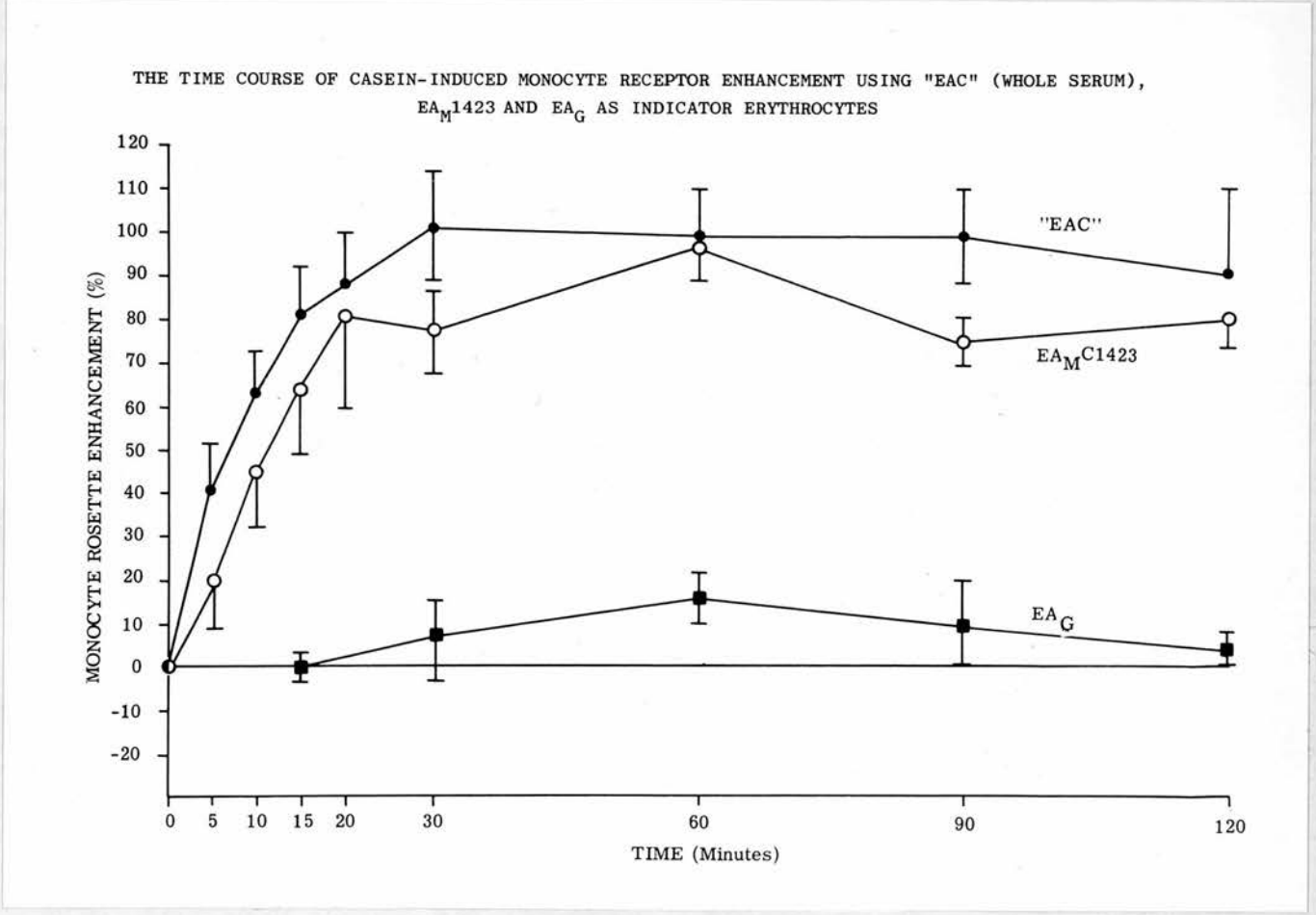


Fig. 15

The time course of the percentage enhancement of monocyte rosettes by casein using sensitised erythrocytes coated with complement or IgG. (●——●) EAC, (○——○) EAC1423b, (■——■) EA$_G$. Each point represents the mean ± 1 S.E.M. of three experiments. The inidcator red cells were EAC at $^1/_{700}$ dilution of AB serum, EAC1423b with 400 molecules C3 per red cell and EA$_G^{rab}$ at $^1/_{20}$ dilution of IgG.

value. Extending the length of incubation with casein up to 180 minutes, 240 minutes or overnight had no further effect on Fc receptors.

As the dose response and time-course studies were essentially similar using EAC or EAC1423b, EAC were used as indicator cells in further experiments on the modulation of C3b receptors on human monocytes.

The effect of increasing doses of casein on the rate and degree of monocyte complement receptor enhancement is shown in Fig. 16. The degree of enhancement and the rate at which enhancement occured was dependent on the concentration of casein. Increasing the concentration of casein from 1 $mg.ml^{-1}$ to 2 $mg.ml^{-1}$ increased the rate of monocyte rosette enhancement three-fold. Further increase in the concentration of casein up to 3 $mg.ml^{-1}$ had no apparent effect on the rate of complement receptor enhancement.

2.3 Effect of formyl methionyl peptides on monocyte complement receptor expression

A number of formyl methionyl peptides were also tested for their effects on monocyte complement receptors (Fig. 17). With f-Met-Leu-Phe activity was observed at 10^{-7} and 10^{-8} $mol.l^{-1}$ with little enhancement at 10^{-9} $mol.l^{-1}$. F-Met-Met-Phe and f-Met-Phe were less active than f-Met-Leu-Phe but both gave enhancement at 10^{-5} and 10^{-6} $mol.l^{-1}$ but not 10^{-7} $mol.l^{-1}$. The unformylated peptides Met-Leu-Phe and Met-Met-Phe were virtually inactive.

2.4 Time course of complement receptor enhancement by f-Met-Leu-Phe

Enhancement of monocyte complement receptors by f-Met-Leu-Phe was/...

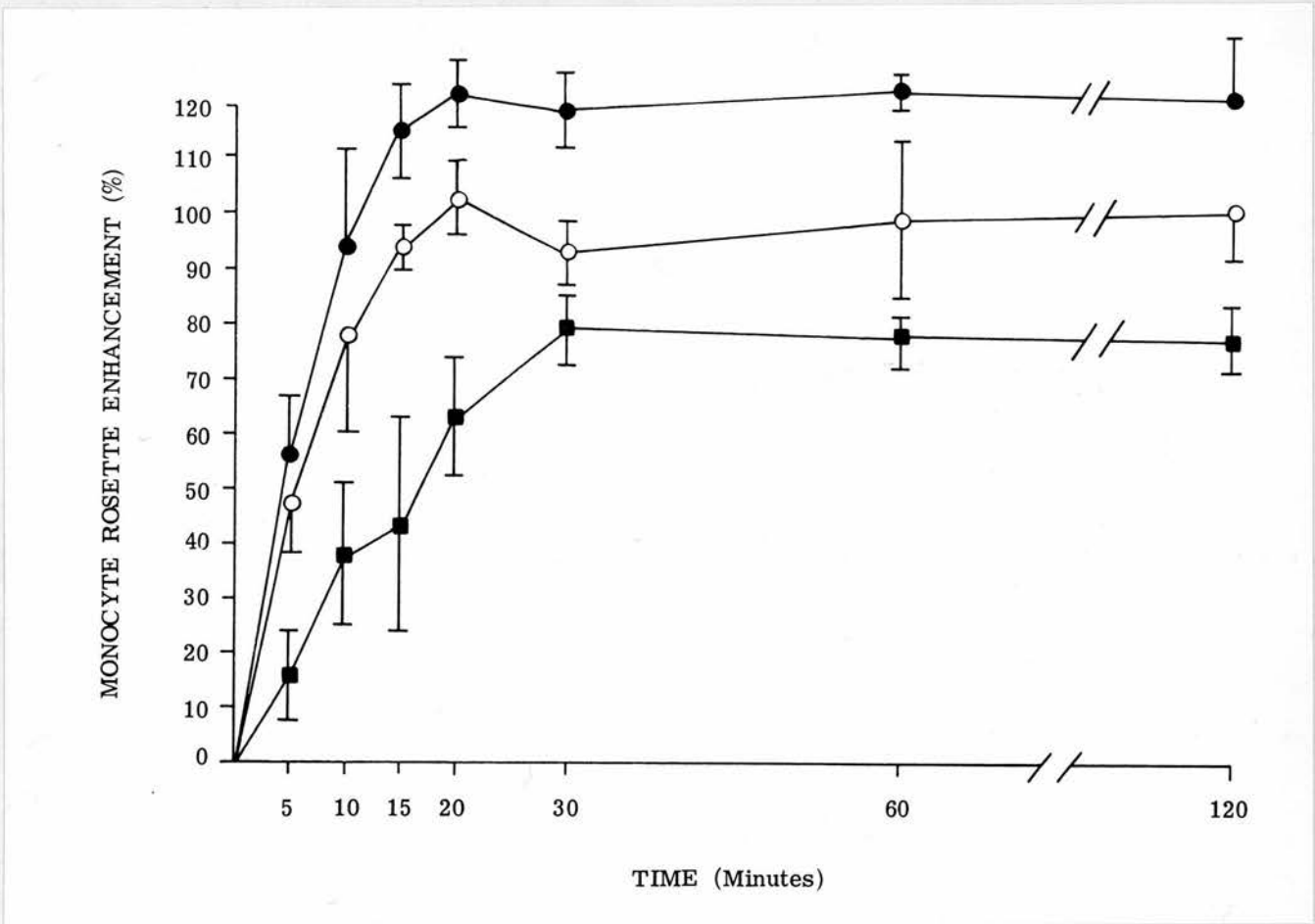


Fig. 16

The time course of monocyte complement receptor enhancement by increasing doses of casein. (●——●) 3mg.ml^{-1} casein, (○——○) 2mg.ml^{-1} casein, (■——■) 1mg.ml^{-1} casein. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. The indicator red cells were EAC (prepared with whole serum as a source of complement) at $^{1}/_{700}$ dilution of AB serum.

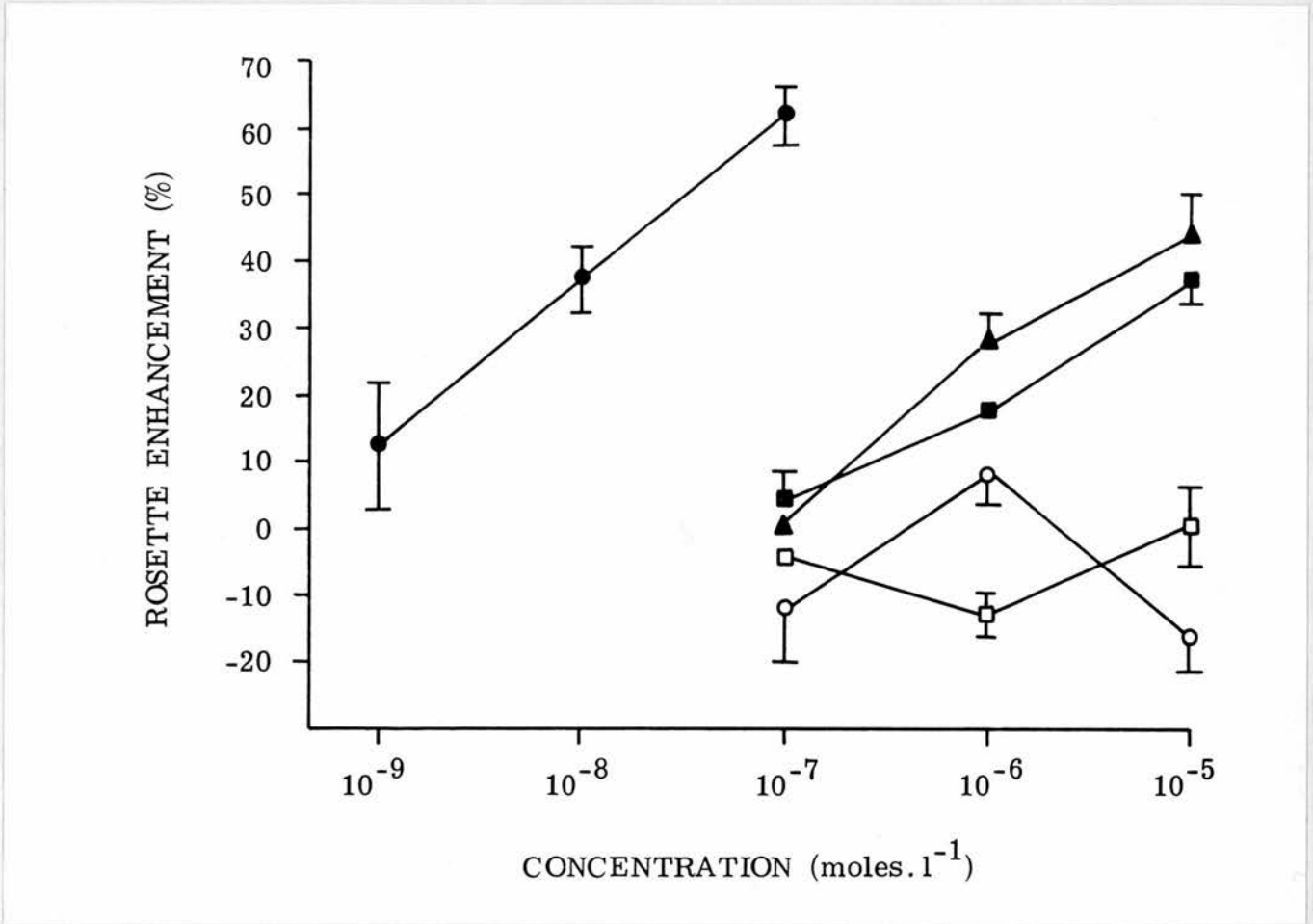


Fig. 17

The effect of formyl-methionyl peptides on the expression of monocyte complement receptors. (●——●) F-Met-Leu-Phe, (○——○) Met-Leu-Phe, (■——■) F-Met-Met-Phe, (□——□) Met-Met-Phe, (▲——▲) F-Met-Phe. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. The indicator red cells were EAC as in Fig. 16.

was also dependent on the time of incubation. Fig. 18 compares the effects of f-Met-Leu-Phe on human peripheral blood monocytes, neutrophils and eosinophils. The time course for monocytes is similar to that shown with casein (Fig. 15) with maximal effects observed by 30 minutes, after which there was no further increase. The degree and rate of complement receptor enhancement was greatest with neutrophils and least with eosinophils.

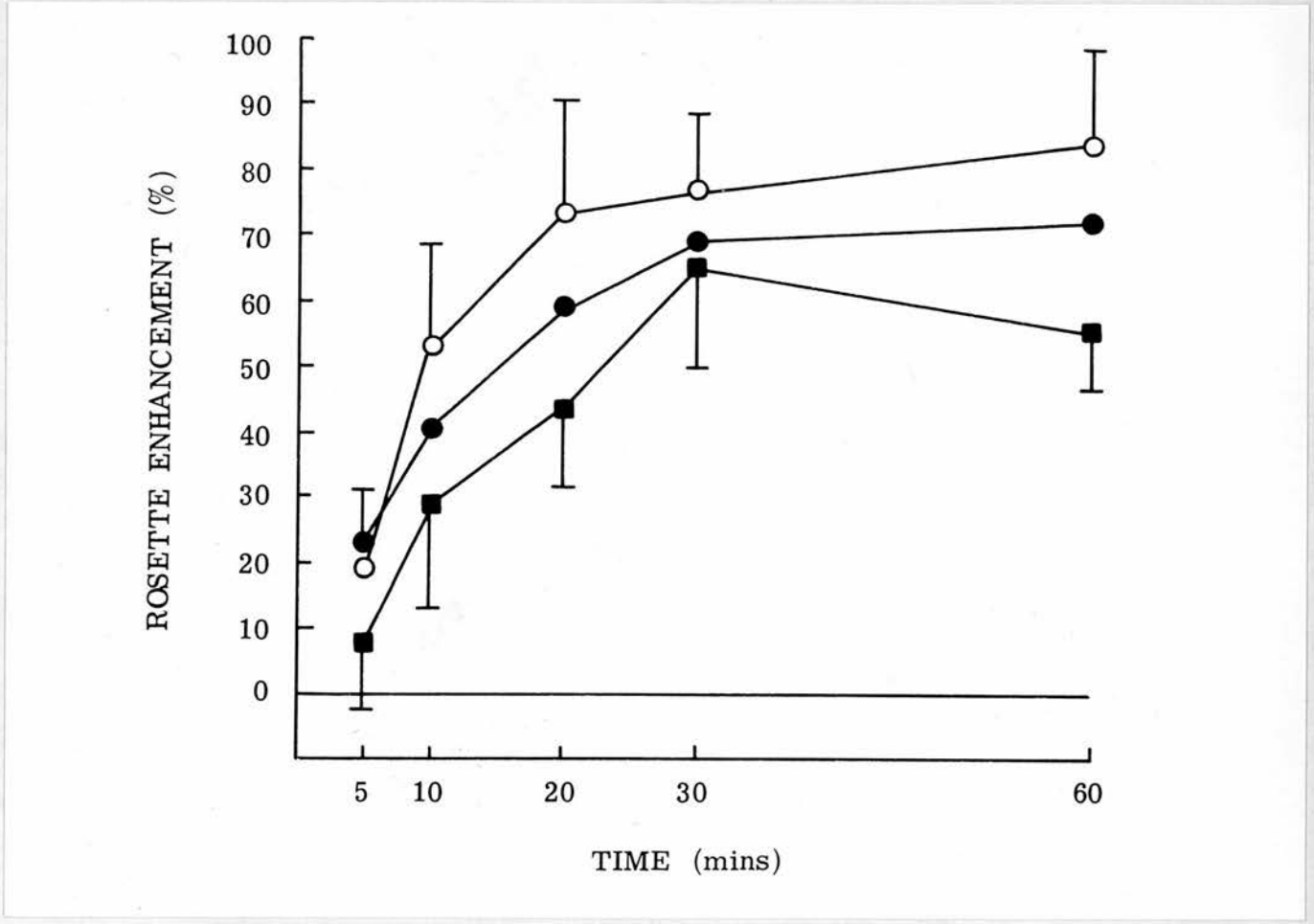


Fig. 18

The time course of complement receptor enhancement by F-Met-Leu-Phe at 10^{-7} moles.l^{-1} for human monocytes (●——●) and eosinophils (■——■) and 10^{-8} moles.l^{-1} for human neutrophils (o——o). Each point represents the mean $\pm$ 1 S.E.M. of four experiments. The indicator cells were EAC(R3) at $^{1}/150$ dilution for monocytes, $^{1}/10$ dilution for eosinophils and $^{1}/25$ dilution for neutrophils, of R3.

3.0 EFFECT OF OTHER CHEMOATTRACTANTS ON HUMAN MONOCYTE COMPLEMENT RECEPTORS

Other recognised monocyte chemoattractants were tested for their capacity to enhance monocyte complement receptors. Supernatants from lymphocytes stimulated with PHA (see materials and methods) increased the numbers of monocyte rosettes in a dose-dependent fashion (Fig. 19). In contrast, unstimulated lymphocyte supernatants had significantly less effect when tested at comparable dilutions. At $^{1}/_{8}$ dilutions the stimulated lymphocyte supernatant enhanced monocyte rosettes by $84 \pm 5\%$ compared to the unstimulated supernatant which only enhanced rosettes by $39 \pm 7\%$ at the same dilution. ($p < 0.05$)

A chemoattractant elaborated from C. parvum 10390 also increased the expression of complement receptors on human monocytes in a dose-dependent fashion (Fig. 19). At a $^{1}/_{4}$ dilution of this factor there was a $95 \pm 10\%$ increase in monocyte complement rosettes. ($p < 0.05$)

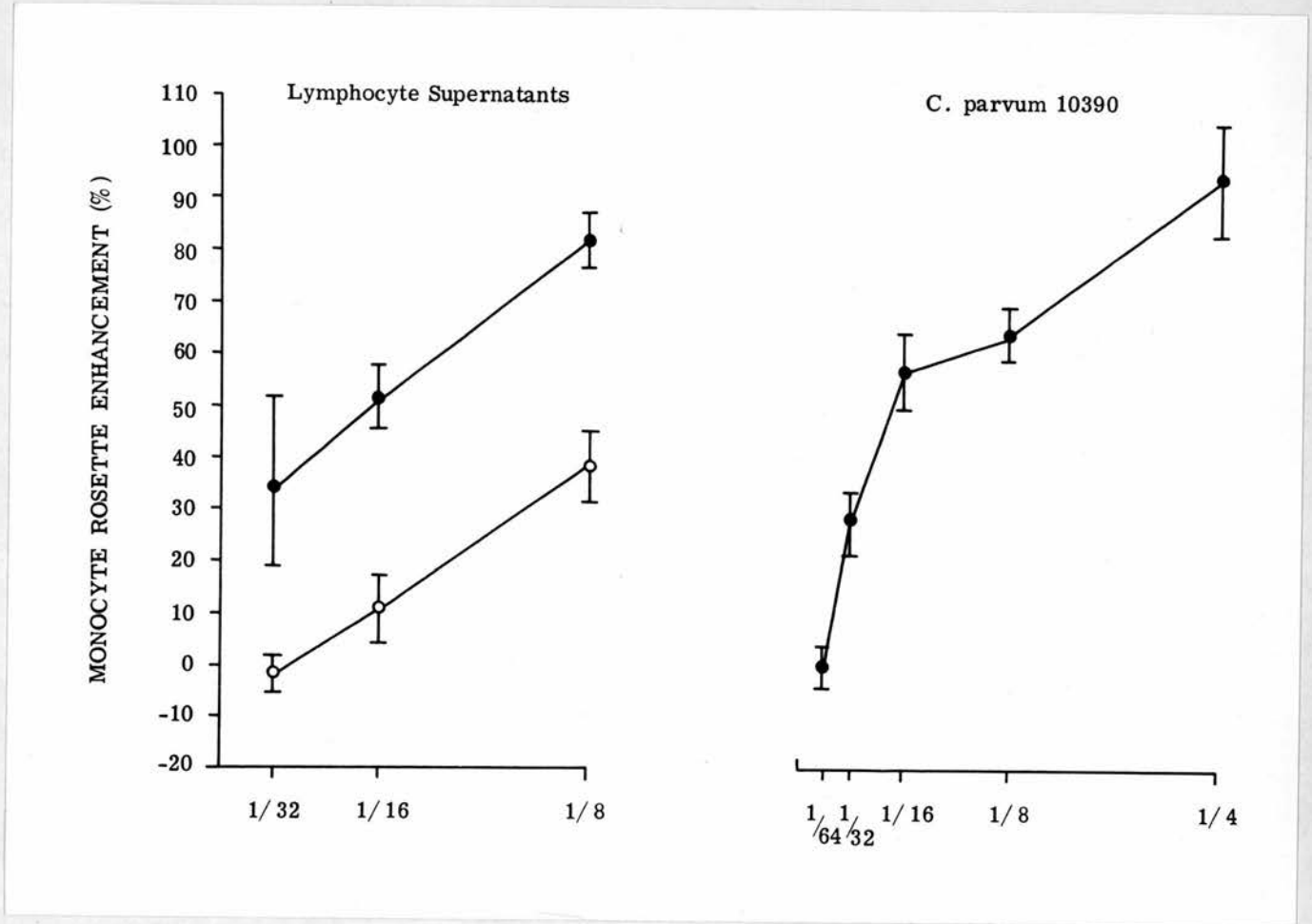


Fig. 19

The effect of stimulated (●——●) and unstimulated (o——o) lymphocyte supernatants, and C. parvum 10390 supernatant on the expression of monocyte complement receptors. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. The indicator red cells were EAC as in Fig. 16.

4.0 EFFECT OF A CHEMOKINETIC AGENT, HUMAN SERUM ALBUMIN, ON MONOCYTE COMPLEMENT RECEPTOR EXPRESSION

Human serum albumin which is known to be chemokinetic but not chemotactic was tested for complement receptor enhancement by human monocytes (Fig. 20). At doses which are chemokinetic (0.5 to 3.0 $mg.ml^{-1}$), there was a dose-dependent increase in complement receptor expression except at the highest dose used which gave less enhancement than the second highest dose (2 $mg.ml^{-1}$).

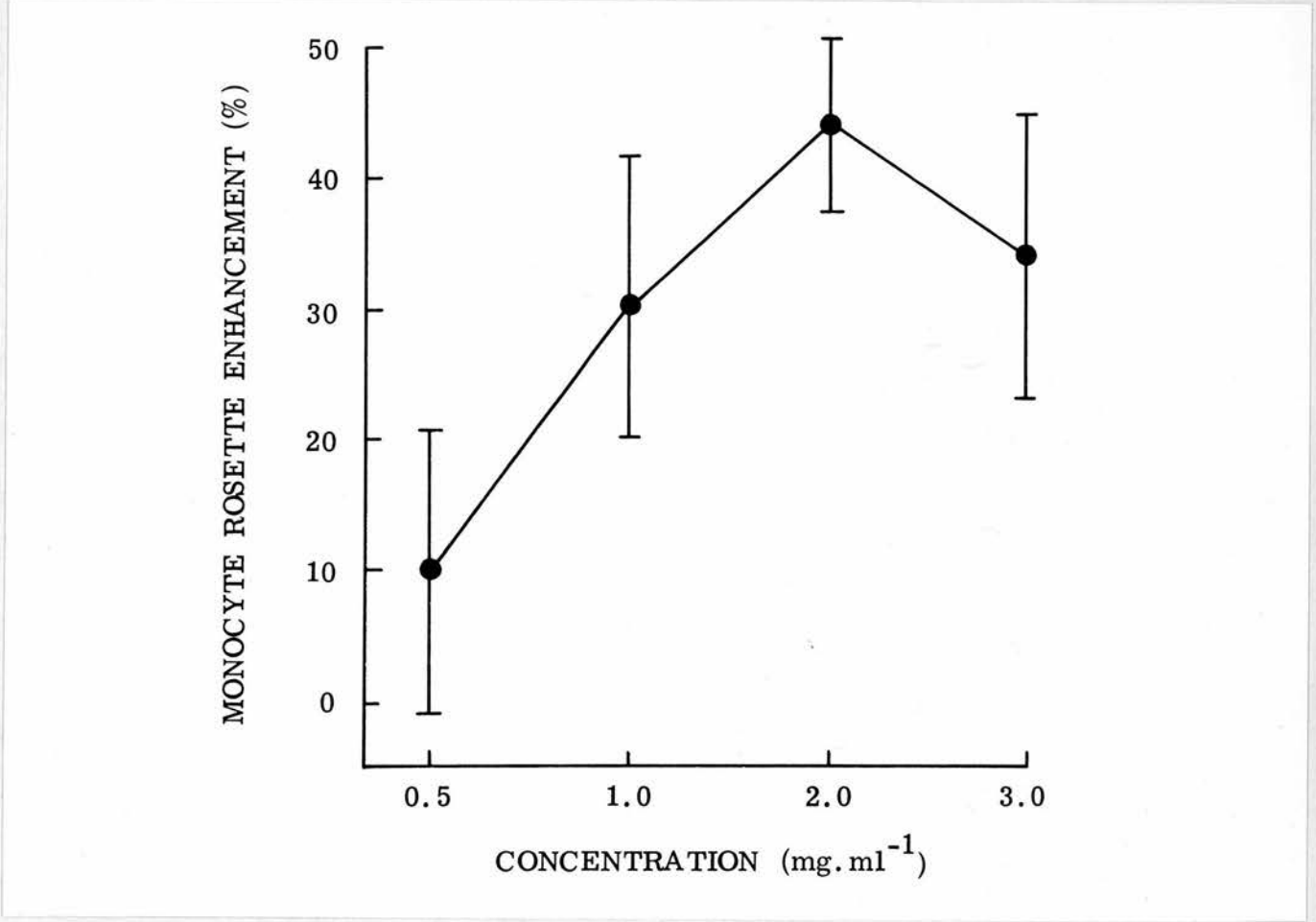


Fig. 20

The effect of human serum albumin on the expression of monocyte complement receptors. Each point represents the mean $\pm$ 1 S.E.M. of five experiments. The indicator red cells were as in Fig. 16.

5.0 EFFECT OF CHEMOATTRACTANTS ON MOUSE PERITONEAL AND GUINEA PIG ALVEOLAR MACROPHAGES

Complement receptors on mouse peritoneal and guinea pig alveolar macrophages were increased following incubation with the chemoattractants f-Met-Leu-Phe and casein (Figs. 21, 22 and 23) for 30 minutes at 37°C. F-Met-Leu-Phe enhanced the percentage of mouse macrophage C3b rosettes in a dose dependent fashion from 10^{-9} moles.l^{-1} to 10^{-11} moles.l^{-1} (Fig. 21). Higher doses of f-Met-Leu-Phe had less enhancing effects. Peak activity, at 10^{-9} moles.l^{-1} f-Met-Leu-Phe, gave a mean value for enhancement of 81 $\pm$ 10%. Complement receptors on mouse macrophages were also increased following incubation with casein at a concentration of 2 and 3 mg.ml^{-1} (Fig. 22). In contrast f-Met-Leu-Phe had no effect on EA_G^{rab} (Fc) rosettes (Fig. 21).

The effects of f-Met-Leu-Phe and casein were also studied on mouse peritoneal macrophage monolayers. As shown in Table III the results are essentially similar to those found using mouse macrophage suspensions. F-Met-Leu-Phe increased macrophage complement receptors to the greatest extent at a concentration of 10^{-9} moles.l^{-1} (74 $\pm$ 22%). Casein also enhanced the receptors at a concentration of 2 and 3 mg.ml^{-1}. At the highest concentration a greater degree of enhancement was observed with the mouse monolayers compared to the mouse macrophage suspensions (73 $\pm$ 9 and 48.5 $\pm$ 10 respectively). (p = N.S.)

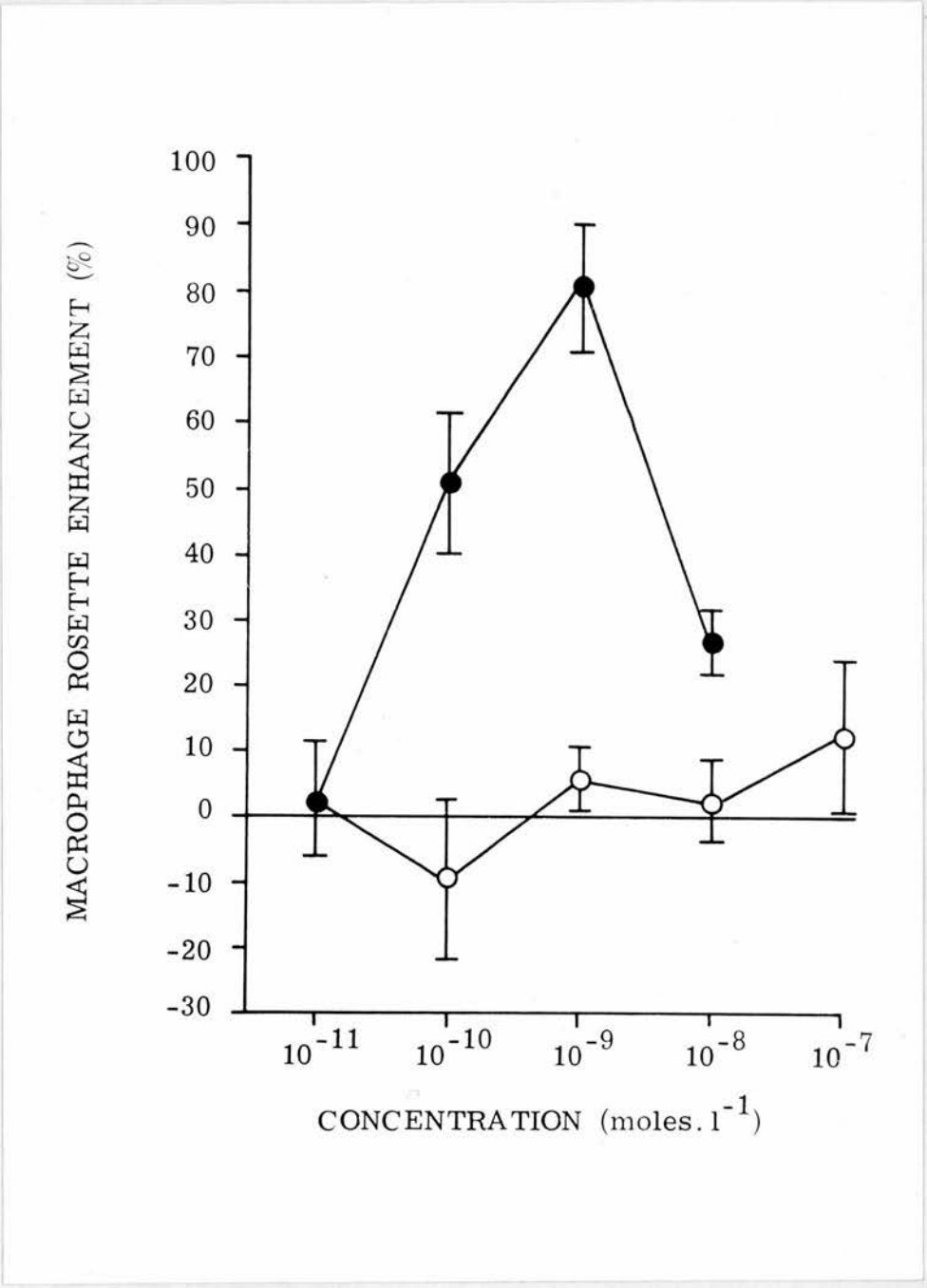


Fig. 21

The effect of F-Met-Leu-Phe on the expression of mouse peritoneal macrophage complement receptors (●——●) and Fc (IgG) receptors (o——o). Each point represents the mean ± 1 S.E.M. of three experiments. The indicator red cells were EAC(R3) at $^{1}/_{50}$ dilution of R3 and EA_{G}^{rab} at $^{1}/_{50}$ dilution of IgG.

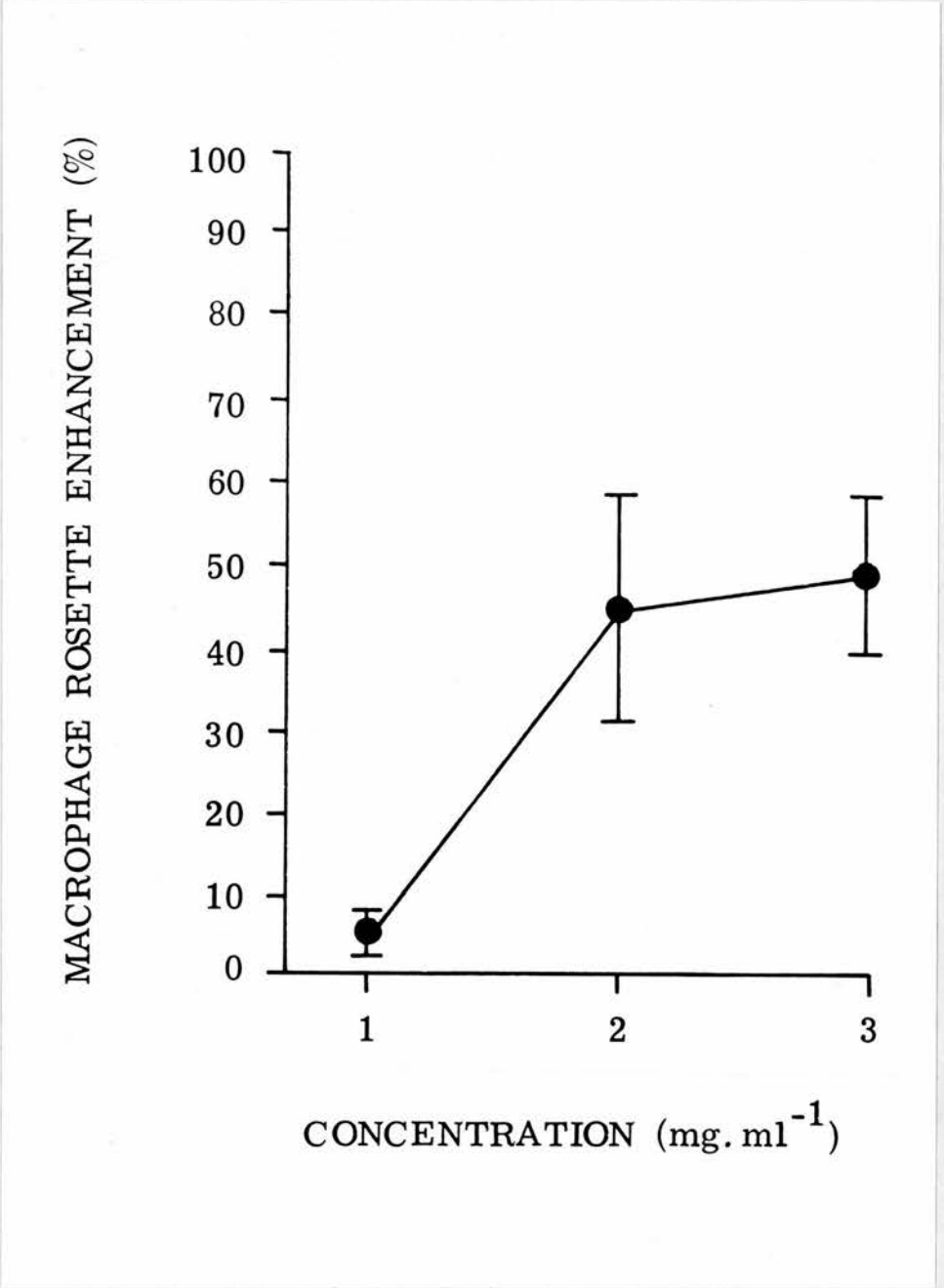


Fig. 22

The effect of casein on the expression of mouse peritoneal macrophage complement receptors. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. The indicator red cells were EAC(R3) as in Fig. 21.

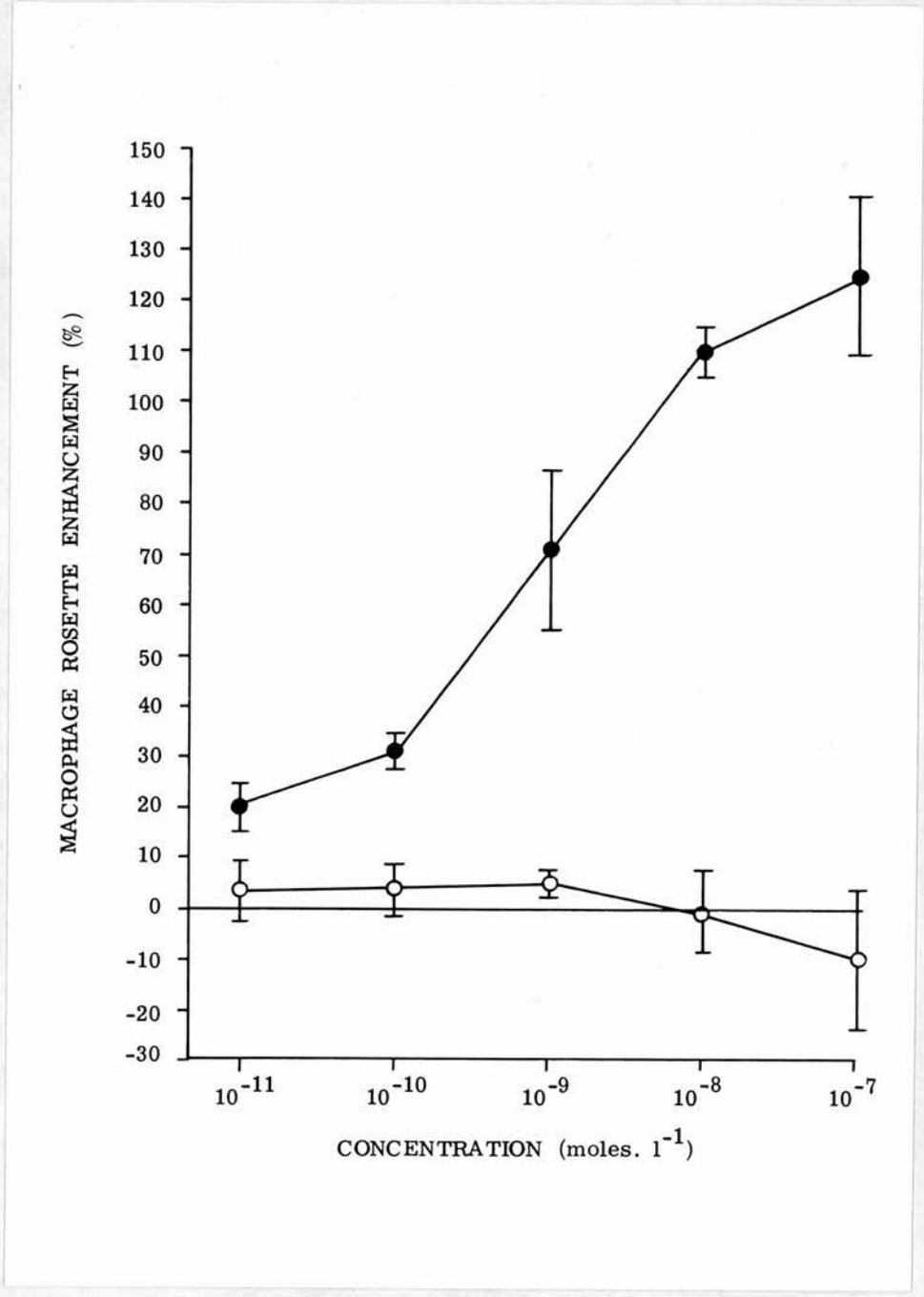


Fig. 23

The effect of F-Met-Leu-Phe on the expression of guinea pig alveolar complement receptors (●——●) and Fc (IgG) receptors (o——o). Each point represents the mean $\pm$ 1 S.E.M. of three experiments. Indicator red cells were EAC(R3) at $^{1}/_{2}$ dilution of R3 and EA_{G}^{rab} at $^{1}/_{40}$ dilution of IgG.

CONCENTRATION		ENHANCEMENT %
f-Met-Leu-Phe	10^{-11} moles.l^{-1}	-11 ± 6
	10^{-10} "	19 ± 13
	10^{-9} "	74 ± 22
	10^{-8} "	27 ± 17
	10^{-7} "	- 3 ± 12
Casein	1 mg.ml^{-1}	- 3 ± 11
	2 "	47 ± 8
	3 "	73 ± 9

TABLE III

The effect of f-Met-Leu-Phe and casein on complement receptor expression on mouse peritoneal macrophage monolayers. The results represent the mean of 3 experiments ± 1 S.E.M.

6.0 SUMMARY

The chemoattractants casein and f-Met-Leu-Phe markedly enhanced the expression of complement receptors on human monocytes, mouse peritoneal and guinea pig alveolar macrophages in a dose-dependent fashion. Increases in monocyte complement receptors by both chemotactic factors were also shown to be time dependent. The time course of enhancement of monocyte complement receptor enhancement was compared to that found with human neutrophils and eosinophils. Other known chemotactic agents were also tested for their capacity to increase complement rosettes on monocytes. Both lymphokines produced by human lymphocytes and a chemotactic factor elaborated by C. parvum were found to enhance the expression of complement receptors.

A chemotactic agent, human serum albumin, also increased complement receptors on human monocytes. In contrast to the increases observed with complement receptors, Fc receptors on human monocytes, mouse peritoneal and guinea pig alveolar macrophages were unaffected by casein or f-Met-Leu-Phe.

SECTION VI -

EFFECTS OF CHEMOATTRACTANTS ON THE EXPRESSION OF OTHER TYPES OF RECEPTORS ON MONONUCLEAR PHAGOCYTES

1.0 EFFECT OF f-Met-Leu-Phe ON HUMAN MONOCYTE RECEPTORS FOR THE Fc PORTION OF IgM

As described in Section 1, Part 3.0 human monocytes do not normally express Fc receptors for IgM but apparently a small percentage of the monocytes ($14 \pm 1\%$) have cryptic receptors which are revealed after neuraminidase treatment. Experiments were carried out to investigate the possibility that incubation with chemoattractants might similarily reveal these receptors. As shown in Table IV 10^{-7} moles.l^{-1} f-Met-Leu-Phe did not alter the expression of Fc receptors for IgM on untreated human monocytes. The effects of f-Met-Leu-Phe were also examined on monocytes after treatment with neuraminidase. Again no significant difference was detected.

TREATMENT	ROSETTES %
Control	3 ± 1
f-Met-Leu-Phe, 10^{-7} moles.l^{-1}	2 ± 1
Neuraminidase	14 ± 1
Neuraminidase + f-Met-Leu-Phe, 10^{-7} moles.l^{-1}	12.5 ± 3

TABLE IV

The effect of neuraminidase and f-Met-Leu-Phe on monocyte rosette formation with EA_M^{rab}. The results represent the mean of 3 experiments ± 1 S.E.M.

2.0 EFFECT OF f-Met-Leu-Phe ON HUMAN MONOCYTE RECEPTORS FOR C3b´

As described in Section III, Part 2.0 human monocytes from rosettes with EAC1423b´, prepared by either incubation of EAC1423b cells with purified human KAF or with EDTA-chelated serum. The latter indicator cells were used to investigate the effects of f-Met-Leu-Phe on C3b´ receptors. The results in Table V show that at the highest concentration tested (10^{-7} moles.l^{-1}) f-Met-Leu-Phe had a slightly inhibitory effect on the expression of C3b´ receptors on human monocytes (-11 $\pm$ 1%). At 10^{-8} and 10^{-9} moles.l^{-1} f-Met-Leu-Phe no alterations in C3b´ receptors were observed.

CONCENTRATION moles.l^{-1}	% CHANGE FROM CONTROL
10^{-9}	2 ± 2
10^{-8}	5 ± 8
10^{-7}	-11 ± 1

TABLE V

The effect of f-Met-Leu-Phe on monocyte rosette formation with EAC1423b´. The results represent the mean of the 3 experiments $\pm$ 1 S.E.M.

3.0 EFFECTS OF f-Met-Leu-Phe AND CASEIN ON STAPH. ALBUS BINDING TO MOUSE PERITONEAL MACROPHAGES

The effect of increasing doses of f-Met-Leu-Phe on Staph. albus binding to mouse peritoneal macrophages is shown in Fig. 24 and Table VI. A dose dependent inhibition of Staph. albus binding was observed when the cells were pre-exposed to varying dilutions of f-Met-Leu-Phe at 37°C for 30 minutes. At the highest concentrations tested 10^{-7} and 10^{-8} moles.l^{-1}, less inhibition was observed. Bacterial binding was also reduced by casein in a dose dependent fashion (Fig. 25). However pre-exposure of the macrophage monolayers to f-Met-Leu-Phe or casein at 4°C (Table VI) had no effect on Staph. albus binding.

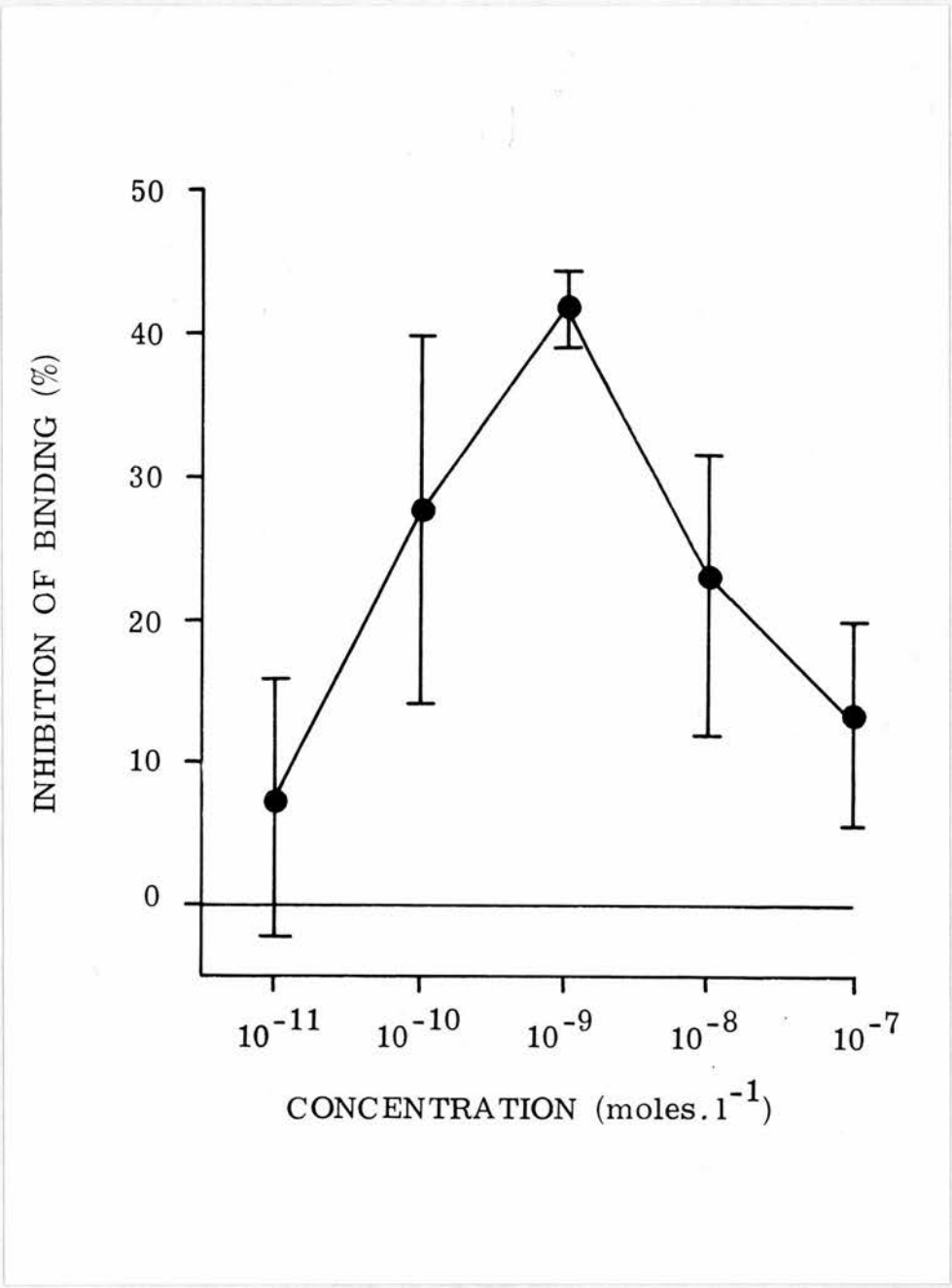


Fig. 24

The effect of F-Met-Leu-Phe on the binding of Staph. albus by mouse peritoneal macrophages. The percentage inhibition of binding is calculated as in Fig. 12. Each point represents the mean $\pm$ 1 S.E.M. of four experiments.

CONCENTRATION OF CHEMOATTRACTANT		INHIBITION OF Staph. albus BINDING (%) AT 4°C
f-Met-Leu-Phe	10^{-11} moles l^{-1}	13 ± 11
	10^{-10} "	3 ± 9
	10^{-9} "	0.5 ± 9
	10^{-8} "	10 ± 15
	10^{-7} "	16.5 ± 15
Casein	1 mg ml^{-1}	10 ± 8
	2 "	7 ± 5
	3 "	11.5 ± 19

TABLE VI

The effect of prior incubation of Staph. albus binding to mouse peritoneal macrophages. The results represent the mean of 3 experiments ± 1 S.E.M.

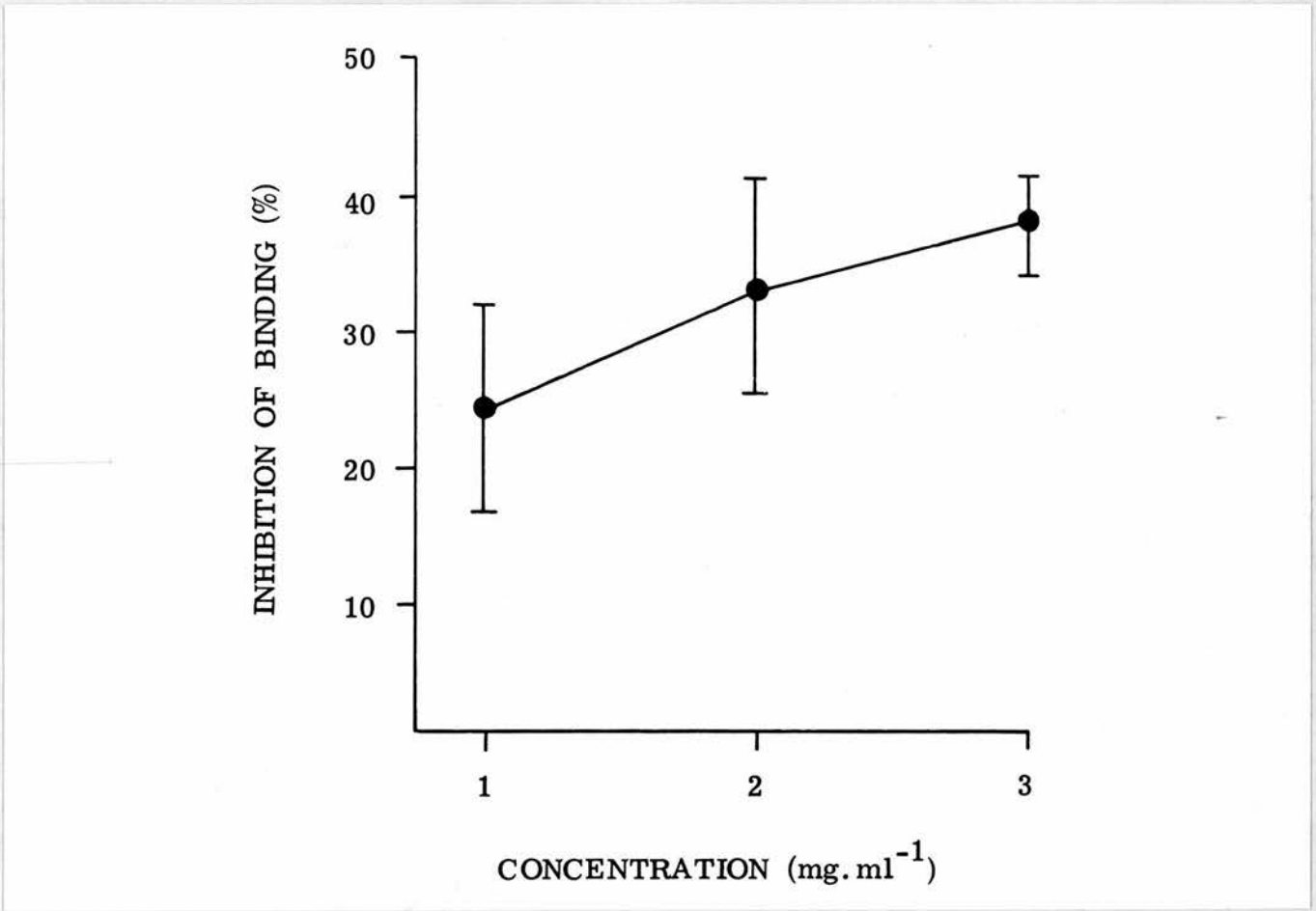


Fig. 25

The effect of casein on the binding of Staph. albus by mouse peritoneal macrophages. The percentage inhibition of binding is calculated as in Fig. 12. Each point represents the mean $\pm$ 1 S.E.M. of three experiments.

4.0 SUMMARY

Neither Fc receptors for IgM or C3b′ receptors on human monocytes were affected by the chemoattractant f-Met-Leu-Phe. Both f-Met-Leu-Phe and casein inhibited the expression of "lectin-like" receptors on mouse peritoneal macrophages in a dose dependent fashion.

SECTION VII -

EFFECTS OF MEDIATORS ON MONOCYTE COMPLEMENT RECEPTORS

1.0 EFFECTS OF PROSTAGLANDINS, 5-HYDROXYTRYPTAMINE, BRADYKININ AND ISOPRENALINE

In this series of experiments the monocytes were incubated with various substances for 1 hour at 37°C. Prostaglandins E_1, E_2 and $F_{2\alpha}$ were tested for their effects on monocyte C3b receptors (Fig. 26). Prostaglandin E_1 (PGE_1) showed significant enhancement at 10^{-6}, 10^{-5} and 10^{-4} moles.l^{-1} with peak activity at 10^{-6} moles.l^{-1} ($48 \pm 7\%$). ($p < 0.005$) Prostaglandin E_2 (PGE_2) significantly increased monocyte complement receptor expression at concentrations from 10^{-7} moles.l^{-1} to 10^{-4} moles.l^{-1} and the greatest activity was observed at 10^{-7} moles.l^{-1} ($62 \pm 15\%$). ($p < 0.05$) Significant enhancement of rosettes by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was only observed at 10^{-4} moles.l^{-1}. ($p < 0.05$)

5-Hydroxytryptamine (or seratonin) (5-HT) increased monocyte complement receptors in a dose-dependent fashion (Fig. 27). At 10^{-5} and 10^{-4} moles.l^{-1} enhancement was significantly different from control values. A concentration of 10^{-4} moles.l^{-1} gave an increase of $61 \pm 6\%$. ($p < 0.01$)

Bradykinin was tested for its effect on complement receptor expression (Fig. 27). A peak in activity was observed at 10^{-6} moles.l^{-1} ($42 \pm 10\%$) (p = N.S.) with lower enhancement observed at 10^{-5} and 10^{-4} moles.l^{-1}.

The effects of isoprenaline were tested over a range of concentrations from 10^{-7} to 10^{-4} moles.l^{-1} (Fig. 27). No significant effects on EAC rosettes by human monocytes were observed.

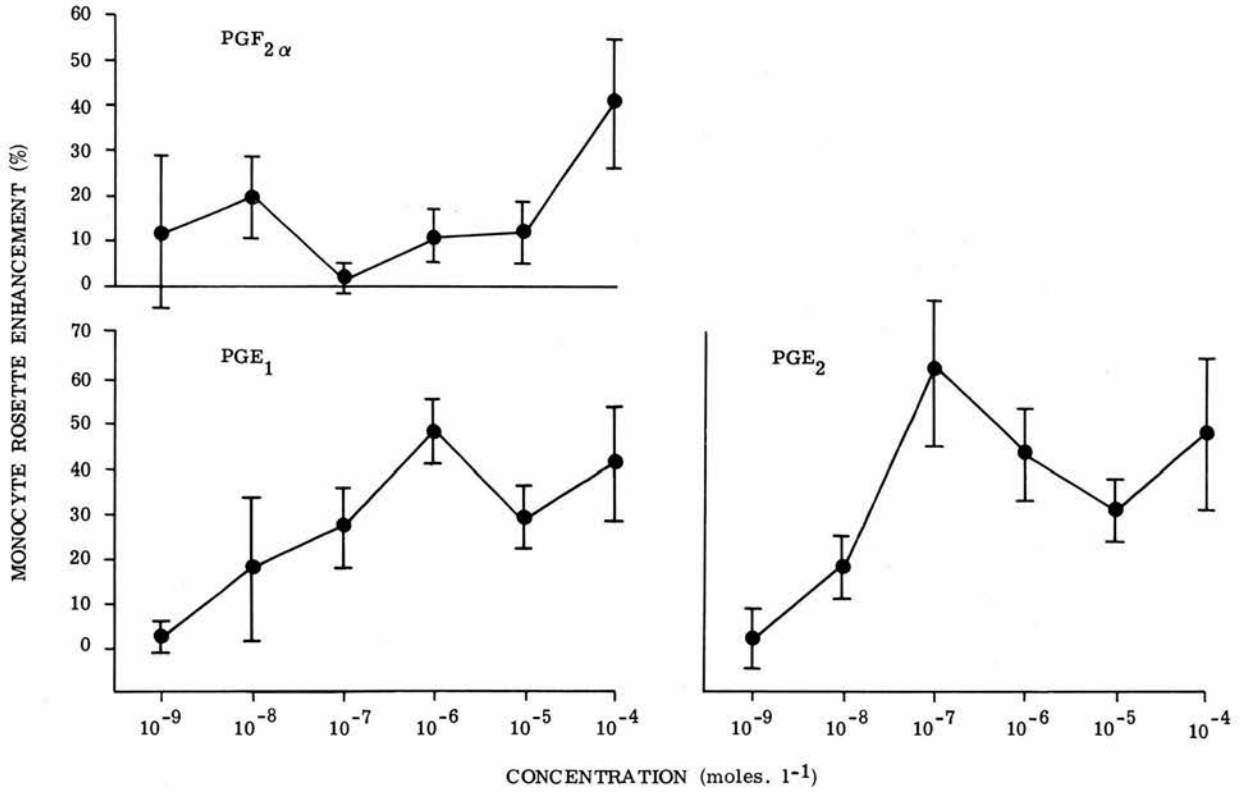


Fig. 26

The effect of prostaglandins $PGF_{2\alpha}$, PGE_1 and PGE_2 on the expression of human monocyte complement receptors. Each point represents the mean $\pm$ 1 S.E.M. 10^{-4} to 10^{-6} moles.l^{-1} - five experiments. 10^{-7} to 10^{-9} moles.l^{-1} - four experiments. Indicator red cells were EAC as in Fig. 16.

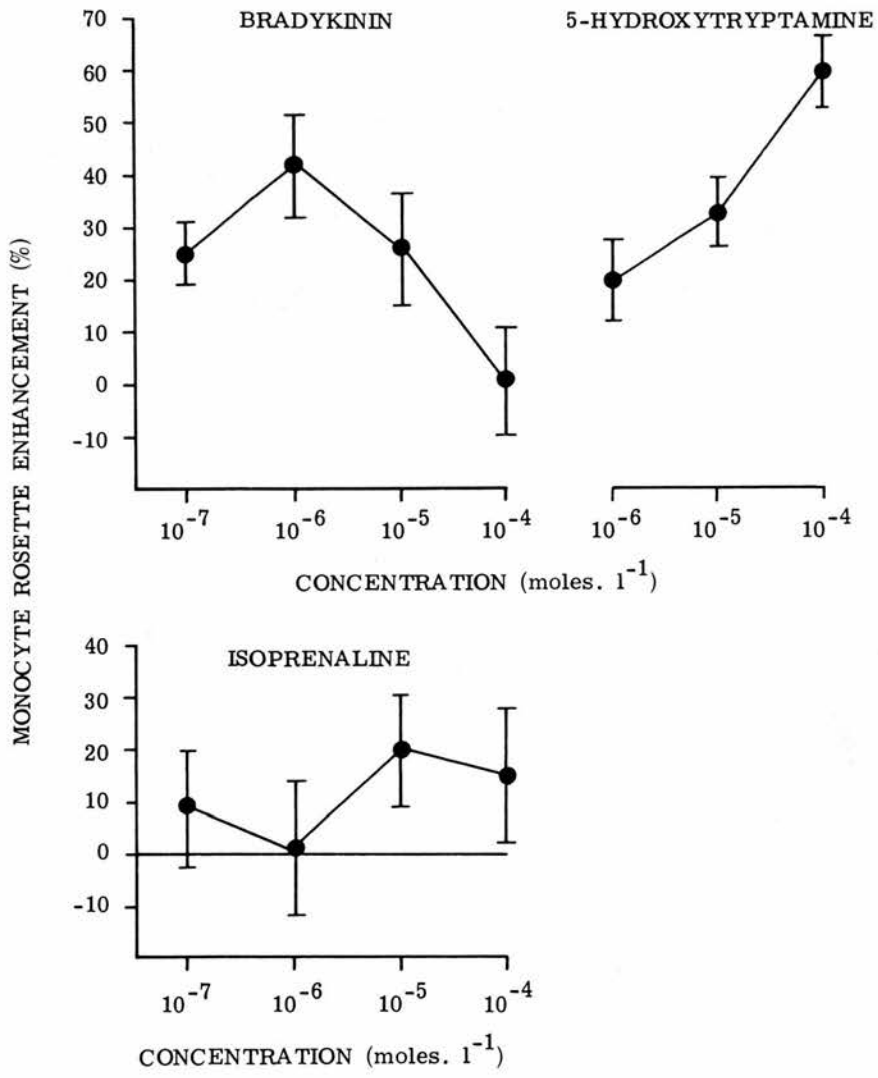


Fig. 27

The effect of various pharmacological mediators on human monocyte complement receptor expression. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. Indicator red cells were EAC as in Fig. 16.

2.0 EFFECTS OF HISTAMINE ON COMPLEMENT RECEPTORS ON MONOCYTES OF ATOPIC AND NON-ATOPIC INDIVIDUALS

2.1 Introduction

A study on the expression of histamine receptors on human peripheral blood monocytes from atopic and non-atopic individuals was carried out by an Honours Student, Lesley M. Smart, following the description of these receptors on guinea pig alveolar macrophages and other phagocytes (Diaz et al., 1979). Individuals were patch tested to determine whether they were atopic or non-atopic using common allergens such as house dust mite, grass pollen, cat hair and egg. Monocytes were also obtained from atopic individuals attending the Skin Clinic, Royal Infirmary, Edinburgh. Concurrently with this study the effect of histamine on monocyte complement receptor expression was studied. Although preliminary studies had shown that the response to histamine was generally small there was a marked enhancement of complement receptors by this agent in a few individuals. The reason for such variations between apparently healthy individuals was unclear but the possibility that it might be related to their atopic state was explored.

2.2 Effect of increasing concentrations of histamine

The effect of histamine on atopic and non-atopic individuals is shown in Fig. 28. Nine atopic and seven non-atopic individuals were assessed for modulation of monocyte complement receptor expression by histamine with a range of concentrations from 10^{-7} to 10^{-4} moles.l^{-1}. A considerable spread of results was obtained particularly with the atopic/...

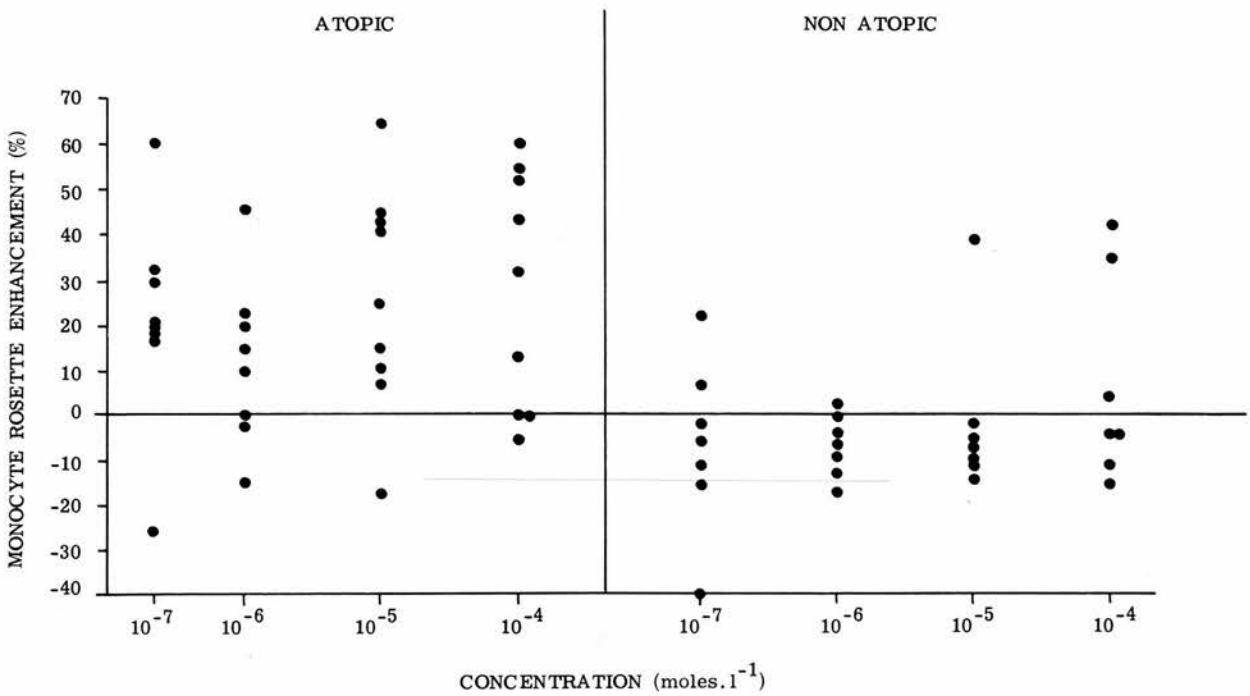


Fig. 28

The effect of histamine on human monocyte complement receptor expression from atopic and non-atopic subjects. Each point represents the result from one individual. Indicator red cells were EAC as in Fig. 16.

atopic group. The effects of histamine did not show obvious dose-dependency with any individual. It was observed that histamine at every concentration tested decreased the expression of complement receptors on the monocytes of the majority of non-atopic individuals. Enhancement of complement receptors by a value of greater than 20% was only observed at 4 points. Three out of the four were the results obtained from one individual. The 4th point (42% enhancement at 10^{-7} moles.l^{-1} histamine) was obtained from a second individual who also showed positive enhancement at 10^{-6} and 10^{-7} moles.l^{-1} (12% and 8% respectively).

The results obtained with the atopic individuals were very different. At all concentrations of histamine tested the expression of complement receptors on monocytes was enhanced albeit to varying degrees with a few notable exceptions. The number of EAC rosettes was reduced at 10^{-7}, 10^{-6} and 10^{-4} moles.l^{-1} histamine by 26%, 2% and 18% respectively in the case of one individual. Another person showed fewer EAC rosettes than the control value at 10^{-6} and 10^{-4} moles.l^{-1} (15% and 5% respectively). A concentration of 10^{-4} moles.l^{-1} histamine had no effect on the expression of complement receptors on monocytes of 2 other individuals, one of whom also showed a lack of response at 10^{-6} moles.l^{-1}. The vast majority of points were found to lie between 10 and 65% enhancement.

A similar series of experiments were also carried out with human neutrophils (results not shown). Again there was a wide variation in the results and the effects of histamine were not dose dependent. However no obvious differences between the response of neutrophils from non-atopic and atopic individuals was observed.

3.0 SUMMARY

The effects of prostaglandins, seratonin, bradykinin and histamine on human monocyte complement receptor enhancement were examined. PGE_1 and PGE_2 increased EAC rosette formation as did $PGF_{2\alpha}$ but the latter only showed activity at the highest dose tested. Monocyte rosettes were also increased by 5-Hydroxytryptamine and bradykinin. In contrast isoprenaline had no apparent effect at any of the doses tested. Histamine had different effects on different individuals. Monocytes from atopic people tended to have increased complement receptor expression after incubation with histamine whereas monocytes from non-atopic individuals tended to show decreases in EAC rosette formation.

SECTION VIII -

FURTHER STUDIES ON THE PHENOMENON OF COMPLEMENT RECEPTOR ENHANCEMENT

1.0 INTRODUCTION

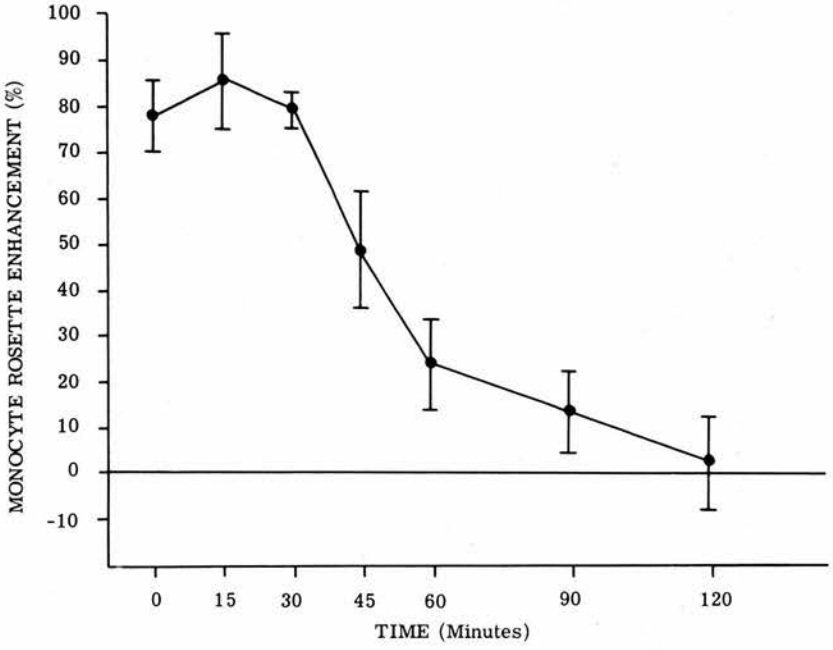
Possible explanations for the mechanisms of complement receptor enhancement has been discussed in previous reports (Anwar and Kay, 1977b,1978) and included "membrane unfolding", "receptor externalisation" and "subunit association".

In the previous sections it has been shown that chemotactic factors enhance the expression of complement receptors on a variety of phagocytes including human peripheral blood monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages. Other receptors including Fc receptors for IgG and IgM, and C3b' receptors were unaffected and lectin-like receptors were reduced by chemoattractants. Having established the optimal conditions for monocyte complement receptor enhancement with casein and f-Met-Leu-Phe, these chemotactic agents were used to explore the phenomenon further in order to gain more insight into the mechanism of increased receptor expression. The following studies investigated the effects of temperature and also examined the possibility that complement receptor enhancement might be a reversible phenomenon. Some comparisons were made with human neutrophils.

2.0 EFFECTS OF WASHING CELLS AFTER INCUBATION WITH CHEMOTACTIC FACTORS

Monocyte complement receptor enhancement appeared to be reversible (Fig. 29). Following 30 minute incubation with 3 $mg.ml^{-1}$ casein at 37°C, the cells were washed and resuspended in medium 199. After about 30 minutes there was a gradual decrease in monocyte rosette enhancement and by 120 minutes there was no discernible difference from the control value. Similar results were obtained with f-Met-Leu-Phe (10^{-7} $moles.l^{-1}$) (Fig. 30), although for the first 30 minutes after removal of the chemoattractant there was an apparent rise in complement receptor expression before the number of rosettes declined to control values at 120 minutes.

Complement receptor enhancement by human peripheral blood neutrophils was also found to be reversible following incubation with f-Met-Leu-Phe (10^{-8} $moles.l^{-1}$) (Fig. 31). Neutrophil rosette enhancement decreased more rapidly than that observed with monocytes and by 30 minutes had returned to control levels.



(Monocytes were preincubated with 3 mg/ml of casein for 30 min at 37°C, centrifuged, the supernatant removed and the cells resuspended in medium 199 to the original volume and incubated at 37°C for various time intervals as shown)

Fig. 29

Reversibility of monocyte complement receptor enhancement with $3mg.ml^{-1}$ casein. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. The indicator red cells were EAC as in Fig. 16.

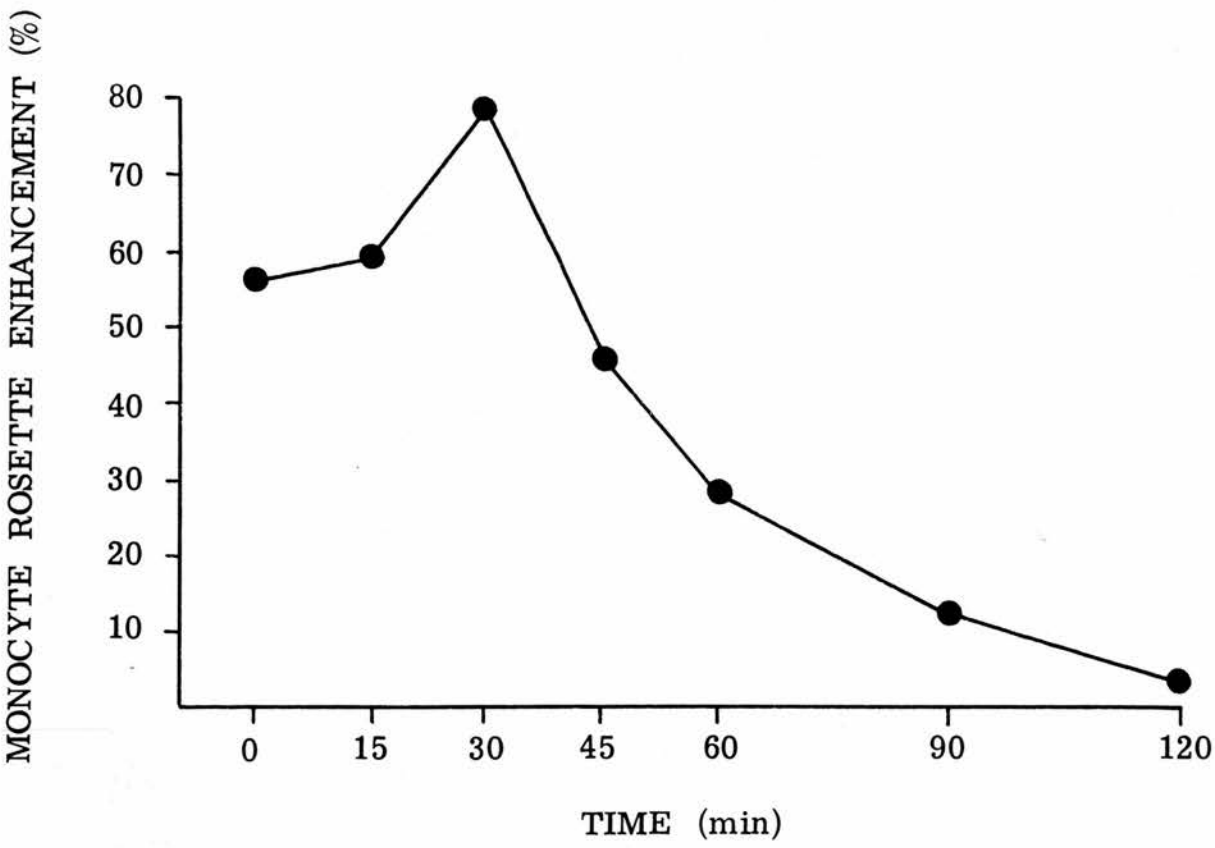


Fig. 30

Reversibility of monocyte complement receptor enhancement with 10^{-7} moles.l^{-1} F-Met-Leu-Phe. Each point represents the mean of two experiments. The indicator red cells were EAC as in Fig. 16.

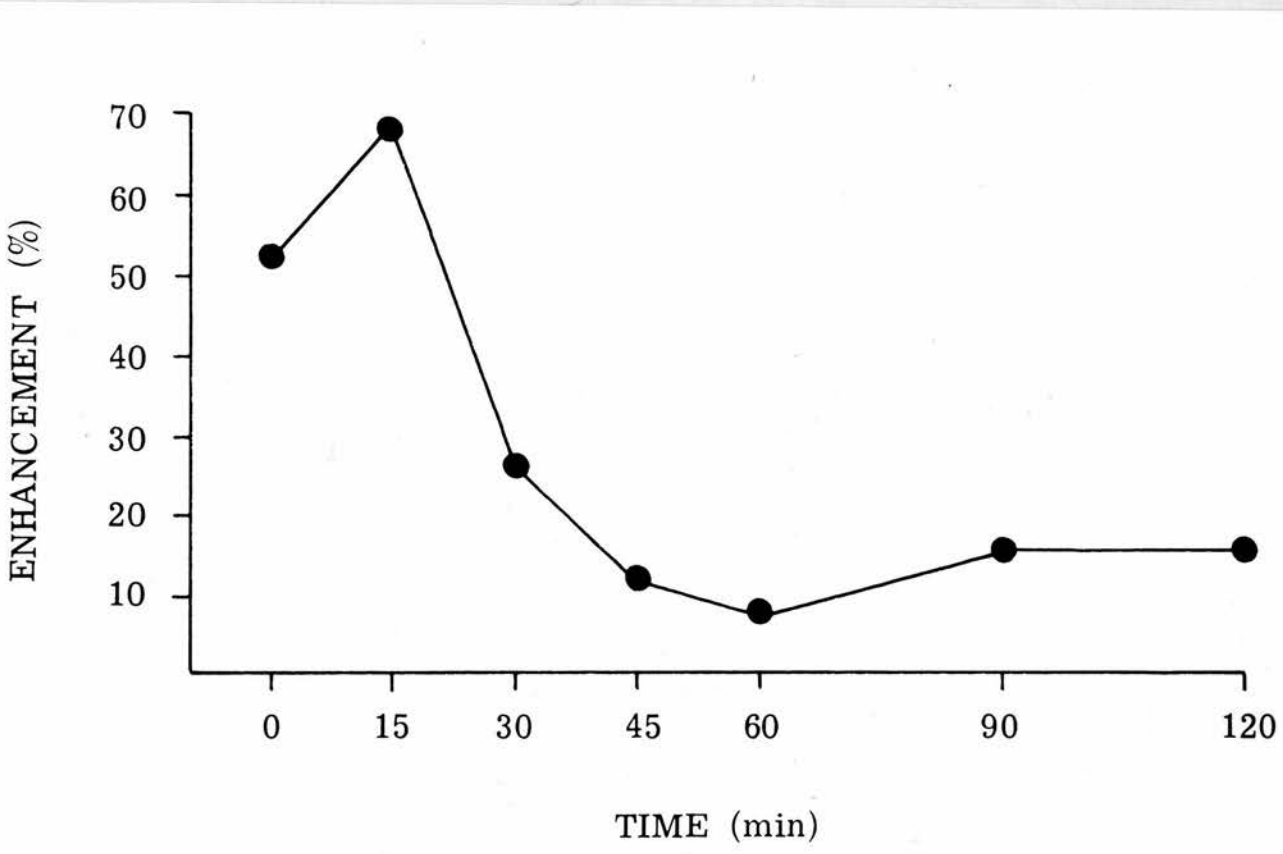


Fig. 31

Reversibility of neutrophil complement receptor enhancement with 10^{-8} moles.l^{-1} F-Met-Leu-Phe. Each point represents the mean of two experiments. The indicator cells were EAC(R3) at $^{1}/_{25}$ dilution of R3.

3.0 TEMPERATURE DEPENDENCE OF COMPLEMENT RECEPTOR ENHANCEMENT BY CHEMOTACTIC FACTORS

In these experiments the leukocytes were incubated with the chemotactic factors at varying temperatures for 30 minutes.

Monocyte complement receptor enhancement was temperature dependent as shown in Fig. 32. At 0°C there was no increase in the percentage of rosettes formed with any of the concentrations of casein used. However at 23, 30 and 37°C, enhancement was observed and increased proportionally with the rise in temperature. Similar results were obtained with f-Met-Leu-Phe (Fig. 33). At 0 and 23°C little enhancement was observed with any of the concentrations of f-Met-Leu-Phe used. However at 30 and 37°C enhancement occured with 10^{-7} and 10^{-8} moles.l^{-1}, a greater increase being observed at 37°C.

Neutrophil complement receptor enhancement was also temperature dependent (Fig. 34). There was no increase in the number of rosettes formed with any of the concentrations of f-Met-Leu-Phe at 0 and 23°C. At higher temperatures (30 and 37°C) enhancement was observed and increased proportionally with the rise in temperature.

In order to investigate the possibility that lowering the temperature was decreasing the rate of enhancement rather than the degree, time course studies at varying temperatures were carried out as shown in Fig. 35. At 37°C monocyte complement receptor enhancement by f-Met-Leu-Phe (10^{-7} moles.l^{-1}) remained virtually constant over the 120 minute incubation period. At 30°C there was a gradual rise from 45% at 30 minutes to 67% at 120 minutes. Increasing the incubation time with f-Met-Leu-Phe at 0 and 23°C had no effect on the degree of enhancement observed.

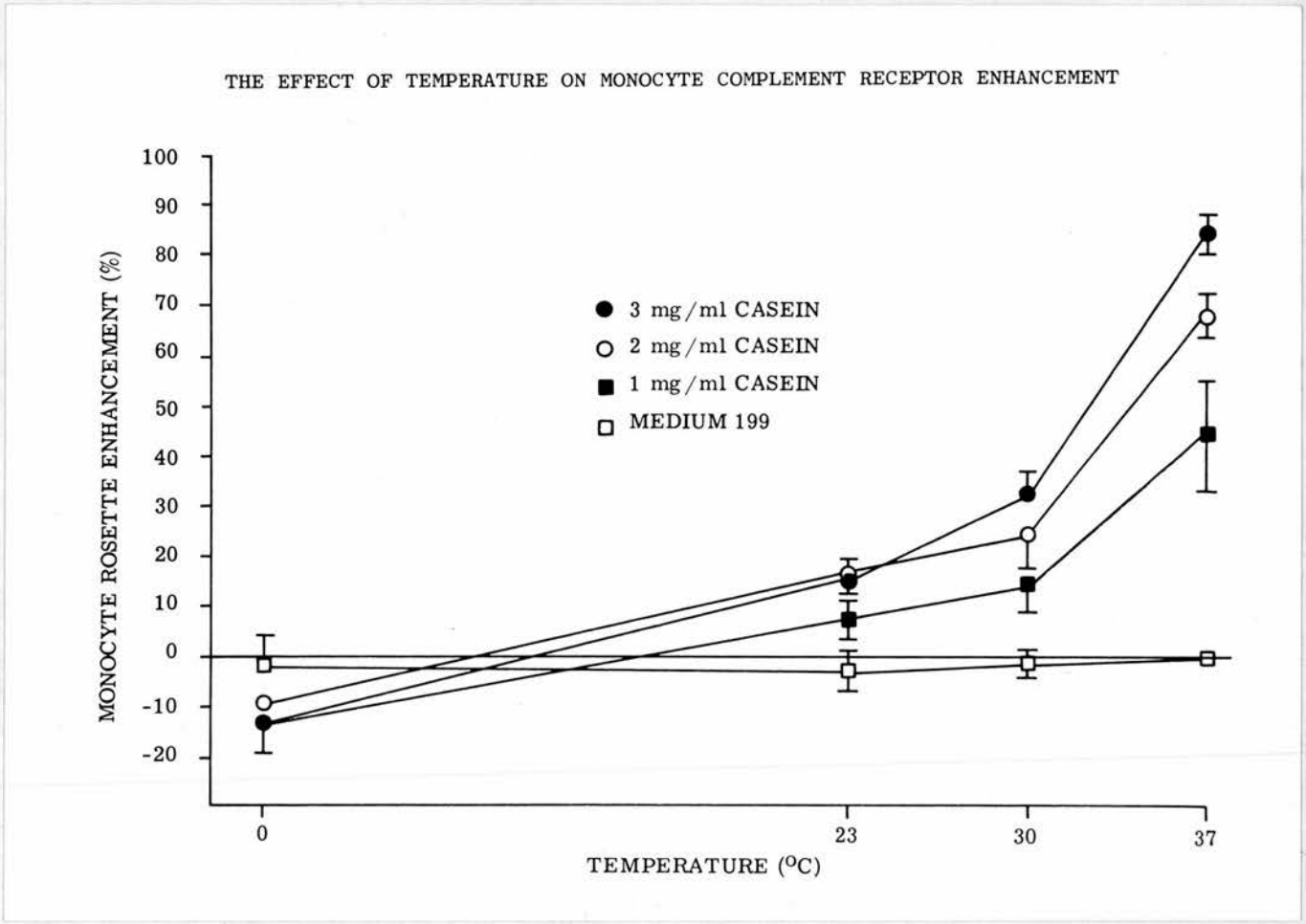


Fig. 32

The effect of temperature on monocyte complement receptor enhancement. (●——●) 3mg.ml^{-1} casein, (○——○) 2mg.ml^{-1} casein, (■——■) 1mg.ml^{-1} casein, (□——□) medium 199. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. The indicator cells were EAC as in Fig. 16.

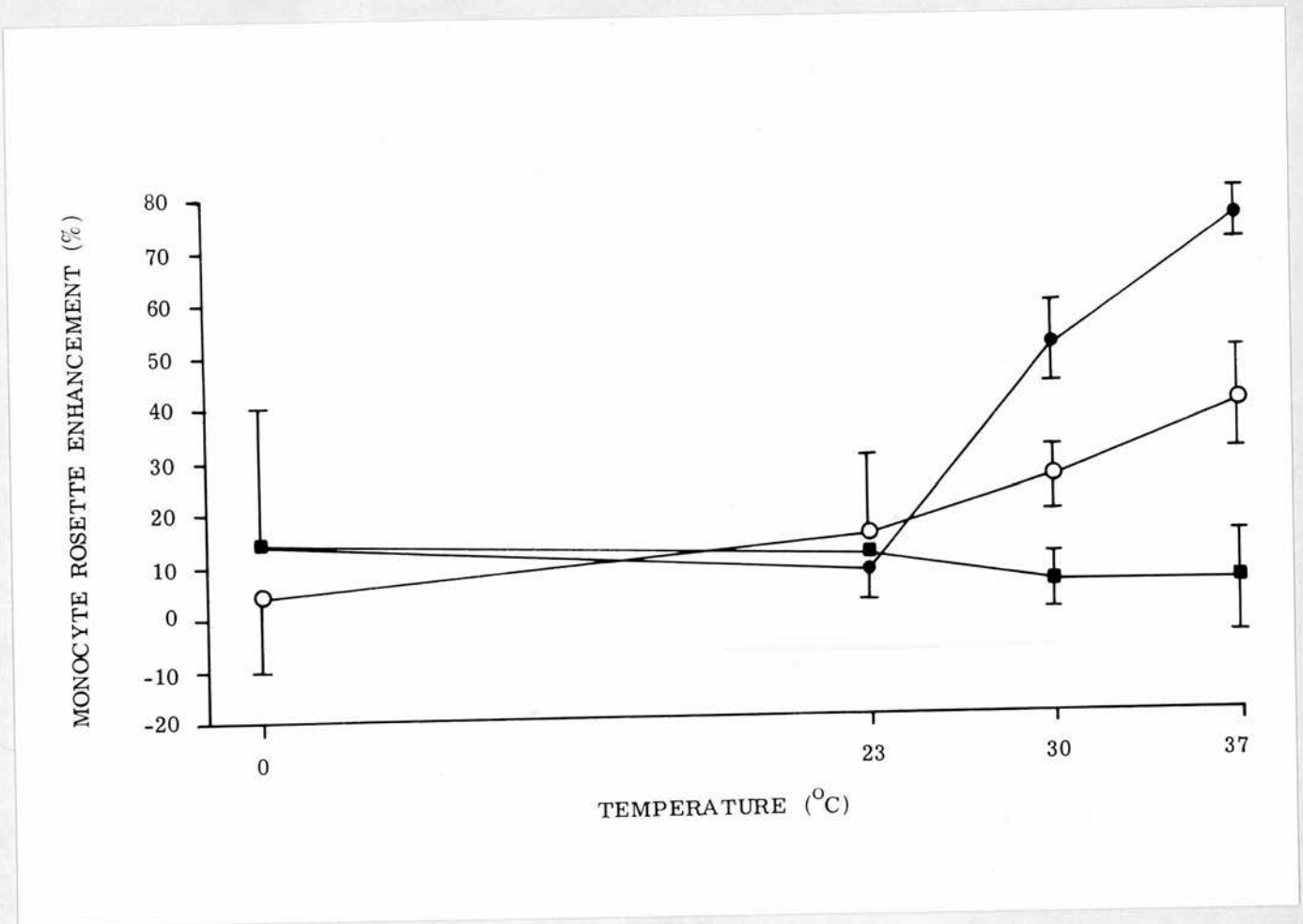


Fig. 33

The effect of temperature on monocyte complement receptor expression. (●——●) 10^{-7} moles.l^{-1}, (o——o) 10^{-8} moles.l^{-1} and (■——■) 10^{-9} moles.l^{-1} F-Met-Leu-Phe. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. Indicator red cells were EAC(R3) at $^{1}/_{150}$ dilution of R3.

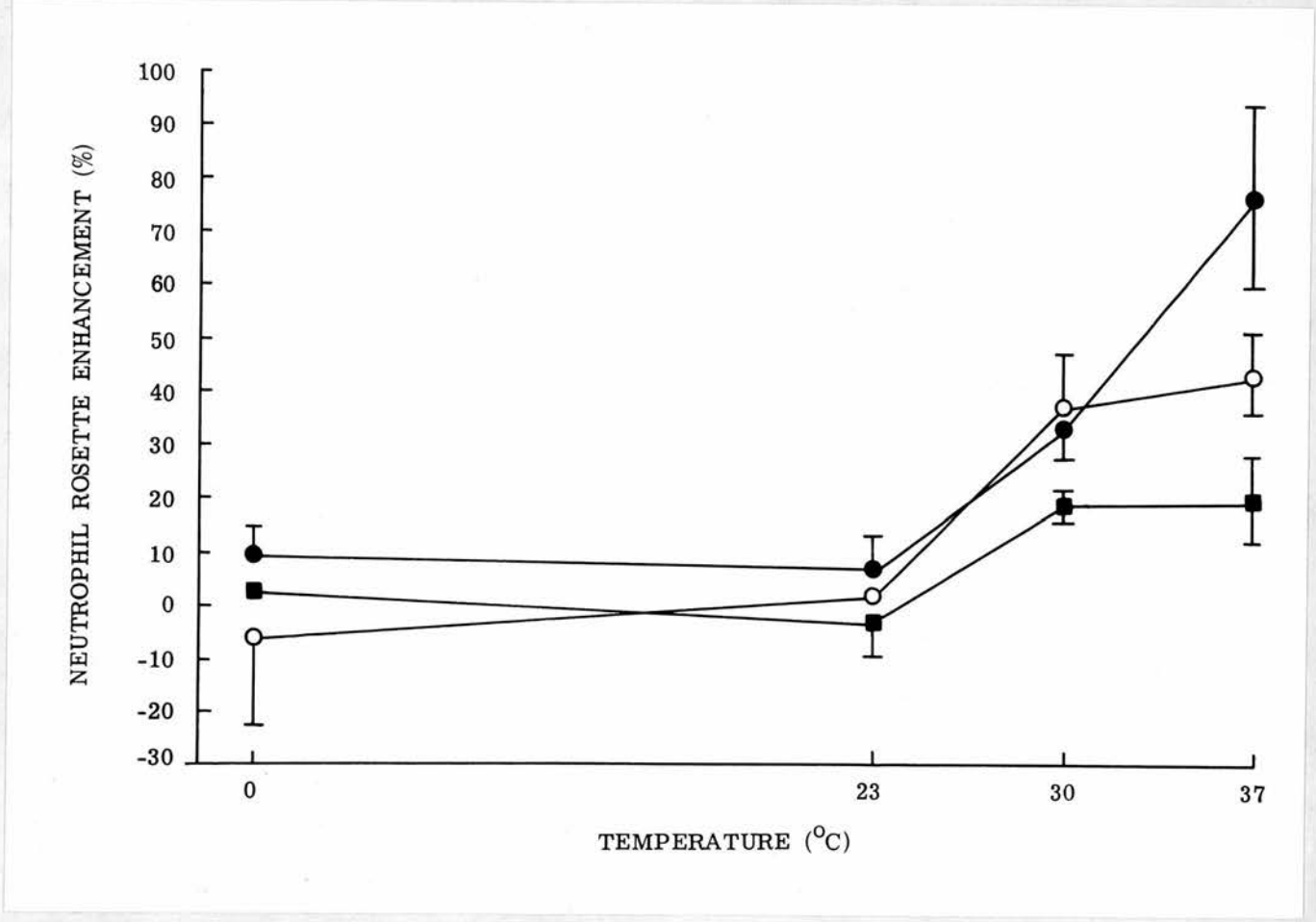


Fig. 34

The effect of temperature on neutrophil complement receptor enhancement. (●——●) 10^{-8} moles.l^{-1}, (o——o) 10^{-9} moles.l^{-1} and (■——■) 10^{-10} moles.l^{-1} F-Met-Leu-Phe. Each point represents the mean $\pm$ 1 S.E.M. of three experiments. Indicator red cells were EAC(R3) as in Fig. 31.

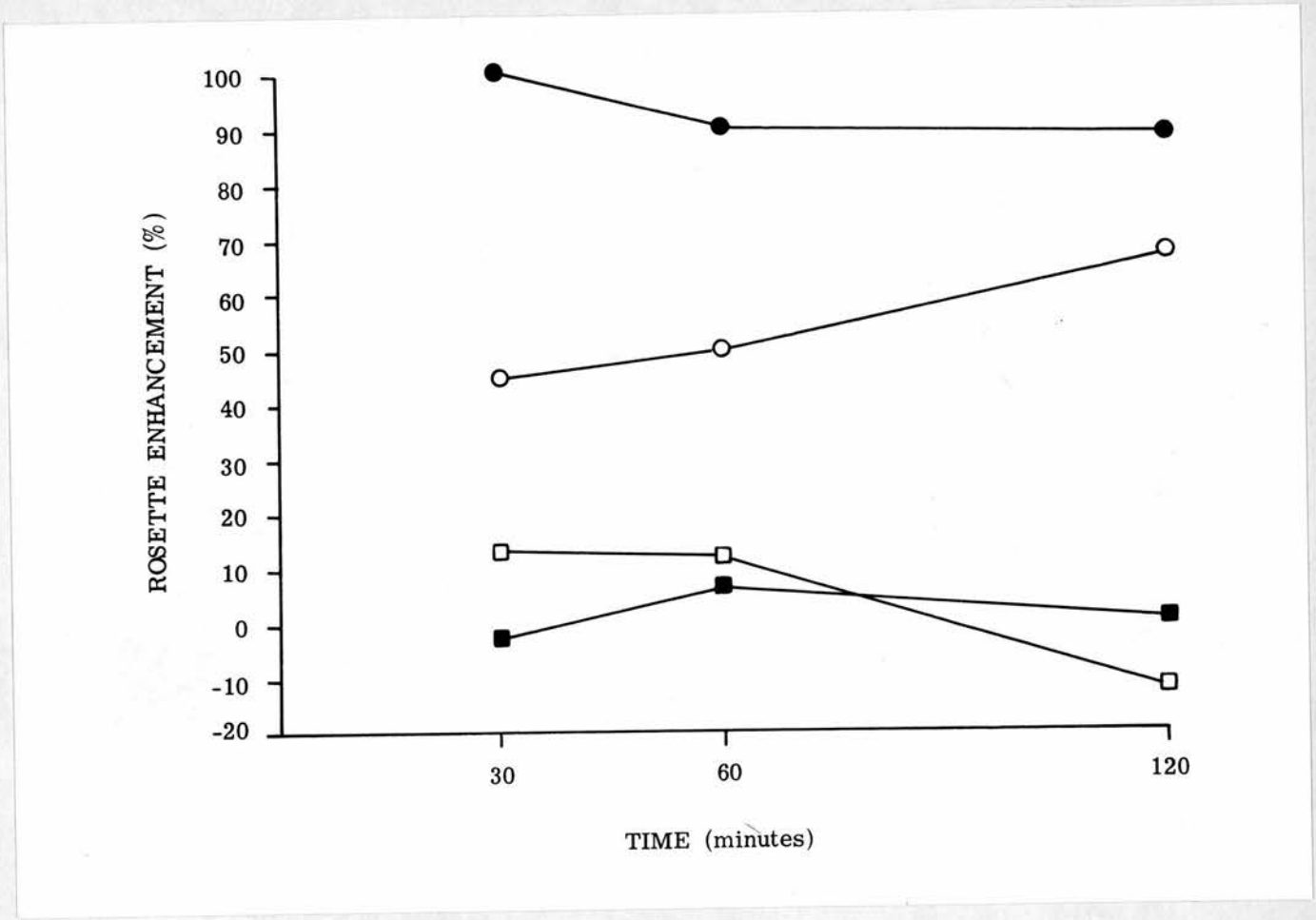


Fig. 35

The time course of monocyte complement receptor enhancement at 37°C (●——●), 30°C (○——○), 23°C (■——■) and 0°C (□——□). Each point represents the mean of two experiments. Indicator red cells were EAC(R3) as in Fig. 33.

4.0 SUMMARY

Washing the monocytes after enhancement with f-Met-Leu-Phe or casein and allowing the cells to recover in medium led to a gradual return in complement receptor expression to basal levels. Similar results were obtained with human neutrophils.

Enhancement of complement receptors on human monocytes and neutrophils was temperature dependent and occurs mainly in the temperature range of 30 to 37°C. The lower level of enhancement observed at 30°C was partly due to a decrease in the rate at which enhancement occured.

CHAPTER V - DISCUSSION

1.0 RECEPTORS FOR IMMUNOGLOBULIN, COMPLEMENT AND BACTERIA ON HUMAN MONOCYTES, MOUSE PERITONEAL MACROPHAGES AND GUINEA PIG ALVEOLAR MACROPHAGES

1.1 Techniques

Using the rosette technique, receptors for immunoglobulin and complement were demonstrated on various mononuclear phagocytes and using a binding assay "lectin-like" receptors were also detected on these cells. The use of these methods for the investigation of various cell membrane markers is well established. For most of the experiments using the rosette technique, the cells were in suspension and smeared onto glass slides before fixation and staining. The ratio of indicator sheep red blood cells to leukocytes was important because they are present in the background and if too many were present it became impossible to distinguish individual rosettes. This problem did not arise when adherent cell monolayers were used since non-attached red cells were simply washed away. In the latter studies leukocytes with 2 or more attached indicator red cells were counted as positive rosettes whereas with the smears 3 or more red cells attached were scored as positive. The disadvantages of using monolayers were (a) it lengthened the time of the experiment and therefore increased the possibility of lowering the viability of the cells; (b) change in membrane activity was likely and in fact with the same ratio of red cells to leukocytes, less complement was required to coat the red cells for detection of complement receptors on monolayers, than on the same cells in suspension to obtain the same percentage of rosetting cells; (c) it was possible that selection of a more adherent cell population might occur.

"Lectin-like" receptors were detected on human peripheral blood leukocytes, mouse peritoneal and guinea pig alveolar macrophages by overlaying/...

overlaying bacteria (Staph. albus) on monolayers of these cells, and after incubation at 4°C the non-attached organisms were washed away. Monolayers of cells were used despite the disadvantages discussed above because after interacting the phagocytes and bacteria in suspension, it proved very difficult to remove sufficient non-attached organisms to obtain a clear background. Even with the monolayers, preparations at a bacterial concentration greater than 10^{10} organisms.ml^{-1} the presence of bacteria in the background made it difficult to estimate binding. Glass coverslips in wells of tissue culture plates were used in these assays instead of flying coverslips in W.R. tubes as employed previously (H. Ogmundsdottir, Ph.D. thesis, 1979). This improved method made experimental manipulations considerably easier and faster. Because the absence of serum was very important in these investigations of binding of unopsonised bacteria to phagocytes, the cells were initially adhered to the coverslip with no serum present. This meant that with the human monocyte and mouse peritoneal macrophage preparations, contaminating lymphocytes also adhered (W. McBride, personnal communication). The majority of these lymphocytes, presumably mainly B cells, were removed by washing the monolayers after 1 hour incubation at 37°C with medium also at 37°C, since it was noted that washing with medium at 4°C did not remove the lymphocytes. It is perhaps surprising that both eosinophils and neutrophils should also adhere so readily to glass since it is generally considered to be a functional property of macrophages and is used as a separation method. However eosinophils and neutrophils share many functions in common with mononuclear phagocytosis. Both of these functions probably involve adherence phenomena, in chemotaxis to the substratum (tissue cells in vivo) and in phagocytosis to the ingesting particle.

Care/...

Care was taken to grow the bacteria, Staph. albus, under virtually identical conditions each time a fresh batch was prepared. However variations did occur in the degree of binding obtained and occasionally little or no binding to leukocytes was observed. The only obvious differences between batches was that different preparations of horse digest broth for culturing the bacteria was unavoidably used. This suggests that the environment in which the bacteria grow may affect the surface properties of the organisms and these considerations may be important in terms of pathogenesis, if the invading organisms grow in a particular environment which does not facilitate the synthesis of binding ligands for the phagocyte receptors, then the organism may be able to grow unchecked.

Rosette smears and coverslips were usually stained with May Grunwald/Giemsa. The use of this stain for monolayers instead of the Gram method as used by H. Ogmundsdottir (Ph.D. thesis, 1979) allowed the observer to recognise more readily the morphology of the cells. For some of the rosette experiments the monocytes were stained with non-specific esterase (Yam et al., 1971) so that monocytes could be more easily distinguished from lymphocytes. However in order to prevent lysis of the indicator sheep cells glutaraldehyde fixation was used instead of the recommended formaldehyde/acetone buffer and the intensity of the stain was reduced (methanol destroys esterase activity). In the experiments where the cells were incubated at 37°C before rosetting, staining with non-specific esterase was often very weak presumably because the enzyme involved is water soluble and may be leached from the cell. Low density staining meant that the slides were difficult to count and this problem together with the relative ease of the May Grunwald/Giemsa stain prompted the author to use the latter/...

latter stain wherever possible. Although it was more difficult to distinguish monocytes from lymphocytes using this stain, comparison of the two methods by 4 independent observers revealed no statistically significant differences in differentiating monocytes from lymphocytes. Because of the obvious morphology of neutrophils there was no difficulty in counting neutrophil rosettes in smears or on coverslips. Previous work by Anwar and Kay (1977a, b, 1978) had investigated the presence of Fc and complement receptors on eosinophils and had used May Grunwald/Giemsa to stain these cells. However counting eosinophil smears under the X 25 objective was very difficult and the X 100 objective had to be used which made counting very slow and laborious. The use of chromotrope which specifically stains eosinophils red and tartrazine as a counter-stain for the indicator red cells enabled the author to easily distinguish eosinophils from other granulocytes.

1.2 Fc (IgG) receptors

Sheep red cells sensitised with rabbit IgG (EA_G^{rab}) formed rosettes with human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages in a dose-dependent fashion with increasing doses of antibody (Fig. 4). Since this is a heterologous system, comparisons between the different cell types must be treated with caution. These results are in agreement with Huber et al. (1969) for human monocytes, Mantovani et al. (1972) for mouse peritoneal macrophages and Rhodes (1975b) for guinea pig alveolar macrophages in that all 3 cell types possess Fc (IgG) receptors. Rabbit IgG binds to both classes of Fc receptors (IgG_{2a} and IgG_{2b}) on mouse peritoneal macrophages (Unkeless, 1980).

Optimal/...

Optimal experimental conditions were determined for the detection of Fc (IgG) receptors on human monocytes. Although more monocytes expressed Fc receptors at 37°C than at 4°C (Figs. 2 and 3), even by 5 minutes, varying degrees of phagocytosis had occured and thereafter the percentage of positive cells decreased. It is possible that instability of EA_G^{rab}-monocyte rosettes at 37°C may be as a result of shedding of the receptor into the medium as has been demonstrated for Fc receptors on lymphocyte membranes at 37°C (Sandor et al., 1979) or it could arise from micropinocytosis of small pieces of the attached red cells, the remainder of the red cells breaking away as has been suggested by Brown and Nelson (1973). However as observed here, the percentage of cells phagocytosing EA_G^{rab} increased as rosetting decreased, suggesting that this was the main cause of the diminished binding. If the medium in which the monocytes were incubated at 37°C contained shed Fc receptor material it might be expected to inhibit Fc rosette formation by other Fc receptor bearing cells. The present studies did not examine this possibility. The expression of Fc receptors at 4°C is in contrast to the results of Passwell et al. (1978) who found that the binding of EA_G^{hum} to human monocyte Fc receptors required metabolic energy whereas cytophilic antibody binding did not. These workers prepared monocyte monolayers and the indicator cells and sensitising antibody used were ORh^+ human erythrocytes and human anti-D antibody. Binding of EA_G in this system required Ca^{2+} and Mg^{2+} ions whereas other workers have found that neither cation was necessary (Lay and Nussenzweig, 1969; Davey and Ascherton, 1967). Although Passwell et al. (1978) found that colchicine inhibited rosette formation with EA_G Atkinson and Parker (1977) showed that it had no effect on rabbit alveolar macrophages. It is possible that cells which have adhered/...

adhered to glass may have different binding capacities. The appearance of prominent microtubules within the cytoplasm of adherent monocytes is presumably associated with enhanced spreading abilities and the topography of the membrane may be altered as a result, as evidenced by the irregular ruffled plasma membrane of these cells. Functionally anti-D antibody behaves as an incomplete antibody since the distribution of D antigen on Rh^+ human erythrocytes is such that it prevents effective haemagglutination. Conversely anti sheep red cell antibody readily agglutinates sheep erythrocytes suggesting that the density of antigen and therefore antibody is considerably greater in this system than with the anti D-Rh^+ human erythrocyte system. The formation of macrophage-EA_G rosettes occurs only when there is ligand-receptor interactions between many macrophage Fc receptors and the erythrocyte bound antibody. This union is dependent upon the density of bound antibody on the red cells and also, probably, on the ease with which the macrophage Fc receptors can move in the plane of the membrane. In the sheep erythrocyte system there is rosetting at 4°C because although Fc receptor mobility is low, there is a high density of erythrocyte-bound antibody. At 4°C, in the human Rh^+ system, both erythrocyte-bound antibody density and the Fc receptor mobility are low and thus rosetting cannot occur.

At the highest possible concentration of antibody 100% positive Fc rosetting cells were never obtained. There is a limit to the amount of antibody which can be deposited on the indicator sheep cells, since it must be subagglutinating. This problem could be overcome by using ox erythrocytes and antiox antisera (Hallberg et al., 1973) because ox erythrocytes are able to bind more molecules of antibody per cell than sheep erythrocytes, or by using certain monoclonal anti sheep red cell antibodies/...

antibodies (Kerbel, 1980) which were completely incapable of directly haemagglutinating these cells. Other more sensitive assays including immunofluorescence or radiolabelled antibody could be employed. However sheep red cells are readily available and the rosette assay is relatively easy to perform and inexpensive. It is possible that the antibody may have a lower affinity for Fc receptors than those used in other systems.

In the present studies the presence of cytophilic antibody on the mononuclear phagocytes tested, was not investigated. By blocking Fc receptors such antibody may prevent binding of EA_G^{rab} to the monocytes or macrophages. When mononuclear leukocytes were isolated directly from whole human blood using Ficoll-Hypaque they had cytophilic antibody on their surfaces (Alexander et al., 1978b) and this decreased the binding of immune complexes compared to cells which had been washed prior to separation. It is not clear why Ficoll-Hypaque should cause cytophilic antibody to bind tightly to Fc receptors when it usually binds with low affinity. The author isolated human monocytes with Ficoll-Hypaque at 4°C unlike Alexander et al.(1978b) who performed their separations at 22°C. It is possible that cytophilic antibody does not bind to the same degree at 4°C under these conditions. Also, particulate complexes (EA_G^{rab}) were used rather than soluble immune complexes to detect Fc receptors. The former might be expected to bind with greater affinity than the smaller immune complexes (Phillips-Quagliata et al., 1971). Ögmundsdóttir and Weir (1976) found no evidence for cytophilic antibody on mouse peritoneal macrophages. So although the presence of some cytophilic antibody on human monocytes may account for the limited number of Fc rosettes it is unlikely to account for these findings on mouse peritoneal macrophages. It is not known whether guinea pig alveolar/...

alveolar macrophages possess such antibody on their surfaces but it seems unlikely since they were washed prior to use and were not separated on Ficoll-Hypaque.

There may be a population of human monocytes, mouse peritoneal macrophages or guinea pig alveolar macrophages which express Fc receptors poorly, either immature cells or possibly as yet unidentified subsets. However van Furth et al. (1980) found that 92% human monocytes and 100% mouse peritoneal macrophages expressed Fc receptors and Rhodes (1975b) reported that 80% guinea pig alveolar macrophages expressed Fc receptors. It is possible that if the mononuclear phagocytes had been incubated with EA_G^{rab} coated with the highest concentration of antibody, at 37°C rather than 4°C, slightly more cells would have rosetted, as indicated by the temperature dependence studies (Table I). However with all 3 cell types, there does appear to be heterogeneity of expression of Fc receptors which is in agreement with Norris et al. (1979) for human monocytes, Rhodes (1975b) for guinea pig alveolar macrophages and Pelus et al. (1981) for mouse peritoneal macrophages. Heterogeneity may be a reflection of different populations of cells or may be related to stages of cellular differentiation (maturation). Although in the present work there are insufficient points on the dose response curves (Fig. 4) to indicate the existence of sigmoidicity, in view of the work by Rhodes (1975) it seems likely that the curve is of sigmoid form. Such sigmoidal curves are often indicative of co-operative effects and therefore may be a consequence of co-operative interactions between Fc receptors as suggested by Tizard (1971) and Leslie and Alexander (1980). High avidity cells among the resident macrophage population may have been stimulated by naturally/...

naturally occuring irritants or pathogens and therefore show increased expression of Fc receptors (Zuckerman and Douglas, 1979). The observed heterogeneity of Fc receptors may reflect functional differences within macrophage populations. For instance Norris et al. (1979) found that the monocyte population which was larger in size and Fc receptor positive also showed high ADCC activity whereas the small monocytes which were mainly Fc receptor negative were inactive against sensitised red blood cell targets.

1.3 Fc (IgM) receptors

Sheep red cells sensitised with IgM were also tested for their capacity to bind to human monocytes at 37°C (Fig. 5) and at 0°C (results not shown). Under the experimental conditions described in this thesis there was no significant rosette formation by human monocytes and it was therefore decided to use these EA_M^{rab} cells in the preparation of complement coated intermediates to investigate the presence of complement receptors on various phagocytes.

The presence of Fc receptors for IgM on human monocytes has been described by Haegert (1979a) using a direct plaque forming assay. He found that neuraminidase treatment increased the expression of these receptors and they became detectable by a rosette assay (Haegert, 1979b). In the present work the author, using an essentially similar technique was able to detect fewer cryptic Fc (IgM) receptors on human monocytes ($14 \pm 1\%$ compared to $33 \pm 11\%$). The IgM used may have a lower affinity for Fc (IgM) receptors than the IgM used by Haegert. Another possibility is that phagocytosis of latex particles as employed by Haegert may have altered the expression of cryptic IgM receptors. Ingestion of latex particles is known to decrease certain plasma/...

plasma membrane constituents including Fc (IgG) receptors (Werb and Cohn, 1972; Doughaday and Douglas, 1976) and conceivably may increase the expression of Fc (IgM) receptors. Although neuraminidase treatment may have simply increased the stickiness of the monocyte membrane and thus led to enhanced binding of EA_M^{rab}, neither EA_G^{rab} nor EAC rosette formation were affected by neuraminidase treatment.

It has been suggested by Haegert (1979b) that these receptors may be involved in IgM dependent ADCC. Because the monocytes had phagocytosed latex particles, ingestion of EA_M^{rab} would not have been possible. Under the experimental conditions described in this thesis (20 minutes at 37°C) no phagocytosis was observed. Lay and Nussenzweig (1969) have described binding of EA_M^{mouse} by mouse peritoneal macrophages but very little phagocytosis was observed in contrast to that found with EA_G^{mouse}. However glycogen elicited murine macrophages do phagocytose EA_M^{mouse} (Mantovani, 1981). It may be significant that rosetting and phagocytosis did occur in this system when homologous IgM antibody was used.

Since fluid phase IgM inhibited rosette formation after neuraminidase treatment, these cryptic receptors must also recognise cytophilic IgM. Rhodes (1973) found that guinea pig splenic macrophages possess an Fc receptor for monomeric IgM. Thus normal monocytes and macrophages may have receptors for IgM which could passively arm these cells and bind antigen, thereby initiating phagocytosis or cell mediated cytotoxicity. Neuraminidase treatment of human peripheral blood neutrophils led to deminished cell viability and the formation of large clumps of cells and therefore the presence of cryptic Fc (IgM) receptors on these cells could not be investigated (normal neutrophils did not bind EA_M^{rab}). The present studies did not examine the presence of these receptors on other macrophages or other peripheral blood leukocytes./...

leukocytes. The small percentage of monocytes which possessed Fc (IgM) receptors may be a subpopulation of cells and further investigation into their expression of other receptors such as Fc (IgG) would be useful.

1.4 Complement receptors

Experiments using the rosette technique were performed to detect the presence of membrane receptors for various complement components on human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages. IgM coated sheep red blood cells (EA_M^{rab}) were used in preparing all the complement coated intermediates. The IgM was purified from whole rabbit immune serum as outlined in the Material and Methods by initial ammonium sulphate precipitation, Sephadex G-200 chromatography and further purification of the ascending IgM peak by Sepharose-4b-Protein A in order to remove any possible remaining contaminating IgG. This ensured that there was no possibility that any IgG was present and was confirmed by immunodiffusion. Thus Fc receptors could not be detected using these red cell intermediates.

Diluted AB serum was used for most of the experiments as a source of complement. However zymosan depleted serum (R3) which removes the terminal complement components and therefore allows more C3 to be deposited on the red blood cells, was used for the detection of complement receptors on certain leukocytes instead of diluted AB serum.

EAC formed by either method was immune adherence positive (see Materials and Methods, Part 6.0) indicating that C3b on the surface of the sheep red cells was present. However Newman et al. (1980) using R3 as a source of complement has shown that C3b´ was also present, presumably/...

presumably because both C3b1NA and β1H were present in the serum. Harrison and Lachmann (1980) using fresh human serum as a source of C3b1NA and β1H showed that fluid phase C3b was degraded by C3b´. Prolonged incubation (between 3 and 24 hours) with purified C3b1NA and β1H cleaved C3b´ into C3c and C3d by a contaminating DFP-sensitive protease. However prolonged incubation with fresh human serum did not lead to C3d production although aged human serum does contain C3c. Similarly Law et al. (1979a) using C3b and β1H contaminated with serum proteases observed the conversion of EAC3b into EAC3b´ and EAC3d (if DFP was not present). Using $^{1}/_{10}$ dilution of human AB serum chelated with 0.1M EDTA, EAC3b cells were converted into EAC3b´ but not EAC3d. Thus although proteases are present in serum which are capable of degrading EAC3b´ to EAC3d they are not normally present in sufficiently high concentrations. Similar degradation mechanisms for the conversion of EAC3b to EAC3b´ must also exist in both human AB serum and R3. However with monocytes in particular very dilute serum was used ($^{1}/_{700}$ dilution of AB serum or $^{1}/_{150}$ dilution of R3) and this may be sufficient to dilute out the effects of C3b1NA/β1H. With lower dilution of AB serum or R3 these components may become more important. The extent of degradation could be assessed with anti-C3c and anti-C3d and this may allow the proportions of C3b, C3b´ and C3d on the surface of the red cells to be calculated. Since AB serum or R3 treated red cells were immune adherence positive these indicator cells are probably mainly detecting C3b receptors. Although C3b´ and C3d may also be present, the surface of the red cells should be more akin to the in vivo state than the in vitro situation using purified components added sequentially, especially since the alternate pathway is also activated in the serum system/...

system and the amplification loop would lead to more dense clusters of C3b on the surface of the red cells (Muller-Eberhard and Gotze, 1972).

Human monocytes formed rosettes with erythrocytes sensitised with IgM and AB serum in a dose-dependent fashion using increasing concentrations of AB serum (Fig. 7). At the lowest possible dilution complement receptors were only detected on human monocytes and not on guinea pig alveolar macrophages or mouse peritoneal macrophages. This is in agreement with Dierich et al.(1974) who suggested that mouse macrophage complement receptors did not recognise C3b presumably because of species differences in the structure of either the C3b components or the C3b receptors or both. However by using R3, complement receptors could be demonstrated on both mouse peritoneal macrophages and guinea pig alveolar macrophages. At $^{1}/25$ dilution, 64% human monocytes formed rosettes with EAC(R3) whereas at $^{1}/10$ dilution 55% mouse peritoneal macrophages and only 17% guinea pig alveolar macrophages were complement receptor positive by this method. Comparison between these 3 cell types is difficult because there may be species variations as suggested above and in fact using mouse serum ($^{1}/2$ dilution), 60% mouse peritoneal macrophages were complement receptor positive and using guinea pig serum ($^{1}/1200$ dilution) 37% guinea pig alveolar macrophages formed rosettes with EAC (guinea pig). Human neutrophils and eosinophils also express fewer complement receptors than human monocytes (Anwar and Kay, 1977a). There results are in agreement with Ehlenberger and Nussenzweig (1977) for human monocytes and Mantovani et al. (1972) for mouse peritoneal macrophages. Although complement receptors have been detected on alveolar macrophages from various species/...

species including human (Reynolds et al., 1975) and rabbit (Atkinson et al., 1977), they have not been reported on guinea pig alveolar macrophages to the author's knowledge.

Optimal conditions were determined for the detection of complement receptors on human monocytes. Unlike Fc receptors, complement receptors were not expressed at 4°C, less than half the cells which could be shown to be complement receptor positive at 37°C, showed EAC binding at 23°C. This may be a reflection of the dependence of complement receptors on the fluidity of the membrane as suggested by Lay and Nussenzweig (1968). In contrast Fc receptor expression does not appear to be affected by membrane fluidity changes. Fc receptors have been shown to be able to move in the plane of the plasma membrane unlike complement receptors (Michl et al., 1979). Cytochalasin B decreased binding to Fc receptors (Atkinson and Parker, 1977) but has less effect on EAC binding to complement receptors although other cytochalasins had a greater inhibitory influence (Atkinson et al., 1977). Petty et al. (1981) found that complement receptors appeared to be arranged in clusters in the membranes of human peripheral blood monocytes and neutrophils and unlike other membrane components were relatively immobile. These observations suggested that Fc and complement receptors are coupled to the underlying cytoskeleton differently.

It has been reported in the literature that only activated macrophages (Bianco et al., 1975) and cultured human monocytes (which are presumed to differentiate into macrophages and become activated) (Newman et al., 1980) phagocytose complement coated erythrocytes. The low percentage of human monocytes which were observed eventually phagocytose EAC at 37°C may represent systemically activated monocytes. Such/...

Such activated cells have been suggested to occur in patients with cancer (Rhodes, 1977). C3b receptors on human monocytes were also detected using purified human complement components instead of AB serum. With either method at the highest concentration of complement on the red cells, not all monocytes formed rosettes. Similarly mouse peritoneal and guinea pig alveolar macrophages also did not appear to be 100% positive. This may be a reflection of the amount of C3 deposited on the red cells and more sensitive assays such as immunofluorescence or radiolabelled complement components may be able to detect more complement receptor positive cells. As with Fc receptors, this may represent a population of cells which express complement receptors poorly or not at all. However van Furth et al. (1980) reported that 88% human monocytes and 100% mouse peritoneal macrophages expressed complement receptors. The cells involved may be immature cells or unidentified subsets. Whether the populations of phagocytes which express Fc receptors poorly overlap with the populations which express complement receptors poorly is not known.

As with Fc receptors there was a direct relationship between the amount of C3 deposited on the red cells and the number of monocytes/macrophages forming rosettes (Figs. 7, 8 and 9). Again this may reflect heterogeneity of cells within the mononuclear phagocyte populataions with respect to complement receptor numbers or affinity or both. As far as the author knowns no reports in the literature have described heterogeneity of complement receptor expression on phagocyte subpopulations unlike those for Fc receptors. These differences in complement receptor expression may be important with regard to functional differences within a given population of cells.

Human/...

Human monocytes have been reported to express C4 receptors (Ross and Polley, 1975) although 18,000 molecules of C4 were required to obtain 70% monocyte rosettes compared to 2,000 molecules of C3. However although 6,000 molecules of C4 per cell were used to detect C4b receptors on human monocytes (an amount expected to give about 20% monocyte rosettes from Ross and Polley (1975)), no rosettes were formed. Possibly more C4 molecules per cell were required in this system but time and expense limited further investigation. In the present studies no experiments were undertaken to investigate the relationship between C3b and C4b receptors on human monocytes. Ross and Polley (1975) showed that the same receptor on human lymphocytes recognised both complement components since fluid phase C3, C3b and C4 inhibited both EAC14b and EAC1423b binding to lymphocytes.

It should be noted that the amount of C4 used to prepare it EAC1423 intermediates was limited (400 effective molecules per red cell) so the rosettes observed with these intermediates were thought to be entirely C3 dependent and it is highly unlikely that C4 even contributed to a limited extent in the formation of rosettes (especially considering that no rosettes were observed when the concentration of C4 was increased 15 fold). Receptors for C1 or C2 were not detected on human monocytes thus confirming the results of Gigli and Nelson (1968).

Recently Ross and Rabellino (1979) have reported the presence of $C3b'$ receptors on human monocytes and immature neutrophils and in the present study, the presence of $C3b'$ receptors on human monocytes was confirmed. $C3b'$ coated erythrocytes were prepared by using either purified KAF (or C3bINA or Factor I) or with $^{1}/_{10}$ dilution of AB serum chelated with EDTA (which inhibits both the classical and alternate pathway/...

pathways by removing Mg^{2+} ions) as a source of KAF according to the method of Law et al. (1979a). Observations by the author suggests that human monocytes may have higher affinity for C3b´ coated cells compared to C3b coated cells since monocytes rosettes had more erythrocytes bound per cell and they appeared to be more tightly bound as judged by microscopic examination.

Phagocytosis of C3b´ coated particles by cultured human monocytes has been reported (Newman et al., 1980) and since cell bound C3b in serum is probably rapidly cleaved to C3b´ by C3bINA (Law et al., 1979a; Carlo et al., 1979) C3b´ receptors on human monocytes may have more physiological relevance under these situations. Mature neutrophils do not appear to have C3b´ receptors probably because of the appearance of elastase on their surfaces as they mature, which converts C3b´ on the target particles to C3d for which neutrophils do not have receptors. However they do possess C4b receptors (Ross and Polley, 1975; Anwar and Kay, 1977a) and these may be funtionally important in vivo since C4b which can be cleaved by C3bINA (Ruddy and Auste, 1971; Cooper, 1975) may be partially protected from C3bINA action by the formation of either a short lived complex with C2 (Cooper, 1975) or with the low molecular weight C4b binding protein (Nagasawa and Stroud, 1978). Thus the C4b sites may be preserved for a sufficient length of time to allow binding of antigen-antibody complexes to the immune adherence (C3b and C4b) receptor bearing phagocyte. The presence of C5b receptors on human neutrophils (Segerling and Opferkuch, 1977) may be important in vivo for similar reasons. However as discussed in the Introduction, Part 8.0, natural activators of the alternate pathway of complement such as bacteria and yeast, have a high affinity for factor B and a low/...

low affinity for β1H (Pangburn et al., 1980; Kazatchkine et al., 1979) unlike erythrocyte target cells which are more likely to participate in the lytic pathway of complement. It seems probable that with both yeast and bacteria, C3b can act as an effective opsonin despite the presence of C3b1NA. Therefore both C3b and C3b′ receptors on human monocytes (or other phagocytes) are important in different situations - alternate pathway activators being removed via C3b receptors and nonactivators which have been coated with complement via the classical pathway, being removed by C3b′ receptors.

C3b′ is very susceptible to cleavage by proteolytic enzymes and EAC1423d was prepared by treating EAC1423b′ with a low concentration of trypsin. Human monocytes did not form rosettes with these indicator cells, confirming the results of Ross and Rabellino (1979). However Carlo et al. (1979) found that EAC3d attached to human monocytes.

1.5 Lectin-like receptors

Optimal conditions were determined for the binding of unopsonised bacteria, Staph. albus, to human peripheral blood leukocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages. Even at the highest possible concentration of organisms human leukocytes did not show more than 25% binding (Fig. 11). This may be a reflection of the assay system in that there was a limit to the number of bacteria which could be overlaid or it may be that only a small subpopulation of these cells express "lectin-like" receptors. Similar results were also found with mouse peritoneal blood monocytes (Dougherty, Stewart, Glass and Weir, unpublished observations). At 10^8 bacteria.ml^{-1}, the degree of binding for peritoneal macrophages from CF1 mice and guinea pig alveolar macrophages plateaued (Fig. 11) and although both these cell/...

cell types showed a much higher level of binding than did peripheral blood leukocytes, 100% binding was never observed. Similarly mouse peritoneal macrophages from other species did not express "lectin-like" receptors on all their cells (Glass et al., 1981b). There may be subpopulations which do not express these receptors or express low affinity receptors. Work in progress in this laboratory has shown that mouse peritoneal macrophages which have been elicited by an intraperitoneal injection of C. parvum (0.25mg/mouse) 4 days previously had significantly lower expression of "lectin-like" receptors as measured by Staph. albus binding. This suggests that the altered expression may represent the influx of monocytes from the blood which apparently have lower levels of receptors. The macrophages present in the peritoneal exudate population which are actually activated may or may not have altered receptor expression compared to resident peritoneal macrophages. Further work to investigate these possibilities (by separating out the putative subpopulations) is currently being undertaken. These results are in general agreement with those found by Ögmundsdóttir et al. (1976) and Freimer et al. (1978) who found that "lectin-like" receptors were present on C3H mouse peritoneal macrophages. Lectin-like receptors were demonstrated by the binding of various bacteria including Staph.albus at 4°C for 2 hours which the author assumed would be the optimal conditions for the other phagocytes tested for the presence of these receptors. However it is possible that binding at 37°C may have indicated that more cells expressed these receptors especially since H. Ögmundsdóttir (Ph.D. thesis, 1979) showed that they were temperature dependent in that more bacteria bound per cell at higher temperatures. Also, different/...

different phagocytes may preferentially recognise strains of bacteria other than Staph. albus. In the present studies no experiments were undertaken to investigate these possibilities.

Staph. albus binding to C3H mouse peritoneal macrophages was inhibited by certain monosaccharides (Freimer et al., 1978) and similar experiments were carried out with various other phagocytes (Figs. 12 and 13). D-glucose and D-galactose which are present in the cell wall of Staph. albus, reduced the attachment of this micro-organism to all phagocytes tested except neutrophils at 10 and 20 mM concentrations. No obvious differences in the inhibition patterns were observed. Higher concentrations than 20 mM had variable effects which were probably due to nonspecific alterations of the leukocyte membranes rather than specific effects on the lectin-like receptors. Van Oss (1978) suggested that the inhibitory effect of glucose on the phagocytic activity of neutrophils was due to the influence of this monosaccharide on cell shape. He observed that concentrations greater than 11 mM led to the withdrawal of pseudopods and the cells became spherical. Glucose may have increased the negative charge on the surface of the neutrophil membranes and therefore increased repulsive forces. It should be noted that both the glucose and galactose solutions were iso-osmolar and 20 mM is only 4 times the normal level in body fluids. At the highest concentration of sugars used, 100% inhibition was never observed, and was usually between 20 and 50%. Binding of bacteria may take place by other sugar components when one of them is blocked although experiments in which more than one sugar was added at the same time to the macrophage monolayers were inconclusive (J. Stewart, personnal communication). The bacteria may also be binding to the phagocyte surface by mechanisms independent of the recognition of these sugars. Non-

specific/...

specific binding involving hydrophobic and other membrane biomechanical forces may contribute to the adherence observed (Wilkinson, 1976). In some bacteria such as E. coli, S. typhimurium and C. parvum lectins are present in the bacterial cell wall itself. These are specific for mannose and appear to bind to that sugar on tissue cells (Ofek et al., 1977; Bagg et al., 1981). It is also probable that oligosaccharides would be more inhibitory in the present system because more interactions between ligand and receptor could be generated. Such considerations are bourne out by the findings of Schlepper-Schaffer et al. (1980). They found that desialated orosomucoid was 30 to 40 times more inhibitory than monosaccharides in reducing the binding of neuraminidase treated erythrocytes to rat peritoneal macrophages.

Attachment of Staph. albus to human neutrophils (and guinea pig peritoneal neutrophils (Glass et al., 1981b)) was not inhibitable by D-glucose at any of the concentrations tested suggesting that the "lectin-like" receptors on neutrophils may be different from these on other cell types. It is likely that the neutrophil receptors have a different role from those on macrophages and as suggested above they may preferentially recognise bacteria other than Staph. albus. Neutrophil "lectin-like" receptors may be important in the early stages of the inflammatory response in the removal of non-opsonised bacteria whereas macrophage "lectin-like" receptors are likely to be involved in the presentation of antigen leading to an immune response. Co-workers and the author have found that binding of Staph. albus to mouse peritoneal macrophages can be blocked by pretreatment of the monolayers with conventional and monoclonal antisera directed against Ia antigens of the H-2 system (Stewart et al., submitted for publication). The role Ia antigens in the co-operative cell interactions in the immune response

(see/...

(see Introduction, Part 4.0) is in support of the antigen presenting role of the "lectin-like" receptors on macrophages and their association with Ia antigens.

The *in vivo* importance of bacterial recognition by "lectin-like" receptors is also suggested by studies on diabetic mice (Weir *et al.*, 1981). Increased blood glucose levels appear by partially blocking the "lectin-like" receptors on leukocytes to make the animals more susceptible to infection by a glucose terminated mutant of *S. typhimurium* associated with a reduced ability of mouse peritoneal macrophages to bind this organism (Freimer *et al.*, 1978).

Although peripheral blood neutrophils expressed a comparatively low level of "lectin-like" receptor expression it is possible that neutrophils in an inflammatory response may express more receptors or receptors with higher affinity for non-opsonised bacteria. The differences between neutrophil and macrophage receptors with respect to sugar specificity may also be because they recognise different species of bacteria. This is borne out by the results of D. Kinane (unpublished observations) who found that unopsonised *N. gonorrhoea* adhered to 70% human peripheral blood neutrophils and only to 30% human monocytes at the same concentration of organisms (10^8 *N. gonorrhoea*.ml^{-1}). This is probably not surprising in view of the fact that neutrophils are the major phagocytic cells involved in the inflammatory response to obligate extracellular bacteria as typified by pyogenic *streptococci* and *staphylococci*. Mononuclear phagocytes on the other hand are of great importance in the defence against both obligate and facultative intracellular parasites such as *Mycobacterium leprae* and *Toxoplasma gondii*.

Eosinophil/...

Eosinophil "lectin-like" receptors appeared to have the lowest affinity for Staph. albus of all the cell types tested. Eosinophils also express fewer Fc and complement receptors than neutrophils or mononuclear phagocytes (Anwar and Kay, 1977a). This may explain their relatively poor phagocytic response which is slower than neutrophils in terms of the rates of ingestion (reviewed by Kay, 1974) and therefore it is unlikely that eosinophils play a major role as phagocytic cells. At present evidence suggests that eosinophils are important in immediate-type hypersensitivity reactions and they may be the principle cytotoxic killer cells in helminthic disease (reviewed by Kay, 1979). These processes presumably involve recognition mechanisms enabling contact between eosinophils and target cells such as Schistosoma mansoni which may depend not only on immunoglobulin and complement (Anwar and Kay, 1979) but also on "lectin-like" receptors recognising putative sugar-specific residues on the helminth surface. Antibodies directed against Schistosoma mansoni cross reacted with glycoprotein antigens (with an approximate 2:1 ratio of carbohydrate:protein) of Fasciola hepatica suggesting that similar glycoproteins are also expressed on the surface of S. mansoni (Hillyer and Sagramoso de Ateca, 1979). This hypothetical recognition system could be important in innate resistance to helminth infections which all mammalian species exhibit to some extent. Ellner and Mahmoud (1979) have shown that human monocytes have the capacity to kill S. mansoni, in vitro independently of antibody and complement.

The relationship between the "lectin-like" receptors described in this thesis and similar receptors reported by other workers (Stahl et al., 1978; Kolb and Kolb-Bachofen, 1978; Muchmore and Blaese, 1979) is not clear especially with respect to specificity and enzyme inhibition studies/...

studies and also probable differences in function (see Introduction, Part 12). For instance desialated sheep red blood cells bind to a trypsin sensitive receptor on human monocytes which may recognise D-galactose expressed on the surface of the desialated erythrocytes although Czop et al. (1978) did not actually show that this was the mechanism of attachment. These desialated red cells also bind to rat peritoneal macrophages and Kupffer cells by a pronase-sensitive galactose-specific "lectin-like" receptor (Kolb and Kolb-Bachofen, 1978; Nagamura and Kolb, 1980). Staph. albus and desialated erythrocytes may be recognised by the same receptor but some initial experiments by the author and co-workers were equivocable. Certain cell types appear to express receptors for Staph. albus and not for desialated red cells, and sugar inhibition studies with desialated erythrocytes did not confirm sugar specific binding.

Bacterial attachment through "lectin-like" receptors to mouse peritoneal macrophages is followed by phagocytosis when the preparations are allowed to warm up to 37°C (Weir and Ögmundsdóttir, 1980). It is not known whether the receptors on the other phagocytes examined in this thesis also mediate phagocytosis. Peripheral blood leukocytes express markedly fewer receptors than other cell types tested and possibly insufficient ligand-receptor interactions may occur to allow phagocytosis via the "zippering mechanism" of Griffin et al. (1975b) to take place. Although complement is not present in the experimental system used, the possibility that complement components produced by macrophages and also expressed on their surfaces (Brade and Bentley, 1980) are involved in the phagocytosis of unopsonised bacteria cannot be excluded. Michl et al. (1976) was able to distinguish between phagocytosis via Fc and complement receptors and phagocytosis by nonspecific receptors/...

receptors for latex and zymosan (which may in fact be specific sugar recognising receptors, see Introduction, Part 12). They found that 2-deoxyglucose inhibited phagocytosis through Fc and complement receptors but had no effect on ingestion of unopsonised particles. Since internalisation via complement receptors is clearly a separate event from phagocytosis by other receptors it would seem probable that ingestion of unopsonised bacteria is indeed independent of complement. However phagocytosis by "lectin-like" receptors and complement receptors may be synergistic as has been suggested for Fc and complement receptors (Ehlenberger and Nussenzweig, 1977). Putative "lectin-like" receptors on human monocytes which recognise desialated erythrocytes were found to act synergistically with complement receptors in inducing phagocytosis of complement coated desialated sheep red cells (Czop et al., 1978).

2.0 EFFECTS OF CHEMOATTRACTANTS AND OTHER PHARMACOLOGICAL MEDIATORS ON MONONUCLEAR PHAGOCYTES

2.1 Effects of chemotactic factors on C3 and Fc receptor expression

Using the rosette technique it was shown that the expression of complement but not Fc receptors on human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages were enhanced by chemoattractants in a dose- and time-dependent fashion (Figs. 14-23). These observations confirm and extend the previous studies on the eosinophil (Anwar and Kay, 1977b, 1978) which indicated that complement receptor enhancement by agents which promote cell locomotion might be a general biological phenomenon of phagocytic cells.

A major disadvantage of using the rosette technique in these studies, is the difficulty in measuring the degree of binding between the phagocyte membrane and individual indicator erythrocytes. For instance treatment with various chemoattractants not only increased the percentage of monocytes having 3 or more adherent red cells but the erythrocytes appeared to be more firmly attached than that observed in the untreated controls and also more red cells were bound per cell. This suggests that chemotactic factors may increase the numbers of complement receptors per cell or the affinity of the receptors for their ligands or both. The percentage of monocyte complement rosettes can be plotted against the concentration of complement coating the indicator red cells as shown in Figs. 7, 8 and 10. By comparing the profiles of such graphs using control monocytes/macrophages and chemoattractant stimulated cells, further information into the alterations of complement receptor expression and avidity may be obtained. Possible alterations in the numbers of red cells bound per phagocyte could also be examined. However the indicator cells are relatively large and steric hinderance effects may be/...

be important. More sensitive assays possibly using radiolabelled complement components would overcome these problems and Scatchard plots could be drawn which would allow the number of receptors per cell and the affinity of these receptors to be calculated. Initial attempts using fluid phase C3 and radiolabelled anti-C3 were unfortunately unsuccessful possibly because the radiolabel ^{125}I was not properly associated with the antibody.

Dose response curves and the time course of enhancement of human monocyte complement receptors by casein using EAC or EAC1423b as indicator cells were very similar (Figs. 14 and 15) suggesting that CR_1 expression is enhanced by chemotactic factors.

It should be noted that the amount of complement of IgG on the indicator red cells was limited to give about 25-35% rosettes in the untreated (control) monocyte/macrophage preparations. Even when optimal conditions were used for complement receptor enhancement it was unusual to achieve greater than 75% rosettes. In other words the degree of enhancement was not directly proportional to the control level of complement rosettes. Whether the control level of monocyte rosettes was 20% or 50%, chemotactic factors such as casein or f-Met-Leu-Phe increased the expression of complement receptors to a similar level i.e. between 60 and 70% rosettes. This suggested that not only was there a population of monocytes which express complement receptors poorly as discussed previously in Part 1.4 but possibly the same population also has a diminished response to chemotactic factors as well. Similar results were also found with mouse peritoneal macrophages. These results are essentially in agreement with those of Cianciolo and Snyderman (1981) who found that there is a subpopulation of human monocytes, consisting of about 60% of the total population of monocytes, which/...

which is capable of responding to any chemotactic factor. The remaining monocytes were unresponsive to all chemotactic factors. The patterns of enhancement by f-Met-Leu-Phe on each of the 3 cell types studied was different. Guinea pig alveolar macrophages showed the highest response although this was probably a reflection of the relative paucity of complement receptors on these cells (see Discussion, Part 1.5). With all the other phagocytes examined the amount of complement on the red cells was limited to give between 25 and 35% rosettes, but only 17% guinea pig alveolar macrophages expressed complement receptors with the highest possible concentration of R3. However after incubation with 10^{-7} moles.l^{-1} f-Met-Leu-Phe, 37% of these cells formed rosettes with EAC(R3) suggesting that there was a population of guinea pig alveolar macrophages which only expressed complement receptors after treatment with f-Met-Leu-Phe (at least under the conditions used here since it may be significant that 37% of these cells formed rosettes with EAC prepared with guinea pig serum).

Mouse peritoneal macrophages required a lower concentration of f-Met-Leu-Phe to elicit a response than that required by guinea pig alveolar macrophages or human monocytes. However at 10^{-8} moles.l^{-1}, mouse peritoneal macrophages had a diminished response to f-Met-Leu-Phe whereas human monocytes and guinea pig alveolar macrophages had a higher response at this dose than at 10^{-9} moles.l^{-1} (Figs. 17, 21 and 23). High dose inhibition was also observed with human monocytes at 10^{-10} moles.l^{-1} both in terms of chemotaxis and further complement receptor expression (results not shown). Internalisation of ligands and post receptor blockade may lead to deactivation of cells so that they can no longer respond to chemotactic factors (Vitkauskas et al., 1980). This is probably important in retaining cells at the site of inflammation. Paradoxically/...

Paradoxically high dose inhibition of complement receptor enhancement would be disadvantageous in a local inflammatory response; however this observation is possibly an artefact of the in vitro system. It can be envisaged that in vivo macrophages will arrive at an inflammatory focus with a high level of complement receptor expression as a result of stimulation by chemoattractants. Thus maximal complement receptor expression will have been attained by the time these cells are exposed to inhibitory concentrations of chemotactic factors. In the in vitro experimental system employed the cells with relatively low levels of complement receptors were immediately exposed to an inhibitory dose of chemoattractant and would thus be prevented from attaining further complement receptor enhancement. Alternatively it may be that in vivo such high levels of chemotactic factors may not occur. It should be noted that such high levels of chemoattractants did not decrease cell viability as assessed by trypan blue dye exclusion.

Casein on the other hand, was not as effective as f-Met-Leu-Phe in inducing complement receptor enhancement on mouse peritoneal macrophages as it was on human monocytes (Fig. 16 and 22). The observed differences in response to chemotactic factors by each of the cell types may be a reflection of the number of chemotactic receptors present on their surface membranes.

As discussed previously there may be changes in membrane properties after the cells have adhered to a substratum. However essentially similar results were obtained when mouse peritoneal macrophages were incubated with f-Met-Leu-Phe in suspension or as monolayers (Figs. 21 and Table III) although at the highest concentration used (10^{-8} moles.l^{-1}) a greater degree of enhancement was observed with the mouse macrophage monolayers compared to the suspensions. Thus these adherent cells do

not/...

not show high dose inhibition unlike the same cells in suspension. Although it is not as yet known whether other cells do not show high dose inhibition when they are adherent, this observation gives further credence to the idea that high dose inhibition may not be important *in vivo*. Wilkinson and Allan (1980) have made *in vitro* observations of human monocytes moving in response to chemotactic factors and have concluded that locomotion is a substratum-determined phenomenon (although sensing of the chemotactic gradients is not). Chemotaxis, phagocytosis and adherence by leukocytes involve similar processes in that in all three cases protrusions of motile membrane are formed. Dierich *et al.* (1977) have found that chemotactic factors had to be surface bound before cells would undergo chemotaxis *in vitro* and they suggested that *in vivo* cells would move up a concentration gradient of chemoattractant by systemically attaching and dissociating from surface-bound chemotaxins. Thus the response of adherent macrophages to chemotactic factors may be more relevant to the *in vivo* situation.

Heterogeneity of populations of monocytes and other macrophages was first described by Walker (1976b) and varying responses to chemotactic factors by different populations of monocytes has been demonstrated (Arenson *et al.*, 1980; Cianciolo and Snyderman, 1981). The ability of monocytes and macrophages to respond to chemoattractants and show enhanced expression of complement receptors appears to be another functional difference between putative subsets of macrophages.

F-Met-Leu-Phe binds to neutrophils very rapidly (Aswanikumar *et al.*, 1977; Williams *et al.*, 1977) and within 5 minutes most of the receptor sites are occupied. However binding of F-Met-Leu-Phe to guinea pig peritoneal macrophages appeared to be slower, saturating at about 20 minutes (Snyderman and Fudman, 1980). Complement receptor enhancement is also relatively rapid (Figs. 15, 16 and 18) and would appear/...

appear to be an almost immediate consequence of chemotactic factors interacting with their receptors on the leukocyte membrane. The effects of f-Met-Leu-Phe on human leukocytes appears to depend on the cell type involved (Fig. 18). Neutrophils were maximally enhanced at 10^{-8} moles.l^{-1} (Kay et al., 1979) whereas monocytes required a higher concentration (10^{-7} moles.l^{-1}) (Fig. 17) and eosinophils also required this higher dose of f-Met-Leu-Phe to show a maximum response. Neutrophil complement receptor enhancement was the most rapid and eosinophil, the slowest. These differences in rates may reflect the kinetics of chemotactic factor binding. The observed increase of complement receptor expression by human eosinophils with f-Met-Leu-Phe was much faster than that reported by Anwar and Kay (1978) for ECF-A or histamine. The number of chemotactic factor binding sites present on the phagocyte membrane may determine the rate of response as is indicated by the results shown in Fig. 16. Since the rate of enhancement of human monocyte complement receptors was directly proportional to the concentration of casein, this suggests that the number of ligand-receptor interactions determines the rate as well as the degree of response to chemotactic factors. The slower rate of enhancement by eosinophils may also be a reflection of the lower density of C3b receptors found on these cells (Anwar and Kay, 1977a).

The capacity of casein to attract human monocytes and other leukocytes in directional and random migration is well established (Keller and Sorkin, 1967b) and it therefore seemed reasonable to use this agent in many of the experiments described in this thesis. However a number of other agents with chemotactic activity for monocytes gave comparable increases in the percentages of complement rosettes (Figs. 17 and 19). These included supernatants from lymphocytes cultured in the presence of phytohaemagglutinin/...

phytohaemagglutinin (PHA) (Snyderman et al., 1972), a supernatant from C. parvum (NCTC 10390) (Wilkinson et al., 1973) and several formyl methionyl peptides (Showell et al., 1976). All these chemoattractants have very different physicochemical properties; casein is an amphipathic protein with a molecular weight of about 20,000 daltons (Wilkinson, 1972), the C. parvum derived chemotactic factor appears to be lipid in nature (Russell et al., 1976), the chemotactic agent in the supernatant of PHA stimulated lymphocytes is a protein with a molecular weight of 12,500 daltons (Altman et al., 1974), and the formyl methionyl peptides are relatively small molecular weight (200-300 daltons) amphipathic chemoattractants (Wilkinson, 1979). Snyderman et al. (1972) found a certain amount of monocyte chemotactic activity in supernatants from unstimulated lymphocytes. In the present studies the highest concentration of the unstimulated supernatants also gave enhancement of monocyte complement rosettes. Kay et al. (1979) have shown that f-Met peptides promoted monocyte migration in vitro and it appears that both chemotaxis and complement receptor enhancement occured in parallel. This is indicated by the observation that increasing the concentrations of f-Met peptides promoted concomitant increases in both the degree of complement receptor enhancement and the number of monocytes migrating through micropore filters. In contrast the unformylated peptides Met-Leu-Phe and Met-Met-Phe were inactive in both biological systems suggesting that the specificity for formylation applies to both complement receptor enhancement and locomotion. Studies on the relative chemotactic activities of various f-Met peptides have previously been reported for neutrophils (Showell et al., 1976) - f-Met-Leu-Phe > f-Met-Met-Phe > f-Met-Phe and similar results were obtained in these studies (Kay et al., 1979). However/...

However no previous work comparing the relative potencies of these peptides for human monocyte chemotaxis had been undertaken. With monocytes about 100 fold more of these peptides was required to give comparable biological effects to that of the neutrophil. Furthermore with human monocytes f-Met-Phe had similar potency to f-Met-Met-Phe both in cell locomotion and rosette enhancement. This is in contrast to the report by Showell et al. (1976) in which F-Met-Met-Phe was 1900 fold more active than F-Met-Phe in promoting chemotaxis of rabbit neutrophils. These observations all support the view that there is a direct relationship between cell locomotion and enhancement of complement receptors. It has been shown that casein (Keller and Sorkin, 1967b), the f-Met peptides (Showell et al., 1976) and the C. parvum chemotactic factor (Russell et al., 1976) promote directional migration as well as increasing the rate of cell movement. Human serum albumin which is chemokinetic but not chemotactic (Wilkinson et al., 1977) also increased complement receptor expression on human monocytes (Fig. 20) suggesting that complement receptor enhancement is dependent on the chemokinetic principle of chemoattractants rather than the chemotactic principle. Thus agents which increase cell movement are important in inducing increased expression of complement receptors on phagocytic cells.

The apparent inability of casein or f-Met-Leu-Phe to alter Fc receptors for IgG on human monocytes, mouse peritoneal macrophages or guinea pig alveolar macrophages (Figs. 15, 21 and 23) is in agreement with previous observations on the eosinophil where chemoattractants affected the expression of complement but not Fc receptors (Anwar and Kay, 1977b, 1978). Similarly Goetzl et al. (1980) showed that HETE's increased/...

increased complement but not Fc receptor expression on human eosinophil and neutrophil membranes. The reasons for these apparent differences between complement and Fc receptors is not clear at present but may be explained by the experimental procedure. For instance optimal Fc (IgG) rosettes were obtained at 4°C whereas 37°C is used for detection of complement receptors. Also, in the time course experiment with human monocytes (Fig. 15) a slight increase in Fc receptors was found but this was not statistically significant. It is possible that there may only be changes in affinity and not changes in numbers of Fc receptors and this may not be detected in the present assay system. Further studies using more sensitive techniques (e.g. immunofluorescence or radiolabelled IgG) will be required before more definite conclusions can be reached. Recent work by Capron et al. (1981) has shown that eosinophil Fc receptors are increased following stimulation with the chemotactic factors, ECF-A and histamine whereas neutrophil Fc receptors are often decreased after incubation with these substances. The reasons for these discrepancies between this work and previous work by Anwar and Kay (1978) and Goetzl et al. (1980) are not clear especially since they were using the same assay system. Capron et al. (1981) suggested that the eosinophils which they were using were from patients with slight eosinophilia whereas those which Anwar and Kay (1978) used were from patients with more severe eosinophilia and there may have been more immature cells present, possibly with deficient Fc receptors. However this does not explain the observations that Fc receptors on macrophages are not affected by chemoattractants. It may be that although Fc receptors are enhanced by chemotactic factors, they are also shed during the incubation period with the chemotactic agents and/...

and therefore no increase in Fc receptor expression would be observed. Fc receptor shedding by human lymphocytes has been reported (Sandor et al., 1979) and there appeared to be two populations of Fc receptors, one which was shed and one which remained on the membrane. It may be that chemotactic factors preferentially increase Fc receptors which are then shed and/or increase the shedding rate. Detection of Fc receptor material in the medium (by blocking Fc rosettes of Fc receptor bearing cells) would clarify this point.

Fc receptors for IgM and C3b´ receptors on human monocytes are also apparently unaffected by chemoattractants (Tables IV and V). Since increases in these receptors were not observed using red cells sensitised with IgM, it is further confirmation that increased C3b rosette formation is due to enhanced complement receptors (CR_1) and not Fc (IgM) receptors. Thus chemotactic agents do not expose cryptic binding sites of the Fc portion of IgM unlike Vibrio cholerae neuraminidase which apparently had this effect on rabbit and human monocytes (Haegert, 1979a, b; this thesis, Fig. 5). Since no enhancement of rosettes with EAC1423b´ cells was detected, presumably CR_3 are not affected nor are cryptic CR_3 expressed after incubation with chemotactic agents.

Thus there appears to be a selective enhancement of C3b receptors (CR_1) by chemotactic or chemokinetic agents.

Following incubation with certain lymphokines, cultured macrophages were found to have augmented the functional capacity of their complement receptors in that the cells could phagocytose via complement receptors as well as bind complement coated particles (Griffin and Griffin, 1980). It is possible that this lymphokine has similar effects as chemotactic factors/...

factors as observed in the author's system. Increasing the numbers of complement receptors on the surface of the macrophages and thereby increasing the number of points of attachment may allow phagocytosis to occur via the "zipper mechanism" as suggested by Griffin et al. (1975b) and Shaw and Griffin (1981) for Fc receptors. It is not as yet known whether chemotactic factors also increase phagocytosis of EAC by monocytes and macrophages as well as enhancing the binding of these indicator red cells.

Since lymphocytes are also present with some of the cell preparations chemotactic agents may be exerting their effects on the lymphocytes and inducing lymphokine production which may then have an enhancing effect on the complement receptors of other leukocytes present. However this is highly unlikely since (a) no lymphocytes are present in the granulocyte preparations and their complement receptors are enhanced by chemoattractants and (b) lymphokine production by lymphocytes requires 2-3 days in culture, whereas the effects of chemotactic factors are evident within 5 minutes of incubation.

2.2 Effects of chemoattractants on "lectin-like" receptors

Concomitantly with the increase in complement receptors on mouse peritoneal macrophages, the expression of "lectin-like" receptors is decreased. The dose response curves for enhancement of complement rosettes (Figs. 21 and 22) and inhibition of binding of Staph. albus (Fig. 24) by f-Met-Leu-Phe and casein are very similar, suggesting that there is a direct relationship between these events. In view of the established specificity of the "lectin-like" receptors for monosaccharides such as glucose and galactose (Figs. 12 and 13) it is unlikely/...

unlikely that the receptor is being directly inhibited by the formylated peptide or casein. The "lectin-like" receptor and the binding sites for these chemotactic factors may be closely related and internalisation of the chemoattractant receptors (Vitkauskas et al., 1980; Niedel et al., 1979) may also lead to the loss of the "lectin-like" receptor. Many cell surface receptors are greatly reduced in number (down regulation) after exposure of the cells to their ligand particularly in the case of hormones and other substances inducing considerable changes in their target cells (Tulkens et al., 1980). Mouse peritoneal macrophages elicited by intraperitoneal injection of C. parvum 4 days previously also have decreased lectin-like receptor expression (Dougherty, Glass, Stewart and Weir, unpublished observations) suggesting that activated macrophages may also have decreased expression of these receptors. However it is not as yet clear whether the effects observed are due to the increased influx of blood monocytes which have fewer "lectin-like" receptors or whether activated macrophages do have decreased expression. Stahl et al. (1980) have shown that mannose-specific "lectin-like" receptors on rat alveolar macrophages are continuously being recycled from the plasma membrane to lysosomal membranes and back again to the plasma membrane, either in the presence or absence of ligand. It is possible that the "lectin-like" receptors described here also undergo recycling and it may be that chemotactic factors increase both the rate of recycling and the intracellular pool such that there is an apparent decrease in surface receptors. When both the ligand (bacteria) for the lectin-like receptor and the chemotactic factor are present together, this could lead to increased phagocytosis of bacteria.

2.3/...

2.3 Effects of pharmacological mediators on complement receptor expression

The effects of various pharmacological mediators on monocyte complement receptors were also examined.

Prostaglandins are involved in inflammatory reactions and are synthesised and secreted by macrophages (Bray, 1980; Bonney et al., 1980). In vivo prostaglandins of the E-series may be particularly important since they regulate leukocytes under physiological conditions (Gordon et al., 1976) and would appear to be the major prostaglandin produced by macrophages in response to inflammatory stimuli (Bonney et al., 1980; see also Introduction, Part 4.0). They bind to human leukocytes possibly via specific receptors (Bourne and Melmon, 1971) and influence various macrophage functions. For instance PGE_2 enhanced the specific activity of 5´ nucleotidase and acid phosphatase in cultured human monocytes particularly in a subpopulation of monocytes which were low density cells with a low specific activity of 5´ nucleotidase. This subpopulation of cells also secreted substantial amounts of PGE_2 compared to high density macrophages and it appeared that this endogenous PGE_2 was involved in the activation of monocytes in vitro (Picker et al., 1980). Prostaglandins E_1 and E_2 were chemotactic for mouse peritoneal macrophages at relatively high concentrations (3×10^{-6}M) and inhibited spreading and adherence at lower concentrations (3×10^{-8}M) (Cantarow et al., 1978). However Gallin et al. (1978) found that PGE_1 inhibited monocyte chemotaxis at a concentration of 1.4×10^{-5}M. Phagocytosis of IgG coated particles by mouse peritoneal macrophages was enhanced by PGE_1, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and PGA_1 (Razin et al., 1978). In the present studies $PGE_1 E_2$ and $F_{2\alpha}$ all had enhancing effects on monocyte/...

monocyte complement receptors (Fig. 26). At 10^{-6}M, PGE_1 showed maximal activity whereas PGE_2 had peak activity at 10^{-7}M. Thus these results are as might have been expected from the chemotactic response to these agents (Cantarow et al., 1978). It is of interest to note that PGE_1 at 10^{-5} moles.l^{-1} had a diminished effect on monocyte complement receptors and also had a lower enhancing effect on phagocytosis of EA_G by macrophages (Razin et al., 1978) and also inhibited monocyte chemotaxis in vitro (Gallin et al., 1978). These results are in contrast to those of Atkinson et al. (1977) who found that 3 x 10^{-5}M PGE_1 caused slight inhibition of adherent rabbit alveolar macrophages. The contrary results observed may be due to species differences or to differences between adherent cells and cells in suspension. $PGF_{2\alpha}$ had no effect on macrophage chemotaxis, adhesion or spreading even at the highest concentrations tested (10^{-5}M) (Cantarow et al., 1978). However it has been shown that $PGF_{2\alpha}$ enhances leukocyte chemotaxis (Hill, 1978) and at 10^{-4} moles.l^{-1} $PGF_{2\alpha}$ was found to enhance monocyte complement receptor expression.

Histamine receptors are reported to be present on guinea pig alveolar macrophages and on other phagocytes (Diaz et al., 1979). Similar results have been found on human peripheral blood monocytes (Lesley M. Smart, unpublished observations). It is of interest to note that histamine apparently increased the phagocytic capacity of rat macrophages (Jancso, 1947). Although the numbers of histamine receptors were not altered in atopic individuals (L.M. Smart, unpublished observations) complement receptor enhancement was apparently altered in these individuals in response to histamine (Fig. 28). Monocytes from normal individuals were generally either unaffected by histamine or showed decreased complement receptor expression whereas many/...

many of the atopic patients had increased complement receptors on their monocytes. Neutrophil complement receptors on cells from atopic and non-atopic individuals showed varying responses to histamine which is in contrast to the results of Anwar and Kay (1978) who found that histamine had no effect on neutrophil complement receptors. The reason for these discrepancies are not clear but in the author's study the neutrophils came from normal individuals and patients with slight eosinophilia whereas those of Anwar and Kay (1978) were from patients with more severe eosinophilia and they may also have other leukocyte defects.

A study by the author and co-workers (Glass et al., 1981a) has suggested that complement receptors may be altered on monocytes in neoplastic disease. The response of monocytes in chemotaxis is diminished in patients with cancer (Boetcher and Leonard, 1974; Hausman et al., 1975), particularly when metastases are also present (Kay and McVie, 1977) and Fc receptors are also altered in cancer patients (Rhodes, 1977). Low molecular weight fractions from human lung cancer specimens, whilst having no effect on complement receptors on normal human monocytes per se, prevented the enhancement of these receptors in response to chemotactic factors. Thus quantitation of complement receptor enhancement might provide a further variable for studies of monocyte function in health and disease.

Bradykinin, a basic nonapeptide, is one of the vasoactive plasma kinins. It is detectable in tissues in experimental anaphylactic shock and has a slower action compared to that of histamine. In vitro at relatively high concentrations (10^{-5} to 10^{-6}M) bradykinin inhibited the spreading of mouse peritoneal macrophages and at lower concentrations (10^{-7}/...

(10^{-7} to 10^{-8}M) it enhanced spreading (Fauve and Hevin, 1977) suggesting that macrophages may have bradykinin receptors. Agents which increase the spreading activity of cells also inhibit chemotaxis (see Introduction, Part 5.10). Although the effects of bradykinin on mononuclear phagocyte chemotaxis have not been reported, at the concentrations which inhibited macrophage spreading (and therefore might be expected to enhance chemotaxis) also increased the expression of human monocyte complement receptors, whereas at 10^{-7}M which increased spreading a lower level of complement receptor enhancement was observed.

Numerous reports have suggested that increased intracellular levels of cyclic AMP (cAMP) inhibit their functions (see Bourne et al., 1974 for a review). Cyclic nucleotide changes may also be involved in the response of leukocytes to chemotactic factors and as discussed below, prostaglandins, histamine, and bradykinin may be mediating their effects on complement receptors by affecting cyclic nucleotide levels in the leukocytes. Simchowitz et al. (1980) has reported that chemoattractants induce a transient increase in intracellular cAMP in neutrophils, although agents which lead to a generalised increase in cAMP are inhibitory in chemotaxis; cGMP agonists, on the other hand, enhanced locomotion (Hill, 1978).

PGE_1 increased cyclic AMP levels in human monocytes (Gallin et al., 1978) and both PGE_1 and PGE_2 have been shown to increase adenyl cyclase activity in human leukocytes (Bourne and Melman, 1972) and peritoneal macrophages (Grunspan-Swirsky and Pick, 1979). β adrenergic receptors have been demonstrated on rat peritoneal macrophages (Ikegami, 1977) and human leukocyte intracellular cAMP levels were increased after isoprenaline had interacted with specific cell membrane β adrenergic receptors/...

receptors linked to membrane bound adenyl cyclase (Bourne and Melman, 1971). Propanolol, a β adrenergic antagonist, inhibited the effects of isoprenaline but not PGE_1 or PGE_2 effects on cAMP levels, and the effects of prostaglandins and isoprenaline were not additive, suggesting that they act on separate receptors but activate the same adenyl cyclase.

Ito et al. (1977) found that β adrenergic agents such as isoprenaline increased mouse splenic lymphocyte complement receptors with peak activity at 10^{-6}M whereas dibutyrl cAMP or aminophylline (a phosphodiesterase inhibitor) had no effect on these receptors. Since these latter 2 agents increase intracellular cAMP concentrations beyond the level of action of adenyl cyclase, these workers concluded that stimulation of this enzyme itself rather than cAMP levels affected complement receptor expression. Histamine increases cAMP levels in leukocytes (Bourne et al., 1974) and inhibits leukocyte chemotaxis (Hill, 1978). Bradykinin increases both cAMP and cGMP levels in lung tissue, although cAMP increases appear to be secondary to prostaglandin production (Stoner et al., 1973). Seratonin increases cGMP levels in human monocytes and also enhances locomotion by these cells (Gallin et al., 1978). Similarly $PGF_{2\alpha}$ also causes increased intracellular levels of cGMP and enhances leukocyte locomotion (Hill, 1978). Thus agents which have been shown to increase cGMP production in leukocytes ($PGF_{2\alpha}$, bradykinin and seratonin) also enhance monocyte complement receptors whereas agents which increase cAMP levels (PGE_1 and PGE_2, histamine and isoprenaline) have varying effects on complement receptor expression. However isoprenaline at 10^{-5}M increases cGMP levels in human neutrophils concomitantly with rises in cAMP levels (Hatch et al., 1977)/...

1977) and at this concentration there is a moderate increase in monocyte complement rosette formation suggesting that isoprenaline may be exerting an effect through cGMP production at 10^{-5}M. The changes in cGMP levels induced by isoprenaline are believed to result from α-adrenergic stimulation by this agent which is a weak α-agonist (Fleisch et al., 1972) and human leukocytes may have α-adrenergic receptors (Logsden et al., 1972). The modulating action of intracellular nucleotides is dependent on the ratio of cAMP/cGMP (Goldberg et al., 1973) and at 10^{-5}M the effects of isoprenaline on cGMP levels may overpower any possibly effects that cAMP is having on complement receptors. The enhancing effects brought about by raising cGMP levels is in contrast to the results of Atkinson et al. (1977) who found 8-bromo cGMP or carbachol (cholergic agonist) had no effect on complement receptors on rabbit alveolar macrophage monolayers. This may be due to species differences or experimental procedure since the cGMP agonists were only incubated with the cells for 10 minutes whereas in the author's studies, the various substances were incubated with the cells for 1 hour.

Prostaglandins E_1 and E_2 enhanced monocyte complement rosettes in contrast to histamine or isoprenaline which either had no effect or decreased complement receptor expression on human monocytes. However prostaglandins may also be chemotactic factors (Cantarow et al., 1978) unlike histamine or isoprenaline and might therefore be expected to have different effects from these latter 2 agents.

The effects of cAMP and cGMP agonists were also tested for their effects on neutrophil complement receptor enhancement by submaximal doses of f-Met-Leu-Phe (Duncan, Glass, Stewart and Kay, unpublished observations). cGMP agonists increased f-Met-Leu-Phe enhancement whereas cAMP/...

cAMP agonists inhibited enhancement. These results together with the results presented in this thesis suggest that cyclic nucleotide levels modulate phagocyte responsiveness to chemotactic factors in chemotaxis (see Introduction, Part 5.12) and may also directly modulate complement receptor expression. Chemotactic factors increase lysophophatidylcholine in leukocyte membranes by stimulating phospholipase A_2 (Hirata et al., 1979) and this has been shown to have a direct effect on guanyl cyclase activity (Shier et al., 1976). Thus increased cGMP levels or simply stimulation of guanyl cyclase by chemotactic factors or cGMP agonists may modulate the expression of complement receptors. Further investigation into the role of cAMP levels or stimulation of adenyl cyclase is necessary to elucidate their involvement in complement receptor expression.

2.4 Possible mechanisms of receptor modulation

As described elsewhere (Anwar and Kay, 1978) possible explanations of the mechanisms of complement receptor enhancement include "membrane unfolding", "receptor externalisation" and "subunit association". F-Met-Leu-Phe increases the cell volume of human neutrophils and this is associated with an increase in cell surface area (O'Flaherty et al., 1977) which may be due to unfolding of membrane pleats or ruffles. Thus more complement receptors may be exposed. However it might be expected that other receptors would be likewise increased, and this is not the case since Fc receptors are apparently unaffected and "lectin-like" receptors are decreased. Electron microscope studies could be carried out to elucidate the role that increased cell surface area may play in modulating receptor expression. Functional complement receptors may be composed of inactive subunits which require aggregation induced/...

induced by chemotactic factors for efficient binding. Multiple ligand-receptor cross-links are necessary for the binding of opsonised particles (Phillips-Quagliata et al., 1971; Shaw and Griffin, 1981). Kaplan et al. (1978) and Michl et al. (1979) have shown that complement receptors are not normally mobile in the plane of macrophage membranes. However with thioglycollate-induced macrophages, these receptors are mobile and are also able to lead to phagocytosis in a similar way that a T cell lymphokine induces complement receptor mediated phagocytosis (Griffin and Griffin, 1979, 1980). These differences between CR_1 and Fc receptors in mobility may explain why chemotactic factors have no effects on Fc receptors since they are able to aggregate and bind more efficiently in the absence of any stimulating agent. Complement receptors, on the other hand, may require an extra signal in order to show enhanced receptor expression and augmented phagocytic capabilities. The generation of new receptors is considered unlikely since optimal enhancement occurs within 60 minutes. However Kohn and Twarog (1979) have shown that new synthesis of complement receptors on Null cells can occur within 15 minutes following stimulation with cAMP agonists. So possibly chemoattractants may increase de novo synthesis and insertion of new complement receptors into the plasma membrane. Experiments with protein synthesis inhibitors could clarify this issue. Apart from increases in numbers of CR_1s it is also possible that chemotactic factors may increase the binding affinity of these receptors for their ligands, possibly by causing steric changes in these receptors.

Low density lipoprotein receptors on human fibroblasts are constantly being recycled by insertion and endocytosis into and from coated pits in the plasma membrane (Goldstein and Brown, 1977); mannose specific "lectin-like" receptors are also recycled in a similar/...

similar manner (Stahl et al., 1980). If complement receptors are also turned over in a similar fashion, chemotactic factors might increase membrane recruitment of complement receptors either by enhancing the insertion or blocking the endocytic removal of these receptors. It has previously been suggested that the disappearance of the bacterial cell wall sugar recognising "lectin-like" receptors may be due to internalisation along with the chemotactic factor binding site. C3b receptors and Fc receptors may also be internalised along with chemoattractants. Increased CR_1 expression and the apparent inability of f-Met-Leu-Phe and casein to alter the expression of Fc receptors may be due to the resistance of these receptors (in contrast to "lectin-like" receptors) to lysosomal enzyme degradation in endocytic vesicles. These receptors may be rapidly recycled and re-expressed on the surface of the macrophage. Temperature dependence studies (Figs. 32 - 35 and Table VI) indicate that human monocyte and neutrophil complement receptors are not enhanced at 4°C and mouse peritoneal macrophage "lectin-like" receptors were not inhibitable at 4°C as would be predicted if recycling was involved in the modulation of these receptors. However pinocytosis of plasma membrane is directly proportional to temperature (Silverstein, 1977) and Figs. 32 - 34 show that this is not the case with complement receptor enhancement. Instead there is a marked change in the slope of the graph between 23°C and 30°C. Similarly increasing the length of incubation from 30 to 120 minutes (Fig. 35) had no further effects at 23°C or 30°C. Endocytosis alone, also cannot account for the observed increase in complement receptors. An increase in the insertion of newly synthesised complement receptors or receptors already present in a putative intracellular pool, and/or a decrease in removal would also be necessary for the observed effects

on/...

on complement receptor expression. (A recent report by Thrall et al., (1980) has described the appearance of a 54,000 dalton protein and the disappearance of a 44,000 dalton protein in neutrophil membranes following stimulation with f-Met-Leu-Phe or C5a. These membrane alterations may be significant in terms of complement receptor enhancement or inhibition of "lectin-like" receptors.)

Complement receptor enhancement by human monocytes and neutrophils appears to be reversible (Figs. 29 - 31). About thirty minutes after the chemotactic factor is removed, the numbers of complement rosettes returns to basal levels. The continuous presence of chemotactic factor is therefore essential for maintenance of enhanced complement receptor expression and the decreases observed may be due to turnover of the newly expressed receptors, possibly by endocytosis or shedding into the medium. It is possible that the leukocytes may then be capable of responding to the stimuli again or else they may remain deactivated. Vitkauskas et al. (1980) has shown that f-Met-Leu-Phe is internalised after interacting with its receptor and removal of the plasma membrane receptors and post receptor blockade renders neutrophils unresponsive to further stimulation by chemoattractants. In vivo neutrophils are relatively short-lived cells and do not differentiate any further once they enter the tissues and presumably die there. Monocytes, on the other hand, enter the tissues and differentiate into macrophages and may also become activated. Thus monocytes may respond to a further chemotactic stimulus whereas neutrophils may not.

Expression of complement receptors appears to depend on membrane fluidity unlike Fc receptors (Lay and Nussenzweig, 1968). Complement receptor enhancement by monocytes and neutrophils (Figs. 32 - 34) is also temperature dependent and there appears to be a threshold level

below/...

below which it does not occur to any significant degree; only between 23°C and 37°C is a marked increase in complement receptor enhancement observed. A unique fluid state of the membrane may be necessary for the binding of chemotactic factors to the membrane. However binding of radiolabelled formyl peptides to neutrophils does occur at 4°C (Aswanikumar et al., 1977; Williams et al., 1978). It is more likely that membrane fluidity is important in the transduction of the effects of chemotactic factor-receptor interactions on complement receptor expression. Arrhenius plots of enzyme functions in artificial membranes such as (Na^+K^+) ATPase (Kimelberg and Papahadjopoulos, 1972) and in biomembranes such as the lactose permease system of E. coli (Overath et al., 1975) have yielded bi- and tri-phasic plots which seemed to correlate with phase changes in the membranes dependent on the characteristic transition temperatures, Tc, of the surrounding lipid matrix. However most biological membranes are heterogeneous in nature with many different phospholipids in the bilayer interspersed with cholesterol and protein, all of which might be expected to broaden any overall phase change. The sharp change in the degree of complement receptor enhancement between 23°C and 37°C suggests that there might be localised phase changes and the temperature dependence of complement receptor enhancement may be dependent on such localised alterations in membrane fluidity. Thus binding sites for chemoattractants may be intimately associated with the lipid matrix of the leukocyte membrane and may depend on a defined lipid environment in order to express their effects as suggested by Wilkinson (1979) who found that perfringolysin and phospholipase C, which are membrane modifying agents, decreased the responsiveness of neutrophils to f-Met-Leu-Phe.

Membrane/...

Membrane fluidity (Haak et al., 1979) may be altered by f-Met-Leu-Phe following receptor-ligand interaction, possibly by altering phospholipid methylation in the plasma membrane (Pike and Snyderman, 1980; Hirata et al., 1979; Hirata and Axelrod, 1978).

Increases in membrane fluidity may increase complement receptor expression by affecting the local environment of these receptors and by doing so may alter their affinity for their ligand, C3b. Similarly "lectin-like" receptors may show diminished expression because fluidity changes have decreased their affinity for their ligands. Fc receptors which are not so dependent on membrane fluidity may thus be unaffected. Fluidity changes may also affect complement receptor enhancement by increasing receptor mobility in the membrane and thus increasing the probability of adequate cross-linking or possibly if complement receptors are composed of inactive subunits which require aggregation for efficient binding, increased fluidity may facilitate aggregation. The use of radiolabelled complement components and membrane fluidity probes may help to develop further our understanding of the phenomenon of chemotactic factor induced modulation of surface receptors on phagocytes.

3.0 GENERAL CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

Human peripheral blood monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages were all relatively easily obtained in sufficient quantities for the experimental procedures described here. Human monocytes required further separation from granulocytes and red blood cells but the other two cell types only required washing before they could be used. Thus very few manipulations of the cells were necessary, minimising possible selection of subpopulations or decreasing the cell viability.

All three cell types examined, expressed C3b (CR_1), Fc (IgG) and lectin-like receptors although different levels of receptor expression both between the cell types and within the individual populations were evident. Receptors for the Fc portion of IgM were expressed on human monocytes after neuraminidase treatment and their presence on other cell types could be investigated. Lay and Nussenzweig (1969) detected Fc (IgM) receptors on mouse peritoneal macrophages using homologous IgM (these receptors did not require neuraminidase treatment for their expression and were thus not cryptic). Possibly other cell types including human monocytes, may express Fc (IgM) receptors for homologous IgM. The physiological role of these receptors in IgM dependent cell mediated cytotoxicity and phagocytosis could be studied. Human monocytes express C3b and C3b' (CR_1 and CR_3) but not C3d (CR_2) receptors and their presence (or absence) on other cell types could be investigated. The use of homologous complement components may provide more information on such receptors, especially since these would be more relevant to the <u>in vivo</u> role of complement receptors. Mouse peritoneal macrophages (Unkeless, 1980) and guinea pig peritoneal macrophages/...

macrophages (Leslie and Alexander, 1980) probably have distinct receptor sites for two cytophilic subclasses of IgG. Presumably guinea pig alveolar macrophages also possess two types of Fc receptor. However, although human monocytes bind both IgG, and IgG_3 (Alexander, 1980), it is not as yet clear whether these cells also possess different Fc receptors. By developing more sensitive assays, possibly by preparing specific antibodies to the various receptors examined in this thesis, the numbers and affinities of these receptors could be determined and variations between and within populations could be compared. Cell membrane fractionation of these receptors may also give further information into their composition and possibly differences between cell types may be detected. Both C3b and Fc (IgG) receptors have been isolated (see Introduction, Part 9.8 and 10.7) using non-ionic detergent cell lysates and affinity column chromatography. Using similar methods it is hoped that "lectin-like" receptors may also be isolated and characterised.

Different levels of expression of surface receptors may be important with respect to the physiological role of the phagocytes and may reflect their maturation state and also the influence of their environment. Tissue macrophages derive ultimately from peripheral blood monocytes (van Furth et al., 1972; van Furth, 1980) and thus mouse peritoneal and guinea pig alveolar macrophages comprise mainly of more mature cells than the human peripheral blood monocyte population. Within the latter population, promonocytes may also be present (Meuret et al., 1974). All three cell types appear to be heterogeneous in terms of size and function (Walker, 1976b; Rhodes, 1975; Norris et al., 1979; Pelus et al., 1981). Heterogeneity may represent subpopulations or be a reflection of different maturation states of the cells.

Separation/...

Separation of distinct subpopulations on the basis of cell size is possible and work is in progress isolating such subpopulations by counterflow centrifugation using a Beckman J21B centrifuge equipped with an Elutriator rotor as described by Sanderson et al. (1976). This method was used by Norris et al. (1979) for separating human monocytes into functionally distinct subpopulations. Similar differences in function have been described in tissue macrophages. Gorcynski (1976) separated peritoneal exudate cells on bovine serum albumin gradients and demonstrated two distinct subpopulations with different capacities for potentiating antibody or specific cell-mediated immune responses. Studies on receptor expression in such separated subpopulations could be carried out. Fc receptor expression in different subpopulations has been investigated (Norris et al., 1979) particularly with respect to the activation state of macrophages (Moore and McBride, 1980). As far as the author knows there are no reports in the literature on the possible heterogeneity of complement receptor activities within populations or comparisons made between populations such as resident and activated macrophages except on a gross level (Bianco et al., 1975), although Atkinson et al. (1977) noted that small and medium sized rabbit alveolar macrophages formed the most florid rosettes with complement coated erythrocytes whereas the larger and multinucleated cells were poor rosette formers. Such considerations may be important in terms of functional activation of macrophages since thioglycollate elicited murine peritoneal macrophages (Bianco et al., 1975) and lymphokine stimulated mouse peritoneal macrophages (Griffin and Griffin, 1979, 1980) and cultured human monocytes (Newman et al., 1980) all ingest complement coated red cells whereas resident/...

whereas resident or peripheral blood mononuclear phagocytes only bind and do not phagocytose EAC. In an inflammatory response macrophage C3b receptors may be functionally altered so that they can mediate phagocytosis. It is of interest to note that a few human monocytes do ingest EAC and these may represent systemically activated monocytes. Apparently only 37% of guinea pig alveolar macrophages expressed complement receptors and only 17% recognised EAC prepared with human complement. During an inflammatory reaction in the lung, it might be expected that serum proteins including complement would diffuse into the inflammatory site and thus complement receptors may be of great importance under these conditions. Activated guinea pig alveolar macrophages may express more complement receptors and thus be able to remove opsonised particles more readily at the site of injury. Alveolar macrophages are the principle phagocytes of the lung and serve to clear small inhaled particles deposited in the terminal alveoli (Green, 1970). Thus "lectin-like" receptors on alveolar macrophages may play an important role in the clearance of nonopsonised particles including micro-organisms, allergens and potentially toxic pollutants. The level of expression of "lectin-like" receptors on guinea pig alveolar macrophages is relatively high compared to peripheral blood leukocytes though slightly lower than that of mouse peritoneal macrophages.

C3b and "lectin-like" receptors can apparently be modulated by substances which are known to promote migration of phagocytes in vitro. CR_1 but not CR_3 were enhanced by chemotactic agents whereas "lectin-like" receptors were inhibited by the same substances. Fc receptors were apparently unaffected by chemoattractants but possibly more sensitive/...

sensitive assays may detect changes in these surface receptors. Human monocytes, mouse peritoneal macrophages and guinea pig alveolar macrophages each had different responses to chemotactic factors and this may reflect functional differences between these cell types. Within the populations there appeared to be heterogeneous responses possibly by different subpopulations of cells. Again, more information may be obtained through separation of whole populations into subgroups.

Certain pharmacological mediators other than chemotactic agents also apparently enhanced the expression of complement receptors on human monocytes and these results imply probable changes in cGMP levels or guanyl cyclase activity modulating the expression of complement receptors, possibly together with alterations in intracellular levels of cAMP. Temperature dependence studies suggested that membrane fluidity was important both in the expression of complement receptors and in the enhancement of these receptors. However further studies into the mechanism of complement receptor modulation is necessary before more definite conclusions can be drawn. It is not known whether cyclic nucleotide levels are involved in the chemotactic factor modulation of "lectin-like" receptors or whether this inhibition is dependent on membrane fluidity. Further understanding may be achieved by the use of a combination of various techniques which might include cell membrane fractionation, the use of radiolabelled complement components or ligands for the "lectin-like" receptors such as glycoproteins, and membrane fluidity probes.

Complement receptor enhancement may be an important biological phenomenon in terms of the events which surround (1) the directional migration of cells into sites of inflammation and (11) the subsequent adhesion/...

adhesion of leukocytes to particles opsonised with complement i.e. the phagocytes are primed for the adhesion process prior to their actual attachment. However in this study, no attempts were made to investigate the relevance of enhancement.of complement receptors or the inhibition of "lectin-like" receptors to various other recognised cellular processes, for example phagocytosis, release of lysosomal enzymes and K-cell cytotoxicity. Cellular mechanisms dependent on complement receptors may be enhanced whereas those dependent on "lectin-like" receptors may be inhibited.

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PUBLICATIONS

The following work connected with this thesis has been or is about to be published.

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Leucoattractants enhance complement receptors on human phagocytic cells

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SUMMARY

The N-formyl-methionyl peptides, F-Met-Leu-Phe, F-Met-Met-Phe and F-Met-Phe, when tested at differing concentrations, proportionally increased both *in vitro* cell locomotion and the expression of surface receptors for C3b on human peripheral blood neutrophils and monocytes. In contrast, the unformylated peptides, Met-Leu-Phe and Me-Met-Phe, had no chemotactic or complement receptor-enhancing activity at comparable concentrations. Casein and supernatants from human lymphocytes (cultured either in the presence or absence of phytohaemagglutinin), also recognized as chemotactic agents for human neutrophils and monocytes, enhanced C3b receptors on these cells in a similar dose-dependent fashion. These data, taken together with our previous findings with the eosinophil, suggest that in addition to promoting cell locomotion a further biological function of leucoattractants may be their capacity to render complement receptors more freely available thereby increasing the magnitude of adhesion of phagocytic cells to opsonized particles.

INTRODUCTION

We recently reported that eosinophil chemotactic factors (including the ECF-A tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu), histamine and imidazoleacetic acid) markedly enhanced the expression of receptors for complement (C3b and C4) on human eosinophils (Anwar & Kay, 1977 and 1978). Therefore, it seemed important to determine whether other phagocytic cells, such as human neutrophils and monocytes, undergo similar complement receptor enhancement following incubation with substances known to promote their migration *in vitro*. If, in addition to promoting cell locomotion, leucoattractants also render complement receptors more freely available this would provide a mechanism whereby the degree of adhesion of phagocytic cells to opsonized particles was increased. The studies reported here employed synthetic bacterial-associated N-formyl-methionyl peptides, as their chemical structure is clearly defined and potent chemotactic activity well documented (Schiffman, Corcoran & Wahl, 1975; Showell *et al*, 1976). In addition, we examined other recognized human neutrophil and monocyte leucoattractants for complement receptor enhancement. These included supernatants from cultured lymphocytes (Ward, Remold & David, 1969; Snyderman *et al.*, 1972) and casein (Keller & Sorkin, 1967).

MATERIALS AND METHODS

F-Met-Leu-Phe was a gift from Dr Dereck Hudson (Royal Postgraduate Medical School, London). F-Met-Met-Phe, Met-Leu-Phe and Met-Met-Phe were gifts from Miles Laboratories (Stoke Poges). F-Met-Phe was obtained from Sigma Chemical Company (St Louis, Missouri, U.S.A.). Other materials were obtained as follows. Preservative-free heparin (Evans Medical, Liverpool); casein (BDH Chemicals Ltd, Poole); phytohaemagglutinin (PHA-P, Wellcome Reagents Ltd., Beckenham); dextran (Lomodex 70, Fisons Pharmaceuticals, Loughborough); Ficoll and Hypaque (Pharmacia, Uppsala,

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Sweden); ovalbumin five times crystallized (Koch Light Laboratories, Colnbrook); cellulose nitrate filters, 8 and 0·45-μm pore size (Sartorius-Membrane Filters, Gottingen, West Germany); polycarbonate (Nucleopore) 5-μm pore size filters (Neuroprobe, Bethesda, Maryland, U.S.A.).

*Preparation of EAC*1423*b* (*EAC*). This has been described in detail elsewhere (Anwar & Kay, 1977) and was briefly as follows. Dextrose-gelatin-Veronal buffer ($DGVB^{2+}$, pH 7·4) was used for washing sheep erythrocytes (E) during sensitization and coating with various complement components and was prepared by mixing equal volumes of isotonic Veronal-buffered saline (containing 0·0015 M Ca^{2+}, 0·0005 M Mg^{2+} and 0·1% gelatin-Veronal buffer (GVB^{2+})) with 5% dextrose in water containing the same concentration of Ca^{2+} and Mg^{2+}. The IgM fractions of rabbit antisera to sheep red cells were prepared by Sephadex G-200 gel filtration (Shevach *et al.*, 1972). Functionally pure human complement components were added sequentially to EAM^{rab} to prepare C3b-coated cells. The amounts were as follows: 400 effective molecules of C1, 400 of C4, 50 of C2 and 500 (for neutrophils) or 400 (for monocytes) of C3. This amount of C4 was insufficient to give EAC14 rosettes with neutrophils, eosinophils or monocytes (Anwar & Kay, 1977).

In some experiments fresh human AB serum diluted with $DGVB^{2+}$ was used as a source of complement. Equal volumes of EAM^{rab} at a concentration of 1×10^8 cells/ml and human AB serum were mixed and incubated for 30 min at 37°C, washed twice in $DGVB^{2+}$ and resuspended in the same buffer to a concentration of 1×10^8 red blood cells/ml. The dilution of fresh serum was adjusted to give between 20 and 30% of neutrophil or monocyte rosettes and was usually between 1 in 600 and 1 in 1000.

Neutrophils and monocytes. Blood from healthy volunteers was drawn into plastic tubes containing 10 units of preservative-free heparin/ml. The red cells were sedimented with 70% dextran; 1 part dextran to 4 parts blood v/v for 60 min at room temperature. The leucocyte-rich plasma was then layered over Ficoll–Hypaque (specific gravity 1·078) at 4°C in 15 ml plastic conical centrifuge tubes and centrifuged at 4°C for 40 min at 400 g. The pellet, containing red blood cells and granulocytes, was resuspended in 0·82% ammonium chloride as described (Boyle, 1968), to lyse the red cells. Monocytes, usually from the same donors, were separated on Ficoll-Hypaque gradients according to the method of Böyum (1968). The separated cells were finally washed twice in medium 199, pH 7·4, and the cell count adjusted to 2×10^6 cells/ml for neutrophils or 1×10^6 monocytes/ml.

Lymphocyte supernatants. 'Stimulated lymphocyte supernatants' were prepared from suspensions of human peripheral blood mononuclear cells, separated on a discontinuous gradient of Ficoll–Hypaque as described above, and contained 3×10^6 leucocytes/ml in medium 199. Under sterile conditions, and with added penicillin and streptomycin, 9 μl of PHA-P (0·5 μg/ml) were added to 3 ml of the mononuclear cell suspension. The cells were incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO_2. After incubation the supernatants were harvested by centrifugation, divided into portions and stored at −70°C until use. The 'unstimulated lymphocytes' were prepared in an identical fashion with the exception that PHA-P was added at the termination of the 3 day incubation period.

EAC rosettes. Equal volumes of neutrophils (2×10^6/ml) or monocytes (1×10^6/ml) and various concentrations of the leucoattractants under study, or medium alone as control, were mixed and incubated in a shaking water bath at 37°C for 30 min. The cells were then washed twice in medium 199 and the numbers re-adjusted to their original concentration in the same medium. A portion (0·1 ml) of EAC (1×10^8/ml) was added to 0·1 ml of the leucocyte suspension, the mixtures centrifuged at 100 *g* for 10 min at 4°C, and the pellets incubated at 37°C for 30 min. The pellet was gently resuspended and smears prepared on clean glass slides in duplicate. These were dried quickly in air, fixed in 95% methanol and stained with May Grunwald/Giemsa. Leucocytes with three or more adherent sheep red cells were termed rosettes. In each slide 200 neutrophils or monocytes were counted and the increase in the percentage of rosetting cells following incubation with the various leucoattractants was calculated.

Cell locomotion. The capacity of neutrophils or mononuclear cells to migrate towards a concentration gradient of various leucoattractants has been described in detail elsewhere (Kay, Pepper & McKenzie, 1974; Campbell, 1977). For neutrophils (Böyum, 1968), cellulose nitrate filters of 8 μm pore size were employed using an incubation time of 90 min at 37°C. The cell count was adjusted to 2×10^6/ml in medium 199 containing 0·5% ovalbumin at pH 7·4. Only those cells which had migrated across the entire thickness of the micropore were counted. Cells were counted under a high power (×40) objective and the average number in five fields was recorded (mean cell count). Monocyte chemotaxis was measured using the double filter technique (Campbell, 1977) with the exception that the 'Boyden chamber' supplied by Neuroprobe, Inc., Bethesda, Maryland, was used instead of the Sykes-Moore type of chamber. In all experiments with neutrophils or monocytes the values obtained from the controls (medium 199 alone) were subtracted from the values obtained with the various agents being tested.

RESULTS

The percentage increases in C3b rosettes and the numbers of neutrophils migrating through the micropore filters were directly related to the concentrations of the formyl-methionyl peptides (Fig. 1). The magnitude of these two biological events appeared to increase proportionally with increasing concentrations of the peptides. With F-Met-Leu-Phe appreciable activity was observed from 10^{-8} to 10^{-10} mol/l, but not at 10^{-11} mol/l. With F-Met-Met-Phe neutrophil chemotaxis and complement receptor

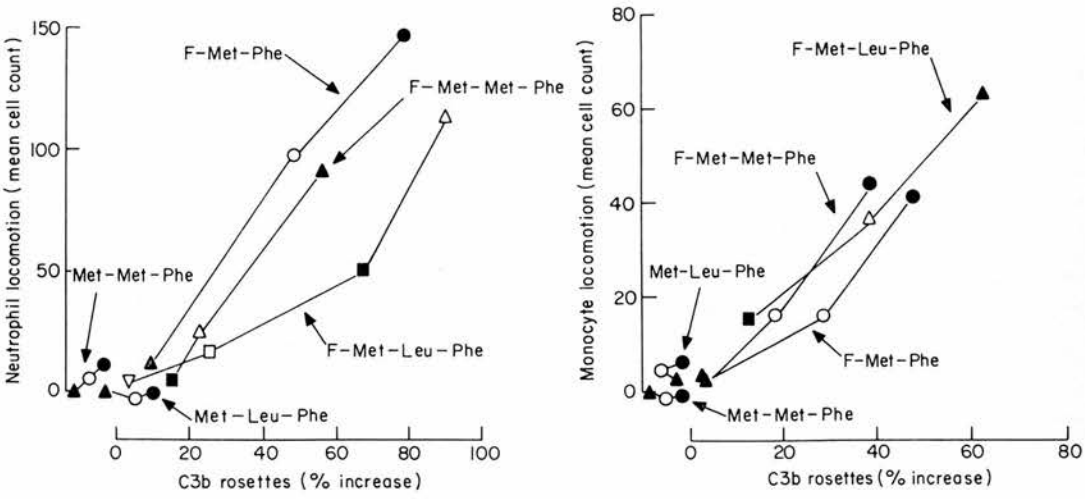

FIG. 1. The association between the percentage increase in neutrophil C3b rosettes and neutrophil locomotion by formylated and unformylated methionyl peptides. The symbols represent the concentration of the peptides in mol/l and are as follows: 10^{+5} (●), 10^{-6} (○), 10^{-7} (▲), 10^{-8} (△), 10^{-9} (■), 10^{-10} (□) and 10^{-11} (▽). The results are the mean values of three experiments (five experiments with F-Met-Leu-Phe). Receptor enhancement and chemotaxis were performed on cells from the same volunteers (with the exception of two experiments with F-Met-Leu-Phe).

FIG. 2. The association between the percentage increase in monocyte C3b rosettes and monocyte locomotion by formylated and unformylated methionyl peptides. The symbols represent the concentrations of the peptides in mol/l and are as follows: 10^{-5} (●), 10^{-6} (○), 10^{-7} (▲), 10^{-8} (△) and 10^{-9} (■). The results are the mean values of three experiments. Receptor enhancement and chemotaxis were performed on cells from the same volunteers.

TABLE 1. Human neutrophil and monocyte complement receptor enhancement by lymphocyte supernatants and casein. N = neutrophils, M = monocytes. The results are the mean ± s.e. of three experiments. The lymphocyte supernatants and casein were tested for neutrophil and monocyte chemotactic activity and gave results comparable to that described in our previous study (Kay & McVie, 1977)

		C3b Rosettes (% increase)			
		Dilution			
		1:4	1:8	1:16	1:32
Lymphocyte supernatant (PHA-stimulated)	N	64±4	49±4	34±2	16±8
	M	—	84±5	52±7	−2±3
Lymphocyte supernatant (unstimulated)	N	69±3	33±5	25±5	1±5
	M	—	39±7	11±7	−2±3
		Concentration (mg/ml)			
		3·0	2·0	1·5	1·0
Casein	N	60±11	64±6	34±3	23±5
	M	97±12	76±17	—	39±4

enhancement were observed at 10^{-7} aud 10^{-8} mol/l and with F-Met-Phe at 10^{-5} and 10^{-6}. The unformylated peptides, Met-Leu-Phe and Met-Met-Phe, gave virtually no responses either with neutrophil C3b-receptor enhancement or chemotaxis.

Similar results were obtained with human blood monocytes (Fig. 2) although the concentrations of the formyl-methionyl peptides required to demonstrate chemotaxis and complement receptor enhancement were higher than those required for the neutrophil. With F-Met-Leu-Phe activity was observed at 10^{-7} and 10^{-8} mol/l with little enhancement and cell locomotion at 10^{-9} mol/l. With F-Met-Met-Phe and F-Met-Phe a clear effect on cell locomotion and rosette expression was demonstrable at 10^{-5} and 10^{-6} mol/l, but not at 10^{-7} mol/l. The unformylated peptides, Met-Leu-Phe and Met-Met-Phe, gave very low values in monocyte complement receptor enhancement and monocyte chemotaxis.

Casein, previously shown to be a potent chemoattractant for neutrophils and monocytes (Keller & Sorkin, 1967), also enhanced C3b receptors on these cell types (Table 1). Similar results were obtained with cultured lymphocyte supernatants. A greater amount of neutrophil and monocyte chemotactic activity was found in cultures prepared in the presence of PHA, although the 'unstimulated' lymphocyte supernatants also promoted the migration of human leucocytes, as was previously shown (Snyderman *et al.*, 1972). Similarly, complement receptor enhancement was greater with 'stimulated' as compared to 'unstimulated' supernatants for both neutrophils and monocytes (Table 1).

The time-course of complement receptor enhancement on neutrophils and monocytes was similar, maximal effect being observed between 30 and 60 min. Irrespective of whether the indicator red cells were EAM with whole serum as a source of complement, or EAM and purified C1, C4, C2 and C3, the percentage increase in rosettes either as a function of time or the concentration of chemoattractant, was virtually identical for both neutrophils and monocytes.

DISCUSSION

In this report we have shown that formyl-methionyl peptides, in addition to promoting cell migration *in vitro*, also increased the expression of C3b receptors on neutrophils (Fig. 1) and monocytes (Fig. 2). We have therefore, confirmed previous reports on the leucoattractant properties of these compounds (Schiffman *et al.*, 1975; Showell *et al.*, 1976) and shown that in addition they have a previously undescribed biological activity—complement receptor enhancement. Both activities, i.e. leucoattraction and complement receptor enhancement, occurred in parallel since increasing concentrations of the peptides promoted concomitant increases in both the degree of receptor enhancement and the numbers of neutrophils or monocytes migrating through the micropore filters. In contrast, the unformylated peptides, Met-Leu-Phe and Met-Met-Phe, were inactive in both biological systems suggesting that the specificity for formylation applies to both complement receptor enhancement and locomotion.

Enhancement of complement receptors by 'chemotactic factors' was first described using human eosinophils (Anwar & Kay, 1977, 1978). In these reports we showed that receptors for C3b and C4 (but not C3d or IgG (Fc)) were 'enhanced' in the sense that previously recognized eosinophilotactic agents such as the ECF-A tetrapeptides, histamine or imidazoleacetic acid produced an increase in the percentage of eosinophils forming rosettes with the appropriate indicator red cells. As described elsewhere (Anwar & Kay, 1978) possible explanations of the mechanisms of complement receptor enhancement include 'membrane unfolding', 'receptor externalization' and 'subunit association'. The generation of 'new' receptors during the incubation with chemotactic factors was considered unlikely since optimal enhancement of eosinophil complement receptors was found to be between 30 and 60 min. A similar time course for complement receptor enhancement on neutrophils and monocytes has also been observed with neutrophils and monocytes (Glass, E.J., Salter, D.McG. & Kay, A. B., unpublished observations).

The term 'leucoattraction' and 'cell locomotion' has been used only in the sense that the various agents which we tested produced migration of neutrophils and monocytes towards a gradient across a micropore filter. Although we have drawn the general conclusion that agents which promote cell locomotion also enhance complement receptors on the leucocytes they chemoattract, we cannot say whether this effect is related to their chemotactic and/or chemokinetic principles. Showell *et al.* (1976), using the 'chequer-

board' titration method previously described (Zigmond & Hirsch, 1973) to distinguish true chemotaxis from chemokinesis, were able to show that F-methionyl peptides caused directional migration as well as increasing the rate of cell movement. In order to establish whether the chemotactic and/or chemokinetic property is necessary for complement receptor enhancement on human leucocytes further studies will be required using substances which increase random migration, but are not chemotactic and *vice versa*. For instance, Wilkinson *et al.* (1977) reported that mouse lymph node lymphocytes responded in chemokinesis, but not chemotaxis, to human and bovine serum albumins.

The relative activities of the formylated peptides used in this study were, for neutrophils, F-Met-Leu-Phe > F-Met-Met-Phe > F-Met-Phe. Similar results, in terms of relative potencies were observed in the study by Showell *et al.* (1975) in which chemotaxis and lysosomal enzyme release from rabbit neutrophils were tested. With monocytes about one hundred-fold more F-Met-Leu-Phe, F-Met-Met-Phe or F-Met-Phe was required to give comparable biological effects to that of the neutrophil (Figs 1 and 2). Furthermore in the human monocyte studies (Fig. 2), F-Met-Phe had a similar potency to F-Met-Met Phe in both cell locomotion and rosette enhancement. This is in contrast to the report of Showell *et al.* (1976) in which F-Met-Met-Phe was found to be 1900 times more active than F-Met-Phe in promoting chemotaxis of rabbit neutrophils.

We have not yet had the opportunity to examine fully the effects of chemotactic agents on cell surface markers other than those for complement. However, in a previous report we showed that eosinophilotactic agents had no apparent effect on IgG (Fc) receptors (Anwar & Kay, 1977 and 1978) and recent preliminary data indicate that on neutrophils and monocytes these receptors are also unaffected by leucoattractants (Salter, D.McG. & Glass, E.J., unpublished observations). Also, we have yet to ascertain whether, like eosinophils, complement receptor enhancement on neutrophils and monocytes applies only to C3b and C4 and not C3d.

It is tempting to speculate that complement receptor enhancement may be an important biological phenomenon in terms of the events which surround (i) the directional migration of cells into sites of inflammation and (ii) the subsequent adhesion of leucocytes to particles opsonized by complement, i.e. that leucocytes are primed for the adhesion process prior to their actual attachment. The N-formyl-methionyl peptides and the other leucoattractants tested (Table 1) enhanced C3b receptors on those cells in which they induced locomotion. Therefore, the present work, taken together with our previous findings with the eosinophil (Anwar & Kay, 1977 and 1978), supports the general hypothesis that leucoattractants enhance complement receptors on human phagocytic cells.

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Enhanced expression of human monocyte complement (C3b) receptors by chemoattractants

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SUMMARY

The capacity of various leucoattractants to enhance, or unfold, receptors for complement (C3b) on human blood monocytes has been studied. A number of recognized monocyte chemoattractants including casein, supernatants from *C. parvum* 10390 and from human lymphocytes (cultured either in the presence or absence of phytohaemagglutinin) and the formyl-methionyl peptides, F-Met-Leu-Phe, F-Met-Met-Phe and F-Met-Phe, increased the percentage of monocytes which formed rosettes with IgM-sensitized sheep erythrocytes coated with complement. Comparable results were achieved irrespective of whether purified components or whole serum was used as a source of complement. In contrast, there was no significant increase in EAG (Fc) rosettes with those doses of casein which gave enhancement of C3b receptors. A small degree of complement receptor enhancement was observed with histamine but the unformylated peptides, Met-Leu-Phe and Met-Met-Phe, were without apparent effect. Maximal receptor enhancement was obtained at 30 min but when the leucoattractant was removed, enhancement was reversible, returning to normal values in approximately 120 min. Monocyte complement receptor enhancement increased with temperatures between 0°C and 37°C.

These data (1) confirm and extend our previous findings on leucoattractant-induced enhancement of complement receptors on human monocytes; (2) indicate that the phenomenon may have potential as a clinical test for monocyte function both in health and disease.

INTRODUCTION

We recently reported that complement receptors on human eosinophils were enhanced, or unfolded, following incubation with various pharmacological mediators to which they migrate in chemotaxis and/or chemokinesis. A number of eosinophilotactic agents, including the eosinophil chemotactic factor of anaphylaxis (ECF-A) tetrapeptides (Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu), histamine and one of its major catabolites, imidazoleacetic acid, selectively enhanced receptors for complement (C3b and C4) on human eosinophils in a dose- and time-dependent fashion (Anwar & Kay, 1977a; Anwar & Kay, 1978). Later, in a short report we established that human neutrophils and monocytes undergo a similar enhancement of complement receptors following incubation with substances to which they are known to migrate *in vitro* (Kay, Glass & Salter, 1979). This suggested that complement receptor enhancement by leucoattractants may be a general biological phenomenon which might provide a mechanism whereby the degree of adhesion of phagocytic cells to opsonized particles was increased.

For these reasons it seemed important to provide further information on leucoattractant-induced complement receptor enhancement by various cell types and in the present report the monocyte has been studied in detail. Thus, we have confirmed and expanded our original observations by studying the

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effect of leucoattractants on both complement and IgG receptors, by undertaking time course experiments over a broad concentration range, examining a number of agents which promote monocyte migration and determining whether the phenomenon is reversible and temperature-dependent.

MATERIALS AND METHODS

Materials. Materials were obtained as follows. Casein (BDH Chemicals Limited, Poole, Dorset); phytohaemagglutinin (PHA-P, Wellcome Reagents Limited, Beckenham, Kent); *C. parvum* 10390 supernatant was a gift from Dr W. McBride (Department of Bacteriology, Medical School, University of Edinburgh); histamine acid phosphate (BDH Chemicals Limited, Poole, Dorset); F-Met-Leu-Phe was a gift from Dr Derek Hudson (Royal Postgraduate Medical School London), F-Met-Met-Phe, Met-Leu-Phe and Met-Met-Phe were gifts from Miles Laboratories (Stoke Poges, England); F-Met-Phe was obtained from Sigma Chemical Company (St Louis, Missouri, USA); preservative-free heparin (Evans Medical, Liverpool); Ficoll and Hypaque (Pharmacia, Uppsala, Sweden).

*Preparation of EA, EAC*1423b *and 'EAC'*, This has been described elsewhere (Anwar & Kay, 1977b; Anwar & Kay, 1978) and was briefly as follows. Dextrose–gelatin–veronal buffer ($DGVB^{2+}$ pH 7.4 was used for washing sheep erythrocytes (E) during sensitization and coating with complement. The buffer was prepared by mixing equal volumes of isotonic Veronal-buffered saline (containing 0·0015 M Ca^{2+} 0·0005 M Mg^{2+} and 0·1% gelatin-Veronal buffer GVB^{2+}) with 5% dextrose in water containing the same concentration of Ca^{2+} and Mg^{2+}. The IgM and IgG fractions of rabbit antisera to sheep red cells (A) were prepared by Sephadex G-200 gel filtration (Shevach *et al.*, 1972). The IgG fraction was purified further by DEAE-52 anion exchange chromatography (Fahey & Terry, 1978). Either IgG or IgM was used in the sensitization of E for the preparations of EA^{rab}_{G} or EA^{rab}_{M} respectively. The antibody concentration of EA^{rab}_{M} selected was that which gave optimal sensitization for haemolysis but no agglutination. For the preparation of EA^{rab}_{G}, the antibody concentration was diluted so as to give approximately 30% monocyte rosettes. Functionally pure human complement components were added sequentially to EA^{rab}_{M} to prepare C3b coated cells (EAC1423b). The amounts were as follows: 400 effective molecules of C1, 400 of C4, 50 of C2 and 400 of C3. This amount of C4 was insufficient to give EAC14 rosettes with monocytes (Anwar & Kay, 1978). In some experiments fresh human AB serum was also used as a source of complement ('EAC'). Equal volumes of EA^{rab}_{M} at a concentration of 1×10^8 cells/ml and human AB serum, diluted with $DGVB^{2+}$, were mixed and resuspended in the same buffer to give a final concentration of 1×10^8 red blood cells/ml. The dilution of fresh serum was adjusted to give between 20 and 30% monocyte rosettes and was usually between 1 in 600 and 1 in 1000.

Monocytes. Blood from healthy donors was drawn into plastic tubes containing 10 units of preservative-free heparin/ml. Monocytes were separated on Ficoll–Hypaque cushions according to the method of Böyum (1968). The separated cells were washed twice in Dulbecco's PBS (modified) (pH 7·2–7·4), containing preservative-free heparin (2 units/ml) and resuspended in medium 199, pH 7·4, to a concentration of 1×10^2 monocytes/ml.

Lymphocyte supernatants. A cell suspension containing up to 90% lymphocytes was obtained using defibrinated blood as described by Böyum (1976). The separated cells were resuspended to a concentration of 3×10^6 lymphocytes/ml in medium 199. Under sterile conditions, and with added penicillin and streptomycin, 9 μl of PHA-P (0·5) μg/ml) were added to 3 ml of the mononuclear cell suspension. The cells were incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO_2. After incubation the supernatants were harvested by centrifugation, aliquoted and stored at −70°C until use. The unstimulated lymphocyte supernatants were prepared in an identical fashion with the exception that PHA-P was added at the end of the 3-day incubation period.

Complement (EAC) and IgG (EA_G) rosettes. Equal volumes of monocytes (1×10^6/ml) and various concentrations of the leucoattractants under study or medium alone as a control, were mixed and incubated in a shaking water bath usually at 37°C for 30 min. The cells were then washed twice in medium 199 and the numbers readjusted to their original concentration in the same medium. A portion (0·1 ml) of EAC or EA_G (1×10^8/ml) was added to 0·1 ml for 10 min at 4°C and the pellets incubated at 37°C for 20 min for EAC and 0°C for 30 min in the preparation of EA_G. The pellet was resuspended gently in medium 199 containing 1% formal-saline and smears prepared on clean glass slides in duplicate. These were dried quickly in air, fixed in methanol and stained with May–Grünwald/Giemsa or fixed in 2·5% glutaraldehyde and stained using α-naphthyl acetate histochemistry according to the method of Yam, Li & Crosby (1971). Comparison of these two methods by four independent observers revealed no statistical significance in differentiating monocytes from lymphocytes.

RESULTS

Casein enhanced the percentage of monocyte C3b rosettes in a dose-dependent fashion (Fig. 1) irrespective of whether 'EAC' or EAC1423 were used as indicator cells. In contrast there was a small but insignificant increase in the percentage of EAG (Fc) rosettes. With the highest dose of casein the mean values for enhancement of Fc receptors was 13% whereas with 'EAC' and EAC1423 the increase was 92% and 100% respectively.

Enhancement of complement receptors by casein was also dependent on the time of incubation.

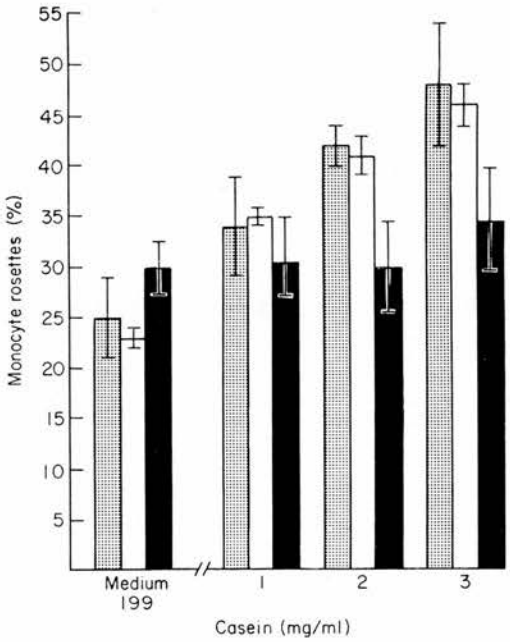

FIG. 1. The effect of casein on monocyte C3b and IgG rosettes. (□) EA_M1423, (▨) 'EAC', (▨) EA_G. Each column represents the mean ± s.e.m. of three experiments. Casein was dissolved in distilled water and the pH raised to 11 with 1 N NaOH. The pH was then adjusted to 7·4 with 1 N HCl. Nine parts of the casein solution were then added to one part of 10-times concentrated medium 199 with HEPES buffer, pH 7·4.

Maximal effects were observed at about 30 min after which there was no further increase (Fig. 2). Similar results were obtained irrespective of whether 'EAC' or EAC1423 were used as indicator red cells. A small but insignificant increase in Fc receptors was also observed in this time course study and was maximal after approximately 60 min. By 120 min this small increase had returned to the original value.

As the dose–response and time–course studies were essentially similar using either 'EAC' or EC1423, 'EAC' were used as indicator cells in further experiments.

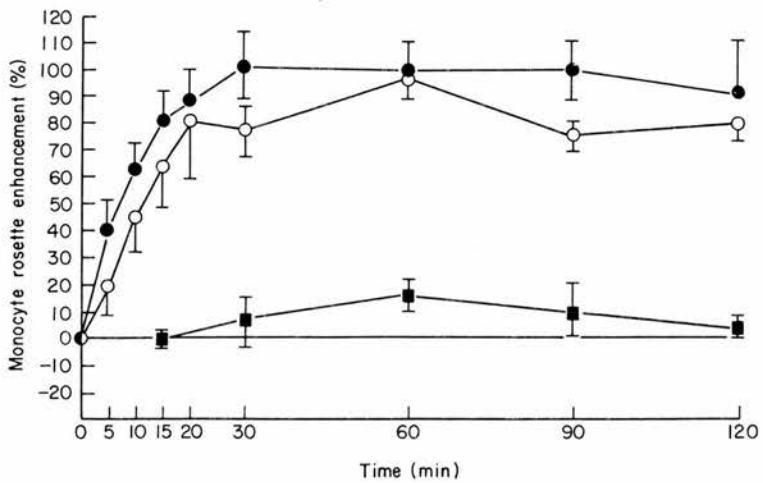

FIG. 2. The time–course of the percentage enhancement of monocyte rosettes by casein using sensitized erythrocytes coated with complement or IgG. (●—●) 'EAC', (○—○) EA_MC1423, (■—■) EA_G. Each point represents the mean ± s.e.m. of three experiments. The casein was prepared as in Fig. 1.

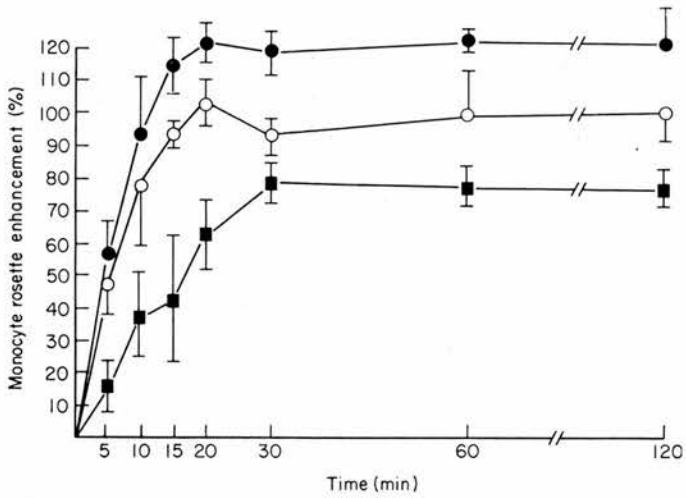

FIG. 3. The time-course of monocyte complement receptor enhancement by increasing concentrations of casein. (●—●) 3 mg/ml casein, (○—○), 2 mg/ml casein, (■—■) 1 mg/ml casein. Each point represents the mean ± s.e.m. of three experiments. The indicator red cells were 'EAC' (prepared with whole serum as a source of complement).

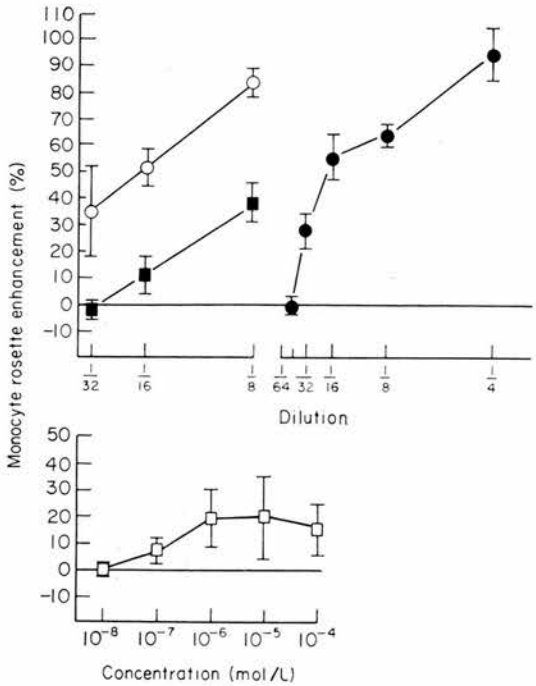

FIG. 4. The effect of various leucoattractants on the expression of monocyte complement receptors. (●—●) *C. parvum* 10390, (○—○) 'stimulated', (■-■) 'unstimulated', (□-□) histamine. Each point represents the mean ± s.e.m. of three experiments, except for histamine in which four experiments were performed. The *C. parvum* 10390 supernatant was prepared from formaldehyde-killed *C. parvum* organisms which had been washed twice in 0·9% NaCl, lyophilized and reconstituted in distilled water. The reconstituted material had a concentration of 7 mg/ml dry weight which represented 3×10^{11} organisms. The indicator red cells were 'EAC' as in Fig. 3.

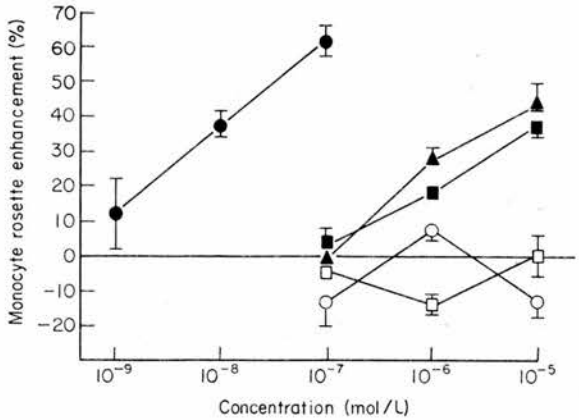

FIG. 5. The effect of formyl-methionyl peptides on the expression of monocyte complement receptors. (●—●) F-Met-Leu-Phe, (○—○) Met-Leu-Phe, (■—■) F-Met-Met-Phe, (□—□) Met-Met-Phe, (▲—▲) F-Met-Phe. Each point represents the mean ± s.e.m. of three experiments. The peptides were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 10^{-2} mol/l and further diluted in medium 199 to the required concentrations. At the highest dose used (10^{-5} mol/l) the concentration of DMSO was approximately 10^{-2} mol/l and at this concentration had no effect on cell viability as determined by trypan blue exclusion and locomotion *in vitro* through micropore filters (Kay, Glass & Salter, 1979). The indicator red cells were 'EAC' as in Fig. 3.

The effect of increasing doses of casein on the rate and degree of enhancement of monocyte complement receptors is shown in Fig. 3. The degree of enhancement and the rate at which enhancement occurred was dependent on the concentration of casein. Increasing the concentration of casein from 1 mg/ml to 2 mg/ml increased the rate of monocyte rosette enhancement three-fold. Further increase in the concentration of casein up to 3 mg/ml had no apparent effect on the rate of complement receptor enhancement.

Other recognized monocyte chemoattractants were tested for their capacity to enhance monocyte complement receptors. Supernatants from lymphocytes stimulated with PHA increased the numbers of monocyte rosettes in a dose-dependent fashion (Fig. 4). In contrast, unstimulated lymphocyte supernatants had significantly less effect when tested at comparable dilutions. A leucoattractant elaborated

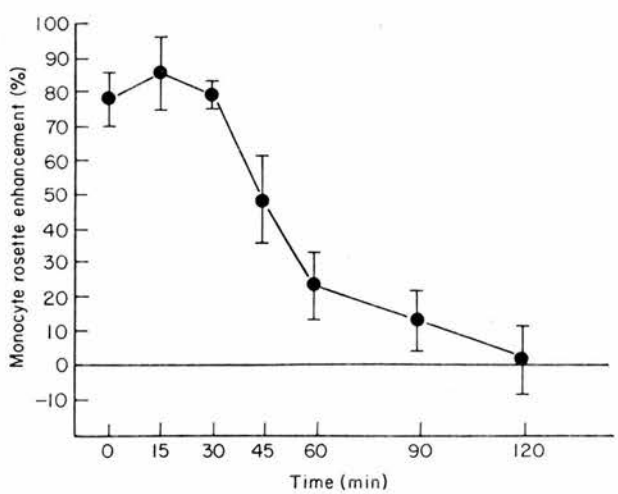

FIG. 6. Reversibility of monocyte complement receptor enhancement. Each point represents the mean ± s.e.m. of three experiments. Monocytes were pre-incubated with 3 mg/ml of casein for 30 min at 37°C, centrifuged and the cells resuspended in medium 199 to the original volume and incubated at 37°C for various time intervals as shown. The indicator red cells were 'EAC' as in Fig. 3.

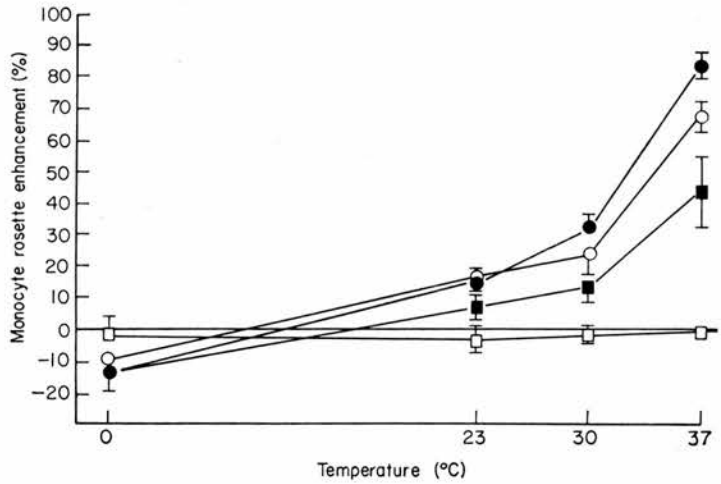

FIG. 7. The effect of temperature on monocyte complement receptor enhancement. (●—●) 3 mg/ml casein, (○—○) 2 mg/ml casein, (■—■) 1 mg/ml casein, (□—□) medium 199. Each point represents the mean± s.e.m. of three experiments. The indicator red cells were 'EAC' as in Fig. 3.

from *C. parvum* 10390 also had a similar effect. Histamine gave a slight increase in the percentage of monocyte complement rosettes at doses of 10^{-5} and 10^{-6} mol/l.

A number of formyl-methionyl peptides were also tested for their effects on monocyte complement receptors (Fig. 5). With F-Met-Leu-Phe activity was observed at 10^{-7} and 10^{-8} mol/l with little enhancement at 10^{-9} mol/l. F-Met-Met-Phe and F-Met-Phe were less active than F-Met-Leu-Phe but both gave enhancement at 10^{-5} and 10^{-6} mol/l but not at 10^{-7} mol/l. The unformylated peptides, Met-Leu-Phe and Met-Met-Phe, were virtually inactive.

Monocyte complement receptor enhancement appeared to be reversible (Fig. 6). Following 30 min incubation with casein the cells were washed and resuspended in medium 199. There was a gradual decrease in monocyte rosette enhancement and by 120 min there was no discernible difference from the controls. The experiments were reported with F-Met-Leu-Phe (10^{-7} mol/l) and gave virtually identical results.

Monocyte complement receptor enhancement was also temperature dependent as shown in Fig. 7. At 0°C there was no increase in the percentage of rosettes formed with any of the concentrations of casein used. However, at 23, 30 and 37°C, enhancement was observed and increased proportionally with the rise in temperature. The increase in monocyte complement receptor enhancement was particularly marked between 30°C and 37°C. The higher concentrations of casein gave correspondingly increased rosette formation at these temperatures.

DISCUSSION

In a recent short report we established that leucoattractants enhance complement receptors on human neutrophils and monocytes (Kay, Glass & Salter, 1979), and that these observations, taken together with previous studies on the eosinophil (Anwar & Kay, 1977a; Anwar & Kay, 1978) indicated that complement receptor enhancement by agents which promote cell locomotion might be a general biological phenomenon. In the present report we have studied details of complement receptor enhancement on the blood monocyte. It seemed important to extend our knowledge of complement receptors to human monocytes since cells of the monocyte/macrophage series are not only specialized phagocytic cells but also synthesize and secrete a variety of products into their immediate microenvironment. In addition, quantification of the degree of complement receptor enhancement might provide a further variable for studies of monocyte function both in health and disease.

The rosette method for identifying cell surface markers is a well established technique. However, a major disadvantage, which applies particularly to the present study is the difficulty in measuring the degree of binding between the monocyte membrane and individual indicator erythrocytes. For instance,

treatment with the various leucoattractants not only increased the percentage of monocytes having three or more adherent red cells but the red cell binding itself appeared far more 'firm' than that observed in the untreated controls. Techniques that facilitate measurements of the binding affinity are required to appreciate the full extent of the complement receptor enhancement phenomenon.

It should be noted that in the present experiments the amount of complement on the red cells was limited to give approximately 25–35% rosettes in the untreated (control) monocyte suspensions. Even when the amount of complement was increased or when optimal conditions were used for complement receptor enhancement, it was unusual to achieve greater than 75% monocyte rosettes. This suggested that there might be a population of monocytes which express complement receptors poorly. They may be either immature cells or possibly an as yet unidentified subset.

The capacity of casein to attract human monocytes in directional and random motion is well established (Keller & Sorkin, 1967) and, therefore, it seemed reasonable to use this agent in the majority of experiments. However, a number of other agents with chemotactic activity for monocytes gave comparable increases in the percentages of complement rosettes (Figs 4 and 5). These included supernatants from lymphocytes cultured in the presence of PHA (Snyderman *et al.*, 1972), a supernatant from *C. parvum* 10390 (Wilkinson, O'Neill & Wagshaw, 1973) and several formyl-methionyl peptides (Showell *et al.*, 1976). Snyderman *et al.* (1972) found a certain amount of monocyte chemotactic activity in supernatants from unstimulated lymphocytes. In the present study the highest concentration of the unstimulated supernatants also gave enhancement of monocyte complement rosettes. These observations all support the view that there is a direct relationship between cell locomotion and enhancement of complement receptors (Kay, Glass & Salter, 1979).

The apparent inability of casein and F-Met-Leu-Phe to alter receptors for IgG on the monocyte is in agreement with previous observations on the eosinophil in which it was found that chemoattractants affected the expression of complement but not Fc receptors (Anwar & Kay, 1977a; Anwar & Kay, 1978). The reasons for these apparent differences between complement and IgG receptors is not clear at this time but may be explained by the experimental procedure. For instance, optimal IgG rosettes are obtained at 0°C whereas 37°C is used for complement. Also, in the time course experiment (Fig. 2) a slight increase in Fc receptors was found but this was statistically insignificant. Although it seems likely that IgG receptors are not enhanced to the same degree as complement receptors, further studies using different techniques for studying cell surface markers (i.e. immunofluorescence) will be required before firm conclusions can be drawn.

We have considered the possibility that our observations might have been the result of unfolding of receptors for IgM (Fc), rather than for complement, in a similar way that *Vibrio cholerae* neuraminidase exposes cryptic sites for the Fc portion of IgM on rabbit and human monocytes (Haegert, 1979). It is unlikely that our present findings can be explained in this way since, at least with human neutrophils and eosinophils, receptor enhancement was observed with $EA_MC1423b$ and EA_MC14 but not with $EA_MC1423b'$ (i.e. C3b cells treated with the C3b inactivator (Law, Fearon & Levine, 1979)). Thus not all complement intermediates prepared with EA_M react in receptor enhancement (Anwar & Kay, 1978). Comparable experiments with monocytes using red cells coated with various complement components are in progress as are blocking studies with IgM.

Although we have drawn the general conclusion that agents which promote cell locomotion also enhance complement receptors, we cannot say with certainty whether this effect is related to their chemotactic and/or chemokinetic principles. It has been shown that casein (Keller & Sorkin, 1967), the F-Met peptides (Showell *et al.*, 1976) and *C. parvum* chemotactic factor (Russell *et al.*, 1976) promote directional migration as well as increasing the rate of cell movement. In order to establish that the chemotactic, rather than the chemokinetic, principle is required for complement receptor enhancement, further studies using substances which increase random migration, but are not chemotactic, are required. As discussed elsewhere (Anwar & Kay, 1978) possible explanations of the mechanisms of complement receptor enhancement include 'membrane unfolding', 'receptor externalization' and 'subunit association'. The generation of new receptors was considered unlikely since optimal enhancement, at least with casein, was achieved by 30 to 60 min.

Complement receptor enhancement on the monocyte (and also the neutrophil (E. J. Glass, unpublished observation)) appears to be both reversible (Fig. 6) and temperature-dependent (Fig. 7). Although these findings do not support any single mechanism (see above) they may be useful observations in our endeavours to provide insight into the mechanism of complement receptor enhancement. For instance, it is reasonable to suppose that during receptor enhancement there is an alteration in membrane fluidity which is both reversible (Fig. 6) and probably occurs in the upper temperature range, i.e. between 30°C and 37°C (Fig. 7). By the use of a combination of various techniques which might include cell membrane fractionation, the use of radiolabelled complement components and membrane fluidity probes we hope to develop further our knowledge of this phenomenon.

At the present time one of our principal interests relates to the possibility of complement receptor enhancement as a useful clinical laboratory test. For instance, although the response to histamine is usually small there is a marked enhancement of monocyte complement receptors by this agent in a few individuals (E. J. Glass, unpublished observation). The reasons for such variations between apparently healthy individuals are presently unclear but the possibility that it may be related to their atopic state is being explored. Also, the effect of tumour-derived products on complement receptor enhancement by normal monocytes is the subject of study in progress (Glass & Kay, 1979), details of which will be the subject of a separate report.

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Inhibition of monocyte complement receptor enhancement by low molecular weight material from human lung cancers

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SUMMARY

We have studied the effect of dialysates from lung cancer homogenates to alter both the expression of complement (C3b) receptors *per se* and also to inhibit leucoattractant-induced enhancement of complement rosettes on monocytes from healthy individuals. Enhancement and enhancement-inhibition by tumour extracts were compared with material derived from normal lung excised some distance from the tumour. There was no significant difference between tumour homogenate (TH) and normal lung homogenate (NLH) in terms of enhancement of complement rosettes *per se*. In contrast, TH produced a dose- and time-dependent inhibition of leucoattractant-induced enhancement of C3b rosettes which was significantly different from that obtained with NLH. This enhancement-inhibition was observed with four undifferentiated, four squamous and three adenocarcinomas of lung. The degree of enhancement-inhibition was not related to the type of tumour or varying accompanying histological features such as necrosis and the degree of infiltration with inflammatory cells. Following gel filtration on Sephadex G-50 each type of cancer gave a major peak of inhibitory activity which eluted with molecules having an apparent molecular size of approximately 3,000 daltons. A second larger peak (8,000–10,000 daltons) was also detectable with extracts from the undifferentiated and adenocarcinomas. These results support previous findings, mainly from experimental animals, indicating that 'anti-macrophage/monocyte principles' are elaborated from certain tumour types.

INTRODUCTION

It is a widely-held view that cells of the lymphoid series, particularly mononuclear phagocytes, are principal effector cells in tumour immunity. Although the role of lymphoid cells in immunological surveillance of tumours in general, and of macrophages in particular, is controversial, it seems likely that some tumours, at some stage in their development, are susceptible to destruction by mononuclear phagocytes. Macrophages are prominent in syngeneic animal tumours (Evans & Alexander, 1972) and, in mice, can inhibit tumour growth when inoculated together with tumour cells (Bennett, 1965). Macrophages can also enhance the inhibition of tumour progression mediated by BCG (Hopper & Pimm, 1976).

A number of investigators have reported that tumour-derived material from experimentally-induced neoplasms inhibit macrophage function both *in vivo* and *in vitro*. In general, the inhibitory activity has been associated with molecules having a molecular size of less than 10,000 (Fauve *et al.*, 1974; North, Kirstein & Tuttle, 1976; Pike & Snyderman, 1976; Nelson & Nelson, 1978; Normann & Cornelius, 1978). As a result of these observations in animals and studies in man which indicate

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that monocyte locomotion is defective in certain solid tumours such as genito-urinary neoplasms and malignant melanoma (Boetcher & Leonard, 1974; Hausman *et al.*, 1975), we have attempted to establish whether human tumours elaborate 'anti-macrophage' principle(s) comparable to those previously described in mice and rats. We chose to study extracts from lung cancers since fresh material was readily available and because our previous work indicated that the advanced stage of this disease was also associated with a defect in the locomotion of peripheral blood monocytes (Kay & McVie, 1977).

Rather than employing inhibition of monocyte locomotion as our test system we have used a newly developed assay of monocyte function – complement receptor enhancement (Glass & Kay, 1980). 'Complement receptor enhancement' is the term we have given to increased expression (or unfolding) of C3b receptors on human leucocytes following incubation with leucoattractants. The phenomenon was first described with eosinophils and 'eosinophilotactic' agents such as histamine and ECF-A tetrapeptides (Anwar & Kay, 1977, 1978). However, we have also shown that complement receptor enhancement is a property of human neutrophils and monocytes following incubation of these cells with various chemoattractants such as formyl-methionyl peptides, lymphokines and casein (Glass & Kay, 1980; Kay, Glass & Salter, 1979). Thus complement receptor enhancement is induced by leucoattractants and occurs in parallel with cell locomotion. We believe that this apparent increase in the density of complement receptors may be indicative of a general biological phenomenon whereby the degree of adhesion of phagocytic cells to opsonized particles is increased.

In the present report we provide evidence that dialysates from a number of human lung cancer homogenates, when compared to dialysates from normal lung, inhibit casein-induced enhancement of monocyte complement receptors. Furthermore, the inhibitory principles appear to have a similar molecular size to material previously identified from animal tumours and which inhibited macrophage chemotaxis.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: casein (BDH Chemicals Ltd, Poole, England); preservative-free heparin (Evans Medical, Liverpool, England); Ficoll and Hypaque, Sephadex G-50 (Pharmacia, Uppsala, Sweden).

Methods

Homogenates from lung cancer and normal lung controls. Fresh surgical specimens were obtained at thoracotomy and placed directly into sterile plastic bags. A portion of approximately 5 g was removed from the tumour mass in such a way as not to jeopardize the histological diagnosis. A portion of normal tissue of comparable size to the tumour was taken from the periphery of the lobe or bronchopulmonary segment. Portions of the normal lung, as well as the tumour mass, were prepared for routine histopathological sections. The unfixed tissue was either stored at -80°C or prepared immediately in the following way: samples of tumour, or normal lung, were cut into small fragments of approximately 200–400 mg and placed in 5–15 ml of PBS. The materials were frozen and thawed six times, centrifuged at 1,800 ***g*** for 10 min to remove particulate matter and the supernatants dialysed against PBS (twice the volume) for 18 hr at 4°C. The protein concentration of the dialysates was determined by the Coomassie blue method and the samples divided into portions of approximately 1 ml and stored at -80°C until use.

The various histological features of the tumours were assessed by two independent pathologists and graded (0, $\pm$, +, ++, +++) as shown in Table 1. The portions of normal lung from the periphery of the sections were free of microscopically detectable tumour cells.

Preparation of EA, EAC1423b and 'EAC'. This has been described in detail elsewhere (Anwar & Kay, 1977, 1978) and was briefly as follows. Dextrose–gelatin–veronal buffer ($DGVB^{2+}$, pH 7·4) was used for washing sheep erythrocytes (E) during sensitization and coating with complement. The buffer was prepared by mixing equal volumes of isotonic veronal-buffered saline (containing 0·0015

M Ca^{2+}, 0·0005 M Mg^{2+} and 0·1% gelatin–veronal buffer, GVB^{2+}) with 5% dextrose in water containing the same concentration of Ca^{2+} and Mg^{2+}. The IgM and IgG fractions of rabbit antisera to sheep red cells (A) were prepared by Sephadex G-200 gel filtration (Shevach *et al.*, 1972). The IgG fraction was further purified by DEAE-52 anion exchange chromatography (Fahey & Terry, 1978). Either IgG or IgM was used in the sensitization of E for the preparations of EA_G^{rab} or EA_M^{rab} respectively. The antibody concentration of EA_M^{rab} selected was that which gave optimal sensitization for haemolysis but no agglutination. For the preparation of EA_G^{rab}, the antibody concentration was diluted so as to give approximately 30% monocyte rosettes. Functionally pure human complement components were added sequentially to EA_M^{rab} to prepare C3b-coated cells (EAC1423b). The amounts were as follows: 400 effective molecules of C1, 400 of C4, 50 of C2 and 400 of C3. This amount of C4 was insufficient to give EAC14 rosettes with monocytes (Anwar & Kay, 1978). In most experiments fresh human AB serum was also used as a source of complement ('EAC'). Equal volumes of EA_M^{rab} were used at a concentration of 1×10^8 red blood cells/ml. The dilution of fresh serum was adjusted to give between 20 and 30% monocyte rosettes and was usually between 1 in 600 and 1 in 1,000.

Monocytes. Blood from healthy donors was drawn into plastic tubes containing 10 units of preservative-free heparin/ml. Monocytes were separated on Ficoll–Hypaque cushions according to the method of Böyum (1968). The separated cells were washed twice in Dulbecco's PBS (modified) (pH 7·2–7·4), containing preservative-free heparin (2 units/ml) and resuspended in medium 199, pH 7·4, to a concentration of 1×10^6 monocytes/ml.

Complement (EAC) and IgG (EA_G) rosettes and rosette enhancement. For the experiments described in Table 2 equal volumes of monocytes (1×10^6/ml) and various concentrations of casein or medium alone were mixed and incubated in a shaking water bath, usually at 37°C for 30 min. The cells were then washed twice in medium 199 and the numbers readjusted to their original concentration in the same medium. A portion (0·1 ml) of EAC or EA_G (1×10^8/ml) was added to 0·1 ml of cells

Table 1. Histological features of undifferentiated, squamous and adenocarcinomas from human lung

	Accompanying histological features				
Patient	Necrosis	Viable tumour	Fibrous tissue	PMNs	Chronic inflammatory cells
Undifferentiated					
1. Large cell	+	++	+	0	±
2. Large cell	±	++	++	±	+
3. Small cell	+	++	++	0	±
4. Small cell	++	+	±	0	±
Squamous					
5. Moderately well differentiated	++	+	±	±	+
6. Poorly differentiated	±	++	+	±	+
7. Poorly differentiated	±	++	++	+	±
8. Poorly differentiated	++	++	+	++	+
Adenocarcinoma					
9. Well differentiated	±	++	±	++	+
10. Poorly differentiated	±	++	+	+	+
11. Moderately well differentiated	0	+++	++	0	0

0 = Nil; ± = very slight; + = slight; ++ = moderate; +++ = marked. The tumour type was assessed independently by two histopathologists.

Table 2. Enhancement of monocyte complement rosettes by casein

	Rosettes (%)		
	'EAC'	EA_M1423b	EA_G
Medium 199	25±4	23±1	30±3
Casein			
1 mg/ml	34±5	35±1	31±4
2 mg/ml	42±2	41±2	30±4
3 mg/ml	48±6	46±2	34±5

Red cell intermediates were sensitized with IgM and prepared either with whole serum ('EAC') as a source of complement or with purified components (EA_M1423b) or with IgG (EA_G). The results represent the mean ±1 s.e.m. of three experiments.

and centrifuged at 100 ***g*** for 10 min at 4°C and the pellets incubated at 37°C for 20 min for EAC and 0°C for 30 min in the preparation of EA_G. The pellet was gently resuspended in medium 199 containing 1% formal–saline and smears prepared on clean glass slides in duplicate. These were dried quickly in air, fixed in methanol and stained with May–Grünwald/Giemsa or fixed in 2·5% glutaraldehyde and stained using α-naphthyl acetate histochemistry according to the method of Yam, Li & Crosby (1971). Comparison of these two methods by four independent observers revealed no statistical significance in differentiating monocytes from lymphocytes.

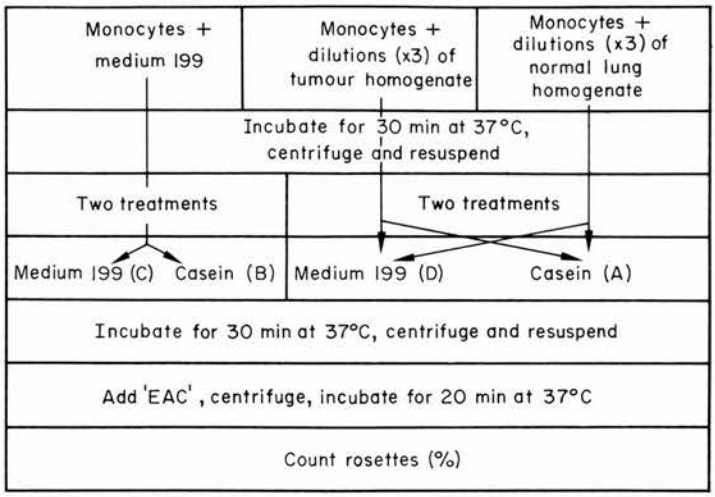
Inhibition of casein-induced enhancement by tumour or normal lung homogenates. Monocytes were prepared as described above and divided into 14 portions of 0·1 ml each containing 1×10^6 monocytes/ml. The experimental design is shown in Fig. 1. Two tubes were required for 'enhancement by casein alone' [(B − C/C) × 100] and this was performed as described for Table 2 (but with the addition of a 30-min incubation period at 37°C, after which the cells were centrifuged (300 ***g*** for 10 min at 4°C) prior to the addition of 0·1 ml of medium 199 (C) or casein (B) – Fig. 1). Monocyte suspensions were also incubated with dilutions of tumour extract (25 μg protein/ml – two tubes, 50 μg/ml – two tubes, 100 μg/ml – two tubes) and normal lung homogenates (25 μg protein/ml – two tubes, 50 μg/ml – two tubes, 100 μg/ml – two tubes). Three of the monocyte preparations treated with tumour extract (i.e. 25, 50 and 100 μg/ml) and three treated with normal lung (i.e. 25, 50 and 100 μg/ml) were then incubated with either medium 199 or casein (3 mg/ml). Enhancement, and enhancement-inhibition were calculated as described in Fig. 1.

Gel filtration. Samples (0·75 ml) of either tumour or normal lung extract were applied to a column of Sephadex G-50 (1·5 × 100 cm). The column was calibrated with molecular markers (blue dextran, cytochrome *c* and vitamin B_{12}) in phosphate-buffered saline (pH 7·2) and 1-ml fractions collected. Every third fraction was tested for inhibition of casein-induced monocyte complement receptor enhancement using 0·1-ml amounts as described in Fig. 1.

RESULTS

Enhancement of complement rosettes

The capacity of casein, a recognized monocyte leucoattractant, to enhance the percentage of monocyte C3b rosettes is shown in Table 2. The effect was dose-dependent and demonstrable when either 'EAC' or EA_M1423b were used as indicator erythrocytes. In contrast, there was a small but statistically insignificant increase in the percentage of EA_G (Fc) rosettes. When expressed as a percentage increase, enhancement of Fc receptors by the highest dose of casein was only 13%



$$\text{Enhancement by casein alone} = \frac{B-C}{C} \times 100$$

$$\text{Enhancement by tumour or normal lung alone} = \frac{D-C}{C} \times 100$$

$$\text{Inhibition of casein-induced enhancement by tumour or normal lung} = 100 - \frac{(A-C)}{(B-C)} \times 100$$

Fig. 1. Schematic outline of the methodology for monocyte complement receptor enhancement and enhancement inhibition by tumour and normal lung homogenates.

whereas with 'EAC' or EA_M1423b the increases were 92 and 100% respectively. Because of the similar results obtained using either 'EAC' or EA_M1423b in terms of the dose response by casein (and also, as reported elsewhere, as a function of time (Glass & Kay, 1980)) for convenience 'EAC' were used as indicator cells in further experiments.

Inhibition of enhancement

A dialysable extract from a homogenate of an adenocarcinoma of the bronchus was tested both for its effect on the percentage of monocyte complement rosettes and for its capacity to inhibit casein-induced monocyte complement receptor enhancement. The tumour extract, at concentrations of 25, 50 and 100 μg protein/ml, was compared with microscopically normal lung from the same resected specimen. Neither the tumour homogenate (TH) nor the normal lung homogenate (NLH) significantly influenced the percentage of rosettes with 'EAC'. In contrast, the TH inhibited casein-induced enhancement in a dose-dependent fashion which at the highest concentration (100 μg protein/ml) was significantly different from NLH ($P < 0{\cdot}05$).

The time course of inhibition enhancement by TH and NLH from another adenocarcinoma of the bronchus is shown in Fig. 2. The percentage increase was maximal at 30 min with no further inhibition being observed up to 2 hr at which time the experiment was terminated. Inhibition by NLH was considerably less and when expressed as the mean of three experiments was virtually negligible.

Various histological types

Undifferentiated. The effect of TH and NLH from four patients with undifferentiated carcinoma of the bronchus on monocyte complement rosettes and casein-induced enhancement is shown in Fig. 3. The percentage enhancement of the normal donor monocytes by casein was comparable to that achieved in the experiments described in Table 2. Both TH and NLH promoted some complement receptor enhancement *per se* although the differences between them, at the three concentrations tested, were not significant. Furthermore, the dose–response with NLH was 'flat'

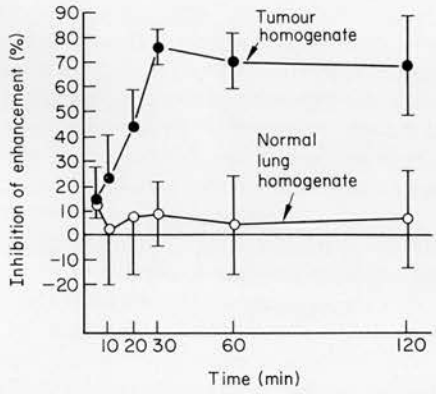


Fig. 2. The time course of inhibition of leucoattractant-induced enhancement of monocyte complement receptors by tumour and normal lung homogenates. Each point represents the mean ±1 s.e.m. of three experiments and the experimental design is shown in Fig. 1. The material was derived from an adenocarcinoma of lung.

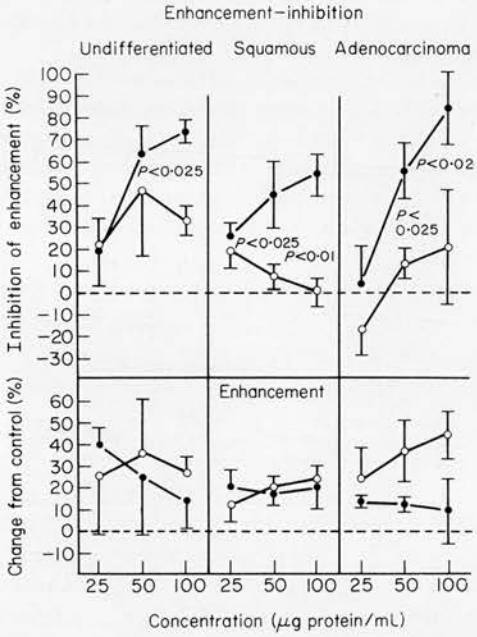


Fig. 3. Inhibition of leucoattractant-induced enhancement of monocyte complement rosettes by homogenates from various lung cancers. (●) Tumour and (○) normal lung homogenate. Each point represents the mean ±1 s.e.m. of four undifferentiated tumours, four squamous and three adenocarcinomas of lung. The *P* values were calculated from Student's t-test. The experimental design is outlined in Fig. 1.

and with TH there was apparent inhibition at higher doses. In contrast, TH when compared to NLH gave significant *inhibition* of casein-induced enhancement at the highest concentration tested. The inhibitory effect of TH increased in proportion to the concentration of TH. Some inhibition of enhancement was observed with NLH but the dose–response was not linear, less effect being observed at 100 μg than with 50 μg protein/ml. The individual data (not shown) indicate that although there was considerable variation in the amount of inhibition achieved by the four undifferentiated tumours, the general pattern of increased enhancement inhibition by TH, as compared to NLH, was sustained.

Squamous. Comparable results were obtained with four squamous carcinomas of the bronchus (Fig. 3). With these tumours the difference between TH and NLH in terms of their effects on receptors *per se* was negligible although with both materials there was a slight increased percentage change from control but this did not appear to be related to the concentration of the extracts. By contrast, *inhibition* of casein-induced enhancement by these tumour extracts increased with dose and, at both the 50 and 100 μg concentrations, was significantly different from NLH. NLH from the squamous tumour resections gave very little enhancement-inhibition, even at the higher concentrations.

Adenocarcinoma. Three adenocarcinomas of the bronchus were tested in a comparable manner (Fig. 3). The NLH from these resections gave a dose-dependent increase in monocyte complement rosettes *per se*, but the effects were not significantly different from TH. With enhancement-*inhibition* there was a marked and statistically significant difference between TH and NLH at the 50 and 100 μg protein concentrations. The individual results (not shown) indicate that the variations in observed effects were wide, but the general pattern of dose-dependent inhibition of casein-induced enhancement by TH, as compared to NLH, was sustained.

Gel filtration of homogenates

TH and NLH from one undifferentiated (patient 1), one squamous (patient 5) and one adenocarcinoma (patient 11) were passed separately through a column of Sephadex G-50, previously calibrated with molecular markers. Fractions were tested for inhibition of enhancement as described in the methodology (Fig. 4).

Virtually all the fractions tested, irrespective of whether they were TH- or NLH-derived, gave inhibition of enhancement although in some the values were below zero. Because of the high 'background' activity with the normal lung homogenates only those peaks having maximal enhancement-inhibition of 40% or more were considered as having appreciable biological activity. Using this 'cut-off' point it could be seen that with the undifferentiated tumour there were two peaks of activity of approximately 3,000 and 8,000 daltons respectively. The squamous tumour also had a

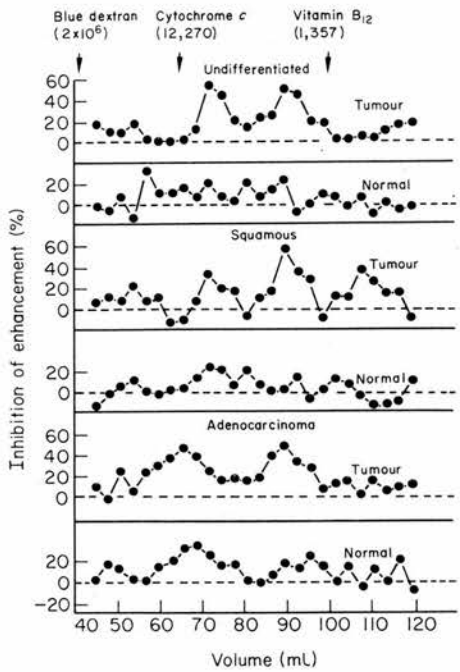


Fig. 4. Sephadex G-50 gel filtration of homogenates from lung cancer and normal lung. The points are the mean of three assays of inhibition of leucoattractant-induced enhancement.

peak at 3,000 and the adenocarcinoma had inhibitory activities at 3,000 and 10,000 daltons approximately. Thus all three tumour types had a peak molecular weight at 3,000 and the undifferentiated and adenocarcinoma had activities between 8,000–10,000 daltons. NLH from each tumour type did not give peaks of greater than 35% and in most measurements it was less than 20% enhancement-inhibition.

DISCUSSION

The main purpose of the experiments reported here was to determine whether factors derived from human lung cancer affected leucoattractant-mediated increase in complement receptor 'density' of blood monocytes from healthy individuals. Although we have provided evidence for the presence of such inhibitory principles we cannot say with certainty whether they are tumour-associated or tumour-derived. It is of note that there did not appear to be an association between the degree of inhibition and the histological features of the tumours (Table 1). For example, tumour nos. 3, 4 and 11 had little or no associated chronic inflammatory cells or infiltration with polymorphonuclear leucocytes but they all gave considerable inhibition of enhancement of complement receptors. Similarly, tumour nos. 2 and 11 had little or no necrosis but considerable inhibitory activity could be derived from the tumour mass. Thus the inhibitory properties of the tumour dialysate would not appear to be directly attributable to the degree of acute and chronic inflammation, necrosis or fibrosis associated with the neoplastic lesion.

We have so far only examined the effect of TH on casein-induced complement receptor enhancement on the blood monocyte and are unable to say whether a similar effect would be demonstrable (a) with other blood leucocytes such as neutrophils and eosinophils and (b) with other appropriate C-receptor-enhancing chemoattractants. Because of limitations in the supply of tumour extract we felt that priority should be given to preliminary studies on the possible heterogeneity of the activities as assessed by gel filtration chromatography. As shown in Fig. 4 each tumour contained at least two peaks of inhibitory activity although NLH did not give inhibition of $>40\%$. Taking 40% inhibition as a reasonable cut-off point it can be seen that the only substantial peaks of inhibitory activity are in the tumour extracts. All three tumour types shared one peak which eluted just before the vitamin B_{12} molecular marker and had an estimated molecular size of 3,000 daltons. The undifferentiated and adenocarcinoma tumour extracts both had a higher peak of inhibitory activity at approximately 8,000–10,000 daltons. The squamous tumour also had a peak at 8,000–10,000 daltons but maximal activity was just $>40\%$. This aspect of the study clearly requires expansion to include more detailed protein-separation techniques.

It is generally agreed that there is no clear association between clinical prognosis and the histological types of various forms of lung cancer. There was no evidence from the present study that the degree of inhibitory activity was related to the histological type.

There is formidable literature on the relation between macrophages and cancer and, as mentioned in the Introduction, there are a number of animal studies which support the view that tumours elaborate anti-macrophage material. Snyderman and his colleagues reported that mouse tumours inhibited macrophage mobilization in the peritoneal cavity and that a number of cell lines produce a factor of molecular weight between 6,000–10,000 daltons which inhibits macrophage chemotaxis *in vitro* (Pike & Snyderman, 1976; Snyderman & Pike, 1976). The agent was heat-stable and had peak inhibitory activity 3 days after administration. When the factor was injected together with tumour cells into syngeneic mice there was an increase in tumour dissemination when compared to the effect of the tumour alone. Fauve *et al.* (1974) have demonstrated that mouse teratocarcinoma and other tumour cells in culture elaborate a factor of between 1,000 and 10,000 which inhibits local inflammation *in vivo*, and these same cells *in vitro* were found to prevent direct contact by normal peritoneal macrophages. Nelson & Nelson (1978) have described a similar factor produced by murine fibrosarcoma cells which depressed delayed-type hypersensitivity reactions *in vivo* and inhibited macrophage chemotaxis *in vitro*. It is believed to be glycopeptide in nature and associated with an RNA fragment. North *et al.* (1976) reported that in the serum of tumour-bearing mice there was a dialysable factor which depressed macrophage-mediated resistance to infection.

In the present report we have described low molecular weight material derived from human lung cancers which inhibits leucoattractant-induced enhancement of complement receptors on normal human monocytes and in this respect may be analogous to factors described in animals which depress macrophage function. Further studies are required to characterize these principles more fully and to explore the possibility that active immunity to them may have therapeutic potential.

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