

ALLERGY AND THE NEUTROPHIL

by

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SUMMARY

The experiments and work outlined in this thesis are divided into two major topics. The first concerns the identification and measurement of a high molecular weight neutrophil chemotactic activity (HMWNCA) in biological fluids. HMWNCA had previously been described in the serum of asthmatic patients following airways obstruction provoked by a number of stimuli. The characterisation of this activity as a high molecular weight, neutral polypeptide with finite properties on anion-exchange chromatography involved time consuming biochemical techniques using conventional large volume columns. These methods held two major problems. The first was that the time involved in column chromatography made it impossible to perform large scale experiments to investigate the time course of HMWNCA release. The second problem was that the large column volumes were responsible for a considerable amount of sample loss.

It was felt that, in order to perform more detailed investigations into the time course of HMWNCA release both into serum and from immunologically challenged lung fragments, it was necessary to design a method that would reliably identify the factor by its known physico-chemical properties (i.e. molecular size, anion-exchange properties and iso-electric point), in a much shorter period of time than that used in previous methods.

Using a combination of fast protein and high performance liquid chromatography, I have described such a method. Although there are difficulties with this technology, (as I have tried to

point out throughout this thesis), it does identify NCA with the same anion exchange properties as HMWNCA and with a molecular size of > 440,000 daltons.

Using this method, I have described the release of HMWNCA into human nasal secretions of atopic individuals following nasal challenge with specific allergen, but not with methacholine. Both methods of challenge produced significant rhinorrhoea. Thus it would appear that HMWNCA was released following an interaction of specific allergen with sensitised mediator cells in the upper airways.

Using a modification of the same chromatographic methods, I have described the time course and dose-dependence of HMWNCA release from human lung fragments challenged with a rabbit antibody to human IgE. HMWNCA was identifiable under similar optimal conditions as those required for histamine release from isolated human lung mast cells.

The other major topic described in this thesis concerns some of the characteristics of the interaction between neutrophils and pollen grains. This followed on from a series of experiments originally designed to investigate the role of complement in the binding of granulocytes to particulate allergens in vitro. It was found that both heated and unheated serum contained a factor (human granulocyte/pollen binding factor (GPBP)) which promoted such adherence; this was later identified as human serum transferrin. The work in this thesis describes the optimum conditions for this reaction and investigates the role of the transferrin receptor in the adherence. Specifically, GPBP-induced binding was time-,

temperature- and concentration-dependent. Maximal adherence was observed at 2 hours, and the optimal temperature for adherence was 37°C. Concentrations of GPBP as low as 1.25 µg/ml promoted significant neutrophil adherence, but eosinophils, monocytes and lymphocytes did not participate in the binding reaction even at high concentrations of GPBP. Neutrophils adhered to a wide variety of pollen grains, but not to inert beads of a similar size. The reaction was Mg^{++} , but not Ca^{++} dependent, and was not inhibited by a monoclonal antibody to the transferrin receptor. A purified commercial transferrin reacted in all respects like GPBP in these pollen binding studies.

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AIMS AND OBJECTIVES.

A. High molecular weight neutrophil chemotactic activity
(HMWNCA).

- (i) To devise a rapid method of partially purifying HMWNCA from human serum and human lung tissue.
- (ii) Using this method, to optimise the conditions for release of HMWNCA from immunologically challenged human lung fragments.
- (iii) To determine whether HMWNCA is a preformed mediator of hypersensitivity.
- (iv) To determine whether HMWNCA is present in nasal secretions induced by local allergen challenge in patients with allergic rhinitis.
- (v) To attempt to produce a monospecific antibody to human HMWNCA.

B. Neutrophil-pollen grain interactions.

To investigate the characteristics of a serum factor which promotes adherence of neutrophils to pollen grains in vitro.

ABBREVIATIONS

HMWNCA	High Molecular Weight Neutrophil Chemotactic Activity
NCA	Neutrophil Chemotactic Activity
EIA	Exercise-Induced Asthma
FPLC	Fast Protein Liquid Chromatography
HPLC	High Performance Liquid Chromatography
LT	Leukotriene, e.g. LTB_4
NHS	Normal human serum
PAF	Platelet Activating Factor
PG	Prostaglandin, e.g. $\text{PGF}_{2\alpha}$
Tx	Thromboxane, e.g. TxB_2

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CHAPTER ONE

NEUTROPHILS AND THEIR ROLE IN ALLERGIC DISEASE

Introduction

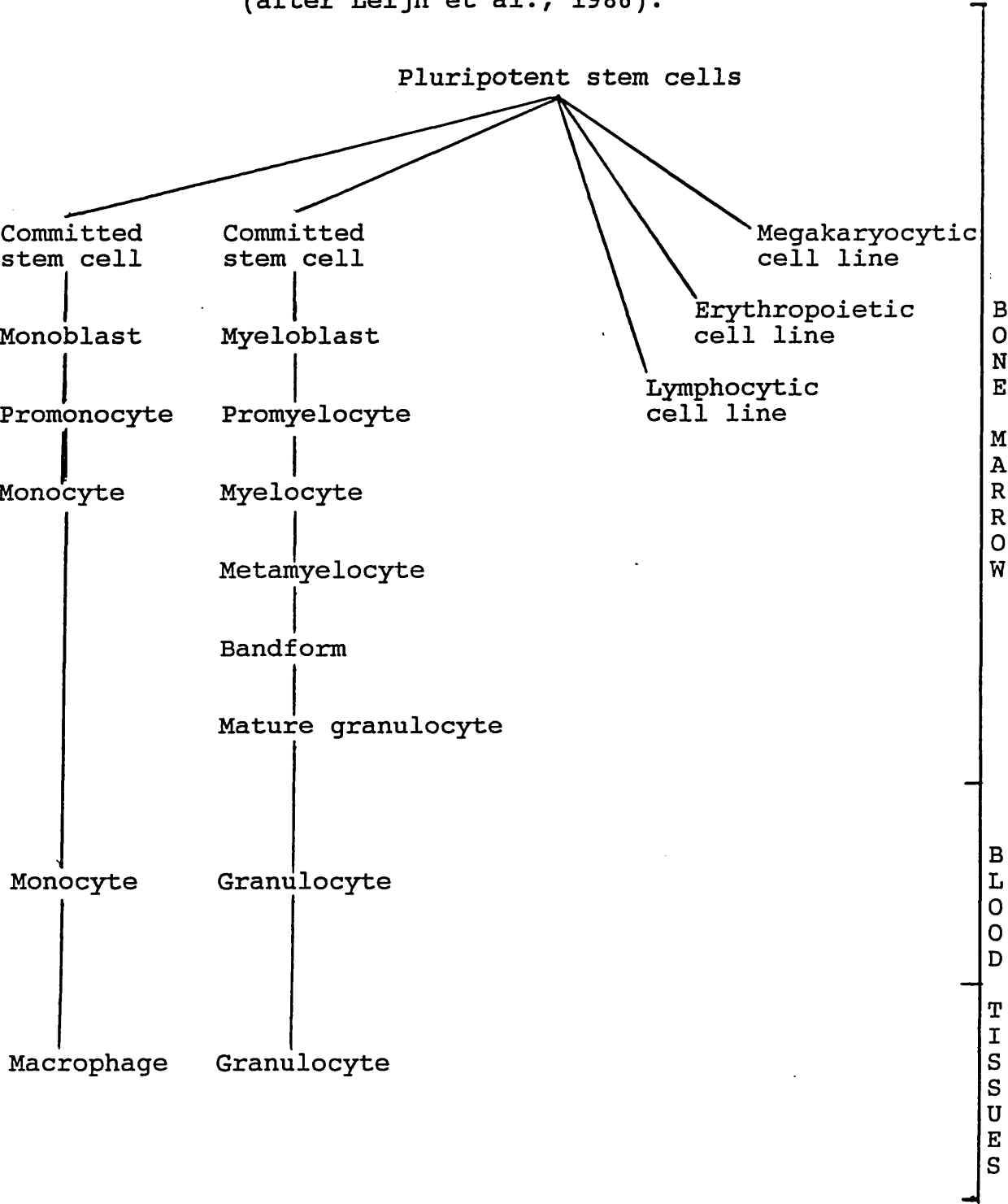
Neutrophils play a central and critical role in acute inflammatory reactions, being attracted to the site of tissue damage and inflammation by factors which promote chemokinesis and chemotaxis. At the site of inflammation, they play a part in the destruction and disposal of foreign material, be it bacterial or parasitic, both by formation of partial reductive products of oxygen, i.e. superoxide anion, and by phagocytosis. In addition, and to the detriment of the host, they also "leak" some of their phagosomal contents, many of which may lead to further host tissue damage.

Neutrophils are phagocytic cells, derived from the same common haemopoietic cell line as the monocyte-macrophage system (Figure 1.1). Together, these cells comprise the most numerous types of phagocytic cells in higher organisms, and are sometimes termed "professional" phagocytes.

(i) Neutrophil morphology and metabolism

The neutrophil leucocyte, the most abundant form of leucocyte in human blood, is a cell of approximately 12-15 μm diameter, containing a multi-lobed nucleus and numerous cytoplasmic granules. The nucleus is coarsely clumped and contains between 2 and 5 lobes inter-connected by thin strands of nuclear material (Dale, 1984). Five to ten percent of normal granulocytes contain certain nuclear irregularities including pockets, bridges, appendices and fibrillar bodies (Laszlo et al., 1977).

Figure 1.1 Differentiation of neutrophils from the
haemopoietic stem cell.
(after Leijh et al., 1986).



As the nucleus does not contain a nucleolus, it is unable to synthesise any new ribosomes. In addition, there is very little incorporation of labelled thymidine into the DNA, which suggests that there is no replication or DNA repair-synthesis.

The main energy-producing pathway in the neutrophil is glycolysis; this results in the conversion of glucose to lactate. Normal granulocytes do not exhibit the Pasteur effect, i.e. they do not decrease their rate of glycolysis in the presence of oxygen (Beck, 1958). If intact neutrophils are incubated with ^{14}C , approximately 80% of the radioactivity is recovered in lactic acid (Silber et al., 1977). However, in cells which are involved in active phagocytosis, approximately 30% of their energy may be derived from the metabolism of glucose via the hexose monophosphate shunt (Dale, 1984). As there are very few mitochondria in granulocytes, the tricarboxylic acid cycle and oxidative phosphorylation can play only a minor part in energy production (Foster et al., 1967).

Neutrophils contain a large amount of glycogen and granulocytes contain most of the glycogen found in leucocytes (Gibb et al., 1949). Neutrophil cytoplasm contains large amounts of actin which can polymerise to form long micro-filaments. These filaments are not arranged in the orderly manner seen in skeletal muscle sarcomeres, but in combination with myosin, ATP, magnesium and gelsolin, are involved in the force generation required for locomotion, phagocytosis and secretion. The actin appears to be arranged in dense networks, with the highest concentrations appearing in cytoplasm adjacent to activated areas of the cell membrane (Wilkinson, 1984).

(ii) Neutrophil heterogeneity.

Although they have a diverse range of functions, until recently neutrophils were thought to form a homogenous population. In recent years, peripheral blood neutrophils have been shown to be heterogeneous in several ways, both functionally and in terms of their surface membrane receptors. It has been known since the 1920s that neutrophils varied greatly in their rates of locomotion (McCuthcheon, 1923), but this was thought to be a measure of differences in cell maturity. More recently, however, neutrophils obtained from clot preparations have been divided clearly into two distinct populations on the basis of their locomotory rates (Howard, 1982). Similarly, Harvath and Leonard (1982) have separated neutrophils into two populations on the basis of their chemotactic responsiveness. Neutrophils also appear to be heterogeneous in their ability to phagocytose opsonised particles; in studies by Gallin (1984) it was found that, in vitro, 20% of adherent neutrophils would not ingest opsonised, heat-killed *Candida albicans*, in conditions considered optimal for phagocytosis.

Neutrophils appear to differ in their cell surface expression of receptors for IgG-Fc fragments. Messner et al. (1970) found that less than 50% of a population of peripheral blood neutrophils formed rosettes with IgG coated erythrocytes, whilst Wong and Wilson (1975), using a more refined technique, found that only 80% of human neutrophils formed rosettes with sheep erythrocytes coated with IgG. These "rosette-forming" and

"non-rosette-forming" subgroups could be separated by centrifugation through Hypaque Conray gradients (Klempner, 1978). Using this technique, it has been shown that the two subgroups also differ markedly in terms of their adhesiveness, chemotaxis and phagocytosis of opsonised bacteria, although there were no significant differences in their rates of random locomotion or ingestion of non-opsonised latex beads. Binding of erythrocytes coated with the purified complement components C4b, C3b, C3d and C3bi was the same in both populations (Whited, 1981). These functional differences were not attributable to different degrees of maturity or artefacts from the cell separation procedure employed (Gallin, 1984).

This functional heterogeneity has also been demonstrated in vivo, in human subjects challenged with a small dose of intravenous endotoxin. Administration of endotoxin was followed 60-90 minutes later by a neutrop^aenia which could be accounted for by depletion of rosette-forming neutrophils, with no changes in the population of circulating non-rosette forming neutrophils (Klempner, 1980). This was thought to be due to the selective stimulation of rosette-forming neutrophils leading to margination of this subgroup only as a result of complement activation. At the time of maximal neutrop^aenia, when the circulation was depleted of the subgroup of rosette-forming neutrophils, peripheral neutrophil chemotaxis was found to be lower than its baseline level (Gallin, 1984).

Similar heteogeneity has been described in the neutrophil expression of IgA receptors (Fanger, 1980), although it is not clear whether IgA receptor expression is linked with IgG

receptor expression.

Twenty per cent of normal human neutrophils fail to respond to incubation with endotoxin in vitro by the normal burst of oxidative metabolism, as assessed by nitroblue tetrazolium dye (NBT) reduction (Repine, 1979) and, as the neutropaenic period following haemodialysis is associated with an impairment of neutrophil oxidative metabolism (Cohen, 1982), it has been speculated that there was selective margination of neutrophils with good capacity for oxidative metabolism, possibly the same sub-population of cells which form IgG rosettes in vitro.

Cohen also described a 27% decrease in neutrophil f-met-leu-phe receptors during the neutropenia of haemodialysis. Expression of f-met-leu-phe receptors has been shown to be heterogeneous by binding of a fluorescein-labelled oligopeptide f-met-leu-phe-lys-fluorescein (Seligmann, 1982).

Neutrophils separated by Percoll density gradient centrifugation distribute themselves over a wide range of density layers, but can be roughly divided into high-density (1.085 - 1.10 g/ml) and intermediate density (1.06 - 1.085 g/ml) subgroups. After stimulation of the neutrophils by chemotactic stimuli, the ratio of high:intermediate density subgroups decreased (Pember, 1983). The f-met-leu-phe-induced decrease in neutrophil density was associated with increases in relative mean cell volume.

Monoclonal antibodies have been used to identify neutrophil subpopulations in recent years. Clement et al. (1983) used two monoclonal antibodies produced by hybridoma techniques, termed IB5 and 4D1 to identify two subgroups of peripheral blood

neutrophils comprising 57% and 51% of the total population respectively. The antigens recognised by IB5 and 4D1 were specific for neutrophils and were antigenically distinct.

(iii) Neutrophil granules and their contents

Neutrophil granules are divided into two categories, primary granules and secondary granules, according to their contents and to the stimuli which cause them to release their enzymes (see Table 1.1) (Falloon et al., 1986).

Mature polymorphonuclear cells have a predominance of specific granules (80-90%) compared with azurophilic granules (10-20%) (Bainton et al., 1971). Azurophilic granules are first seen in the promyelocytes and are essentially lysosomes; i.e. membrane-bound granules containing acid hydrolases (de Duve et al., 1966). Although they are numerous in the promyelocyte, primary granules are not formed in the later stages of granulocyte maturation, and so the number in each cell decreases with subsequent cell divisions. During the myelocyte stage, the specific, secondary, granules are formed.

Primary granules contain acid hydrolases, proteases, bactericidal cationic proteins and microbicidal enzymes, whereas specific granules contain lysozyme, collagenase, lactoferrin, vitamin B₁₂-binding protein, histaminase and sialidase (Spitznagel et al., 1974; Bretz et al., 1974; Ringel et al., 1984 and Cross et al., 1985). In recent years, a third granule type has been described; these granules are slightly less dense than specific granules, and contain gelatinase (Dewald et al., 1982).

TABLE 1.1 Contents of human neutrophil granules and their
possible biological activities

PRIMARY GRANULES

<u>Cathepsin G:</u>	Chymotrypsin-like protease activity Complement components Coagulation factors Proteoglycans Neutrophils Platelets Cytotoxic properties Bacteria Parasites Tumour cells Mammalian cells (e.g. erythrocytes)
<u>Elastase:</u>	Elastolytic activity Elastin Basement membrane Trypsin-like protease activity Complement components Coagulation factors Neutrophils
<u>Unspecific collagenase:</u>	Collagenolytic activity Collagen Basement membrane Trypsin-like protease activity Complement components
<u>Myeloperoxidase:</u>	Peroxidase activity Cytotoxicity (bacteria, parasites, tumour cells, mammalian cells) Mast cell activation Inactivation (protease-inhibitors, chemotactic agents, leukotrienes, prostaglandins)
<u>Lysozyme:</u>	Muramidase activity Obscure function

cont.....

Table 1.1, continued

SECONDARY GRANULES

Lactoferrin: Iron-binding properties
 Bacteriostatic
 Hydroxyl-radical (generation,
 prevention)

 Myelopoietic regulation
 Monocyte

 Adherence promotion
 Endothelial cell

Specific
collagenase

B₁₂-binding protein

Lysozyme

TERTIARY GRANULES

Gelatinase

There is considerable evidence that neutrophil granules show some heterogeneity. Acid hydrolases, on sucrose gradient centrifugation, do not band as a single granule component, but display density heterogeneity (Bretz et al., 1974). Kinetic studies of degranulation appear to suggest that myeloperoxidase (MPO) and β -glucuronidase (β GU) are in different granules (Segal et al., 1980; Bentwood et al., 1980). Myeloperoxidase itself seems to exist in several distinct forms that are differentially distributed between different subpopulations of azurophil granules (Kincade et al., 1983). The maturation process of neutrophils seems to be associated with the production of morphologically distinct subpopulations of granules (Scott et al., 1970) with differing staining characteristics. A recent paper describes a method for separation of subpopulations of human neutrophil granules using Percoll density gradients; this method has been used to identify and isolate thirteen granule fractions. Heterogeneity was not only due to maturational differences but also functional differences between neutrophils (Rice et al., 1986).

(iv) Membrane-derived neutrophil mediators

The background to the discovery of the structure and functions of leukotriene B₄ and the platelet activating factor are discussed in Chapter 2. Leukotriene B₄ was first purified and characterised as a product of human and rabbit neutrophils following short-term culture (Borgeat et al., 1979). This mediator has also been described as being released from human peripheral blood neutrophils following incubation with various

stimulants including arachidonic acid and the calcium ionophore A23187 (Borgeat et al., 1979), zymosan (Claesson et al., 1981) and the chemotactic tri-peptide f-Met-Leu-Phe (Salmon et al., 1982).

A lot of the information on the generation of LTB_4 from human neutrophils has come from studies in which neutrophils have been stimulated with the calcium ionophore A23187. LTB_4 production by human neutrophils is maximal 2 minutes after the addition of calcium-ionophore; LTB_4 is then metabolised to 20-hydroxy- LTB_4 or 20-carboxy- LTB_4 (Sun et al., 1984). This stimulus is a very potent one and is non-physiological. Recently, more physiological stimuli have been used to promote LTB_4 release from human peripheral blood neutrophils, including exposure to receptor-ligand interactions (Claesson et al., 1981).

In other studies, neutrophils stimulated with f-Met-Leu-Phe (Clancy et al., 1983) or receptor ligand interactions (Haines et al., 1987) failed to generate LTB_4 unless given exogenous arachidonic acid in concentrations which far exceed the physiological one, for example, 50 μM . Such a concentration is sufficient to stimulate O_2^- production and activate protein kinase C (McPhail et al., 1984), although the activation of protein kinase C is not sufficient a stimulus to trigger the generation of LTB_4 .

Neutrophils appear to be unable to generate the sulfido-peptide leukotrienes LTC_4 , D_4 and E_4 , although they have been shown to produce 5-HETE (Sun et al., 1984). In mixed granulocyte preparations stimulated with A23187, CaCl_2 and reduced glutathione, the amount of LTC_4 generated depended

solely on the relative amount of eosinophils in the preparations (Verhegen, 1984). However, other factors and other cell types may influence the ability of the cell to manufacture leukotrienes. For example, monokines released by lipopolysaccharide-stimulated blood mononuclear cells enhanced the ability of neutrophils to generate LTC₄ and LTB₄ after activation by the calcium ionophore (Dessein et al., 1986).

The pharmacological effects of LTB₄ are outlined in Chapter 2. Of particular interest is that LTB₄, which is produced by neutrophils, has several effects on neutrophils, including enhanced chemotaxis and chemokinesis (Ford-Hutchinson et al., 1980), and moderately enhanced granular enzyme release (Rollins et al., 1983). Other actions of LTB₄ on human neutrophils include aggregation, adherence, deoxyglucose uptake, cAMP stimulation and enhancement of C3b receptors (Bray, 1983).

Human neutrophils are also an abundant source of the platelet-activating factor, PAF acether (Lotner et al., 1980). Resting neutrophils contain 300 pg/10⁶ cells of lyso PAF, which is the direct precursor and early metabolite of PAF acether (Chilton et al., 1983) as detected by mass spectrometry. However, this figure increases 2-3 fold following incubation with the calcium ionophore A23187 (Haroldsen et al., 1987). Release of PAF acether from human neutrophils could be dissociated from that of lysosomal enzymes and is dependent on the presence of Ca²⁺ ions (Betz et al., 1980).

In addition to the stimulation of platelet aggregation, PAF acether also stimulates neutrophils. The injection of purified PAF acether intravenously into rabbits leads to the development

of an acute neutropenia and to the formation of intravascular neutrophil aggregates (McManus et al., 1981).

Synthetic PAF acether induces superoxide production by neutrophils and also induces the release of β -glucuronidase, acid phosphatase and lysosyme (but not cytoplasmic LDH) from these cells (Jouvin-Marche et al., 1982).

(v) Chemotaxis and chemokinesis

In recent years, there has been much work on the kinetic responses of leucocytes to chemoattract^{ant} chemicals. These chemoattractants can act in either (or both) of two ways, by increasing random movement of leucocytes (chemokinesis) or by increasing directional movement of leucocytes (chemotaxis) (Keller et al., 1977). To use more strict definitions, chemokinesis is "a reaction by which the speed or frequency of locomotion and/or the frequency and magnitude of turning of cells or organisms moving at random is determined by substances in the environment". In contrast, chemotaxis is defined as "a reaction by which the direction of locomotion of cells or organisms is determined by substances in their environment".

Chemokinesis can be divided into increased speed or frequency of locomotion (orthokinesis) and increased turning per unit time (klinokinesis). One of the major effects of colchicine, an anti-inflammatory drug, is due to its klinokinetic action; by causing the cells to turn more frequently, it may reduce the persistence of the random or chemotactic "walk" (Allan et al., 1978). Serum albumin has been shown to have a positive orthokinetic effect on neutrophils and lymphocytes when coated

on glass surfaces or micropore filters (Keller et al., 1978).

Substances with negative orthokinetic effects for neutrophils include immune complexes. Wilkinson et al. (1984) have shown that neutrophils moving normally on albumin-covered glass were slowed considerably on glass which had been coated with albumin anti-albumin immune complexes, which may have relevance in the accumulation of neutrophils at an inflammatory focus.

In response to chemotactic stimuli, leucocytes first orientate themselves along the chemotactic gradient, and project a leading lamellopodium in the direction of the chemotactic source (Zigmond et al., 1974). The cells then change morphologically, compacting themselves into a triangular shape. This is followed by directional movement up the chemotactic gradient; once cells have been polarised, they do not increase their velocity, but merely decrease the frequency and magnitude of turning. It has been suggested that neutrophil chemotaxis is not induced by a spatial gradient of chemoattractant alone, but that the orientated temporal component of an attractant signal is essential (Vicker et al., 1986).

Neutrophils can sense certain chemotactic agents, for example, the formulated peptide f.Met-Leu-Phe and the activated 5th component of complement, C5a, by the coupling of ligands on the chemoattractants with specific cell surface receptors. Cells may be able to adapt to high concentrations of chemoattractants and this may be a method for modulating the inflammatory reaction. Zigmond et al. (1979) showed that neutrophils changed their behaviour and morphology, in that they assumed generalised ruffling over most of their surface and

stopped random movement, on being exposed to a formylated peptide. However, these changes were only transient, and further responses were only provoked by increases in chemoattractant concentration.

(vi) Neutrophil phagocytosis and the respiratory burst

Neutrophils and other phagocytic cells are fastidious eaters, avidly devouring some particles with which they come into contact, whilst completely ignoring others. Opsonisation by IgG, with or without complement, is probably the most important factor by which phagocytes recognise phagocytosable particles. IgG molecules bind to particles by their F(ab)₂ sites, leaving the Fc site free on the particle surface. Neutrophils then bind to the exposed immunoglobulin via their Fc receptors (Messner et al., 1970). If the Fc receptors on macrophages are experimentally blocked (with blocking antibodies), phagocytosis of opsonised particles cannot take place (Griffin et al., 1975). Further studies from the same group, using erythrocytes wholly or partially coated with complement or IgG, suggested that the attachment of a particle to specific receptors on the cell membrane is not enough to "trigger" phagocytosis. Instead, the stimulus for phagocytosis requires the sequential, circumferential interaction of particle ligands with cell surface receptors, the so-called "Zipper" theory of phagocytosis. This involves the phagocyte membrane surrounding the particle in a zip-like fashion via its Fc receptors; when the particle is completely surrounded, it lies in a phagosome, the walls of which consist of inverted cell membrane. Nearby

lysosomes then fuse with the phagosome, releasing their contents around the particle, and forming a phagolysosome (Leijh et al., 1986). The manner in which the third component of complement (C3) participates in opsonisation of phagocytosable particles is less clear. There are many reports of C3-coated bacteria and erythrocytes adhering to phagocytes, with rosette formation, but not being ingested (Scribner et al., 1976). Ehlenberger et al. (1977) concluded that both C3b and C3d can act as opsonins in conjunction with IgG. Interaction of particle bound C3b with cell receptors initiates cell/particle binding, whereas cell Fc receptor interactions initiate particle ingestion. Neutrophils, which have no receptors for C3d, respond only to particles opsonised with C3b.

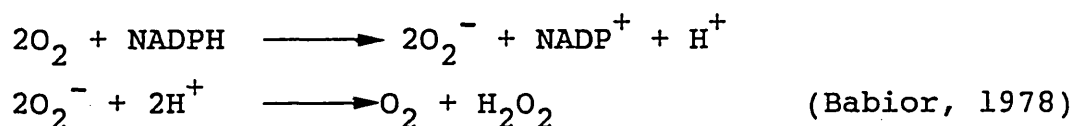
Other serum factors may play a role in opsonisation; both fibrin and the Hageman factor (clotting factor XII) enhance the adhesiveness of neutrophils (Van Oss, 1978).

Non-opsonised particles, including bacteria, can also be phagocytosed by neutrophils. The ability for neutrophils to recognise and engulf bacteria depends on their contact angles, (θ), a measure of their hydrophobicity. Bacteria which are more hydrophobic than neutrophils, for example, *Streptococcus pyogenes*, are readily ingested, whereas more hydrophilic bacteria, for example, group B *Haemophilis influenzae*, resist phagocytosis (Van Oss, 1978).

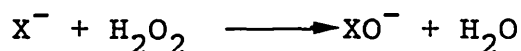
Whereas particle ingestion can take place at +4°C, and so is not an active metabolic process, intracellular killing of ingested bacteria is an energy dependent system, with maximum efficiency at 33-39°C (Leijh et al., 1980). The rate of

bacterial killing in this study depended on the bacteria:cell ratio, being higher when this ratio was lower, but the maximal rates of intracellular killing was between 3.7 and 8.5×10^5 bacteria per 5×10^6 granulocytes.

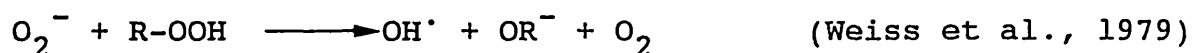
Neutrophils in their resting state consume very little oxygen, even in oxygen-rich environments (Karnovsky, 1968), but dramatically increase their oxygen consumption on phagocytosis. This process provokes a metabolic burst in neutrophils (Badwey et al., 1980), characterised by increased oxygen consumption and the production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), both of which have antimicrobial activity (Babior et al., 1978). Hydrogen peroxide may then further react to form the hydroxyl radical ($OH^\bullet$) and singlet oxygen ($^1O_2$). The reactions of the respiratory burst are as follows:



The toxicity of hydrogen peroxide is greatly enhanced through the action of neutrophil myeloperoxidase, which catalyses the oxidation of halide ions (e.g. Cl^- , Br^- and I^-) to hypo halite ions by H_2O_2 .



Hydroxyl radicals are formed from a reaction between O_2^- and a peroxy compound.



In contrast, Thomas et al. (1986) found that neutrophils alone do not cause peroxidation, implying that the formation of strongly oxidising free radicals is not an intrinsic property of the cell; the addition of exogenous iron salts catalyses per oxidation by already activated neutrophils.

In addition to their antimicrobial activity, these "active oxygen species" have also been implicated in other processes including the cytolysis of some tumour cells (Nathan et al., 1979) and inactivation of leukotrienes B_4 , C_4 , D_4 and E_4 (Henderson et al., 1983). It is possible to directly measure active oxygen species and also to measure the phenomena associated with their production, e.g. oxygen consumption and chemiluminescence. Human recombinant tumour necrosis factor (TNF) is a weak stimulant of neutrophil respiratory burst and degranulation. This effect was enhanced by the presence of unopsonised zymosan (Klebanoff et al., 1986).

Both particulate and soluble stimuli can lead to generation by neutrophils of both H_2O_2 and a distinct peroxide product, probably 5HPETE, detected by phenol red oxidisation which is insensitive to catalase (Masler et al., 1987).

Neutrophils from both guinea pigs and humans, when stimulated by a variety of factors that stimulate the oxidative burst (e.g. f-Met leu-phe, phorbol myristate acetate and opsonized zymosan) generate hydroperoxides which are capable of activating prostaglandin H synthase, the primary enzyme in the pathway to the prostaglandins (Marshall et al., 1986). As H_2O_2 itself only causes tissue damage at high concentrations (Chance et al., 1979), this is a more likely role for neutrophil derived peroxides in inflammatory reactions.

(vii) Neutrophils in allergic asthma and rhinitis

There are many possible sources of neutrophil chemotactic factors in acute allergic reactions, and these are summarised in Table 1.2. In immediate hypersensitivity reactions (in antigen-induced bronchoconstriction and rhinitis for example), the most important of these are probably those derived from mast cells and/or basophils. However, alveolar macrophages have also been shown to secrete a low molecular weight neutrophil chemotactic factor (Gosset et al., 1984) and these cells may also play an important role in the pathogenesis of the inflammatory reaction in asthma. Neutrophil chemotactic activity (NCA) released by stimulated alveolar macrophages has many of the biochemical characteristics of a leukotriene and has been suggested to be LTB_4 . The release of this chemotactic activity can be inhibited by anti-C5 antibody (Robbins et al., 1987) suggesting that C5a may stimulate alveolar macrophages to generate NCA. Neutrophils respond to chemotactic stimuli, not only by increased directional locomotion, but also by other signs of activation. Serum HMWNCA causes release of granule-associated enzyme from cells and also leads to an increase in cell surface membrane receptors for IgG-Fc and C3b (Lee et al., 1983).

There is some evidence of leukocyte activation in experimental models of asthma. Rosette formation by neutrophils with IgG or complement-coated erythrocytes, which is associated with cell surface receptors, is increased after antigen-induced early and late-phase asthmatic reactions, and after exercise-induced bronchoconstriction, suggesting increased membrane

TABLE 1.2 Sources of neutrophil chemotactic substances in allergic reactions. (after Parish, 1986).

PRIMARY	SECONDARY
Direct consequence of allergic response	Indirect augmenting direct allergic changes
Anaphylaxis	Cell derived
Mast cells	Neutrophils
ECF (attracts neutrophils)	HETES; LTB ₄
ECG oligopeptides	Proteases
? NCF (high molecular weight)	Serum activating factor of complement pathway
HETES; LTB ₄	Fibroblast products
Basophils	Epidermal proteinase
ECF	Damaged tissue and red cells
ECF oligopeptides	
Immune-complex mediated	Humoral
C5a	Hageman factor related
IgG; Fc	Kallikrein products
Cytotoxic	Plasmin products
Mast cell/basophil derived	
IgG; Fc; C5a	C-reactive protein
Macrophage and lymphocyte products	
Delayed hypersensitivity	
Macrophages	
Several; undefined	
Lymphocytes	
Leukocyte (neutrophil) chemotactic factor	

receptor expression in asthma (Papageorgiou et al., 1983; Carroll et al., 1985; Durham et al., 1984). Neutrophils also show increased cytotoxicity, as measured by their ability to kill the schistosomulae of *Schistosoma Mansoni* in vitro, in antigen and exercise induced asthma (Kay et al., 1985). Patients with chronic, ongoing asthma show enhanced expression of neutrophil complement receptors, whereas patients successfully treated with oral prednisolone showed no such enhancement (Gin et al., 1985). Neutrophils from patients with severe asthma responded more actively to a known chemoattractant (casein) than did cells from normal controls, and this effect diminished following a course of oral corticosteroids (Gin et al., 1985b).

Cutaneous injection of anti-IgE or allergen produces a dual response in which the skin is infiltrated with first neutrophils and later, eosinophils (Dolovich et al., 1973; Solley et al., 1976). Neutrophils migrate into the inflammatory area within hours and persist, so may be in part responsible for the late phase reaction.

There is no direct proof of the involvement of neutrophils in human late phase asthmatic reactions. Durham et al. (1985) studied peripheral blood leucocyte counts during allergen-induced early and late phase asthmatic responses. Although there was an elevation in eosinophil counts associated with late-phase reactions, there were no consistent changes in peripheral neutrophil numbers. This may, of course, only reflect the smaller numbers of circulating eosinophils; with a small circulating pool, any increase in production is likely to cause a significant increase in total eosinophil numbers. However,

Durham et al. also showed a positive correlation between eosinophil counts and bronchial hyperreactivity, whereas no such relationship was found with neutrophils. In support of the important role of eosinophils and their products in the pathogenesis of asthma, levels of eosinophil major basic protein (MBP) were found to be elevated in subjects admitted to hospital with acute severe asthma, and these elevated levels fell to normal as the clinical state of the patients improved with treatment (Durham et al., 1985b).

In animal models, bronchial hyperreactivity has been found to be associated with increases in the numbers of neutrophils recovered by bronchoalveolar lavage (Fabbri et al., 1983) without accompanying significant changes in lavage macrophages, lymphocytes or eosinophils. Furthermore, hyperreactivity only developed in those animals which developed increases in neutrophils in the epithelium (Holtzmann et al., 1983). The time course of the increase in airways responsiveness was similar to the time course of neutrophil influx into the airway epithelium (Holtzmann et al., 1983b).

Dogs depleted of neutrophils, by treatment with hydroxyurea, did not develop airway hyperresponsiveness after ozone exposure (O'Byrne et al., 1983).

In human models of asthma, some evidence exists to support the role of the neutrophil as a pro-inflammatory cell. Following ozone exposure, normal human subjects experience a transient increase in bronchial airway reactivity to cholinergic stimuli, the magnitude of which correlates with neutrophil counts in bronchoalveolar lavage fluid (Seltzer et al., 1984).

Byssinosis is a chronic respiratory disorder caused by inhalation of cotton dust. In an experimental model of the disease, cotton bract extract inhalation was followed by bronchoconstriction. This was associated with and correlated with an increase in the numbers of polymorphonuclear leukocytes in bronchoalveolar lavage fluid. Moreover, BAL fluid contained three peaks of neutrophil chemotactic activity when assessed by a molecular sieve column (Protein-Pak I60) by HPLC, with corresponding molecular weight of > 25,000, 10,000 and 1,000 daltons respectively (Cooper et al., 1986).

In allergen-induced rhinitis, elevated numbers of eosinophils, but not of neutrophils, have been identified in nasal washings and secretions (Pelikan et al., 1972). Mast cells (Otsuka et al., 1986) and eosinophils (Pelikan, 1983) are prominent in the nasal mucosa of subjects with allergic rhinitis, whilst neutrophils are less plentiful in the absence of superimposed infection (Rappaport et al., 1953). However, neutrophil counts are elevated in the bronchoalveolar lavage fluid of asthmatic subjects compared with normal controls (Takayama et al., 1984).

Activated neutrophils have the capacity to cause some of the pathological features seen in asthma. For example, actively phagocytosing neutrophils, but not resting neutrophils, appear to be able to increase alveolar epithelial permeability. This was assessed using rat alveolar type II cells cultured on collagen coated Millipore filters and measuring potential difference and transepithelial resistance (Sugahara et al., 1986).

Stimulated human neutrophils are cytotoxic for rat alveolar epithelial cells, by a mechanism which appears to be unrelated to the oxidative burst, but which required strong adherence of granulocytes to epithelial cells (Simon et al., 1986). The cytotoxicity was not produced by purified neutrophil granule contents.

Thus the contribution of neutrophils towards the inflammatory reaction in asthma and rhinitis in humans remains far from clear. In this thesis, I shall describe studies on two aspects related to the involvement of neutrophils in allergy; firstly the identification of a potent chemotactic agent for neutrophils in anaphylactic situations, in vitro and in vivo, and secondly the adherence of neutrophils to pollen grains in vitro.

CHAPTER TWO

MAST CELLS AND THEIR MEDIATORS IN IMMEDIATE HYPERSENSITIVITY

(i) Inflammation and allergy.

Allergic diseases (evoked totally, or in part, by IgE-mediated mechanisms) affect many organs and tissues, but in particular the upper and lower respiratory tract, the eyes, the gastro-intestinal tract and the skin. The rapid release of mast cell-associated mediators probably accounts almost entirely for the clinical manifestations of immediate hypersensitivity, i.e. rhinorrhoea, nasal blockage, sneezing, wheeze, pruritis, abdominal discomfort and diarrhoea. After a single exposure to allergen, these symptoms are self-limiting and usually subside spontaneously. With repeated exposure, a prominent local inflammatory response develops. This is associated with more prolonged and protracted symptoms such as bronchial irritability or hyperresponsiveness, permanent nasal congestion, indurated skin lesions and chronic mucoid diarrhoea. Mucus hypersecretion is a characteristic feature of inflammation at all mucosal surfaces.

Inflammation is essentially the response of vascularised tissue to injury and serves to resolve and repair the effect of damage. The causes of inflammation, like those of cell injury, are diverse and include, in addition to immunological reactions such as allergy and auto-immunity, infectious (bacterial, viruses and parasites), physical (burns, radiation and trauma), and chemical agents (drugs, toxins and industrial agents). Acute inflammation is of short duration, characterised by exudation of fluid and plasma proteins (oedema) and leucocyte emigration, with neutrophils being prominent. In contrast,

chronic inflammation is of longer duration with a dense infiltrate of lymphocytes and macrophages. These cell types are found together with proliferation of blood vessels and connective tissue. In allergy and helminthic parasitic disease, the eosinophil is a particularly prominent cell. Both acute and chronic inflammation are associated with some degree of fibrin deposition, with platelet adherence and with the release of platelet products. The basophil is also an inflammatory cell since it is prominent in certain forms of delayed-type hypersensitivity (particularly cutaneous basophil hypersensitivity) and is found in the upper airways in allergic rhinitis. At the present time there is no conclusive evidence that the basophil participates in pathological processes in the lung. Thus the cells emigrating from the blood vessels which contribute to inflammation in general, and allergic inflammation in particular, include the neutrophil, eosinophil, lymphocyte, macrophage and platelet, with the role of the basophil remaining uncertain. In addition, fixed tissue cells such as the mast cell, fibroblast and epithelial cell also participate to a greater or lesser extent.

In clinical experimental models of allergic asthma, using antigen inhalation challenge, the early asthmatic reaction, characterised by an increase in airways obstruction occurring 15-20 minutes following challenge, may be followed, 6-8 hours later, by a more prolonged and severe period of airways obstruction, termed the late-phase asthmatic reaction. Both early and late phase asthma are associated with the release of the mast cell-associated mediators, histamine and HMWNCa, into

the peripheral circulation (Atkins et al., 1977; Lee et al., 1982; Nagy et al., 1982).

The late phase reaction is thought to correspond more closely to clinical asthma than does the early reaction. Late phase asthma is prevented by prior administration of corticosteroids (Durham et al., 1984c), is abolished by successful immunotherapy (Price et al., 1984) and has been found to be associated with the development of increased airways hyperresponsiveness (Cockcroft et al., 1983). In animal models, both late reactions (Abraham et al., 1983; Larsen et al., 1984) and airway hyperreactivity (Holtzmann et al., 1983) are associated with airway inflammation, involving a predominantly neutrophil infiltrate, and it has been speculated that enhanced bronchial hyperreactivity is a direct physiological consequent of this airways inflammation (Holgate, 1986). In humans, the evidence for a link between hyperreactivity and inflammation is less clear. However, eosinophils are present in large numbers in asthmatic sputum, but not in other states of airways obstruction such as chronic bronchitis (Holgate et al., 1985). Late phase asthmatic reactions are associated with elevated numbers of eosinophils in bronchoalveolar lavage fluid (De Monchy et al., 1986) and peripheral blood (Durham et al., 1985). Diaz et al. (1986) have confirmed that eosinophils accumulate in the bronchoalveolar washings of asthmatics with late phase reactions, and have also observed that the number of mast cells in BAL fluid was decreased in late-responders.

Eosinophils may be responsible for some of the pathology of

asthma, particular epithelial damage, by release of their granule-associated enzymes, particularly major basic protein (MBP), and MBP has been identified in the airway mucosal and submucosal layers (Gleich et al., 1984). Eosinophils, on exposure to particles coated with IgG, are stimulated to produce considerable quantities of LTC₄ (Shaw et al., 1985b). Similarly, the coupling of immune complexes of Aspergillus fumigatus and specific antibody to inert beads, and the subsequent exposure of these beads to eosinophils leads to generation of appreciable quantities of LTC₄ (Kay et al., 1986).

It thus appears that eosinophils play an important role in the pathogenesis of asthma in humans, whilst in animal models, and in human skin (Solley et al., 1976), late reactions are more a consequence of neutrophil infiltration. However, neutrophils become activated following both early and late phase reactions as shown by receptor enhancement (Durham et al., 1984; Carroll et al., 1985), and enhanced cytotoxicity (Kay et al., 1985). Thus, although the role of neutrophils in the pathogenesis of allergic airways obstruction has not been fully elucidated, there is some evidence that they may play an important role.

ii. Mast Cells.

a) Morphology and heterogeneity.

Mast cells are characterised by their numerous metachromatic granules; these granules comprise a matrix of sulphated glycosaminoglycan and protein to which are bound several low molecular weight chemicals such as histamine. They are

ubiquitous, being found in all vertebrates and in most tissue sources.

Most of the early studies of mast cells and mast cell-derived mediators were based on the rat peritoneal mast cell (RPMC), because these cells can be easily acquired in large numbers, and can be readily enriched to near purity. RPMC have the characteristics of connective tissue mast cells (CTMC), and contain many uniformly-sized granules per cell. They are approximately 20 μm in diameter and the major proteoglycan present in the granule matrix is heparin.

In more recent years, following studies of mast cells from other sources, including mouse PMC (Siraganian, 1979), P815 mastocytoma cells (Taurog, 1977), guinea pig lung (Pearce, 1977) and human lung (Patterson, 1976), it has been established that mast cells form a heterogeneous population, both morphologically and functionally. Not only do mast cells between species differ, but also mast cells between different tissue sources in the same species.

In rats, a specific type of mucosal mast cell (MMC) occurring in the gastrointestinal tract has been described, which differs in several fundamental ways from the classical CTMC (Enerback, 1981; Wingren et al., 1983).

The main morphological and functional differences between MMC and CTMC are shown in Table 2.1 (taken from Wingren et al., 1983).

The histochemical basis for the original suggestion that MMC were fundamentally different from CTMC involves the fixing and staining properties of the two cell types (Enerback, 1981). MMC

TABLE 2.1 Differences between mucosal mast cells and
connective tissue mast cells.

(from Wingren et al., 1984)

MUCOSAL MAST CELLS (MMC)	CONNECTIVE TISSUE MAST CELLS (CTMC)
Found in mucosal membranes such as lamina propria of GI tract	Found in serosal layers, peritoneal cavity and solid tissues
Small (9.7 μ) cells with few granules of variable shape	Larger cells (19.6 μ) with many uniformly sized granules
Low, age-dependent histamine content (< 2 pg/cell)	High, age-dependent histamine content (< 35 pg/cell)
Low 5HT content (increased by nematode infections)	Age-dependent 5HT content
Soluble granular matrix containing unidentified low sulphated glycosaminoglycan	Less soluble granular matrix containing heparin
Non-secretory but proliferative response to compound 48/80 and polymyxin B	Secretory response to compound 48/80 and polymyxin B
Proliferative response to nematode infections	?
Short life span; half-life approx. 40 days	Very long life span (approx. 6 months)
Thymus-dependency of proliferation	Thymus independency of age dependent proliferation and growth
Main repository of histamine in the gut mucosa	Main repository of histamine in peritoneum and tongue

granules were found to be readily soluble in several fixative materials, rendering the cell subsequently unstainable. For example, formalin fixation renders the MMC difficult to stain by standard procedures (e.g. toluidine blue), but this fixative has no such effect on the staining properties of CTMC.

MMC could be demonstrated readily, however, after fixation in Carnoy's solution, or in low concentrations of formaldehyde or acetic acid. It was thought that this observation may explain the previously noted absence of mast cells in the mucosa of the intestinal wall of laboratory animals. It was also noted that MMC were relatively unaffected by exposure to the histamine liberator, compound 48/80. Since that original observation, CTMC and MMC from the rat have been compared with respect to the actions of a variety of secretagogues. The results of one such study are shown in Table 2.2, from Bienenstock et al., 1986.

MMC in the rat have been shown to be dependent on a thymus derived factor for their growth and proliferation. In the mouse this has been identified as interleukin 3.

The principle proteases of peritoneal mast cells and mucosal mast cells in the rat have been found to be different, and have been named rat mast cell protease (RMCP) I and II respectively. Jarrett et al. (1982) have developed an in vivo method of invoking MMC proliferation in the gut wall of rats by infection with the nematode worm, *Nippostrongylus*. A further challenge with *nippostrongylus* antigen then leads to the release of RMCP II into the peripheral circulation.

TABLE 2.2 Mast cell heterogeneity in the rat.

Secretagogue	Mast cell source	
	Peritoneum	Intestine
Antigen, anti-IgE	++	++
Neutrophil cationic protein, C3 _a , C5 _a , dextran, polylysine	++	?
48/80, Bee venom peptide 401	++	0
Ionophores	++	+ / ++
Substance P	++	+
VIP, somatostatin, bradykinin, neurotensin	++	0
Dynorphin, β -endorphin, neoendorphin	++	0

CTMC have been known for some time to originate in the bone marrow and, more recently, MMC have been shown to develop from the same source. W/Wv mice, deficient in both MMC and CTMC, produced both types of mast cells when treated with bone marrow, spleen cells or anti-Thy1 treated bone marrow or spleen cells. No response was produced in these mice on treatment with thymus cells or thymus gland grafts. These results suggest that MMC are derived from bone marrow, but require a thymic factor for development (Crowle et al., 1984).

Mast cells with both the morphological and the functional properties of MMC are the principle cell type to develop in normal rat bone marrow cultures which are stimulated with factors from antigen or mitogen activated lymphocytes (Haig et al., 1982).

There are major differences in the proteoglycan content of various mast cell types; the presence of chymase and heparin proteoglycan in the rat CTMC and of rat mast cell protease II along with chondroitin sulphate in the mucosal mast cell, helps to distinguish these two cell subtypes from one another.

The heterogeneity of mast cells in humans is not so clearly defined. In patients with allergic rhinitis, nasal scrapings contained predominantly formalin-sensitive mast cells, which therefore resembled MMC. Nasal biopsies showed predominantly these formalin sensitive mast cells in the mucosal layers, while the submucosa contained formalin resistant, (or the connective tissue) type of mast cells. Moreover, the numbers of MMC in the nasal smears and biopsies correlated well with the symptom score of the individual patient (Otsuka et al., 1985). Similarly,

nasal symptoms in patients with allergic rhinitis, but not perennial rhinitis, correlated directly with the degree of peripheral blood basophilia and inversely with blood basophil colony forming units in culture (Otsuka et al., 1986, 1986b).

Flint et al. (1985) investigated the mast cell content of bronchoalveolar lavage fluid in 31 non-asthmatic subjects (7 of whom had carcinoma of the bronchus, 4 of whom had old tuberculous scarring, and 20 of whom had a normal bronchoscopy). Mast cells comprised 0.5 - 3.0% of the total cell population, and these cells had the morphological and histochemical properties of MMC. The cells released histamine on incubation with anti IgE, and the amount of histamine released correlated with the mast cell count. The approximate histamine content of each cell was 1.2 - 1.5 pg, which supports previous data on MMC histamine content (Wingren et al., 1983). When bronchoalveolar lavage was performed in asthmatics and controls, the amount of fluid and total numbers of cells recovered was similar in both groups. However, a greater proportion of the cells recovered from asthmatic patients was of mast cells and eosinophils. There was a relationship between the percentage of mast cells in the BAL cell population and both the degree of bronchial hyper-reactivity and the baseline FEV₁ prior to lavage (Flint et al., 1985b).

Thus in rodents, and to a lesser extent in humans, there is some evidence that mast cell heterogeneity exists, and that mast cells may be roughly divided into two categories, CTMC and MMC. There is no information at the present time on the differences

in glycosaminoglycan and serine proteases between the two cell types in humans, although there is extensive work on this matter in both rat and mice mast cells. Both types of mast cell have been shown to exist in the nose in allergic rhinitis, with the MMC lying more superficially and with therefore the first exposure to inhaled allergen. The interactions and communications between the superficially placed MMC and the more deeply placed CTMC will be of great interest, and may shed more light on the role of mast cells in allergic disease.

b) Activation.

Mast cells and basophils bear IgE molecules, which are bound with high affinity to specific surface receptors for the Fc portion of IgE (Ishizaka et al., 1970; Stanworth et al., 1968). Human mast cell IgE receptors have not been studied as fully as those on basophils, but it appears that the affinity of the mast cell receptors for IgE is as high as those of basophils ($K_a = 10^{10} - 10^{11}$) (MacGlashan et al., 1983). In basophils, the numbers of IgE receptors is related to the serum IgE concentration (Malveaux et al., 1978) and basophils and mast cells possess 10,000 - 500,000 receptors per cell (MacGlashan et al., 1983).

The cells are activated by the cross-linking of these cell-bound IgE molecules by divalent or multivalent ligands, which initiates a complex series of triggering mechanisms which finally results in the release of pre-formed mediators from mast cell granules, and the generation of newly-formed lipid mediators (Ishizaka et al., 1972). Most of the work on the

Since this work was published, the role of phospholipid methylation in mast cell activation has been questioned, (Boam et al, 1984) as other authors have been unable to demonstrate phospholipid methylation in IgE dependent release of histamine from mast cells (Benyon et al, 1986). In addition the changes in cell membrane methylation reported would be too small to account for the physiological responses attributed to them, (Vance et al, 1980). Inhibitors of mast cell histamine release, which were thought to act by inhibiting phospholipid methylation, also inhibit histamine release caused by the calcium ionophore A23187, a mechanism which bypasses phospholipid methylation (Beyon et al, 1984).

The cleavage of phosphoinositides is induced by immunological and non-immunological methods of mast cell activation and this is now thought to be of primary importance in signal transduction during the activation of mast cells.

details of triggering signals for mast cell mediator release has been performed by Dr. Teruko Ishizaka and a brief review of her findings follows. Rabbit antibody against IgE receptors, or its F (ab)₂ fragments, induced an increase of calcium uptake into rat peritoneal mast cells, which was closely followed by histamine release (Ishizaka et al., 1979). This was not achieved by Fab monomer fragments of the anti-IgE receptor antibody, and so it was deduced that only the bridging of mast cell IgE receptors induced an increase in membrane permeability to calcium. This increased calcium flux was found to be preceded by an increase in incorporation of [³H]methyl into phospholipids, which occurred within 50-30 seconds of cell stimulation; it was suggested that increased methylation of membrane phospholipids caused the membrane to be able to take up calcium ions (Ishizaka et al., 1980). The increase in phospholipid methylation was accompanied by an increase in intracellular cyclic AMP, which occurred within 15 seconds of receptor bridging. The reaction and subsequent Ca²⁺ influx and histamine release were suppressed by prior increases in cyclic AMP, and by inhibition of phospholipid methylation (by (s)-isobutyryl-3-deaza-adenosine), suggesting that the activation of both phospholipid methylation and adenylate cyclase are both closely associated and mutually regulated (Ishizaka et al., 1981). The reaction was also suppressed by inhibitors of chymotrypsin and trypsin, suggesting that a membrane-associated proteolytic enzyme(s) may also be activated by receptor bridging, and play an important role in the subsequent events of activation (Ishizaka, 1981b).

The work was repeated using human lung mast cells (prepared by digestion of lung fragments with proteolytic enzymes) and the human basophils which developed in suspension culture of cord blood mononuclear cells (Ogawa et al., 1983). The cells were sensitised with IgE and then challenged with anti-IgE; the same sequence of events was found to occur in human mast cells and basophils as had been described in rat peritoneal mast cells, although these events occur more slowly in the human cells.

(iii) Mast cell mediators.

Introduction.

Type I allergic reactions are the fastest and most explosive form of hypersensitivity. The interaction between antigen and cell bound immunoglobulin, (usually IgE), results in activation of the cell, via pathways which have already been discussed earlier in this chapter.

Activation of the cells results in the active secretion of a number of ready-stored, pre-formed mediators of anaphylaxis. Newly generated mediators are also produced and secreted. Both pre-formed and newly-formed mediators can act directly on smooth muscles, mucus glands and vasculature, and can also play a role in the recruitment of secondary cells, resulting in a more severe inflammatory reaction. In recent years, great progress has been made in the identification of some of the newly-formed mediators and in the potential for mast cells to produce such mediators. The availability of purified human mast cells (Schulman et al., 1982) has helped to answer questions on the origins of these mediators, and on their possible physiological

relevance to allergic disease.

This section will address each mediator, or group of mediators, in turn, with particular emphasis on mediators which have been shown to be released by human or rat mast cells in vitro. Whenever possible, in vivo information will also be included.

a) Histamine.

Histamine was first suggested as a mediator of immediate hypersensitivity by Dale and Laidlaw (1911) and was first associated with tissue mast cells by Riley and West (1953).

Histamine (5[2-aminoethyl]-imidazole) is associated exclusively with mast cells and basophils, and is stored pre-formed in the metachromatic granules of these cells, bound loosely to the proteoglycans that make up the granule matrix. Mast cell granules contain histidine decarboxylase, the enzyme necessary for histamine synthesis. Within the mast cell granule, there is a slow turnover of histamine, which in this situation has a half-life of 23 days, compared with the much shorter half-life of exogenous labelled histamine (Wingren et al., 1983).

Histamine is released from mast cell and basophil granules following cell activation by mechanisms that have been described earlier in this chapter. Loosely, ionically-bound, histamine may be washed from the granules by ions in tissue fluids (Uvnas et al., 1970). Following release, histamine rapidly appears in the blood. Because of its high charge, it does not cross

biological membranes easily, and so is readily metabolised and excreted. Metabolism occurs by two different pathways (Schayer et al., 1956), either oxidation by non-specific diamine oxidase or ring methylation of the amine by histamine N-methyl transferase to form N-methylhistamine which can be further metabolised by monoamine oxidase to methylimidazole acetic acid. The choice of metabolic pathway, and the rate of histamine metabolism shows large variation (Oishi et al., 1983), and within a species there are large differences in histamine metabolism between tissues (Erjavec et al., 1986). N-methylhistamine is a more stable molecule than histamine itself, and whereas measurement of plasma histamine is hampered by contaminating basophils, histamines, short half-life and exogenous histamine production by bacteria, N-methylhistamine has been shown to be a good parameter for histamine release (Kayzer et al., 1985).

Histamine acts by the stimulation of two different receptors, termed the H_1 and H_2 receptors. The H_2 receptor was discovered relatively recently (Ash & Schild, 1966), when it was found that several actions of histamine, in particular the stimulation of gastric acid secretion, could not be inhibited by the classical antihistamine, mepyramine. Ash & Schild then designated the "classical" mepyramine-sensitive receptors as H_1 receptors and the mepyramine-resistant receptors as H_2 receptors. The structure of the receptor has been identified and specific synthetic H_2 receptor antagonists have been synthesised (Black et al., 1972) and have been used to clarify which of the histamine effects are mediated by each receptor type.

Recent studies involving the infusion of histamine into normal volunteers, and using specific H_1 and H_2 receptor antagonists have helped to clarify the situation further (Kaliner et al., 1981, 1982). The results of some of these studies is shown in Table 2.3.

The association between plasma histamine levels and asthma has been a controversial subject for many years. Until recent years, assays for histamine lacked sufficient sensitivity and specificity to show small changes in plasma histamine concentration. This may explain earlier studies in which some workers found elevated levels of blood histamine during acute asthma attacks (De Gara, 1951) whilst others did not (Rose et al., 1950). Using a single isotope radioenzymatic assay, Barnes et al. (1980) found increased plasma histamine levels in asthmatic subjects at night, and Charles et al. (1979) found elevated plasma histamine levels in asthmatics during spontaneous exacerbations of their asthma. Antigen and exercise-induced bronchospasm has been shown to lead to elevated plasma histamine levels by some groups (Lee et al., 1982b; Anderson et al., 1981), but not by others (Deal et al., 1980, McFadden et al., 1977). Ind et al. (1983) reviewed the past evidence linking histamine with asthma and, using a double isotope radioenzymatic assay, found no differences in histamine levels between stable asthmatics, chronic bronchitics, and normal controls, but did find elevated plasma histamine in asthmatic subjects with acute exacerbations of their asthma.

The conflicting reports of plasma histamine in asthma may be due to differences in the techniques used in the measurement of

TABLE 2.3 Histamine H₁ and H₂ actions.
 (after Kaliner et al., 1982).

H ₁	H ₂	H ₁ and H ₂
Smooth muscle contraction	Gastric acid secretion	Vasodilation (hypotension)
Increased vascular permeability	Mucus secretion	Flush
Pruritis	Increases cyclic AMP	Headache
Irritant-receptor stimulation	Inhibits basophil histamine release	
Prostaglandin generation	Inhibits lymphokine release	
Increases cyclic GMP	Inhibits neutrophil enzyme release	
	Inhibits eosinophil migration	
	Inhibits T-lymphocyte-mediated cytotoxicity	

plasma histamine. A double isotope radiometric technique, developed by Brown et al. (1982), is now widely used, and it is to be hoped that this long-standing controversy may be resolved.

b) Proteases and exoglycosidases.

A number of enzymes have been identified in mast cell granules, and these are listed in Table 2.4. On immunological challenge of human pulmonary mast cells, these enzymes are released in parallel with histamine (Schwartz et al., 1981).

The acid hydrolases, β -hexosaminidase, β -glucuronidase and arylsulphatase B are less plentiful than the neutral protease, tryptase, which is the major component of human mast cell secretory granules on the basis of weight (Schwartz, 1984). In contrast, rat connective tissue mast cells contain a neutral protease with chymotrypsin-like activity (Chin et al., 1972). Mast cell tryptase activity can be quantitated by measuring the ability to hydrolyse TAME (Schwartz et al., 1981). The activity of the enzyme is inhibited by diisopropylfluorophosphate, but not by more conventional protease inhibitors, such as α -1-antitrypsin, aprotonin, limabean, soya or ovomucoid trypsin inhibitors.

One of the more important actions of tryptase is to cleave the α -chain of the 3rd component of complement (normally achieved by C3 convertase) to form C3a. C3a causes smooth muscle contraction and increases vascular permeability (da Silva et al., 1967), activates rat mast cells (Cochrane et al., 1968), and causes a wheal and flare reaction when injected into human skin (Lepow et al., 1970). Kinin generating factors have been

TABLE 2.4 **Human mast cell proteases and exoglycosidases**
 (from Cromwell et al., 1986).

MEDIATOR	STRUCTURE	BIOLOGICAL ACTIVITY
Tryptase	Protein	Neutral protease
	144,000 daltons	Trypsin specificity for basic amino acids
	4 subunits,	Inactivates high mol.wt. kininogen
	2A = 37,000 daltons 2B = 35,000 daltons	Cleaves C_3 , generating C_{3b} and C_{3a}
β -Hexosaminidase	Protein	Acid exoglycosidase acting on glycoproteins, glycolipids and glycosaminoglycan
	Tetramer	
	Subunits 27,000 daltons	
β -Glucuronidase	Protein	Acid exoglycosidase acting on glucuronide conjugates and proteoglycan
Aryl sulphatase B	Protein	Acid hydrolase acting on sulphated proteoglycan

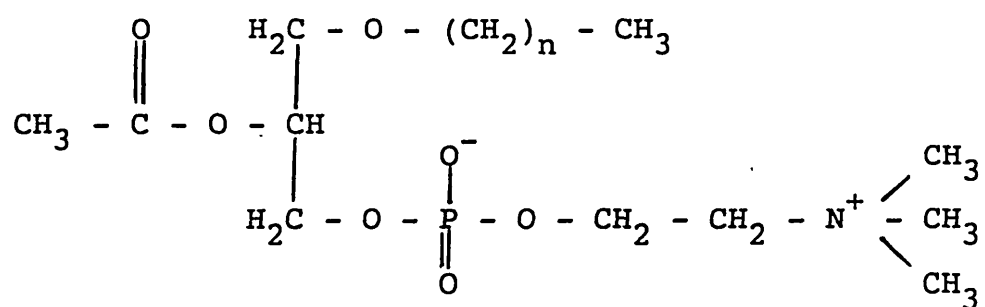
identified in the supernatants of challenged lung fragments. High molecular weight kininogen acts as a cofactor in the intrinsic pathway of blood coagulation and also acts as a source of bradykinin; this factor is destroyed by human lung mast cell tryptase (Schwartz, 1984). This activity, in conjunction with that of heparin, may explain why immediate allergen-induced skin wheals are not associated with the deposition of fibrin as are delayed-sensitivity skin reactions.

c) Platelet activating factor.

It was recognised in 1972 (Benveniste et al.) that, following allergen challenge, sensitised rabbit basophils release a mediator that produces histamine release from platelets. There was considerable difficulty in the purification of this mediator, and thus in the elucidation of its structure, but it was suggested (Benveniste et al., 1977) that it may be 1-lyso-phosphatidyl choline. This theory was later modified, and the structure of 1-0-alkyl-2-0-acetyl-Sn-glycerol-3-phosphoryl choline was proposed (Benveniste et al., 1979) (see Figure 2.1).

In addition to basophils, PAF has been shown to originate from zymosan-treated rabbit neutrophils (Lynch et al., 1979) and from ionophore treated (Lotner et al., 1980) and phagocytosing (Jouvin-Marche et al., 1982) human neutrophils. Platelets were also identified as a source of PAF (Chignard et al., 1979 and 1980). Mencia-Huerta (1979 and 1981) could find no evidence that PAF was released from rat PMCs, either on incubation with the calcium ionophore A23187, or on exposure to phagocytosable

Figure 2.1 Structure of PAF-acether
after Roubin et al., 1983



1-O-alkyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine

particles. PAF may also be released from alveolar macrophages of several species, including humans, and from their precursor cells, blood monocytes (Roubin et al., 1983). Sensitised human lung tissue, challenged with anti-IgE, has been found to generate a platelet-activating factor (Kravis et al., 1975), presumed to be derived from lung mast cells, although it is possible that alveolar macrophages are a major source.

PAF in low concentrations (100 pM) leads to activation and aggregation of rabbit platelets (Demopoulos et al., 1979), although there is great inter-species variation in platelet responsiveness to this agent (Namm et al., 1982). Activation of platelets can be shown by release of serotonin (Henson, 1970), histamine (Benveniste, 1972), a slow reacting substance resembling LTC₄ (Mencia-Huerta et al., 1981b) and prostaglandins (Shaw et al., 1978). Basophil derived PAF was thought to be a mediator of acute anaphylaxis, as injection of antigen into IgE-sensitised rabbits produced basophil degranulation, followed by transient thrombocytopaenia, after which platelets were rendered resistant to PAF (Henson et al., 1977), i.e. desensitisation occurred.

Further in vitro studies have confirmed this desensitising phenomenon and have shown that platelets become desensitised to PAF from all sources, independent of the source of the factor used to activate the cells (Lalau-Keraly, 1982).

Platelets are not the only cell to act as a target for AGEPC; neutrophils also respond by aggregation (McManus et al., 1980), enzyme release and generation of superoxide anions (Jouvin-Marche et al., 1982). Of more relevance to this thesis

is PAF-induced neutrophil chemotaxis (Goetzl et al., 1980). More recently, it has been shown that PAF acether elicits eosinophil and neutrophil chemotaxis in a dose dependent fashion over a concentration range of 10^{-6} to 10^{-9} M (whereas lyso-PAF has minimal activity). PAF was found to be as potent a chemotactic factor for neutrophils as LTB_4 (Kay et al., 1986). PAF is more effective than histamine in enhancing eosinophil-mediated cytotoxicity (MacDonald et al., 1986).

In baboons, intravenous injection of PAF induced a thrombocytopaenia and neutropaenia, with concomitant release of platelet factor 4 (PF_4) and thromboxane B_2 (McManus et al., 1981).

PAF also induces bronchoconstriction when given intravenously to guinea-pigs, by a platelet dependent mechanism, but independent of platelet cyclooxygenase or lipoxygenase activity, and it was thus proposed that PAF plays a role in the bronchoconstriction of asthma (Vargaftig et al., 1980). However, asthmatic subjects are not hyperresponsive to aerosolised PAF; the mediator causes bronchoconstriction in normal subjects and asthmatics alike (Rubin et al., 1987).

In rabbits sensitised to *Alternaria* (using the model designed by Abraham et al., 1983), intratracheal instillation of *Alternaria* extract, and intratracheal instillation of Paf-acether both produced late "asthmatic" responses, documented by a fall in dynamic compliance. *Alternaria* challenge also produced an early decrease in pulmonary compliance, but although some animals had an early response to Paf-acether, there was no overall early reaction in the group (Page et al., 1985). It was

therefore suggested that PAF may be a mediator responsible for the late asthmatic response in humans. In partial support of this theory, it has been shown that intradermal injection of Paf-acether into human skin elicits a biphasic inflammatory reaction, consisting of a predominantly neutrophil infiltrate at 4 hrs and infiltration with lymphocytes and histiocytes at 24 hrs after injection (Archer et al., 1984).

Release of PAF in inflammation has been sparcely documented, probably because of the very short half-life of PAF, and thus difficulties in its measurement. However, lyso PAF (probably the inactive metabolite of PAF) is released by guinea-pig lung fragments following ionophore stimulation (Parente et al., 1985). These results should be interpreted with some caution; as lyso-PAF is both a precursor and a metabolite of PAF, it is not known whether an increase in lyso-PAF really represents an increase in PAF production.

Whether or not platelets become activated during acute asthmatic reactions is a controversial topic. Although several workers have reported platelet activation, shown by elevation of PF_4 and TxB_2 , these factors may be elevated through artefactual means. Studies in which plasma samples were collected meticulously, and in vitro platelet activation was excluded, showed no such increases in plasma PF_4 and TxB_2 (Durham et al., 1984b).

d) Prostaglandins and Thromboxanes.

Prostaglandins were first discovered in the 1930s. Kurzrok and Lieb (1930) found that human semen caused violent

contractions of the human uterus and it was later discovered that minute volumes of human semen when administered to rabbits rendered them severely hypotensive. The substances responsible for these activities were found to be lipid-soluble under acidic conditions. PGE_1 and $\text{PGF}_{1\alpha}$ were first isolated in 1957; meanwhile, an essential fatty acid had been described, deficiency of which led to infertility in rats (Burr and Burr, 1930) or skin disease, consisting of dryness and scaling, in human infants (Hansen et al., 1963).

Linoleic and α -linolenic acid were found to possess essential fatty acid (EFA) activity, and the conversion of linoleic acid to arachidonic acid was demonstrated by Mead et al. (1953), and thromboxane A_2 by Hamberg et al. (1975). Prostacyclin was first isolated and characterised by Moncada et al. (1976). Mass spectrometry and gas-liquid chromatography was used to further elucidate the structures of prostaglandins in the 1960s. Because of the presence of cis-double bonds in the prostaglandin series, it was suggested that prostaglandins were metabolites of arachidonic acid (Bergstrom et al., 1964). Over the next decade, a number of other prostanoids were identified, and the oxidative cascade from arachidonic acid to prostaglandins and thromboxanes was described (Hamberg et al., 1974). Both prostaglandins and thromboxanes are formed from the parent fatty acid by the cyclo-oxygenase enzyme, and are thus referred to as "cyclo-oxygenase products". Of the three parent fatty acids, trienoic (dihomolinolenic), tetraenoic (arachidonic) and pentaenoic, by far the most abundant in animals is arachidonic acid (Crawford, 1983).

The pharmacological activities of cyclo-oxygenase products are extremely diverse. TXA_2 and prostacyclin have directly opposing pharmacological actions in many systems, as shown in Table 2.5. The balance between thromboxane A_2 and prostacyclin synthesis in an individual cell may therefore have significance in the pathophysiology of a wide variety of diseases, particularly thrombosis and ischaemia (Bunting et al., 1983).

The biological actions of prostaglandins are both diverse and complex, and nearly all body functions may be affected. Different prostaglandins may exert opposite effects on the same organ (for example, the PGE group bronchodilates whilst the PGF group bronchoconstricts) and prostaglandins may affect different parts of the same organ in different ways (for example, PGE_2 contracts the corpus of the uterus whilst relaxing the cervix). The same prostaglandin has different effects in different species (e.g. $\text{PGF}_2\alpha$ is hypotensive for cats, but has a mild pressor effect in humans) and the effect may change with increasing doses (e.g. PGE in low doses is pro-inflammatory, but in higher doses is anti-inflammatory). Finally, different prostaglandins within the same group may have opposite effects (e.g. PGE_1 increases intracellular cAMP, whilst PGE_2 decreases it) (Oliw et al., 1983).

With reference to the human lung, and particularly to asthma, $\text{PGF}_2\alpha$ by inhalation causes bronchoconstriction, whereas PGE_1 and PGE_2 causes bronchodilation (Mathe et al., 1977). Asthmatics are particularly sensitive to $\text{PGF}_2\alpha$ (Mathe et al., 1973), and elevated plasma levels of a major metabolite of $\text{PGF}_2\alpha$ have been described following antigen-induced bronchoconstriction (Green

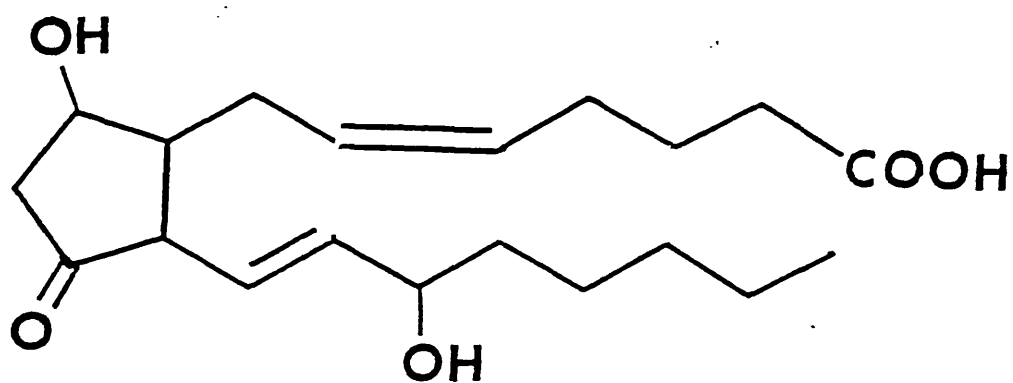
Table 2.5 Opposing and interactive pharmacological
properties of prostacyclin and thromboxane A₂
 (after Whittle et al., 1983).

PROSTACYCLIN	THROMBOXANE
Anti-aggregatory	Pro-aggregatory
Vasodilator	Vasoconstrictor
Bronchodilator	Bronchoconstrictor
Cytoprotective	Cytolytic

et al., 1974). Thromboxane A_2 is also a potent bronchoconstrictor (Hamberg et al., 1975b), whilst PGE_1 is a powerful vasodilator and could contribute to the inflammatory response in asthma.

Using rat serosal mast cells, Roberts et al. (1979) demonstrated that the major cyclo-oxygenase product in these cells is prostaglandin D_2 (PGD_2), the structure of which is shown in Fig. 2.2. TXA_2 , PGD_2 and prostacyclin were found to be generated by human lung fragments on challenge with anti-IgE (Schulman et al., 1981). Studies using isolated human lung mast cells, obtained by enzymatic digestion of human lung tissue (Schulman et al., 1982), showed that the major cyclo-oxygenase product of these cells was also PGD_2 (Lewis et al., 1982; MacGlashan et al., 1983). Release of PGD_2 from human lung mast cells is inhibited by indomethacin, an inhibitor of cyclo-oxygenase enzyme (Peters et al., 1985). Although dexamethasone inhibited prostaglandin generation from whole lung tissue, it failed to influence release of PGD_2 or histamine from human lung mast cells (Schleimer et al., 1983). This is in some ways surprising, as recent work has suggested that the action of corticosteroids as anti-inflammatory drugs is mediated by the synthesis of a polypeptide (lipomodulin) which inhibits phospholipase A_2 and thus impairs the release of arachidonic acid (Flower et al., 1979; Hirata et al., 1980). This discrepancy may be explicable if dexamethasone acts on other inflammatory cells in the lung, whilst not directly affecting lung mast cells.

Figure 2.2 The structure of prostaglandin D₂
(after Bakhle, 1983).



e) Lipoxygenase products.

Slow reacting substance of anaphylaxis (SRS-A) has been recognised as a mediator of anaphylaxis for nearly fifty years (Kellaway et al., 1940; Brocklehurst et al., 1962). In more recent years, SRS-A has been identified as comprising three related leukotrienes, LTC₄, LTD₄ and LTE₄.

Borgeat & Samuelsson (1979) identified five new dihydro-eicosanoids which were formed following stimulation of rabbit neutrophils by the calcium ionophore A23187. These were LTB₄, trans LTB₄ and three other isomers of LTB₄. In constructing a possible pathway for the generation of these substances from arachidonic acid, they postulated a hypothetical intermediary compound, LTA₄. Stimulated mouse mastocytoma cells were found to generate a substance with identical properties to SRS-A; this was identified as 5(S)-hydroxy-6(R)-L-γ-glutamyl-L-cysteinyl-glycinyl-eicosa-7,9-trans,11,14-cis-tetraenoic acid, and was termed leukotriene C₄ (LTC₄) (Hammarstrom et al., 1979). Morris et al. (1979), using mass spectrometric methods, investigated lung-derived SRS-A and found it to consist of LTC₄ and a related material, in which the glutamyl residue was absent, termed leukotriene D₄ (LTD₄). The cysteinyl analogue, leukotriene E₄ (LTE₄) was reported by Lewis et al. (1980).

Although, as discussed in the previous section, the major product of arachidonic acid metabolism in human mast cells is prostaglandin D₂ (PGD₂), both mast cells and basophils are able to produce leukotrienes under appropriate conditions.

Purified human lung mast cells, pre-labelled with arachidonic acid, when stimulated with anti-IgE, were found to generate,

in addition to PGD_2 , significant amounts of LTC_4 , LTD_4 and LTE_4 (Peters et al., 1984). Cells treated with control immunoglobulins released a small quantity of PGD_2 , but no measurable leukotrienes. LTB_4 , and the 6-trans and 6-trans,12-cis, stereoisomers of LTB_4 were also generated on stimulation with anti-IgE, although the LTB series was present in smaller amounts than the sulphidopeptide leukotrienes. There is considerable evidence that mucosal mast cells differ from connective tissue mast cells in their profile of arachidonic acid metabolism. Mouse bone-marrow derived mast cells appear to produce predominantly LTB_4 in preference to PGD_2 (Mencia-Huerta et al., 1983). In addition, small quantities of 5,hydroxyeicosatetraenoic acid (5HETE) were identified in stimulated cell supernatants.

The biological effects of the sulphidopeptide leukotrienes include sustained smooth muscle contraction (Hanna et al., 1981) and bronchoconstriction (Barnes et al., 1984) and an increase in airway mucus production (Marom et al., 1982; Piper et al., 1984). In addition, they increase vascular permeability (Dahlen et al., 1981) and intradermal injection of LTC_4 and LTD_4 into human skin produces vasodilation and results in a wheal and flare reaction (Bisgaard et al., 1982; Camp et al., 1983). They produce coronary vasoconstriction (Piper et al., 1982) and systemic and pulmonary hypotension (Smedegard et al., 1982). In addition, the sulphidopeptide leukotrienes play a part in immunoregulation, by promoting lymphocyte production of gamma interferon (Johnson et al., 1984). LTB_4 is a potent chemoattractant and chemokinetic agent for human neutrophils,

guinea pig eosinophils, human mononuclear cells and rat macrophages (Ford-Hutchinson et al., 1980; Smith et al., 1980). LTB_4 has also been shown to activate human neutrophils, as shown by enhancement of their C_3b receptor expression (Bray, 1981) and enhancement of lysosomal enzyme release (Hafstrom et al., 1981).

Amongst the numerous possible hydroperoxyacids (HPETEs) and hydroxyacids (HETEs) that are intermediates in leukotriene synthesis, human lung fragments appear to produce predominantly 15-HETE (Hamberg et al., 1980). 15-HETE causes slow, histamine-like contractions of human bronchi, although it is less potent in this respect than either histamine or other HETEs, notably 5HETE (Bach, 1982). Peters et al. (1984), however, showed that the principle leukotriene intermediate generated by purified human lung mast cells was 5-HETE rather than 15-HETE. This controversy may either be caused by lipoxygenase production by different cell types in whole lung tissue, or simply by difficulties in the methods used to identify arachidonic acid metabolites.

f) Eosinophil Chemotactic Factor of Anaphylaxis

Kay et al. (1971) showed that passively or actively sensitised guinea pig lung fragments, when challenged with antigen, released a factor which was chemotactic for guinea pig eosinophils. The release of this eosinophil chemotactic factor of anaphylaxis (ECF-A) closely paralleled that of histamine and of SRS-A, in both time course, dose dependence and other requirements for optimum release. This was differentiated from ECF-C (C_5a), derived from zymosan treated serum, by means of its

low molecular weight (approx. 500 daltons). ECF-A was later purified from human lung and identified as a family of tetrapeptides, predominantly Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu (Goetzl et al., 1975). However, these purified individual tetrapeptides were not as potently chemotactic for eosinophils as the crude ECF-A. Further studies showed that the ECF-A tetrapeptides acted exclusively on eosinophils, over a narrow dose range, with great variation in the response of eosinophils between individual donors. No potentiation of activity was found in the presence of other ECF-A tetrapeptides or histamine (Turnbull et al., 1977).

ECF-A is a pre-formed mediator, associated with the granules of purified mast cell populations on cellular sub-fractionation studies (Wasserman et al., 1974). Human leukaemic basophil cells (which are thought to be related to mucosal mast cells) have also been shown to contain ECF-A in a pre-formed state (Lewis et al., 1975). In addition to promoting eosinophil chemotaxis, ECF-A tetrapeptides activate the eosinophil, as shown by enhancement of the eosinophil Fc receptor (Capron et al., 1981). The tetrapeptides also enhance eosinophil cytotoxicity, as shown by enhanced eosinophil-mediated killing of schistosomulae (Anwar et al., 1980; Capron et al., 1981b).

In addition to the low molecular weight ECF-A, (with a molecular size of approximately 500 daltons), Boswell et al. (1978) described eosinophil chemotactic activity associated with rat peritoneal mast cell granules with an apparent molecular weight of 1500-2500 which he termed intermediate weight eosinophil chemotactic factor(s). This activity comprised two

distinct moieties, one with a pI of approximately 5, and the other with a pI of approximately 2.5. These factors were chemotactic and not chemokinetic for eosinophils, and had the capacity to render eosinophils resistant to subsequent chemotactic stimuli (i.e. de-activated the target cells).

Thus the mast cell is capable of producing several factors which are chemotactic for eosinophils, leading to recruitment and activation of the target cells. These factors include histamine, low molecular weight eosinophil chemotactic tetrapeptides, and intermediate weight eosinophil chemotactic factors.

(iv) High Molecular Weight NCA.

Atkins et al. (1977) first described a neutrophil chemotactic activity (NCA) in the serum of asthmatic subjects following antigen inhalation challenge using ragweed antigen. This was not released in response to bronchoconstriction alone, as methacholine-induced bronchospasm was not associated with serum NCA. Although 18 ragweed sensitive patients were studied in all, only 8 of these subjects had a positive reaction ($\geq 20\%$ fall in FEV_1) to both antigen and methacholine (8 patients had a negative reaction to antigen challenge, and 9 patients had a negative reaction to methacholine challenge). In the 8 patients who reacted to antigen and methacholine, serum NCA was only found after antigen challenge.

It was originally thought that the chemotactic activity may be due to chemotactic components of complement, despite the heat-stable nature of the NCA. However, serum complement levels

did not change during or after antigen-challenge, and chemotactic activity was not abolished by preincubation with antisera to C_3 . The NCA described was found to have a molecular weight of over 50,000 daltons by ultrafiltration. No further characterisation of the chemotactic activity was undertaken during that study. Although the findings were novel in that NCA had not previously been described in serum following antigen challenge, the NCA could have been due to a number of factors other than HMWNCA, including fibrinogen fragments and intermediate-molecular weight NCA (Wasserman et al., 1977) which has a molecular weight of 150,000 daltons. The heat stability of the molecule was thought to exclude plasminogen activator, LTB_4 and kallikrein. In a later paper, (Atkins et al., 1978), the authors showed that the release of this NCA following antigen challenge was prevented by the prior administration with sodium cromoglycate (Atkins et al., 1978). It was postulated from these findings that NCA was liberated following an "anaphylactic" reaction, presumably involving mast cells or basophils, although more recently cromoglycate has been shown to act in asthma by effects other than a direct effect on mast cells (Holgate, 1986). Another interesting point in Dr. Atkins' study was that, following antigen challenge, serum NCA reached a peak at 10 minutes, but was still significantly elevated over baseline levels over 24 hours after challenge. It was suggested that this may have some bearing on the occurrence of the late phase reaction, as late phase skin reactions had been shown to be associated with the accumulation of neutrophils, but this prolonged serum NCA has not been reproduced by other studies

(Durham et al., 1984c).

Characterisation of serum NCA was first described by Wasserman et al. (1977); these authors described a high molecular weight NCA with neutral isoelectric point, which appeared in blood collected from the cold challenged arm in patients with cold urticaria, and which was absent from the control arm. In these elegant experiments, serum from the challenged and control arms were compared, and the NCA characterised by gel filtration (Sephadex G200 and Sepharose 4B), anion-exchange chromatography (QAE-Sephadex), and isoelectric focusing (1% sucrose and Sephadex G75). The peak of NCA occurred two minutes following challenge and was therefore earlier than had been observed in asthmatic patients' serum following antigen-induced asthma. However, this may simply reflect the fact that blood was collected directly from the venous effluent of the challenged extremity, whereas in the previous studies, NCA produced locally at the site of the immunological reaction was then required to circulate peripherally before it could be identified in the serum.

Subsequently, HMWNCA was identified in serum following antigen induced late phase reactions (Nagy et al., 1982; Durham et al., 1984) and in exercise-induced early (Lee et al., 1982c) and late (Lee et al., 1983) phase reactions, in addition to solar (Soter et al., 1979) and cholinergic (Soter et al., 1980) urticaria. Further physico-chemical characterisation of the NCA by Lee et al. (1982) revealed that it had a molecular size of approximately 750,000 daltons (by gel filtration on Sephadex G200) and eluted with between 0.15 and 0.3M NaCl on anion

exchange chromatography with DEAE-Sephacel. The isoelectric point of HMWNCA was found to be slightly acidic by chromatofocusing (pI 6.0 to 6.5). The activity of HMWNCA was partially inhibited by the action of the proteolytic enzymes, trypsin and chymotrypsin (Lee et al., 1982c).

In each clinical asthmatic situation, the appearance of HMWNCA paralleled the appearance of histamine in peripheral blood. Early phase reactions, and the early increase in serum NCA, were both inhibited by the prior administration of disodium cromoglycate (DSCG). This evidence was thought to suggest a close association between HMWNCA and mast cell activation, and indeed HMWNCA was postulated to be a mast cell derived mediator of inflammation.

In further support of this theory, it has been demonstrated that sensitised guinea pig lung fragments, when challenged with specific antigen, released a HMWNCA (Schenkel et al., 1982). NCA identical in physiochemical properties to serum HMWNCA has also been identified in the supernatants of human lung fragments, immunologically challenged by reverse anaphylaxis, using anti-human IgE (O'Driscoll et al., 1983). Small amounts of HMWNCA were also released on challenge with control immunoglobulins, although the activity in control supernatants was very much less than that in challenged supernatants. An interesting and unexplained point in that study is that lung derived HMWNCA appeared to be more heterogeneous than serum HMWNCA; on gel filtration of actively challenged lung supernatants, there were three distinct peaks of chemotactic

activity, one of which corresponded to serum HMWNCA (molecular weight approx. 650,000 daltons) and two of which appeared to have a higher molecular weight. Time course experiments were also undertaken; over a 12 hour period following anti-IgE challenge, no further NCA was released after the initial 15' peak. It was suggested that the appearance of serum HMWNCA during the late phase reactions to antigen or exercise challenge was therefore not due to the spontaneous secondary degranulation of lung mast cells.

Although these studies supported the mast cell as a major source of HMWNCA, there remained no definite proof that mast cells released NCA, or that they were the only source of this mediator. Rat serosal mast cells, on stimulation with rabbit anti-rat F(ab)₂ antisera, release a neutrophil chemotactic factor which elutes in the void volume of Sephadex G200 gel, although the precise molecular weight of this activity was not elucidated (Center et al., 1980).

CHAPTER THREE

GENERAL MATERIALS AND METHODS

(i) Buffers

Tyrodes

NaCl (137 mM)

Glucose (5.5 mM)

HEPES (10 mM)

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.4 mM)

$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.5 mM)

Human serum albumin (0.03%)

KCl (2.7 mM)

pH 7.35 (HCl)

$\pm \text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (0.9 mM)

Phosphate-buffered Saline (PBS)

NaCl (100 mM)

NaH_2PO_4 (12.8 mM)

Na_2HPO_4 (37 mM)

pH 7.35 (HCl)

Polyanion SI starting buffer

Triethanolamine (0.05 M)

pH 7.8 (HCl)

Polyanion SI eluting buffer

PSI, containing NaCl (0.5 M)

HPLC buffer

Triethanolamine (0.05 M)

pH 7.3 (HCl)

Tris/HCl

Tris (hydroxymethyl) methylamine (0.025 M)

pH 7.8 (HCl)

SDS-PAGE (Laemmli, 1970)

A (lower gel)

Tris, pH 8.8	1.5 M
--------------	-------

B (30% acryamide)

Acrylamide	29.2%
------------	-------

Bis acrylamide	0.8%
----------------	------

C (Stacking gel)

Tris, pH 6.8	0.5 M
--------------	-------

D (Sample buffer)

SDS	3%
-----	----

β -mercaptoethanol	5%
--------------------------	----

Buffer C	12.5%
----------	-------

E (Sample buffer)

SDS	3%
-----	----

β -mercaptoethanol	5%
--------------------------	----

Buffer C	12.5%
----------	-------

Glycerol	20%
----------	-----

Bromphenol blue

F (Reservoir buffer)

Glycine	72g
---------	-----

Tris	15g
------	-----

10% SDS	50mls
---------	-------

made up to 5l and adjusted to pH 8.3

Electrophoresis Buffers.

Veronal buffer:

Sodium barbitone	21.4g
Barbitone	3.68g
Thiomersalate	0.1g
Distilled water	1 l.

Coomassie stain:

Coomassie blue	5.0g
Ethanol	450 mls
Glacial acetic acid	100 mls
Distilled water	450 mls

Destain:

Ethanol	450 mls
Glacial acetic acid	100 mls
Distilled water	450 mls

All buffers were filtered through 0.45 pure size filters and de-gassed under vacuum prior to use with columns.

(ii) Column chromatography.

A. Gel filtration.

a) Principles.

Gel filtration is a form of column chromatography in which molecules are separated on the basis of their molecular size; it is a commonly used method for determination of molecular weight. The gel filtration matrix acts as a molecular sieve, as it contains "pores" of a known physical size. Very large

molecules are excluded from the pores and so elute rapidly from the column, whereas smaller molecules are able to enter the gel pores and so move more slowly through the column. Thus molecules elute in order of decreasing molecular weight.

In this study gel filtration was performed on either Sephacryl S400 gel, or on Microbondagel using HPLC. Sephacryl S400 (Pharmacia) is a stable rigid matrix suitable for separating molecules in the 5,000 - 1.5×10^6 daltons range. The gel was contained in a vertical glass column and gel filtration was performed with gravity, using a fixed rate pump (Microperspex pump, LKB). Microbondagel is a very rigid matrix pre-packed in metal columns. It is said to be suitable over a similar molecular weight range as Sephacryl S400. In each case, calibration was performed using markers of known molecular weight (Pharmacia).

b) Methods.

Sephacryl S400

Column (bed volume 198 mls) packed with gel (equilibrated with starting buffer, and de-gassed) at 15 mls/hr for 18 hours. Equilibrated at flow volume (10 mls/hr) for 18 hours.

Buffer - PBS

Running temperature $+4^{\circ}\text{C}$

Flow rate 10 mls/hr

Sample size 2 mls

Fraction size 2 mls

Microbondagel (HPLC)

HPLC (LDC); comprising:

Constametric pump Model III G

Injection valve (LDC)

UV monitor - 2138 Unicord S (LKB)

Chart recorder - 2210, 1-channel recorder (LKB)

Fraction collector - 2211 Superrac (LKB)

Three columns were used in series to provide optimal resolution.

a) E-linear

b) E-1000

c) E-linear

Total bed volume: 1.5 mls

Running temperature RT (19°C)

Flow rate 0.5 mls/min

Sample size 100 µl

Fraction size 1.5 mls

B. Ion exchange chromatography.

a) Principles.

Ion exchange chromatography is widely used for the separation of proteins on the basis of their ionic charge. It makes use of specially prepared resins, containing either positively charged groups at pH 7.0 (anion-exchange chromatography, e.g. DEAE Sephacel) or negatively charged groups at pH 7.0 (cation exchange chromatography, e.g. SP Sephadex). Depending on the

ionic charge of protein molecules at a given pH, these protein molecules either adhere to the resin or are excluded from it. Adherent proteins are then washed off by a gradually increasing salt concentration (a process called gradient elution), which has the effect of decreasing the binding of anionic proteins.

b) Methods

In these studies, anion exchange chromatography was performed on DEAE Sephacel in vertical glass columns, or on Polyanion SI 17 columns using the FPLC systems. Cation exchange chromatography was performed on SP Sephadex gel, using vertical glass columns. In each case, the gradient was made with 0-0.5 M NaCl, either using a commercial glass gradient maker (LKB) or by programming on FPLC.

DEAE Sephacel (Anion exchange)

Starting buffer TRIS/HCl, pH 7.8

Bed volume 35 mls

Running temperature +4°C

Flow rate 35 mls/hr

Sample size 10-30 mls

Fraction size 2 mls

Gradient (100 mls) made with Tris/HCl containing 0.5 M NaCl

Polyanion SI 17 (Anion exchange), obtained in pre-packed 35/10 columns.

FPLC system (Pharmacia), comprising:

Two P-500 pumps

Gradient programmer GP-250

Single path monitor UV-1 Control Unit and Optical Unit

Fraction collector FRAC-100

Chart recorder

Buffer - Polyanion SI starting buffer

Bed volume 10 mls

Running temperature RT 19°C

Flow rate 1 ml/min

Sample size 2-20 mls

Fraction size 1-2 mls

Gradient made with Polyanion SI eluting buffer (with
0.5 M NaCl)

SP Sephadex (cation exchange)

Bed volume 30 mls

Running temperature +4°C

Flow rate 35 mls/hr

Starting buffer:- 0.02 M phosphate, 6 M urea, pH 5.3

Eluting buffer:- starting buffer + 0.5 M NaCl

Sample size 20-30 ml

Fraction size 3 mls

After sample application, a 100 mls gradient (0-0.5 M
Na Cl) was applied.

C) Chromatofocusing.

a) Principles.

Chromatofocusing separates proteins on the basis of their
isoelectric point, using specialised chromatofocusing gels.

Samples are applied to the gel under alkaline conditions, under which most proteins adhere to the gel. A gradient of steadily decreasing pH is applied to the column, and proteins elute from the gel with eluent whose pH corresponds to their own isoelectric point, or pI.

b) Methods.

Mono P gel (obtained in pre-packed HR 20/5 columns) was used on the FPLC system.

Starting buffer 0.025 M triethanolamine, pH 8.3
(iminodiacetic acid)

Eluting buffer: Polybuffer 96 (70%)
Polybuffer 74 (30%)
pH 5.0 (iminodiacetic acid)

Bed volume 5 mls

Running temperature - Room temperature

Flow rate - 1 ml/min

Sample size - 1 ml

Fraction size - 2 mls

(iii) Polyacrylamide gel electrophoresis.

a) Principles.

Polyacrylamide gel electrophoresis (PAGE) is a useful method for determination of protein molecular weights, as only small quantities of protein are required, and high resolution is achieved. Sodium dodecyl sulphate (SDS) is commonly used in the process of polyacrylamide gel electrophoresis; this detergent

interacts with proteins to form SDS-protein complexes with their constituent chains. SDS masks the protein charges and the SDS-protein complexes have a constant charge per unit mass. Thus proteins can then be separated electrophoretically purely on the basis of their molecular size (Shapiro et al., 1967).

A stacking gel, made with a lower percentage of acrylamide, is used to maximise protein separation. Protein bands in the resultant gels can be stained with Coomassie blue, or by using a more sensitive silver stain for small quantities of protein.

b) Methods.

15% stacking gel (Buffer A, 25 mls + Buffer B, 25 mls + H₂O, 11.74 mls + ammonium persulphate 6%, 0.25 mls) is degassed and SDS (10%, 500 µl) and TEMED (10 µl) is added. The gel is poured between two glass plates using the Vertical Electrophoresis Apparatus (LKB) and left to polymerise. The presence of oxygen is inhibitory to the polymerisation process (which is catalysed by TEMED) and so the gel is covered with a small layer of butan-2-ol during polymerisation. After approximately 1 hour, the butan-2-ol was removed and 3% stacking gel (Buffer C, 5 mls, Buffer B, 2 mls, H₂O 12.6 mls, ammonium persulphate 6%, 100 µl, SDS, 10%, 200 µl and TEMED 20 µl) was overlaid. A polypropylene "comb" was inserted into the stacking gel to form sample wells, and the gel was left to polymerise overnight. Samples (500 µl) were added to buffer D (125 µl) and incubated at 60°C for 15 minutes to break up disulphide bridges, before the addition of buffer E (125 µl). High molecular weight markers were treated in

the same way. Samples and reference standards (20 μ l) were added to the sample wells, after removal of the comb. The gels were then electrophoresed with a constant current of 20 mA/gel. Gels were then removed, fixed in industrial methylated spirit (50%) and acetic acid (10%) and stained, using a silver stain kit (BioRad).

(iv) Immuno-electrophoresis.

a) Principles.

Immuno-electrophoresis by a variety of methods is an extremely sensitive method which can be used for studying antigenic extracts or antibody production to known antigens. It can also be used to monitor purification techniques of serum proteins by the use of anti-normal serum antibodies.

Rocket electrophoresis (RIE).

This involves electrophoresis of antigen directly into agarose containing antibody, without any prior separation of antigen into its individual components. The area under the precipitation curve is directly proportional to the amount of antigen in the sample (Laurell, 1972).

Crossed immuno-electrophoresis (CIE) (Laurell, 1965).

In this method antigen is separated by electrophoresis in agarose in one direction followed by electrophoresis in a perpendicular direction into antibody-containing agarose. The area under the precipitation peak is proportional to the antigen quantity (Clarke et al., 1968). Intermediate gels, containing specific antibody, may be used, and will selectively precipitate

out antigen components against which the specific antibody is directed.

Immuno-electrophoresis (IE).

Antigen, separated into individual components in one direction is then allowed to diffuse into a parallel "line" well of agarose containing antibody. This method can be used to test the purity of serum proteins following purification methods, when tested against normal serum.

b) Methods.

Rocket immuno-electrophoresis.

Commercially acquired Gelbond (LKB) plastic backing material was coated with 1% agarose in veronal buffer/water; the volume used was 0.125 mls agarose/cm² Gelbond. Antigen sample wells and antibody-containing "windows" were cut using templates, and windows were filled with the appropriate volume of agarose containing antibody. Samples (10-20 µl) were placed in the sample wells and electrophoresis was performed directly into the antibody containing window or 1.5 V/cm overnight. Plates were washed in saline (0.9%) for at least 2 hours, and dried with Whatman filter paper before being stained with Coomassie blue, destained with acetic acid and methanol, and re-dried.

Crossed immuno-electrophoresis.

Clean glass plates (8 x 8 cm) were coated with a thin smear of 0.1% agarose and dried, before being covered with 1% agarose as described above (8 mls). Antibody windows and sample wells were cut from the gel as described above. Antigen (10-20 µl) was

separated by electrophoresis in one direction at 4.5 V/cm for 90 minutes before filling the window with 1% agarose containing antibody. Electrophoresis into the window (at 90° to the first electrophoresis) took place at 1.5 V/cm overnight. Plates were washed and stained as previously described.

Immunoelectrophoresis.

Glass plates were prepared as described above, and cut using a template designed for LIE. Serum (1:3 dilution, 5 µl) and partially purified serum protein (10 µl) were separated in parallel in one direction (4.5 mV/cm, 90 minutes). Antibody-containing agarose was then incorporated into the well, in a line between the two samples, and in parallel with the direction of electrophoresis. Both separated samples were then allowed to diffuse into the antibody-containing gel overnight at room temperature in a humidified atmosphere. Plates were then washed and stained as described above.

(v) Measurement of histamine.

Principle.

Histamine was measured by the method of Siraganian (1974). Histamine is extracted into butan-1-ol from an alkaline aqueous salt solution. It is then recovered by the addition of heptane, being back-extracted into hydrochloric acid. Histamine is incorporated into a fluorescent histamine-0-phthalaldehyde complex following reaction with 0-phthalaldehyde at alkaline pH.

Reagents.

n-butanol and methanol (Rathburn Chemicals, Peebles, Scotland)

n-heptane (BDH, Poole, Dorset)
o-phthalaldehyde (Sigma, Poole, Dorset)
Brij-35 (Sigma)
Histamine dihydrochloride (Sigma)
Nitrogen gas (BOC, London, UK)
All other chemicals, Analar grade (BDH)

Equipment.

Technicon AA II autoanalyser with fluoronepholometer (Technicon, Basingstoke, U.K.)
Primary filter, 355 nm narrow pass
Secondary filter, 462 nm sharp cut

The detailed arrangement of the manifold is described by Cromwell et al. (1986).

Method

Samples are de-proteinised prior to analysis by precipitation with perchloric acid, followed by centrifugation to remove the proteinaceous pellet. o-phthalaldehyde (OPT) solution is made by dissolving 40 mgs OPT in 3 mls methanol and making up to 100 mls with 0.1 M HCl.

Saline/EDTA/Brij in the diagram refers to NaCl (18 g) added to EDTA (0.1 M, 32 mls, pH 7.4) made up to 2 l with distilled water and adjusted to pH 7.4 to which has been added Brij-35 (2 mls).

Sampling takes place at a rate of 30 samples/hour; an 80 second sampling time is followed by a 40 second wash. Results are recorded as peaks on a standard recorder, the height of the

peak reflecting the concentration of histamine. Results are interpreted by the use of a standard dose curve, using histamine dihydrochloride (6.25 - 50 g/ml).

(vi) Neutrophil Chemotaxis Assay.

Principle.

The potency of chemotactic factors is measured by a bioassay, which is a modification of the technique described by Boyden (1962). Neutrophils, under the influence of chemoattractants, migrate through a cellulose nitrate filter, and cells that have migrated through the entire thickness of the filter can be stained and counted microscopically. The technique is described in more detail below. Other methods of assessing chemotaxis include multichamber microassay techniques (Swanson et al., 1977; Falk et al., 1980) and the transendothelial passage of cells across an in vitro blood vessel model (Migliorisi et al., 1984; 1987). These techniques will be discussed and compared in a later chapter.

Materials.

"Top" chambers - "Kay" tubes (TMS Ltd., Dalkeith, Scotland)

"Bottom" chambers - pill pots (lids removed) 13 mm diameter x 28 mm (Camlab, Cambridge, U.K.).

Cellulose nitrate filters, 8 pore-size, 150 thick (Sartorius Instruments Ltd., Belmont, Surrey, U.K.).

Polystyrene container with tightly-fitting lid.

Modified Neubauer haemocytometer.

Cork borers, 13 mm (Gallenkamp, London, U.K.).

Dextran 110 (Fisons, Loughborough, U.K.)

Heparin sodium BP, 1000 U/ml (Paines and Byrne Ltd., Greenford, U.K.)

Ficoll-Paque (Pharmacia, Uppsala, Sweden)

Hanks balanced salt solution (Gibco Europe, Paisley, Scotland)

Microscope with 10 x eyepiece incorporating a 1 x 1 cm graticle and a 40 x objective (e.g. Dialux, Leitz Instruments, Luton, U.K.)

HEPES, ovalbumin (Grade VI), zymosan, all from Sigma, Poole, Dorset, U.K.

Industrial methylated spirit, xylene, Depex mount medium, Harris's haematoxylin, glacial acetic acid, hydrochloric acid, lithium carbonate, ammonium chloride; all from BDH, Poole, Dorset, U.K.

Staining dishes and staining racks (R.A. Lamb, North Acton, London, U.K.)

Stainless steel clips (xx 6201701, Millipore Ltd., London, U.K.)

Glass slides.

Method

Purification of human neutrophils.

Whole blood is sedimented on 20% dextran containing heparin (1 U/ml) to obtain leucocyte-rich plasma, which is then applied to a Ficoll-Hypaque density gradient in the ratio of 2.4:1 and centrifuged at 200g, for 30 minutes to remove mononuclear cells. Contaminating erythrocytes are lysed with ice cold ammonium chloride (0.83%) and the remaining granulocytes are washed twice

in Hanks balanced salt solution (HBSS), pH 7.35, containing 3% HEPES (1 M). Cells are counted in a Neubauer chamber and resuspended to a final concentration of 2×10^6 /ml in HBSS containing ovalbumin (0.4%).

Preparation of assay.

"Top" and "bottom" chambers are washed thoroughly and dried. Nitrocellulose filters are cut with a 13 mm cork borer, and a filter circle is glued to the end of each "top" chamber. Samples to be tested for chemotactic activity (0.8 mls) are placed in the "bottom" chambers in a polystyrene box, lined with a damp sponge with holes cut to take the bottom chambers. Cell suspension (1 ml) is placed in each top chamber and the chamber is immediately but gently lowered into the lower chamber. The polystyrene boxes are sealed and incubated for 90 minutes at 37°C.

Removal and staining of filters.

Top chambers are removed and washed by gentle immersion in distilled water, and then fixed in absolute alcohol for 2 minutes. (This process also has the advantage of dissolving the glue fixing the filters to the chambers). The filters are then placed, outer surface uppermost, on glass slides, and kept in place with metal clips. The staining process is as follows:

- (i) haematoxylin, 5 minutes
- (ii) tap water, 2 minutes
- (iii) HCl, 1%, in 70% alcohol, 2 minutes

- (iv) tap water, 2 minutes
- (v) lithium carbonate (12%), 1-2 minutes
- (vi) tap water, 2 minutes)

They are then dehydrated by dipping in 95% alcohol, followed by immersion in propan-1-ol (2 minutes), xylene/propan-1-ol (equal volumes, 2 minutes) and finally xylene (30 minutes).

They are then mounted with cover slips, using Depex.

Counting method.

Ten random high power fields are counted near the centre of each filter. The surface of the filter is focused on, so that only cells that have completely traversed the filter are counted. Results are expressed as the number of neutrophils in 10 high power fields (neutrophils/10 hpf). All samples are assayed in duplicate and counted blind in coded random order. Zymosan activated serum (R3) is used as a positive control, and is prepared by incubating zymosan (10 mgs, previously boiled in saline solution) with serum (10 mls). The resulting activated serum can be stored in aliquots in liquid nitrogen.

CHAPTER FOUR

HMWNCA IN NASAL SECRETIONS FOLLOWING
ANTIGEN-INDUCED RHINITIS

(i) INTRODUCTION.

Allergic rhinitis is included in the type I category of allergic disease, involving the interaction of antigen with cell-bound IgE. Mast cells and basophils, which are known to bear surface IgE, have been identified in the nasal secretions of patients with allergic rhinitis (Okuda et al., 1983), and are present in far greater numbers than in normal, non-allergic subjects. These basophilic cells resembled both blood basophils and tissue mast cells, and were found to release histamine on incubation with antigen extract.

Hastie et al. (1979) showed that in subjects with allergic rhinitis, nasal blowings contained basophils, though normally in small numbers, whilst mucosal scrapings contained mast cells. In contrast, in patients with colds, or in healthy non-allergic subjects, no basophilic cells were seen.

Mast cells have also been described in the epithelium of some pathological nasal tissues. These include mucosal biopsies of patients with allergic rhinitis (Bryan et al., 1959; Shioda et al., 1966) and nasal polyps.

Friedman et al. (1985) identified mast cells, both resting and actively degranulating, in nasal turbinate mucosa by electron microscopy, although in this study the allergic status of the subjects was not defined.

During the immediate nasal response to antigen challenge, there is a significant increase in the eosinophil count in nasal secretions (Pelikan, 1983). Indeed, the nasal eosinophil count has been used to differentiate allergic rhinitis from viral

rhinitis. An increase in the numbers of neutrophils in nasal secretions has also been described following antigen challenge (Pelikan, 1983), although this is not as dramatic as the rise in eosinophils. However, neutrophils are not commonly found in the mucosa in chronic rhinitis unless there is superimposed infection (Rappaport, 1953). It has been postulated that eosinophils and neutrophils may behave as target cells, and that they may be attracted to the nasal mucosa by chemoattractants released by mucosal mast cells or basophils.

Many chemical mediators have been identified in nasal secretions during both the early and late phase reactions to nasal antigen challenge. Naclerio et al. (1983) have described the release of histamine and of PGD_2 and TAME esterase, in a time dependent fashion, following nasal challenge, with release being maximal 10-15' following challenge corresponding with maximal changes in nasal airways resistance. In a study by Baumgarten et al. (1985), antigen nasal challenge was followed within 10' by the local release into nasal secretions of histamine, TAME esterase, kinins, and both high and low molecular weight kininogens. Challenge of sensitised nasal polyps with ragweed antigen releases histamine, slow reacting substance of anaphylaxis, and eosinophil chemotactic factor of anaphylaxis, and this release is modified by agents affecting the intracellular concentrations of cyclic nucleotides (Kaliner et al., 1973). Moreover, in the same group of patients as in my own study, LTC_4 and to a lesser extent, LTB_4 , was identified in the nasal secretion 10-15' following antigen challenge, but not

methacholine challenge (Shaw et al., 1985).

Although HMWNCA, which is postulated to be a mast cell associated chemical mediator, has been described by many groups in serum, and in the supernatants of stimulated lung fragments (O'Driscoll et al., 1983), it had not been identified in nasal secretions. The aim of the study described was therefore to determine whether heat-stable neutrophil chemotactic activity is generated following antigen induced rhinitis, and if this was so, to investigate further the chemical nature of this NCA.

(ii) MATERIALS AND METHODS

Materials

Methacholine bromide (Sigma, Poole, Dorset, U.K.)

Dermatophagoides pteronyssimus antigen extract (Bencard, U.K.)

Atomiser - De Vilbis No. 15 atomiser, De Vilbis Co., Somerset, PA, USA.

Homogeniser, e.g. Silverson

FPLC system as described in Chapter 3 (ii) B and C

SDS-PAGE as described in Chapter 3 (iii)

METHODS.

Patient selection.

Patients studied gave a history of perennial symptoms of rhinitis, consisting of rhinorrhoea $\pm$ nasal blockage $\pm$ sneezing, and gave a history of symptoms exacerbated by exposure to high levels of dust. All responded to skin prick testing with house

dust mite (*Dermatophagoides pteronyssinus*) antigen with a wheal of > 3 mm after 15 minutes. No patients gave any suggestion of anaphylaxis or asthma on exposure to house dust mite.

Nasal Challenge.

Nasal methacholine and antigen challenges were performed on informed subjects as described by Shaw et al. (1985). In brief, antigen or methacholine was administered by aerosol at room temperature in approximately 0.2 ml volumes to each nostril. During the administration procedure, the patient held his/her breath at maximal inspiration to prevent inhalation of the test compound.

Day 1: Methacholine challenge day.

Each nostril was washed with 4 mls of normal saline, warmed to 37°C, and the washings were discarded.

After five minutes, the procedure was repeated, and the resultant washings were stored on ice, and labelled "pre-wash".

Methacholine bromide (64 mgs/ml, approximately 0.2 mls per nostril) was administered as described above.

At the following time points, each nostril was washed with 4 mls of normal saline (37°C) in 2 ml aliquots:

2', 5', 10', 15', 30'

The resulting washings were collected into 50 ml polypropylene containers on ice, and any extra secretions between washes were collected into similar containers and stored on ice.

Day 2: Antigen Challenge Day.

Pre-washes were performed as on Day 1. After two pre-washes, *D. pteronyssinus* antigen was administered at a concentration which had been shown to produce a positive reaction on previous testing. A positive reaction was defined by the patient producing ≥ 5 sneezes and ≥ 0.5 mls secretions within 10 minutes of challenging. Diluted antigen extracted was administered as described above.

Nasal washes were performed at the same time points as for Day 1.

At all times, medication was readily available to treat any untoward reactions, i.e. β_2 -agonist inhaler and subcutaneous adrenaline. A physician was present at all times with the patient during challenge procedures.

Processing of Nasal Secretions and Washes.

Nasal secretions and washes were pooled such that any secretions formed between two time points was added to the following wash. For example, if a patient collected 0.5 mls of nasal drips between 2 minutes and 5 minutes, this was added to the 5 minute wash.

The total volume at each time point was measured in a volumetric flask, and an equal volume of 12 M urea was added so that the final molarity of urea was 6 M. The urea-treated washes were then homogenised for 30 seconds, and shaken in a 37°C water bath for 1 hour, to allow the urea to fully cleave the mucus glycoproteins in the secretions. Following

incubations, cell debris was removed by centrifugation (10', 4°C), and the resulting supernatant was stored at -20 C prior to analysis.

SP-Sephadex Chromatography.

Nasal washings and secretions were partially purified by cation exchange chromatography in 6 M urea on SP Sephadex gel, as described in Chapter 3.ii.B.

(v) Sephacryl S400 Gel Filtration.

Following partial purification by SP Sephadex cation-exchange chromatography, the peak of NCA from the antigen challenge was pooled, and the corresponding fractions from the methacholine challenge were also pooled.

2 ml of each pool was further purified by Sephacryl S400 gel filtration as described in Chapter 3.ii.A.

Alternate fractions were tested for absorbance at 280 nm and for NCA.

Mono-P Chromatofocusing on FPLC.

Peaks of NCA from antigen challenge samples from S400 chromatography, and equivalent fractions from methacholine challenge were pooled and concentrated to 2 mls by Amicon filtration. Samples were dialysed against starting buffer prior to chromatofocusing on Mono P columns, using the FPLC system, as described in Chapter 3.ii.C. After sample application, the column was washed with starting buffer (10 ml) and then with eluting buffer (47 ml). The pH gradient was self-forming.

Fractions were tested for:

Absorbance at 280 nm

pH

and NCA (after dialysis against PBS)

Fucose Assay

Fucose was measured as a means of quantifying the amount of mucus (i.e. the concentration of mucus glycoproteins) in nasal secretions and in SP Sephadex column fractions. As it was felt that it was necessary to control for any chemotactic effect possibly exerted by mucus glucoproteins and as it was expected that antigen challenge would produce a greater quantity of mucus than would methacholine challenge, nasal secretions were standardised for mucus content prior to any purification step. Fucose has been shown to be a sensitive and specific method of quantifying mucus.

Fucose was assayed by the method previously described in 1955 (Zelliken et al., 1955). In brief, H_2SO_4 (6 parts concentrated H_2SO_4 to 1 part water, 4.5 mls) was added to test solution (1 ml) in a pyrex tube, and heated to 100°C for 10 minutes. After cooling on ice, mercaptoacetic acid (3.3%, v/v, 100 l) was added, and the tubes were left in the dark for 4 hours. A yellow/green compound is formed, which is quantified by measuring the difference between absorbances at 400 nM and at 430 nM.

α_1 -fucose standards (5, 15, 30 and 50 $\mu\text{g}/\text{ml}$) were used to prepare standard curves, which were linear between 5 and 50 $\mu\text{g}/\text{ml}$ of α_1 -fucose.

Protein Estimation.

Nasal secretions from methacholine and antigen challenge were

also standardised by protein estimation, using the Bio-Rad protein estimation kit. Test solution (100 μ l) is added to diluted Bio-Rad solution (4.5 mls) and the intense blue colour formed by proteins is measured after 1-2 hours by measuring absorbance at 595 nm. This is compared with a range of protein standards (ovalbumin, grade VII, Sigma, 0.2 - 1.2 mgs/ml). The Bio-Rad method is based on a biuret reaction which detects the presence of peptide bonds.

Preliminary Experiments.

In view of recent experiments in the laboratory, showing leukotriene release into nasal secretions following antigen-provoked rhinitis, a preliminary experiment was undertaken in which chemotactic activity in nasal secretions was investigated. In this preliminary experiment, no methacholine day was used, instead, pre-challenge wash and 15 minutes post-challenge wash were compared for chemotaxis.

Each nostril was washed with 4 mls normal saline (at 37°C) and the washes were discarded. The "pre"-wash was performed in the same way after five minutes. Antigen (D. pteronyssinus extract 0.1%) was instilled into the nose (0.2 mls per nostril, via a De Vilbis atomiser), and subsequent nasal secretions were collected into a sterile polystyrene container on ice. Fifteen minutes after challenge, the nose was washed again, and the wash was pooled with the collected secretions.

The nasal washings were treated as described earlier, the only difference being that, in these preliminary experiments, 4 M urea was used rather than 6 M urea.

Urea-treated secretions were assayed for NCA in the following dilutions: Neat; 1:5, 1:10.

"Pre" and "post" challenge urea-treated secretions were then purified by cation exchange chromatography on SP Sephadex columns, as described in Section 3.ii.B. Once again, the only difference was that the columns buffer contained 4 M urea instead of 6 M urea. After thorough dialysis against PBS, alternate fractions were assayed for NCA at 20% dilution.

Pools of NCA from SP Sephadex chromatography of "post" sample and the equivalent fractions from the "pre" sample, were lyophilised. 1 mg of each pool was used for polyacrylamide gel electrophoresis (as described in Chapter 2), being electrophoresed in the absence of mercaptoethanol on 6% polyacrylamide vertical slab gel overlaid with a 3% stacking gel. Resulting gels were fixed and stained by the Bio-Rad silver stain technique as described elsewhere.

Pools of activity from SP Sephadex (and their pre-challenge controls) were further purified by gel filtration on Microbondagel columns using HPLC, as described in Chapter 3.ii.A. Lyophilised material (3 mgs) was dissolved in HPLC starting buffer (250 μ l) and filtered (1 μ pore size). Two HPLC "runs" were made for each sample, each run using 100 μ l of dissolved sample. Equivalent 1.5 ml fractions for the same sample were pooled. Fractions (1.5 ml) were collected as previously described, i.e. Fraction II had as its end point the elution time for ferritin (M.Wt. 440,000) and the previous adjacent and the three subsequent fractions were also collected.

Following dialysis against PBS, all fractions were assayed for NCA at 20% dilution.

Further experiments were undertaken to determine the time

course of the release of this NCA following nasal challenge. These time course experiments differed in two major ways from the preliminary experiment:

- (i) A methacholine control day was used
- (ii) Nasal secretions were treated with 6 M urea

On day 1, a methacholine challenge was performed and washes were performed at the time points described earlier, i.e. two pre-washes and 2', 5', 10', 15', 20' and 30' following methacholine challenge.

On day 2, at the same time of day (to control for any diurnal variation), an antigen challenge was performed at a dose which had previously been shown to produce symptoms in the same patient, and washes were performed in an identical way as on day 1.

Nasal secretions were processed as previously described. Following incubation and centrifugation to remove cell debris, the urea- treated washings were freeze-dried under vacuum overnight.

1 mg of each sample was electrophoresed on vertical slab polyacrylamide gels as described in Chapter 2 in the absence of mercaptoethanol. Gels were fixed and stained by the Bio-Rad silver stain technique.

(iii) RESULTS.

Preliminary experiments.

A preliminary experiment was undertaken to ascertain whether any form of NCA was released into nasal secretions

following nasal antigen challenge. A nasal challenge was undertaken on a subject who had previously been shown to produce LTC₄ 15 minutes following challenge with 0.1% D. pteronyssimus antigen. Nasal washings were collected before and 15 minutes following challenge.

Chemotaxis assays of both pre- and post-challenge crude secretions were negative. However, zymosan-treated serum (R₃ serum, 40 l), added to either "pre" or "post" challenge secretions, also failed to induce chemotaxis, although R₃ serum was more effective in pre-challenge than in post-challenge secretions. It was therefore assumed that the viscosity of the nasal secretions, which was caused by the viscous nasal mucus glycoproteins, was interfering with the chemotaxis assay, and possibly masking any chemotactic activity in the secretions.

As human tracheo-bronchial, cervical and nasal mucus proteins have been described as being highly acidic glycoproteins, one way of separating mucus glycoproteins from any less acidic proteins in nasal secretions is by cation-exchange chromatography, performed at such a pH that acidic proteins elute from the gel prior to commencement of a salt gradient. To enable column chromatography of highly viscous secretions, 4 M urea was used to disrupt the tertiary structure of the mucus glycoproteins, thus reducing viscosity.

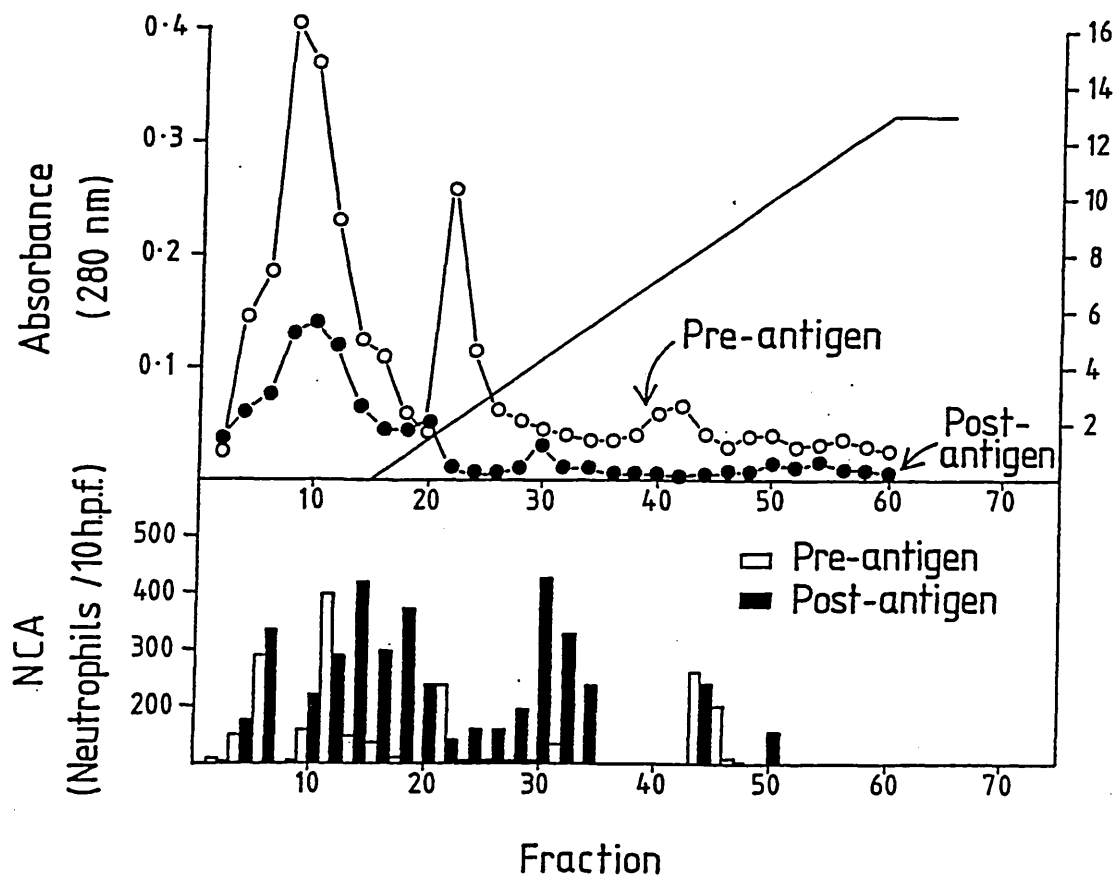
SP-Sephadex C25 gel was used as a strong cation exchanger, and the cation exchanged was Na⁺, provided by on NaCl gradient

The results of the SP Sephadex cation-exchange chromatography are shown in Figure 4.1.

There is a peak of optical density which elutes before the

Figure 4.1

Cation exchange chromatography of nasal secretions before and after the intranasal administration of house dust mite antigen to a patient with perennial rhinitis.



Cation exchange chromatography on SP Sephadex gel performed on nasal washings prior to house dust mite (HDM) challenge (open bars and circles) and 10 minutes following challenge (closed bars and circles). Secretions were solubilised in 4M urea prior to analysis. The top section represents absorbance at 280 nm of eluting fractions (circles) and the salt gradient, from 0 - 0.5 M NaCl is represented by the increasing conductivity of the eluting fractions, shown by the solid line. NCA in the eluting fractions is shown in the lower section.

commencement of the salt gradient, which is associated with a peak of NCA in both the pre- and post-challenge samples. Such a protein peak would be due to highly acidic proteins, and may be associated with mucus glycoproteins present in both pre and post-challenge samples.

The post-challenge sample also exhibits another, discrete peak of NCA, not present in the pre-challenge sample, which elutes from the column with approximately 0.2 M NaCl. Although cation-exchange chromatography gives no precise information on the isoelectric point of eluting proteins, the pH of the running conditions (pH 5.5) and the position of the NCA peak on the NaCl gradient would be compatible with a protein with neutral isoelectric point.

Polyacrylamide gel electrophoresis of this peak of NCA in the post-challenge sample (and its equivalent in the pre-challenge sample) showed a high molecular weight protein band, as shown by silver staining, in the post-challenge sample, that is not present in the pre-challenge sample (results not shown).

Time course experiments were undertaken as described. Maximal symptoms in terms of irritation and sneezing occurred within the first two minutes, although increases in mucus production lasted for 10-15 minutes, and subjective changes in nasal obstruction persisted for up to 30 minutes following antigen challenge. Methacholine challenged produced similar (though not so marked) changes in nasal patency and mucus production, but without the effects of irritation and sneezing.

Urea-treated, lyophilised secretions were subjected to polyacrylamide gel electrophoresis, and the results are shown

in Fig. 4.2.

A faint protein band is visible at times 5, 10, 15 and 20 minutes on the antigen challenge day, which is not present on the methacholine challenge day. High molecular weight protein standards, treated with urea but without mercaptoethanol are also shown on the gels. Using a standard calibration curve, the molecular weight of the protein band noted may be estimated at approximately 650,000 daltons.

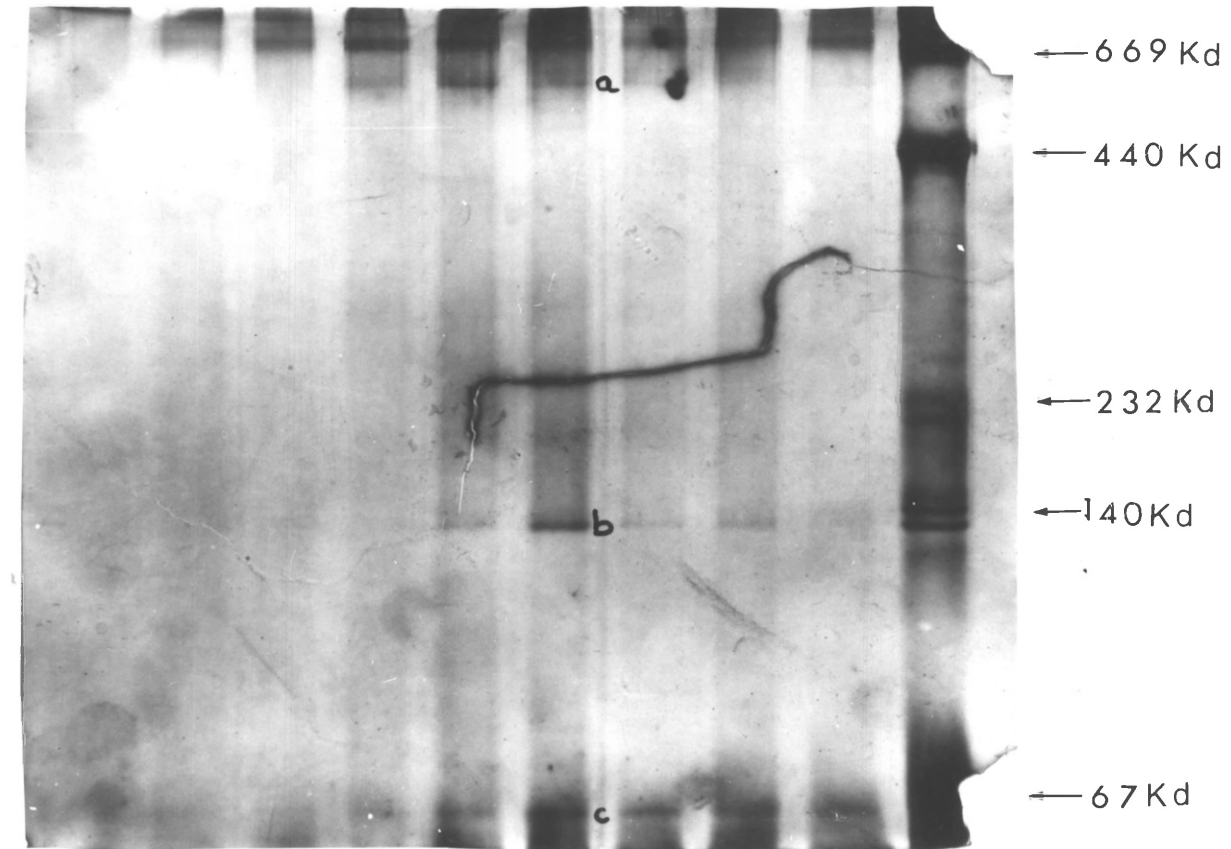
A second time course, performed on patient R.M. gave similar results. Once more, a high molecular weight protein band was visible at times 5, 10, 15 and 20 minutes, seeming maximal at 10 and 15 minutes (results not shown). This band was visible only in the antigen challenge day samples, and was absent in the methacholine challenge day samples. The estimated molecular weight of this protein was approximately 600,000 daltons.

Secretions treated with urea (6 M) collected 15 minutes following antigen challenge of 5 patients were pooled (as the 10 and 15 minute samples seemed to contain the greatest concentration of the high molecular weight material described above). The equivalent samples from methacholine challenge were similarly pooled as a negative control.

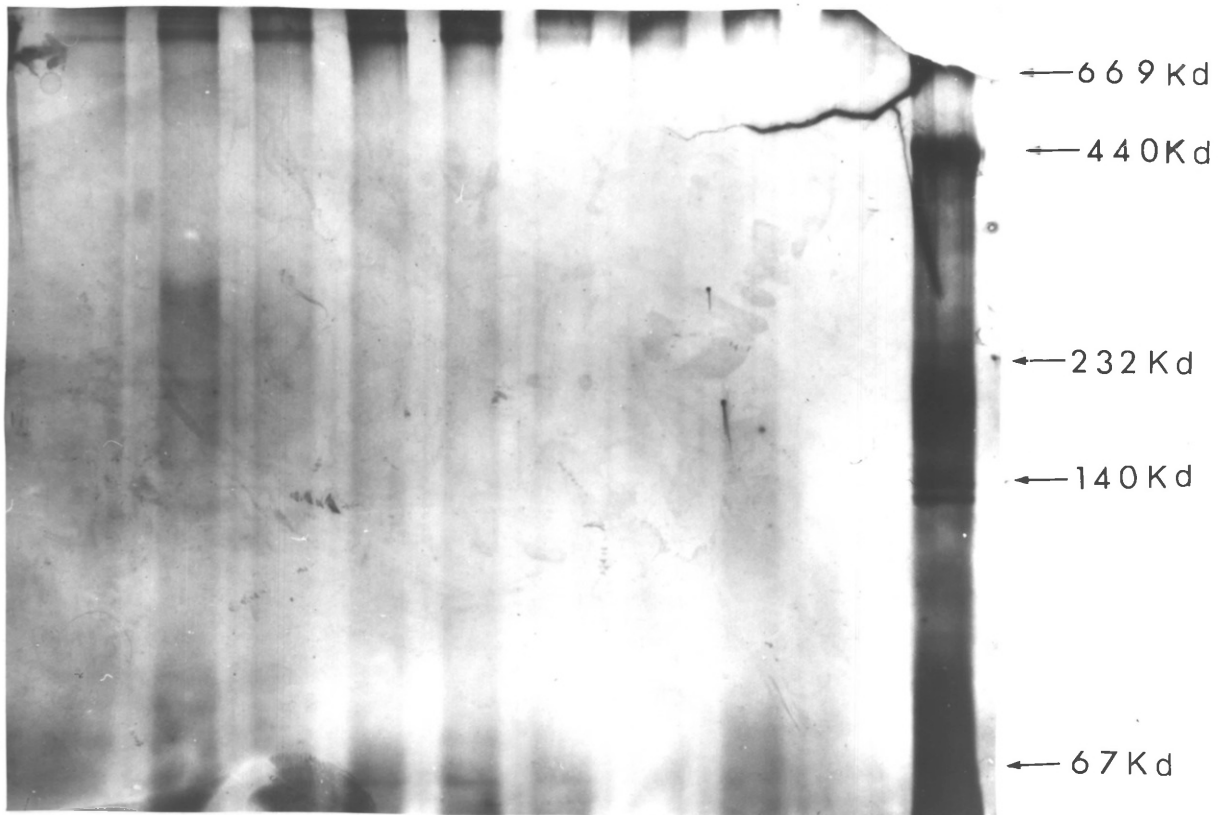
These antigen and methacholine samples (2 ml) were applied separately to SP Sephadex columns. Eluting fractions were tested for NCA, fucose concentration and absorbance (280 nm) and the results from one patient are shown in Fig. 4.3. Although there is some background chemotactic activity "smeared" across the column, there is a definite antigen-induced peak of NCA which is not apparent in the methacholine induced secretions,

Figure 4.2

Polyacrylamide gel electrophoresis of nasal secretions at time intervals following intranasal challenge with methacholine and house dust mite. (For legend, see over).



ANTIGEN



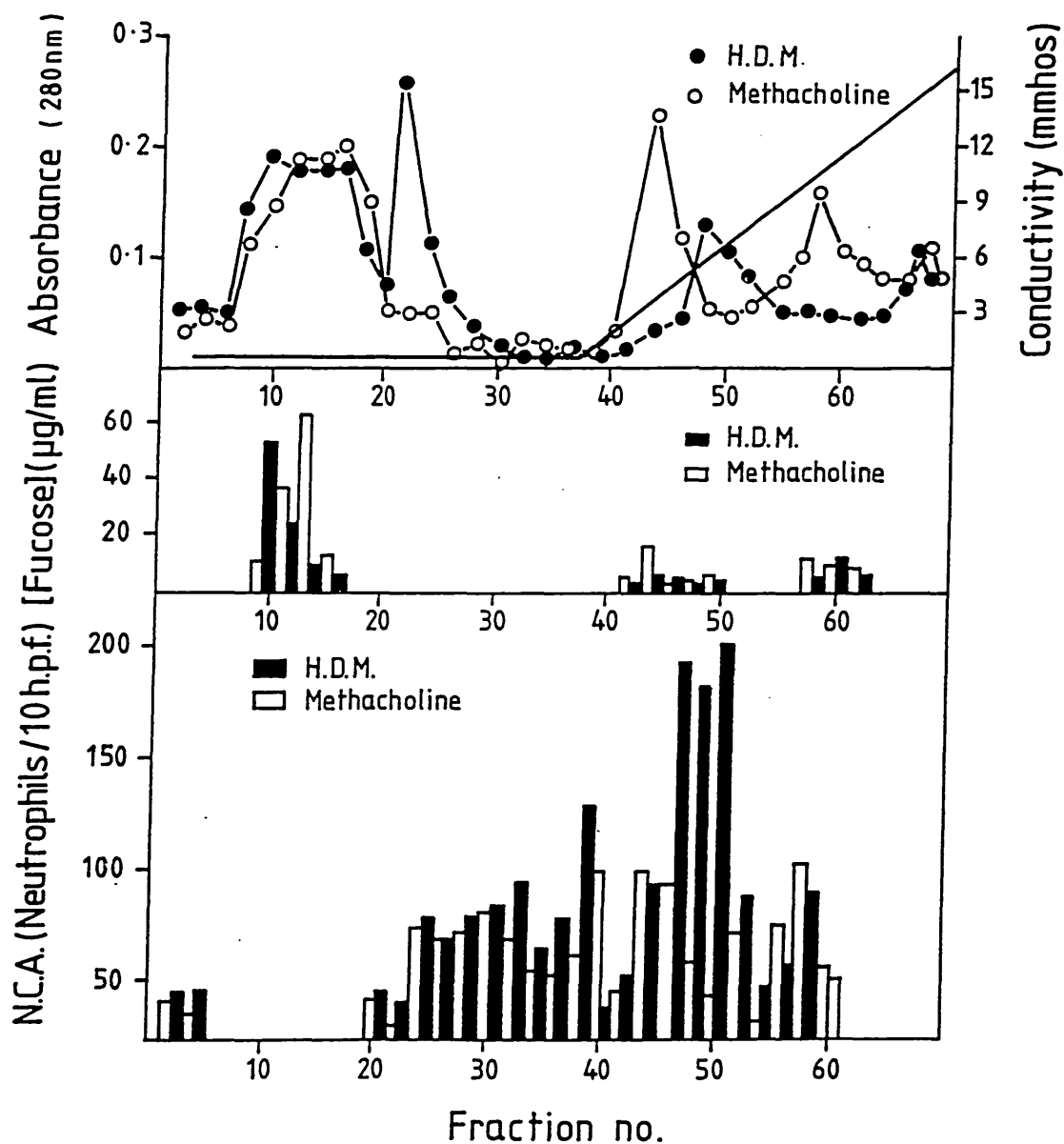
2' 5' 10' 15' 20' 30' 60'
METHACHOLINE

Legend to Figure 4.2.

Polyacrylamide gel electrophoresis was performed on nasal washings obtained prior to intra-nasal challenge (prewash A and B) and 2-30 minutes following intranasal challenge with either (i) methacholine or (ii) house dust mite antigen. The first well contained methacholine or house dust mite antigen alone, and the last well in each plate contained standard high molecular weight markers. Letters a and b depict the high and intermediate weight bands respectively that were found in antigen-induced secretions.

Figure 4.3

Cation exchange chromatography of antigen and methacholine induced nasal secretions (SP Sephadex).



House dust mite (HDM) (solid bars and circles) and methacholine (open boxes and circles) - induced secretions were analysed by cation exchange chromatography (SP Sephadex). The absorbance of the eluting fractions at 280 nm (open and closed circles) and the salt gradient (solid line) are shown in the top section. The middle section shows the fucose content (µg/ml) of the eluting fractions and the lower section the neutrophil chemotactic activity (NCA) in neutrophils per 10 hpf.

and which elutes from the column with approximately 0.2 M NaCl. The major peak of fucose, corresponding with a peak of mucous glycoproteins, elutes prior to the commencement of the salt gradient. Fig. 4.4 shows the cation exchange profiles of NCA from all 5 patients. The background activity is variable, but in each case, an antigen-induced peak of NCA elutes from the column with approx. 0.2 - 0.3 M NaCl.

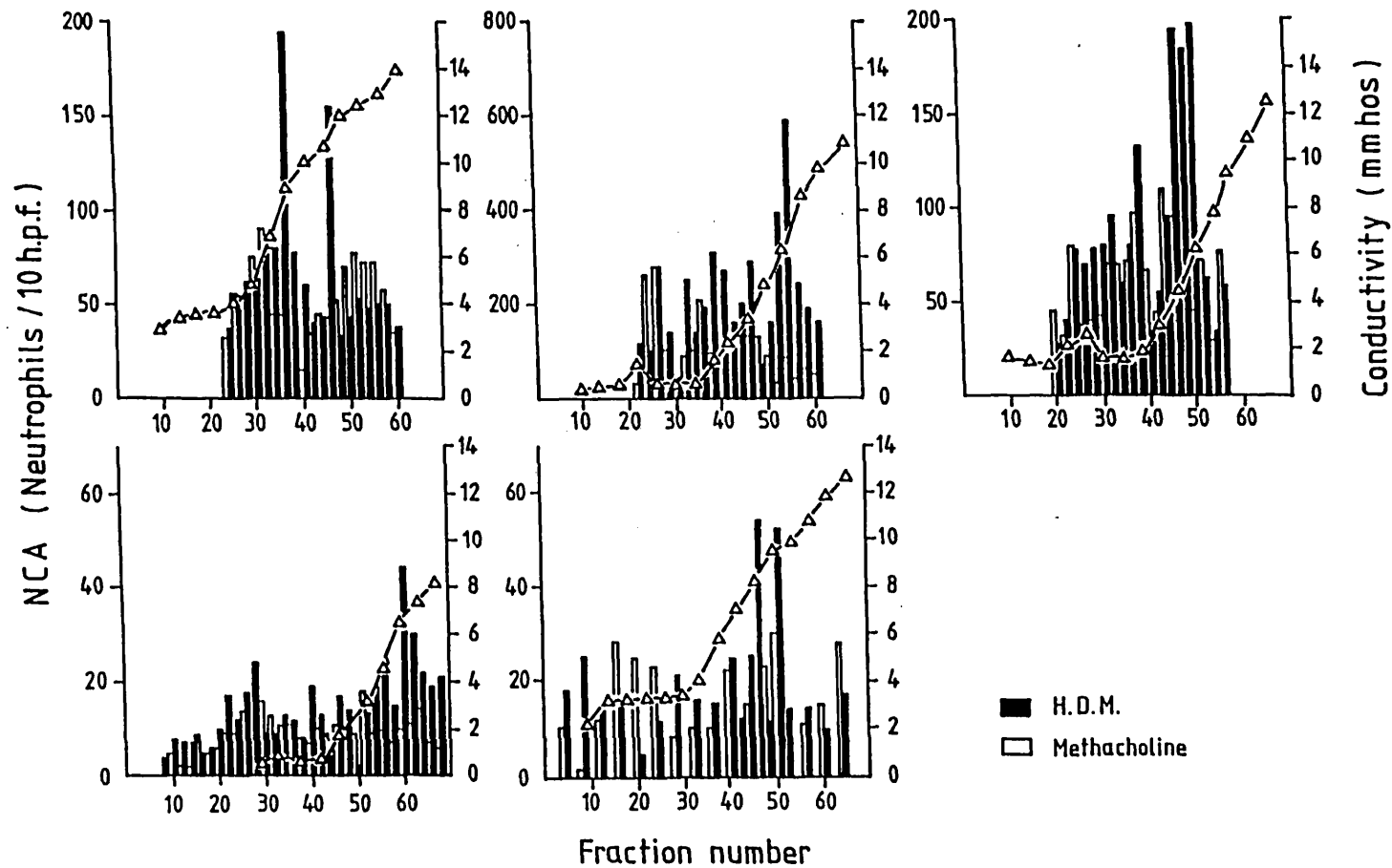
These peaks of NCA from antigen challenge, and their corresponding fractions from methacholine challenge were pooled separately and dialysed free of urea, before being applied to a Sephacryl S400 gel filtration column. The eluting fractions were assessed for neutrophil chemotactic activity and absorbance (280 nm).

The results of gel filtration from 1 patient are shown in Figure 4.5. Although there is great variability in the chemotactic activity from methacholine control fractions, there is a clear peak of activity in the antigen challenge sample which elutes between thyroglobulin (molecular weight 669,000) and ferritin (molecular weight 440,000). The peak of activity does not elute "clearly" in one or two fractions; this may reflect the heterogeneous nature of the eluting protein. The peak of activity is not associated with a peak of optical density.

Fig. 4.6 shows gel filtration profiles from 4 out of the 5 patients tested. In each case there is an antigen induced peak of NCA, not present in the methacholine induced secretions, eluting between thyroglobulin and ferritin, and so being of high molecular weight.

Figure 4.4

Cation exchange chromatography of antigen- and methacholine-induced nasal secretions from five patients.



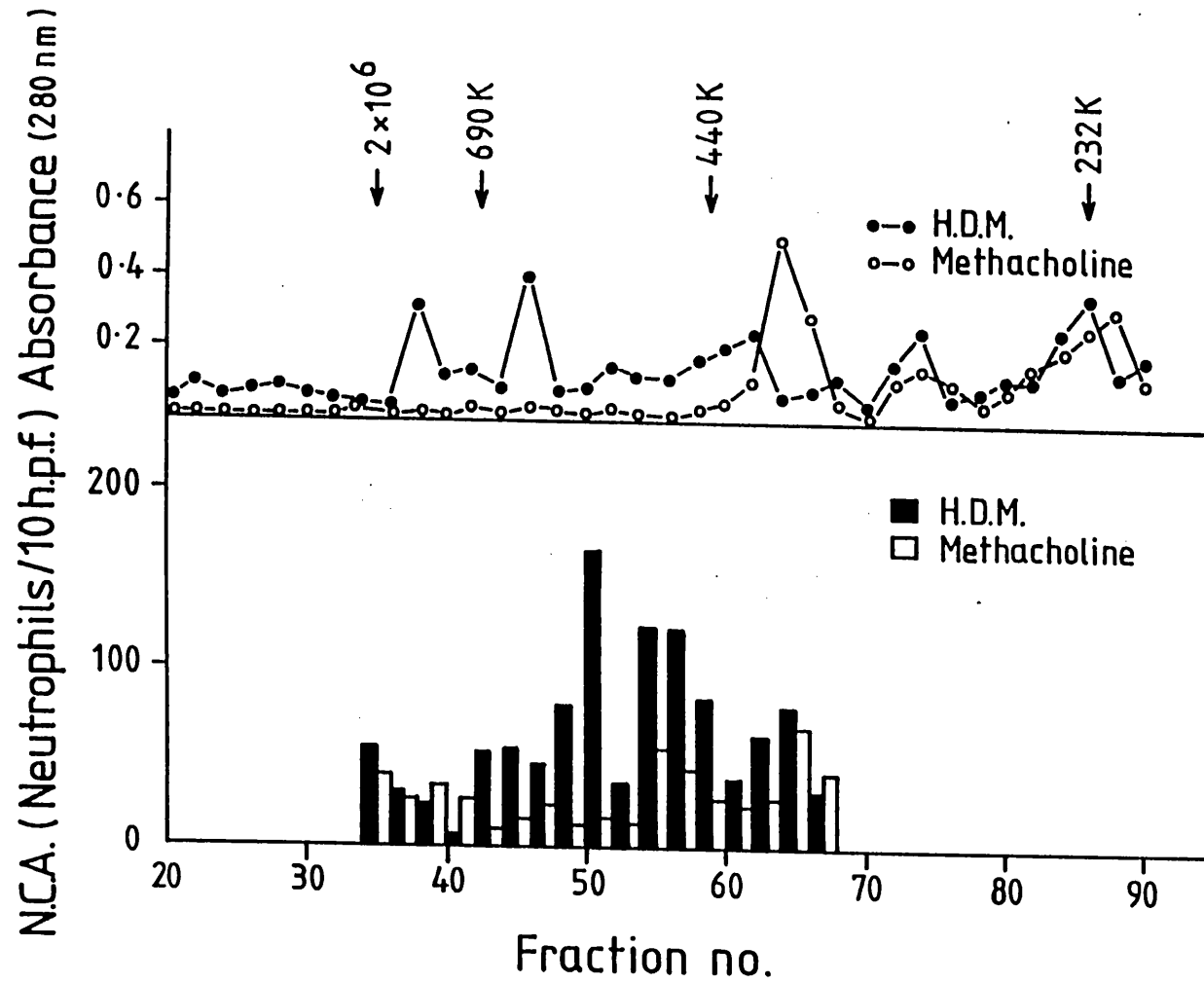
For legend, see over.

Legend to Figure 4.4

Each graph represents antigen and methacholine nasal challenge from one patient. The vertical bars show neutrophil chemotactic activity (NCA) in neutrophils/10 high power fields in eluting fractions when methacholine induced nasal secretions (open bars) and house dust mite (HDM) induced secretions (closed bars) were subjected to cation exchange chromatography on SP Sephadex gel. The conductivity of the eluting fractions (and hence the salt gradient) is represented by open triangles.

Figure 4.5

Gel filtration of antigen and methacholine induced nasal sections on Sephacryl S400.



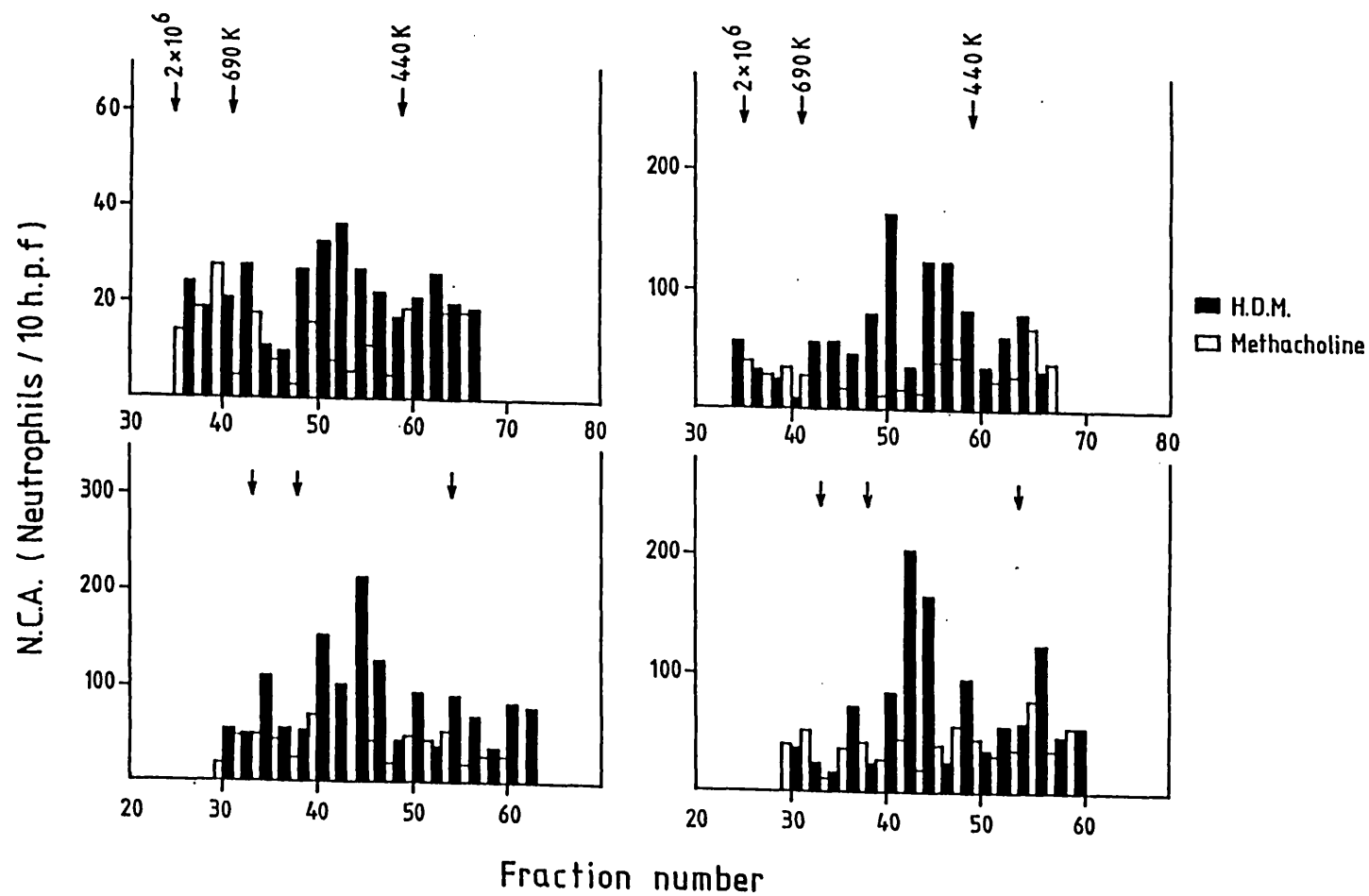
For legend, see over.

Legend to Figure 4.5

Following cation exchange chromatography, NCA from house dust mite (HDM) induced secretions (closed bars and circles) and methacholine induced secretions (open bars and circles) were subjected to gel filtration on Sephacryl S400 gel. The absorbance at 280 nm of the eluting fractions is shown in the top section, along with the position of the high molecular weight markers. The lower section shows the neutrophil chemotactic activity (NCA) in the corresponding fractions, in neutrophils/10 high power fields. Markers used were blue dextran (2×10^6), thyroglobulin (690,000), ferritin (440,000) and aldolase (230,000).

Figure 4.6

Gel filtration of methacholine- and antigen-induced nasal secretions from four patients.



For legend, see over.

Legend to Figure 4.6

Each box represents gel filtration of nasal secretions of one patient. The vertical bars show the neutrophil chemotactic activity (NCA) in eluting fractions from gel filtration of house dust mite (HDM) induced secretions (closed bars) or methacholine induced secretions (open bars). The position of the molecular weight markers Blue Dextran (2×10^6 daltons), thyroglobulin (690,000 daltons) and ferritin (440,000 daltons) is shown above each graph by a series of arrows.

Chromatofocusing of this HMWNCA from 3 patients is shown in Fig. 4.7. In each case, the major peak of NCA elutes with pH 6.5 - 7.0, and so has an isoelectric point (pI) of near neutrality. In one patient there is an additional peak of NCA with a more acidic isoelectric point (pH 5.5).

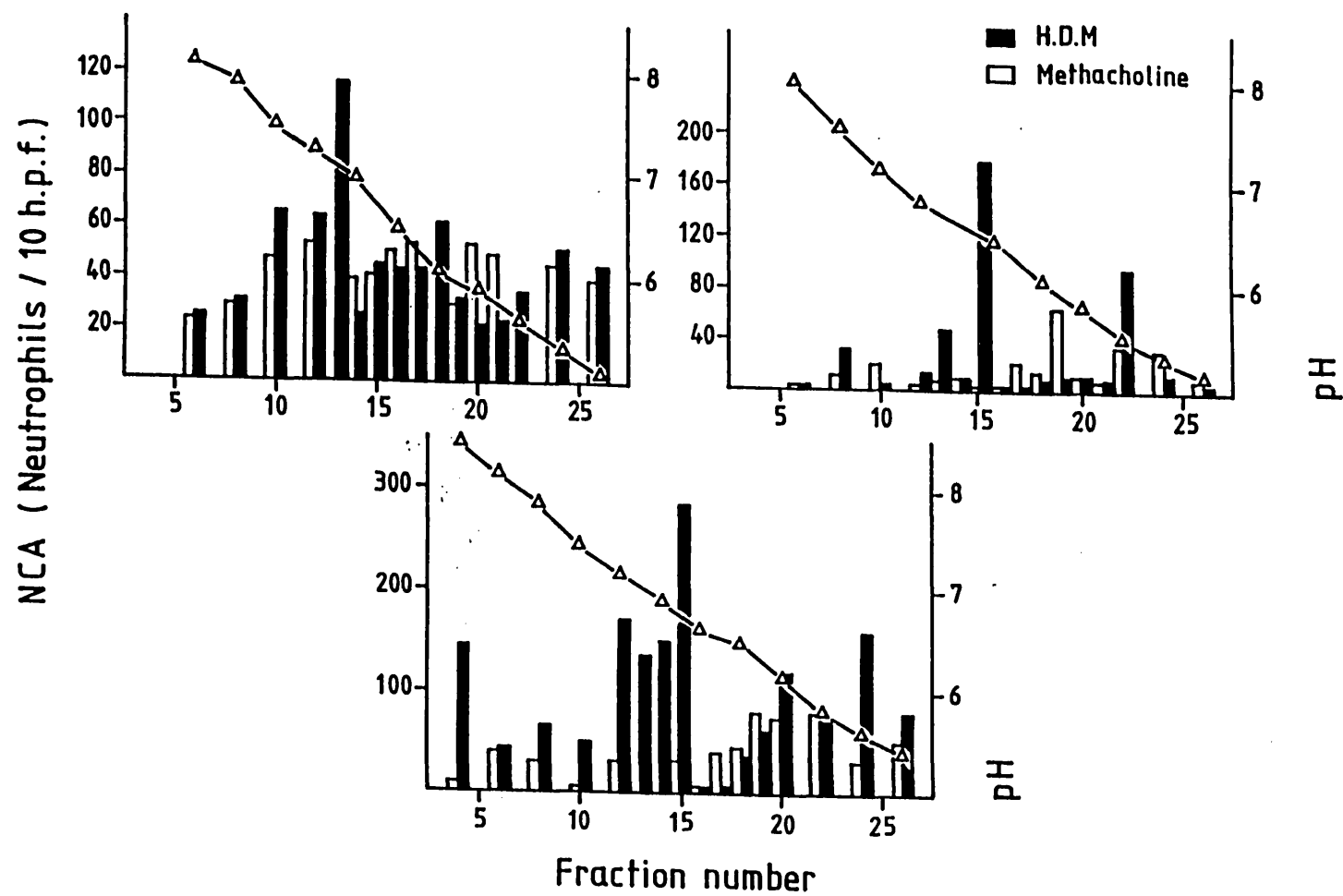
(iv) DISCUSSION.

The number of patients studied was of necessity small, because of the time consuming chemical procedures which were performed on each subject. The subjects involved in the study all suffered from perennial rhinitis, but were completely asymptomatic at the time of the study. All subjects had previously been shown to give a positive reaction to intra-nasal house dust mite antigen as judged by 10 or more sneezes within 5', and by production of > 3 mls mucoid secretions. The subjects were chosen because they had also previously been shown to release LTC₄ and LTB₄ into nasal secretions following HDM nasal challenge (Shaw et al., 1985). In retrospect, it would have been preferable to involve patients with grass pollen induced rhinitis and to study them outside the grass pollen season, so that the external exposure to antigen and therefore the airway hyperreactivity, would have been reduced to a minimum. However, as subjects were asymptomatic, and as no chemotactic activity was found in pre-challenge samples, the problem of background hyperreactivity was ignored.

Nasal reactivity, as measured by the concentration of methacholine or histamine required to produce an increase in nasal airways resistance, is markedly increased in hayfever

Figure 4.7

Chromatofocusing of antigen and methacholine-induced nasal secretions from three patients.



For legend, see over.

Legend to Figure 4.7

Each box represents chromatofocusing of house dust mite (HDM) induced secretions (closed bars) or methacholine induced secretions (open bars) from one patient using Mono P columns on the fast protein liquid chromatography (FPLC) system. The vertical bars represent neutrophil chemotactic activity (NCA) of the eluting fractions in neutrophils/10 high power fields, and the pH of the corresponding fractions is shown by the open triangles.

sufferers during the pollen season, and in patients with perennial rhinitis throughout the year; smaller increases in reactivity are associated with viral illnesses of the "common cold" type (Borum et al., 1983; Gronborg et al., 1983). Patients with rhinitis, but with no history suggestive of asthma, show increased bronchial reactivity as evidenced by increased airway responsiveness to inhaled methacholine (Ramsdale et al., 1985). Although this finding may only reflect the common association of allergic rhinitis and asthma, in which the asthma is sub-clinical, it may also be interpreted as an indication of generalised airway hyperreactivity. My own observation in a single subject has been that following heavy dust exposure, the sensitivity to inhaled HDM antigen was greatly increased, and the dose of HDM antigen required to produce rhinitis was reduced by 100 fold.

The study was designed so that each subject acted as his own control. The control used for antigen inhalation challenge was that of methacholine inhalation challenge; methacholine acts via the parasympathetic nervous system as a cholinergic agonist. Parasympathetic nerves innervate respiratory tract mucus glands, and cholinergic agonists result in the stimulation of glandular activity and have been shown to cause the release of proteins and mucus glycoprotein in vitro (Boat et al., 1975; Shelhamer et al., 1980). In vivo, inhalation of methacholine via the nose causes rhinorrhoea, occurring within 2-5 minutes, which is not accompanied by sneezing or itching. Allergic reactions in the nose cause mucus secretion, accompanied by sneezing and irritation, via complex mechanisms, probably mediated by

interactions between mast cell-associated mediators. Histamine, via its H_2 receptor can stimulate airway mucus release in vitro (Shelhamer et al., 1980). Most prostaglandins (with the exception of PGE_2) stimulate mucus release (Marom et al., 1981) as do lipoxygenase metabolites and LTC_4 and D_4 (Marom et al., 1982).

The object of choosing methacholine as a control was to compare nasal secretions produced by an allergic, presumably mast cell mediated, reaction to those produced by reflex parasympathetic stimulation. However, methacholine challenge has been shown to produce secretions with lower viscosity than those produced by histamine or allergen challenge (Brofeldt et al., 1983). This may be because a pharmacological stimulation of glandular cholinergic receptors (by methacholine) produces a different secretory response from that of nervous stimulation (by allergen and histamine) reflected in differences in the output of mucous glycoproteins and hence in viscosity of nasal secretions. A further criticism of the study is that as histamine concentration was not measured in the secretions, there was no definite marker of mast cell activation. The reasons for not measuring histamine were purely mechanical. The collected secretions, washed out with saline solution, were not homogeneous, and it was felt that taking an aliquot from the crude secretions would not provide a representative sample for histamine estimation. Mucus was dispersed to make a homogeneous solution by chemical rather than mechanical means, using high concentrations of urea. Having transferred the secretions into urea, they were no longer suitable for histamine measurements.

A better design would perhaps be to gently mechanically disperse the mucus and to remove a small aliquot for histamine measurement prior to transfer into urea.

The problems of performing biochemical purification procedures on nasal mucus arise because of its physical, rheological properties. Mucus glycoproteins consist of a series of related glycoproteins with a protein core which is rich in residues of serine and threonine, to which are attached long carbohydrate side chains (Kaliner et al., 1984). These carbohydrate side chains consist predominantly of pentose sugars, especially fucose. The molecular size of airway mucus glycoproteins varies from 400,000 to 20 million daltons (Patow et al., 1984), but this probably reflects the linking of individual subunits to form large polymers. These large polymers adsorb water to form thick gels, and gives mucus its physical properties. The gelatinous nature of the mucus causes problems when applied to columns, as the gel pores quickly become clogged. This leads to slowing down and eventually complete cessation of buffer flow. In these studies, this problem was overcome by chemically disrupting the mucus glycoproteins using 6M urea.

High concentrations of urea act by splitting non covalent forces between protein subunits and therefore destroying the tertiary structure of protein molecules. This has the effect of denaturation of mucus glycoprotein polymers and so reducing the viscosity of mucus. The reaction is reversible (Anfinsen, 1973; Lehninger, 1975) and once urea has been thoroughly removed by dialysis, the proteins spontaneously regain their tertiary structure. It has

been shown that mucus will spontaneously go into solution without having to break up disulphide bonds (Silberberg et al., 1977). However, this process is slow at room temperature, and only speeds up at higher temperatures; even at 40°C spontaneous dissolution takes approximately 24 hours. The nasal secretions were incubated with 6 M urea in a method adapted from Kaliner et al. (1984), and centrifuged to remove the mucus containing supernatant from the pellet which contained epithelial debris, free cells, hairs, etc.

It was observed that nasal secretions collected after even the highest doses of methacholine challenge were not as plentiful or as viscid as those collected after antigen inhalation challenge. It was therefore important to standardise the amount of mucus which was to be subjected to further analysis, to rule out any mucus-associated NCA. This standardisation was achieved by assessing the fucose content of the secretions, using the method described by Gibbon et al. (1955). Having measured the fucose concentration of each sample, the same total quantity of fucose from either antigen or methacholine induced secretions was applied to cation exchange chromatography columns, in the same volume. It would have been preferable to promote more mucus secretion by using higher doses of methacholine, but when very high doses of methacholine were used by inhalation, the resultant nasal obstruction was so great that the yield of nasal mucus was reduced further.

The first chemical process, cation exchange chromatography under acidic running conditions, was chosen so as to remove the mucus glycoproteins. Mucus glycoproteins are acidic, with a pI

varying from 3.5 - 5.1 (Kaliner et al., 1984). Under the acidic running conditions (pH 5.3), they would then be expected to elute directly from the column, without adhering to the gel. In fact, in both methacholine and antigen-induced secretions, a peak of absorbance associated with a peak of fucose concentration was found to elute with the void volume of the column. This corresponded to the nasal mucus glycoproteins which had no neutrophil chemotactic activity per se. In the case of each subject, a peak of NCA appeared in the antigen induced secretions, but not in the methacholine induced secretions; this activity eluted with 0.3 M NaCl. There was a great deal of background chemotactic activity throughout the eluting fractions, which puts doubt on the significance of the observed antigen induced NCA. However, this antigen induced NCA was present in all 5 subjects to a varying degree. In retrospect, it would have been advisable to measure chemotactic activity in more than 1 dilution of each fraction, as it has since been shown that in asthmatic serum, greater dilution of the samples may unmask high levels of NCA (Buchanan et al., 1986). This may be due to high-dose inhibition in the assay itself.

The peaks of chemotactic activity were pooled and the urea removed by thorough dialysis against buffered phosphate. At this stage it was impossible to assess whether treatment with urea had irreversibly damaged the chemotactic factor(s) or whether the urea-treatment was entirely reversible. If the experiments were to be repeated, it would be possible to do this by performing polyacrylamide gel electrophoresis before and

after urea treatment and cation exchange chromatography. This would help to assess any changes in molecular size of the chemotactic protein(s).

In order to estimate the size of the chemotactic activity after the cation exchange chromatography step, gel filtration was performed on Sephacryl S400 gel as described in previous chapters. NCA with an apparent molecular weight of approximately 600,000 daltons was present in antigen-induced, but not in methacholine-induced secretions. Shelhamer (1980) described a mucus glycoprotein formed by human bronchial airways which has a molecular weight of 400,000 (in addition to a larger glycoprotein molecule, with molecular weight > 7,000,000). Both glycoproteins were released from airway strips following immunological stimulation with antigen following passive sensitisation with IgE, and following reversed anaphylaxis using anti-IgE. Molecules with similar physical properties were also found after incubation of airways with exogenous histamine and methacholine. It is not known whether these proteins are chemotactic for neutrophils, and so it is therefore possible that the NCA isolated in our experiments is the same as the smaller mucus glycoprotein previously described. However, there are several reasons why this may not be so; firstly, antigen induced NCA was larger, with a M.Wt. of 600,000 daltons. Secondly, this NCA was not associated with any appreciable fucose, suggesting that it is not a mucus glycoprotein. Finally, although identical quantities of mucus were analysed, NCA was only identified in the antigen-induced secretions.

Similar peaks of NCA, with similar molecular weights, were

identified in 4 subjects. Although there was a variable degree of background activity throughout the eluting fractions and in some subjects, the high molecular weight peak was small, this chemotactic activity was found to be repeatable. The variable background activity could be explained if there was some contamination with mucus proteins, since mucus macromolecules are known to demonstrate anomalous behaviour on gel filtration (Boat et al., 1975).

The final purification step used was chromatofocusing, using Mono P columns on the FPLC system. This procedure was chosen because the HMW-NCA previously described (Wasserman et al., 1977) has a pI of 6.7 - 7.0, and is therefore nearly neutral. It was important to know whether nasal NCA, which had a similar molecular size, also had a similar, near neutral, isoelectric point. In his original paper, Wasserman et al. used both sucrose and thin layer Sephadex G75 plates as isoelectric focusing media. In this study, Mono P columns were chosen as they provide good resolution under moderately high pressures and therefore provide a rapid method for focusing. Nasal NCA from 3 subjects was subjected to isoelectric focusing, and in each case, a peak of NCA was found to elute at pH 6.5 - 7.0. This peak of NCA, which was of high molecular weight, and near neutral isoelectric point, was thus physico-chemically similar to that previously described in asthmatic serum (Lee, 1982; Nagy, 1982), in cold urticaria serum (Wasserman, 1977) and in the supernatants of human lung fragments (O'Driscoll, 1983).

(v) Summary

We have attempted to determine whether HMWNCA was released after specific allergen challenge in patients with allergic rhinitis. Six subjects with perennial rhinitis underwent nasal challenge with an extract of the house dust mite (D. pteronyssinus). Post-challenge but not pre-challenge nasal washings contained a neutrophil chemotactic activity which was released in a time-dependent fashion with maximal activity 10-15 min after allergen administration. In contrast, when the same patients were challenged with methacholine on a control day, negligible quantities of NCA were detected. Secretions obtained by allergen or methacholine challenge were treated with 6M urea standardized for their mucus glycoprotein content, and subjected to cation exchange chromatography (SP-Sephadex), gel filtration (Sephacryl S-400) and chromatofocusing (Mono-P gel). The allergen-induced NCA was associated with molecules of 600,000 daltons molecular weight and neutral iso-electric point. These studies suggest that HMWNCA is a mediator of rhinitis.

CHAPTER FIVE

HMWNCA RELEASE FROM IMMUNOLOGICALLY CHALLENGED
HUMAN LUNG FRAGMENTS

(i) INTRODUCTION.

High molecular weight NCA had been identified in the supernatants of human lung fragments challenged with anti-IgE antibody (O'Driscoll et al., 1983). This activity had an apparent molecular weight of approximately 750,000 daltons, a near-neutral isoelectric point, and eluted from DEAE Sephacel, on anion-exchange chromatography, with 0.2 M NaCl. A similar HMWNCA had also been identified in guinea pig lung fragments, passively sensitised with IgE, and challenged with specific allergen (Schentel et al., 1982). Because of the time-consuming chemical methods necessary to confirm the physico-chemical properties of HMWNCA, and to differentiate it from other possible chemotactic factors, it was not possible in these studies to investigate the characteristics of HMWNCA release, in particular its time-course and dose-dependency.

The object of the work which will be described was to develop a more rapid method for identifying HMWNCA, by means of its molecular size and its behaviour on anion-exchange chromatography, utilising the rapid techniques of FPLC and HPLC. In order to validate these techniques, serum HMWNCA and lung HMWNCA have been compared.

Using these methods, I have proceeded to investigate the time course and dose-dependency of anti-IgE induced HMWNCA release from human lung fragments.

Eosinophil chemotactic factor of anaphylaxis (ECF-A) release from challenged guinea pig lung fragments has been shown to be enhanced by the addition of small concentrations of maleate and

succinate to the incubation mixture (Kay et al., 1971). The effect of these substances on HMWNCA release has also been assessed. In addition, the effects of the presence of several protease inhibitors on the levels of detectable HMWNCA in lung supernatants was investigated.

(ii) MATERIALS AND METHODS.

Materials

Maleate	Sigma, Poole, Dorset
Succinate	Sigma, Poole, Dorset
Ovomucoid trypsin inhibitor	Sigma, Poole, Dorset
Aprotinin	Sigma, Poole, Dorset
Soybean trypsin inhibitor	Sigma, Poole, Dorset
Normal rabbit immunoglobulins	DAKO, High Wycombe, Bucks
Rabbit immunoglobulins to human	
IgE (ε chains)	DAKO, High Wycombe, Bucks
Fine scissors, scalpel, forceps	
37° shaking water bath, 56°C water bath	Grant, Cambridge
Tyrodes buffer, with and without calcium	
(see Chapter 3)	
³ H-leukotriene B ₄	Amersham
FPLC and HPLC as described in Chapter 2.	
Bolting cloth (160 μm pore size)	Boots plc.

Methods.

Processing of Human Lung.

"Normal" human lung tissue was obtained from thoracotomy,

which was usually performed for carcinoma of the bronchus. Lung tissue used in experiments was macroscopically normal, showed no signs of bacterial infection and was not immediately adjacent to the site of carcinoma. The lung was placed promptly into ice cold Tyrodes buffer (without calcium) on ice. Lung tissue was carefully dissected free of pleura, visible blood vessels and airways using a scalpel (number 10 blade) and forceps. Fine scissors were then used to mince the lung parenchyma into fragments approximately 1 mm^3 in size. At all times the lung tissue was kept on ice, and was not allowed to dry. The lung fragments were placed in a plastic sieve, lined with $160 \mu\text{m}$ pore size bolting cloth, and were washed with approximately 2 litres of Tyrodes in 200 ml aliquots, until the washings were visibly clear of blood. The washed lung fragments were then removed from buffer, weighed and aliquoted into sterile polystyrene 25 ml containers, and were re-suspended in Tyrodes buffer containing calcium chloride and allowed to equilibrate for 15 minutes in a water bath at 37°C .

Rabbit immunoglobulins^(IgG) to human IgE or normal rabbit IgG immunoglobulins (as a control) were added, the volume depending on the design of the experiment. After thorough mixing, the lung fragments were left gently shaking in a 37°C water bath for a time period dependent on the design of the experiment. Following this incubation period, the lung suspension was centrifuged ($140g$, $10'$, 4°C) and the resulting supernatant removed and stored at -20°C prior to use.

Processing of Lung Supernatants for Histamine Assay.

Supernatant

Lung supernatant (250 μ l) was added to perchloric acid (50 μ l, 30%) in a 4 ml polypropylene test tube. After thorough mixing, the cloudy suspension was centrifuged (1832g, 10', 4°C) and the resultant de-proteinised supernatant was diluted 1 in 20 (5% dilution) with perchloric acid (5%).

Total Histamine.

After removal of the lung supernatant, remaining lung fragments were resuspended in the same volume of Tyrodes with CaCl_2 and rapidly frozen (in solid CO_2 and alcohol mixture) and re-thawed (in a water bath at 56°C) three times to break up the cells. After centrifugation (570g, 10', 4°C) the resultant supernatant (250 l) was added to perchloric acid (50 l, 30%), mixed thoroughly and centrifuged (570g, 10', 4°C). The resulting de-proteinised supernatant was diluted 1:20 (5% dilution) with perchloric acid (5%).

Diluted supernatants were stored at -20°C prior to use.

Time Course Experiments.

Lung fragments were divided into 5 gm aliquots, and each was resuspended in 10 mls of Tyrodes with CaCl_2 . Following equilibration at 37°C, fragments were challenged with either anti-IgE or normal rabbit immunoglobulins (which acted as a negative control). Lung supernatants were collected at the following time points:

"0'" (i.e. immediately after addition of challenging agent),
5', 10', 15', 30', 60'.

Supernatants were tested for:

- a) Histamine
- b) Crude NCA after heating for 30' at 56°C
- c) NCA after partial purification by anion exchange on FPLC
- and d) NCA after further purification by gel filtration on HPLC

According to the availability of human lung the number of time points tested was varied. Once it had been established that at no time tested did the control normal rabbit immunoglobulins cause NCA release, further experiments were undertaken in which the number of time points with anti-IgE was increased at the expense of the number of time points with control. This was unfortunate, but unavoidable as, for any one lung specimen, the number of aliquots that could be made was limited.

In all, four time course experiments were undertaken, but for the reasons stated above, the time points tested vary between experiments.

Dose-Response Experiments.

Lung fragments were aliquoted as described above and equilibrated at 37°C for 10-15' in Tyrodes with calcium. Anti-IgE or normal rabbit Ig control were then added in the appropriate quantities to form the following final dilutions:

1:2000 (0.05%)

1:1000 (0.1%)

1:500 (0.2%)

1:200 (0.5%)

1:100 (1%)

After thorough mixing, the lung fragment suspensions were left shaking in a 37°C water bath for 15 minutes, after which time the supernatants were removed as described above.

Supernatants were assayed for:

- a) Histamine concentration
- b) Crude NCA after heating for 30' at 56°C
- c) NCA after partial purification by anion exchange on FPLC
- and d) NCA after further purification by gel filtration on HPLC

As described in the previous section, the number of concentrations tested in any one experiment was dictated by the availability of human lung tissue, and thus although dose response experiments were conducted four times, the number of doses tested in each experiment varies.

Maleate and Succinate Experiments.

According to the method described by Kay (1971) maleate (5 mM) and succinate (5 mM) were added to lung fragments immediately prior to challenge with anti-IgE, whilst lung to which no maleate/succinate was added was used as a control. Anti-IgE was used at a 1:200 dilution (as this had been shown to be optimal for NCA release) and supernatants were collected 15' following challenge.

Lung supernatants were tested for histamine release, and NCA (before and after purification steps as described above).

Protease inhibitor experiments.

Ovomucoid trypsin inhibitor, soybean trypsin inhibitor and aprotinin were added to the incubation buffer, at 5 mM and 10 mM concentrations, singly or in combination, prior to challenge of lung fragments with anti-IgE (1:200 dilution). Supernatants were removed and NCA purified by FPLC and HPLC steps as described. HMWNCA (following HPLC step), released in the presence and absence of these protease inhibitors, was compared.

Histamine Assay and NCA Assay.

As described in Chapter 3.v.

NCA Assay.

As described in Chapter 3.vi.

Gel filtration (HPLC).

Columns, buffers and column conditions are described in Chapter 3.ii.A. Samples applied to the HPLC had been partially purified by anion exchange chromatography on the FPLC; they were lyophilised and then re-dissolved in 300 µl of HPLC buffer and then filtered through a 1µ filter prior to injection onto the columns.

S400 Gel Filtration.

As described in Chapter 3.ii.A.

Evaluation of Effect of Lyophilisation on NCA.

Lung supernatant NCA partially purified by Polyanion SI anion

exchange chromatography was divided into two equal aliquots. Aliquot (i) was stored at -20°C prior to NCA assay; Aliquot (ii) was lyophilised in the conventional way, and resuspended in the original volume of PBS. Both aliquots were assayed for NCA at 4 different dilutions: neat; 1:2 (50%); 1:5 (20%); 1:10 (1%). The experiment was performed twice.

Comparison of Sephacryl S400 Chromatography with Gel Filtration using HPLC as methods of HMWNCA analysis.

Lung supernatant NCA, partially purified by Polyanion SI anion exchange chromatography was divided into two equal aliquots.

Aliquot (i) (2 mls) was further purified by Sephacryl S400 gel filtration as described in Chapter 2. The fractions eluting between the molecular weight markers Blue Dextran (M.Wt. 2×10^6) and Ferritin (M.Wt. 440,000) were pooled, and stored at -20°C prior to NCA assay.

Aliquot (ii) (2 mls) was lyophilised, re-dissolved in 250 µl of HPLC buffer, and two HPLC runs were made, each run having a sample size of 100 µl. Allowing for minimal losses during re-dissolving, this effectively used the entire sample. On each HPLC 'run', a 1 ml fraction was collected, the end point of which exactly matched the elution time of Ferritin (440,000 M.Wt.). This process effectively collected any protein eluting which had a molecular weight of above 440,000. The fractions from the two separate runs were pooled, and were then made up to the same volume as that of aliquot (i) using PBS.

Aliquots (i) and (ii) (following gel filtration) were then

assayed for NCA in four different dilutions:

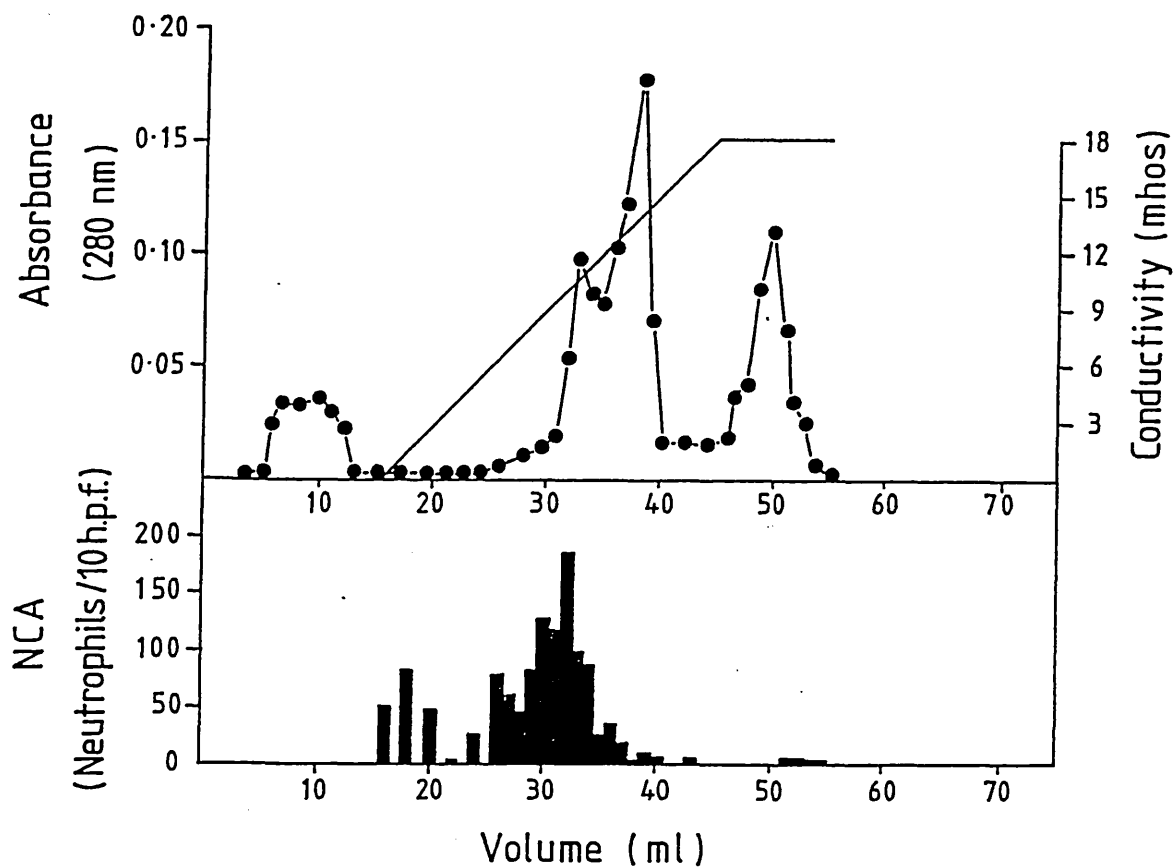
neat; 1:2 (50%); 1:5 (20%); 1:10 (10%)

(iii) RESULTS.

Serum NCA, previously shown to be of high molecular weight by gel filtration on Sephadex S400, was analysed by anion exchange chromatography on FPLC. A peak of chemotactic activity eluted from the column with buffer that had a conductivity of 6 mmols, corresponding with 0.2 M NaCl (see Figure 5.1). This was in close concordance with the known behaviour of serum HMWNCA on conventional DEAE-Sephacel anion exchange chromatography. Heated serum, taken from an asthmatic patient pre- and post-exercise induced bronchoconstriction (EIA) was subjected to the same procedure, and the resulting NCA profile is shown in Fig. 5.2. The post EIA serum shows a NCA which elutes under similar conditions to serum HMWNCA. Heated asthmatic serum, post-antigen challenge was then compared with supernatants of human lung fragments challenged with anti-IgE, using the same FPLC method. The major peak of serum NCA elutes with a conductivity of 8 mhos, whilst the major peak of lung derived NCA elutes with a slightly lower conductivity of 6 mhos, corresponding to NaCl concentrations of 0.3 and 0.15 M respectively. However, although these results seem to show a discrepancy between serum and lung derived NCA, further experiments showed that although this NCA behaved slightly heterogeneously on FPLC columns, it always eluted with buffer containing between 0.15 and 0.3 M NaCl.

Figure 5.1

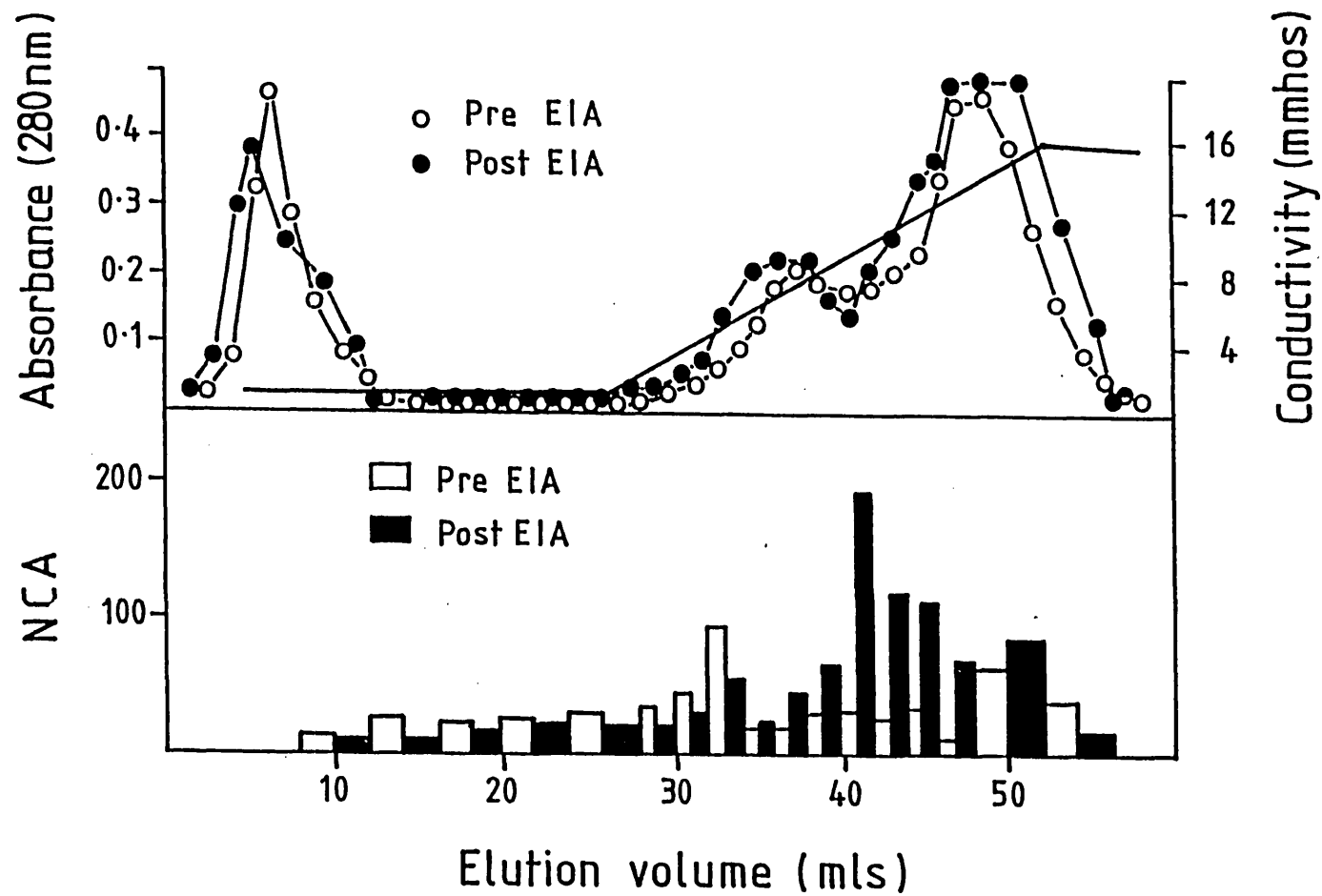
Anion exchange chromatography (FPLC) of serum NCA (from asthmatic serum following antigen induced asthma) partially purified by gel filtration (Sephacryl S400 gel).



The top section shows the absorbance (280 nm) of eluting fractions (closed circles) and the salt gradient (solid line). The lower section represents the neutrophil chemotactic activity (NCA) (neutrophils/10 hpf) of the eluent.

Figure 5.2

Asthmatic serum, pre and post exercise-induced bronchoconstriction;
profile on Polyanion SI anion-exchange chromatography (FPLC)



For legend, see over.

Legend to Figure 5.2

Serum from an asthmatic patient, before (open circles and bars) and 10 minutes after (closed circles and bars) exercise-induced bronchoconstriction (EIA), was analysed by anion exchange chromatography (FPLC). The top section shows the absorbance at 280 nm of eluting fraction (open and closed circles) and the salt gradient (solid line). The lower section shows the neutrophil chemotactic activity (NCA) in the eluting fraction (open and closed bars), in neutrophils/10 hpf.

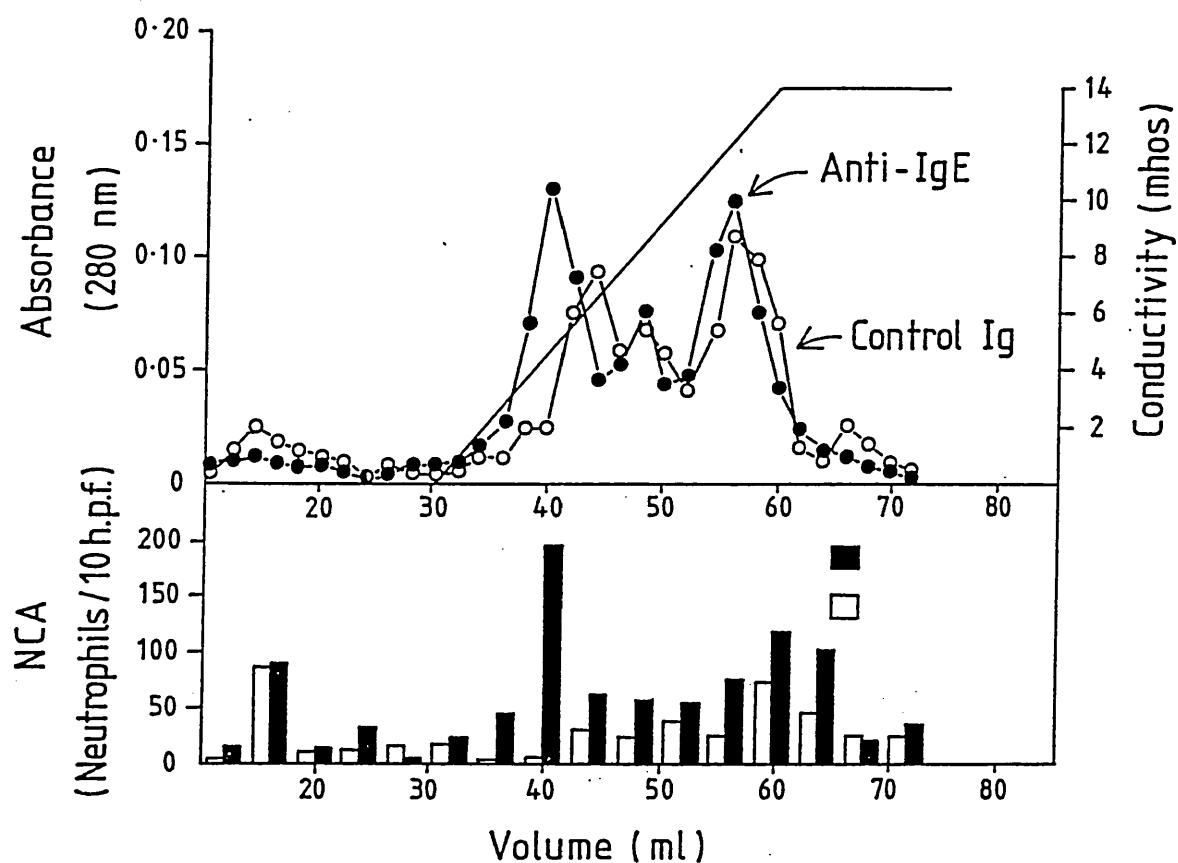
Supernatants of human lung fragments challenged with anti-IgE, were compared with those challenged with control Ig, using FPLC. The results of one experiment are shown in Figure 5.3. NCA eluting with approximately 0.2 M NaCl (conductivity 6 mhos) was present in the anti-IgE stimulated, but not in the control, supernatant.

Serum HMWNCA, obtained by gel filtration (Sephadex S400) of asthmatic serum following antigen challenge, and supernatants of human lung fragments stimulated with anti-IgE, following anion exchange chromatography (FPLC), were subjected to gel filtration on microbondagel columns using the HPLC and the elution profiles are shown in Figure 5.4. It had previously been shown that the optimum resolution in the high molecular weight range was obtained by the use of three columns in series; these consisted of an E-linear column, followed by an E-1000 column followed by a second E-linear column (results not shown). Although a small degree of further resolution was gained by the addition of further microbondagel columns, it was felt that the sample losses incurred by this procedure did not justify it.

Because of the small elution volumes of the column, and the large volumes needed for assaying NCA, sample collection reduced the resolution of the column still further. As a compromise, 1 ml samples were collected, so that sample 2 (shaded box in figures) would contain any high molecular weight activity. The cut-off point for high molecular weight activity was deemed arbitrarily to be 440,000 daltons; this could be readily assessed in every experiment using a transferrin marker of

Figure 5.3

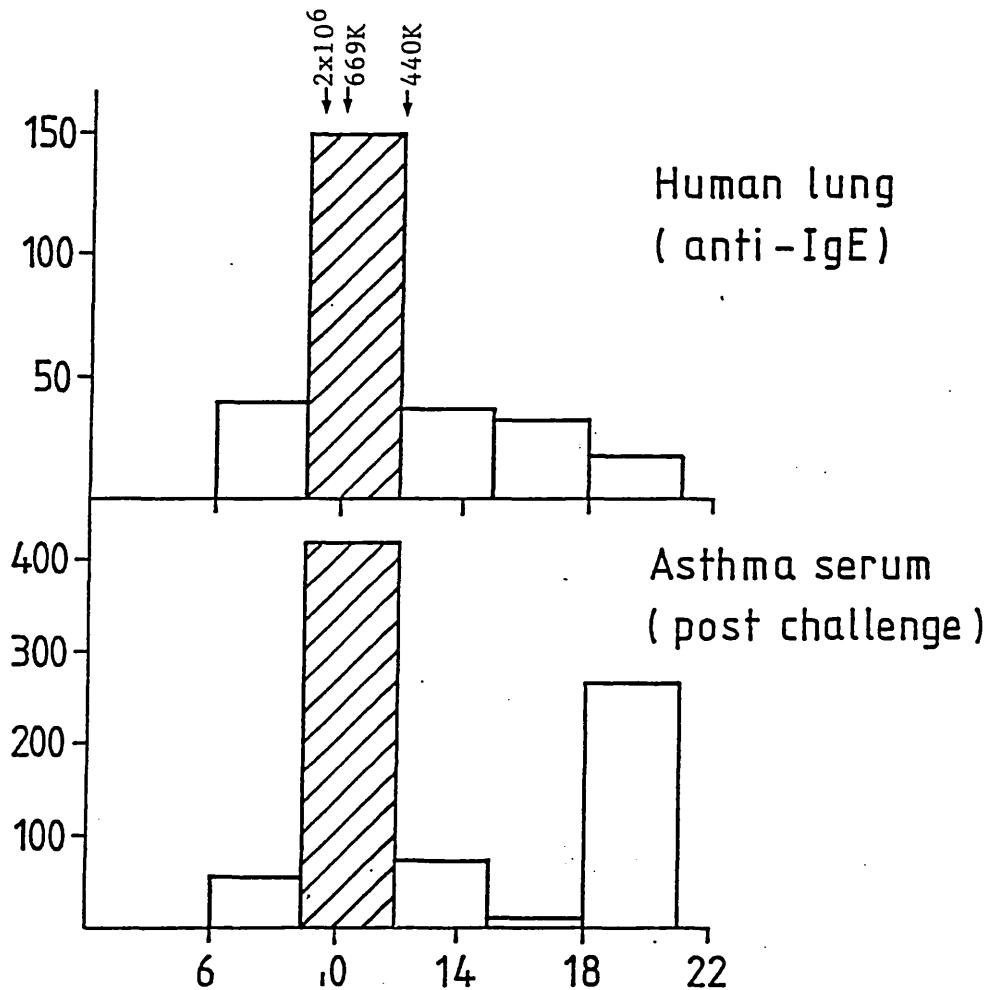
Supernatants from human lung fragments, challenged with anti-IgE or control Ig; profile on anion exchange chromatography (FPLC).



Supernatants from human lung fragments challenged with either anti-IgE (closed circles and bars) or control Ig (open circles and bars) were analysed by FPLC. Absorbance at 280 nm (open and closed circles) and conductivity (continuous line) of the eluting fractions are shown in the top section, whilst neutrophil chemotactic activity (NCA, neutrophils/10 hpf) of the equivalent fractions is shown below.

Figure 5.4

Supernatant from anti-IgE-stimulated human lung fragments and serum NCA compared by gel filtration on HPLC.



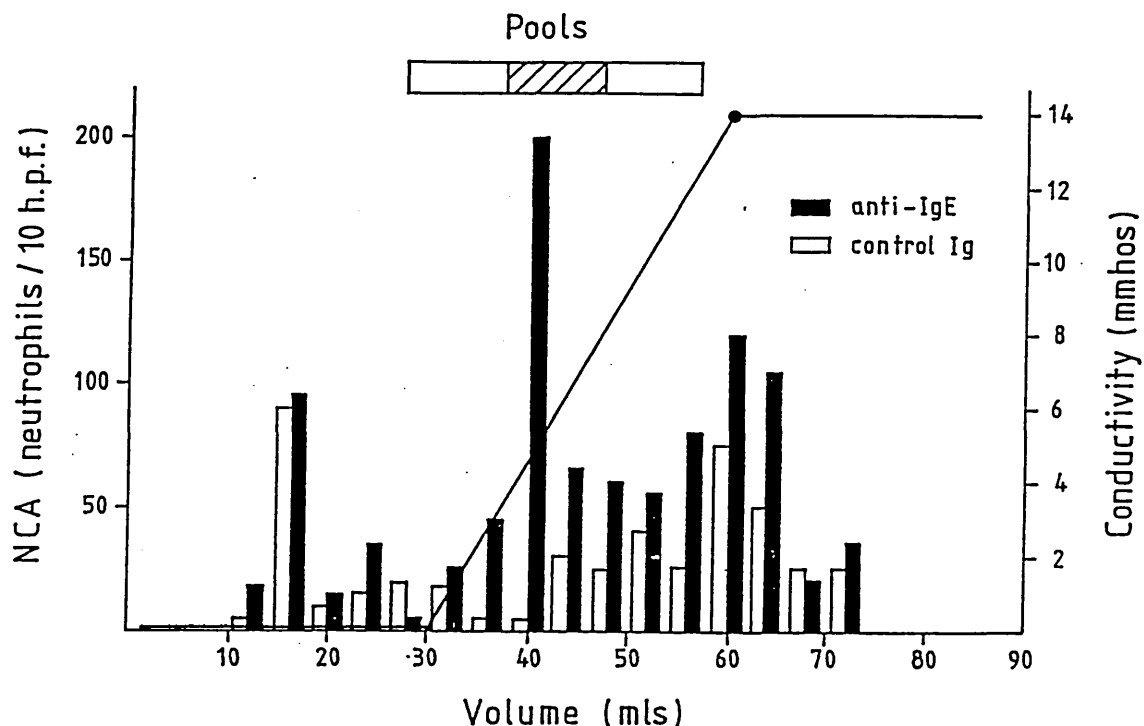
NCA from anti-IgE stimulated human lung fragments (post FPLC) was compared with NCA from asthmatic serum (post Sephacryl S400 gel filtration) using gel filtration on Micronbondagel columns (HPLC). The eluting position of the high molecular weight markers, Blue Dextran, (2×10^6 daltons), thyroglobulin (690,000 daltons) and ferritin (440,000 daltons) are marked above by arrows. The top section shows the neutrophil chemotactic activity (NCA) of eluting fractions from lung-derived NCA, whilst the lower section shows the chemotactic activity in the equivalent fractions from serum-derived NCA.

molecular weight 440,000 daltons, and no other chemotactic factors described have had molecular weights higher than this value, with the exception of fibronectin. Both serum and lung-derived NCA eluted from HPLC columns in the high molecular weight fraction, although serum also contained a low molecular weight NCA, below 150,000 daltons (see discussion). In a single experiment designed to compare the methods of Sephadex S400 gel filtration and HPLC gel filtration in the evaluation of HMWNCA, it seemed that smaller quantities of HMWNCA were obtained from the HPLC method, and that the HPLC method also produced lower molecular weight activity that was not present following the Sephadex S400 method (results not shown). The significance of these results will be considered more fully in the discussion section of this chapter. Thus, although the HPLC method was by no means a perfect method of gel filtration of HMWNCA, it acted as a rapid method for differentiating "high molecular weight" NCA from other lower molecular weight substances. The time-course and dose-response experiments produced large numbers of samples, each of which was analysed by the FPLC and HPLC methods described. In order to reduce the numbers of samples to be assayed for NCA from the FPLC columns, so that FPLC profiles from every sample in each experiment could be analysed simultaneously, the eluting fractions for the FPLC were pooled as shown in Figure 5.5. Pool 2 (shaded box in all subsequent figures) contained any activity eluting with between 0.15 and 0.3 M NaCl; thus the HMWNCA previously described eluted in this fraction.

Figure 5.6 shows the FPLC profiles of one time course

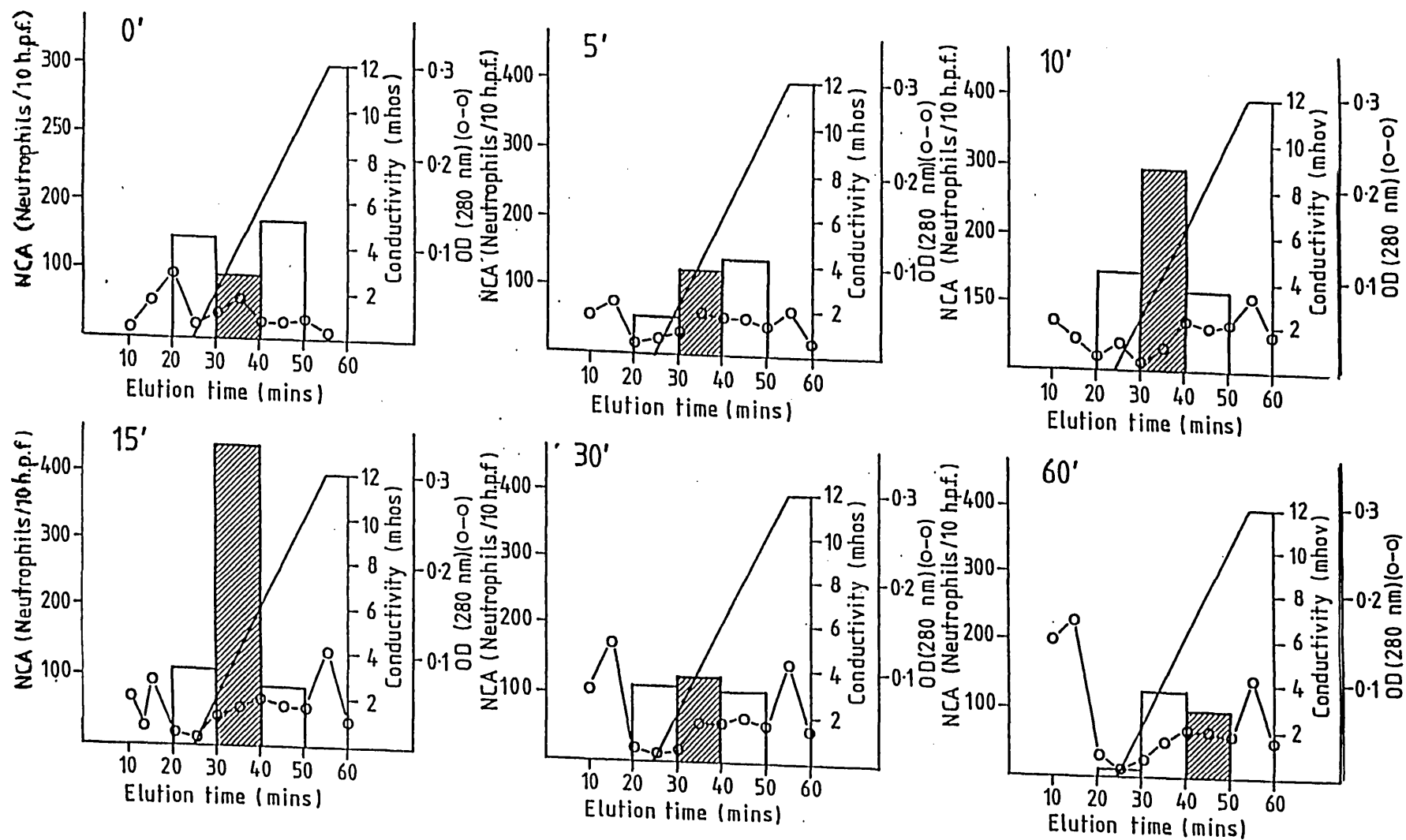
Figure 5.5

Diagram showing the pooling of fractions eluting from anion-exchange chromatography on FPLC of lung-derived NCA.



Supernatants from human lung fragments stimulated with either anti-IgE (solid bars) or control Ig (open bars). The bars represent neutrophil chemotactic activity (NCA) of the eluting fractions whilst the salt gradient is shown by the solid line. Three pools of fractions were collected, as indicated above the diagram. In subsequent time-course and dose response experiments, fractions were thus pooled prior to analysis for NCA.

Figure 5.6 Time course of NCA release from anti-IgE-stimulated human lung fragments - results of anion-exchange chromatography (FPLC)



For legend see over.

Legend to Figure 5.6

Each graph represents anion exchange chromatography (FPLC) of a single supernatant, collected at a specified time (0-60 minutes) following anti-IgE stimulation of human lung fragments. The three bars in each graph represent the neutrophil chemotactic activity (NCA) in the pooled fractions shown in Figure 5.6, the middle shaded fraction being that in which HMWNCA elutes. The absorbance of the eluting fractions (280 nm) is shown by open circles, and the solid line shows the salt gradient.

experiment. . Each graph in Figure 5.6 represents a lung supernatant collected at a single time point (denoted in boxes) following challenge with anti-IgE. There is a time-dependent release of NCA (eluting in the same fractions as would HMWNCA) which is maximal 15 minutes following stimulation with anti-IgE. In contrast, there is no such release following control Ig stimulation.

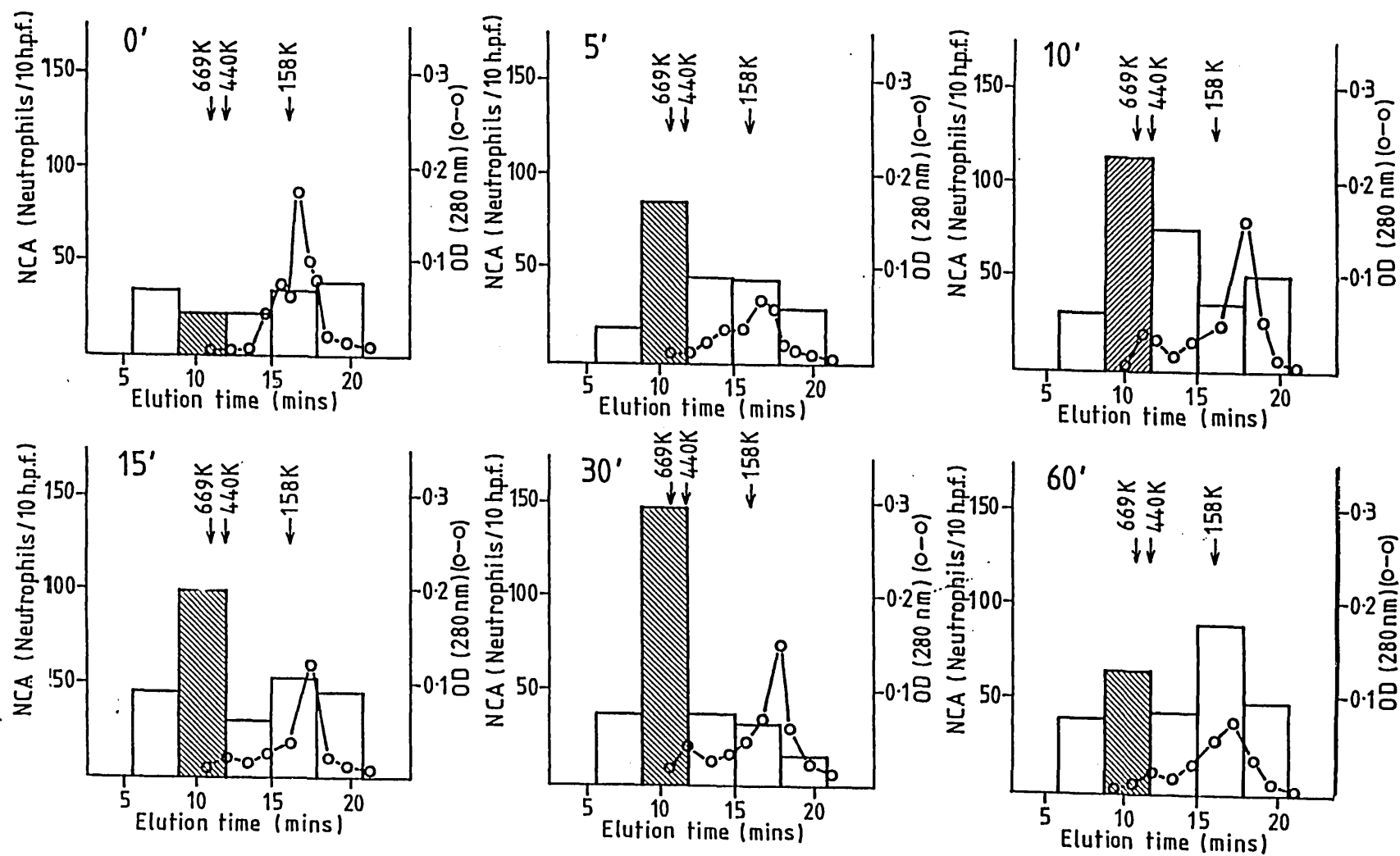
The fractions (shaded boxes) from anti-IgE stimulated lung supernatants were then subjected to gel-filtration on HPLC, and the results are shown in Figure 5.7. The majority of the NCA released at 15 and 30 minutes was of high molecular weight, whilst the 10 minute NCA seemed more heterogenous. Surprisingly, the maximum HMWNCA was contained in the 30 minute sample, in discordance with the FPLC results of the same samples. I think that this discrepancy lies in the poor quality of gel filtration provided by the HPLC system, and will refer to this again in the discussion section.

Dose-response experiments were conducted using 1:1000 (0.1%) to 1:100 (1%) dilutions of anti-IgE and Ig control, and the samples were analysed by FPLC and HPLC as previously described.

The results of one of four experiments are shown in Figure 5.8. HMWNCA, analysed by anion exchange chromatography (FPLC) and then gel filtration (HPLC) is compared with histamine in lung supernatants. Neither HMWNCA nor histamine was produced on challenge with a wide range of concentrations of control Ig, whilst both mediators are maximally stimulated by a 1:200 (0.5%) dilution of anti-IgE.

Because of the variation between individual NCA assays, which

Figure 5.7 Time course of HMWNCA release from anti-IgE-stimulated human lung fragments; results of gel filtration (HPLC)



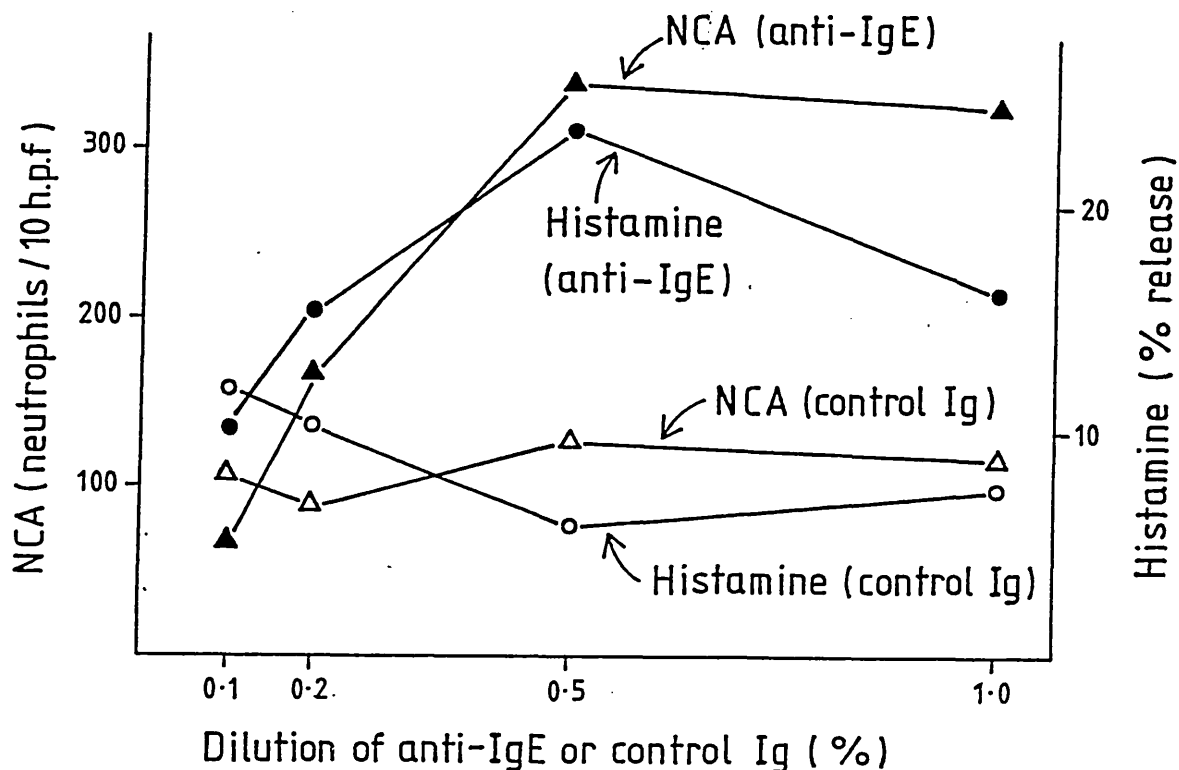
For legend, see over.

Legend to Figure 5.7

Each graph represents the gel filtration of one lung supernatant obtained at a single time-point following anti-IgE stimulation which had already been partially purified by FPLC, as shown in Figure 5.7. The eluting positions of the molecular weight markers thyroglobulin, ferritin and aldolase are shown by arrows above each graph. The absorbance of the HPLC eluent is indicated by open circles, and the neutrophil chemotactic activity (NCA) of eluting fractions is shown by the vertical bars. The shaded bar represents the fraction in which HMWNCA would be expected to elute.

Figure 5.8

Dose-dependence of histamine and HMWNCA release from human lung fragments challenged with anti-IgE or Ig control - results of one experiment.



Human lung fragments were challenged with 0.1 - 1.0% of anti-IgE (solid circles and triangles) or control Ig (open circles and triangles) and supernatants were collected 15 minutes following challenge. HMWNCA (following purification by FPLC and HPLC) is represented by open and closed triangles and is compared with histamine concentrations in crude supernatants (open and closed circles). This graph represents a single dose-response experiment.

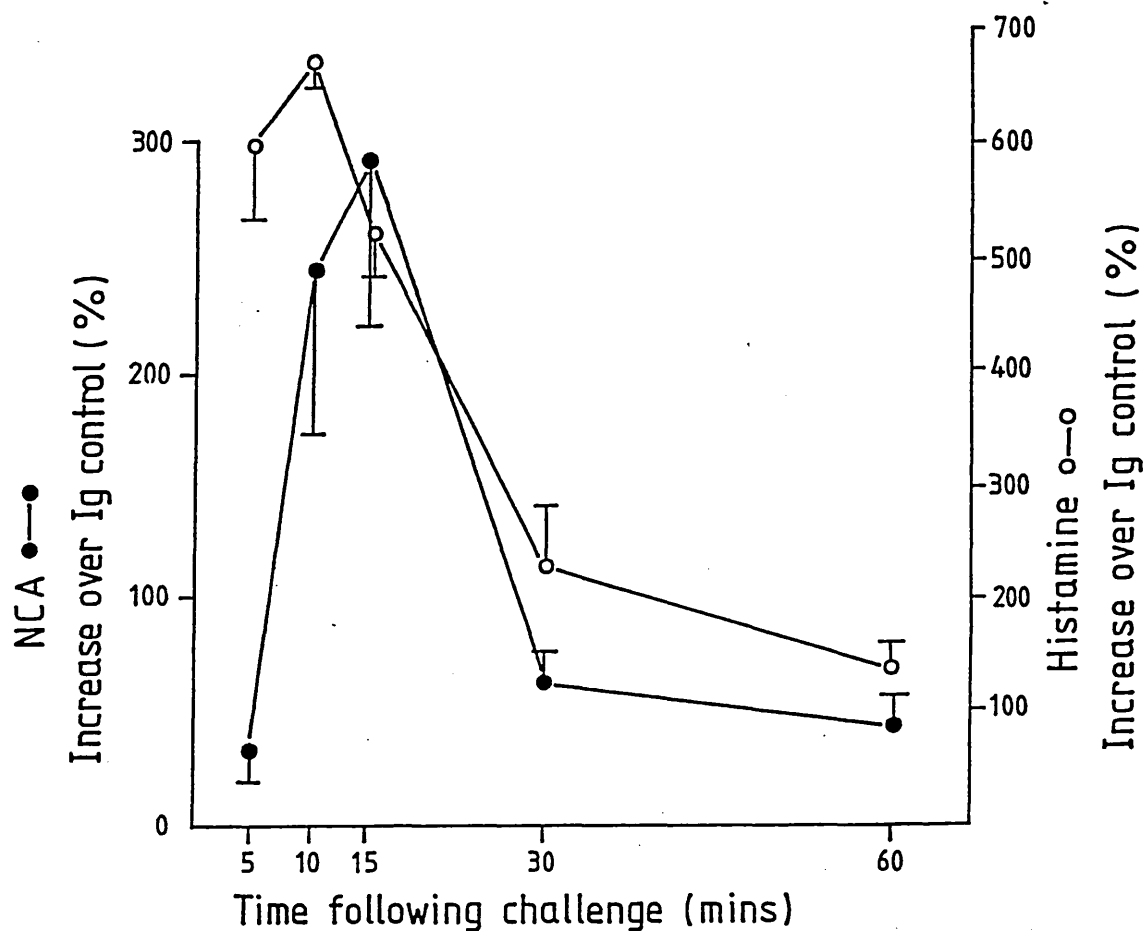
occurred even when neutrophils from the same individual were used in different assays, it was felt to be impossible to pool the "raw" NCA results from different experiments. However, it was desirable to compare the results from different experiments. The solution chosen was to express NCA released by anti-IgE as a percentage change over that released by control Ig under the same conditions. For comparison, histamine release was also expressed in this way. The pooled results of three time-course experiments and four dose-response experiments are shown in Figures 5.9 and 5.10 respectively, the error bars representing the standard error of the mean at each point.

It can be seen that the requirements for histamine release are very similar to those for HMWNCA release. Conditions for maximal HMWNCA release in the pooled experiments are 1:200 (0.5%) anti-IgE dilution, with the supernatant collected 15 minutes following challenge. Maximal histamine release occurs 10 minutes following challenge with the same (0.5%) dilution of anti-IgE. As the method of expressing the results was unusual, being a percentage increase over control results, it was felt that statistical analysis would be inappropriate to these data. Neither maleate nor succinate, nor the presence of the protease inhibitors ovomucoid trypsin inhibitor, aprotinin, or soybean trypsin inhibitor, had any appreciable effect on the release of HMWNCA from anti-IgE challenged lung fragments (results not shown).

To assess whether the HMWNCA recorded was or was not due to LTB_4 attached to a macromolecule, various experiments were performed. $^3\text{H-LTB}_4$ and serum containing $^3\text{H-LTB}_4$ were first

Figure 5.9

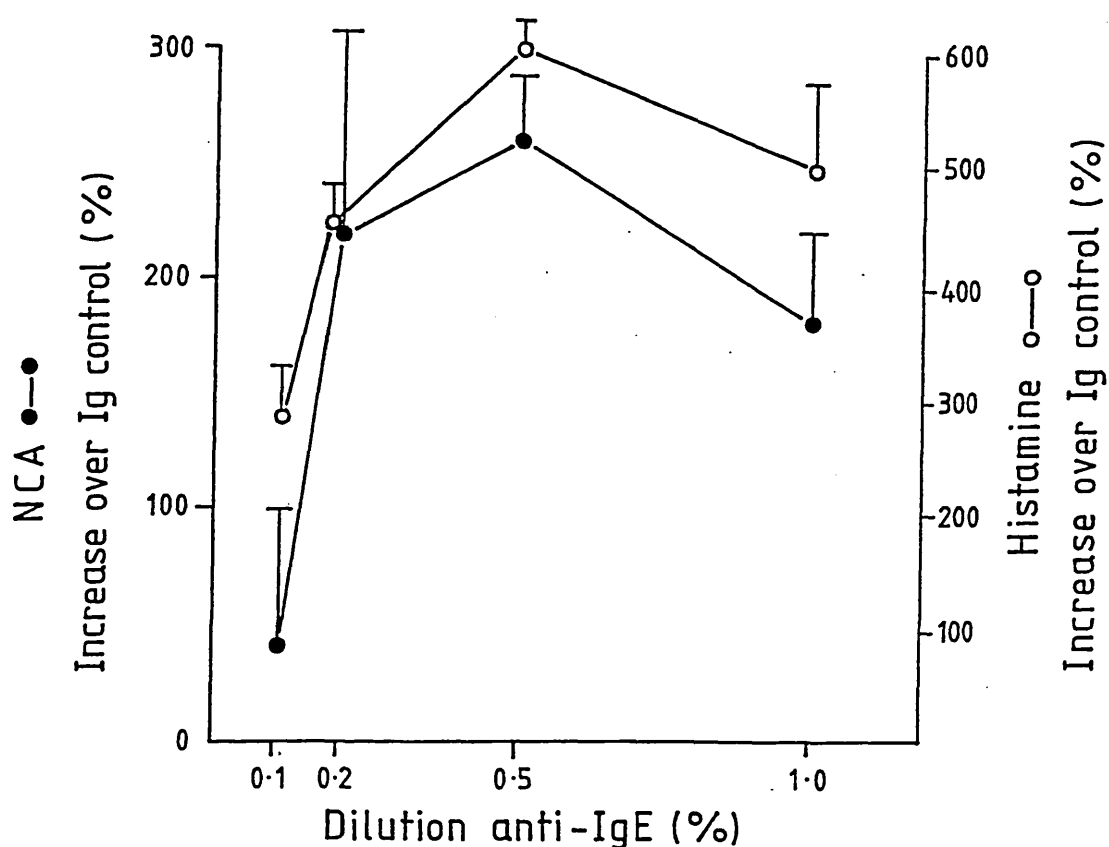
Time course of histamine and HMWNCA release from anti-IgE stimulated human lung fragments - results of three experiments.



Supernatants were collected 0-60 minutes following stimulation of human lung fragments with anti-IgE or control Ig. Histamine release is expressed as percentage increase of histamine in anti-IgE stimulated supernatants over that in control supernatants (open circles). HMWNCA (purified by FPLC and HPLC) is expressed in a similar way. The results are the pooled results from three time course experiments and each point and error bar indicates the mean and standard error of the mean.

Figure 5.10

Dose dependence of histamine and HMWNCA release from anti-IgE stimulated human lung fragments - results of three experiments.



Supernatants from human lung fragments were collected 15 minutes following challenge with 0.1 - 1.0% of either anti-IgE or control Ig. Histamine release (open circles) and HMWNCA (post FPLC and HPLC) release (closed circles) is expressed as the percentage increase of anti-IgE stimulated release over that produced by control Ig. The points and error bars represent the means and standard error of the means of three experiments.

analysed by anion-exchange chromatography on FPLC. $^3\text{H-LTB}_4$ was found to elute as soon as the gradient was applied, with approximately 0 - 0.1 M NaCl, thus differing from HMWNCA, which eluted with 0.15 - 0.3 M NaCl (results not shown). However, in case there was any overlap between these mediators, eluting fractions of heated-lung supernatants from FPLC were analysed by radio-immunoassay for LTB_4 . The experiment was performed twice; although there were very small levels of measurable LTB_4 in the supernatants, this did not overlap with HMWNCA, and were not associated with any chemotactic activity (results not shown).

(iv) DISCUSSION

Crude supernatants of lung fragments challenged with anti-IgE contained both histamine and neutrophil chemotactic activity, as well as eosinophil chemotactic activity. The time course of release of histamine (completion within 10-15 minutes) was slower than would be expected from rat serosal mast cells (completion within 30-60 seconds), but agreed with previous work using isolated human lung mast cells (MacGlashan et al., 1983). In this paper, 40% of total histamine was released 10 minutes following challenge, whilst only 50% had been released by 40 minutes (unfortunately there was no analysis of 15 minute supernatants for direct comparison). Ishizaka et al. (1981) described maximum histamine release 10 minutes after challenge with anti-IgE, whereas Church et al. (1982) described histamine release as being completed within 2-5 minutes of challenge. Mast cells obtained from bronchoalveolar lavage fluid from

asthmatic patients, which have the characteristics of mucosal mast cells, have completed their histamine release within 2 minutes of anti-IgE challenge (Leung et al., 1986). The dose requirements of anti-IgE antibody for histamine release was rather different; MacGlashan et al. (1983) produced 50% histamine release from lung mast cell preparations using 3 g/ml of anti-IgE antibody (approximately equivalent to 1:200 dilution of anti-IgE). In my studies, histamine and NCA release were maximal using a 1:200 dilution of anti-IgE, whereas Church et al. (1982), using a specially prepared goat anti-human IgE, found a linear response of histamine release to anti-IgE dilutions from 1:1000 to 1:10 from dispersed human lung mast cells. In other experiments using enzymatically dispersed lung mast cells, optimum histamine release was only achieved with a 2% (1:50) dilution of anti-IgE (Paterson et al., 1976).

These discrepancies can be explained by the use of different preparations of anti-IgE from different sources. Peters et al. (1984) used goat antihuman IgE, provided by Dr. K. Ishizaka, for his studies, whilst my studies utilised commercially available rabbit antihuman IgE. The differences between the requirements of anti-IgE concentration for histamine release between purified lung mast cells, and chopped lung tissue are probably only due to differences in mast cell concentrations, and the relative inaccessability of lung mast cells in the chopped lung model.

Challenged lung supernatants also contained LTC₄ and small quantities of eosinophil chemotactic activity and LTB₄ (results not shown). Thus the neutrophil chemotactic activity observed in crude supernatants was not solely due to HMWNCA, but was

contributed to by other factors such as LTB_4 , and probably HETEs (although these were not measured). Although all samples were heated at 56°C for 30 minutes, which would be expected to destroy the activity of LTB_4 and other lipoxygenase products, some (albeit reduced) immunoreactive LTB_4 remains following this treatment (Mackay and Shaw, unpublished observations). In order to differentiate between the activity of HMWNCA and that of other factors, the techniques FPLC and HPLC were utilised.

Heated serum and lung NCA behaved similarly on anion exchange chromatography using FPLC. Although there was some variation between the elution profiles of individual samples, NCA eluted in all cases with 0.15 - 0.3 M NaCl. Heterogeneity of lung-derived HMWNCA on ion-exchange chromatography has been described (O'Driscoll et al., 1983). The major peak of NCA in this study eluted with 0.1 - 0.2 M NaCl, but in the published example, further smaller anti-IgE induced NCA peaks also eluted with up to 0.3 M NaCl. Serum NCA in contrast appeared to be less heterogenous, and although both pre- and post-challenge asthmatic serum contained NCA that adhered strongly to the DEAE Sephacel column, the NCA associated with antigen-induced bronchoconstriction eluted as a single peak, with 0.2 M NaCl, in concurrence with the work of Lee et al. (1982). The heterogeneity of NCA on FPLC caused problems in the design of time-course and dose-response experiments. In order to reduce the number of eluting FPLC fractions needing to be assayed for NCA, fractions were pooled as described in the previous section. Fraction 2 would be expected, but could not be guaranteed, to contain any HMWNCA. However, figure 5.7, which shows the FPLC

results of one time course experiment, shows considerable NCA in fraction 3 at the 10 minute time point. Although this could be due to another chemotactic factor, this explanation is unlikely, as no other samples, either in that experiment, or in any other, showed similar activity. It is more likely that HMWNCA "spilled over" into the following fraction owing to its heterogeneity, and this makes interpretation of the results somewhat difficult. However, using this technique, NCA with similar anion-exchange properties to serum HMWNCA was shown to be released in a time- and dose-dependent fashion from human lung fragments upon challenge with anti-IgE. This NCA was unlikely to be due to LTB_4 , as LTB_4 had a different elution profile on FPLC and, in addition, immunoreactive LTB_4 was not discernable in the FPLC fractions containing NCA. Although LTB_4 and isomers were released from the lung fragments, and have been shown to be released from similar challenge of human lung mast cells (Peters et al., 1984), the processes of heat-inactivation and anion-exchange chromatography seem to adequately differentiate the chemotactic activity of LTB_4 from that of HMWNCA. HPLC, although useful as a rapid process of gel filtration, was generally disappointing as a quantitative method for determination of HMWNCA. Some activity was lost during passage through the HPLC columns themselves. In addition, there was a variable amount of low molecular weight activity in HPLC-treated samples. Fig. 5.4 shows the HPLC profile of HMWNCA from asthmatic serum. This NCA had already undergone gel-filtration by Sephacryl S400, and so had been shown to have a molecular weight of approximately 650,000 daltons. However,

HPLC gel-filtration revealed, in addition to the high molecular weight NCA peak, another low molecular weight (< 150,000 daltons) activity. It is, of course, possible that the serum HMWNCA had fragmented before passage on HPLC, but in view of further results with lung supernatants, it seems more likely that the HPLC columns themselves cause some fragmentation of macromolecules. Since the completion of this work, another rapid method of gel filtration, using columns on the FPLC system, has been used in the characterisation of HMWNCA, and this process gives better resolution and more reproducible results (Buchanan et al., 1987). Microbondagel columns were not really designed to measure substances of high molecular weight, being more suitable for the 20-100,000 daltons size range. The lack of resolution made it impossible to accurately assess the molecular size of lung NCA, but enabled us to collect NCA with a molecular weight greater than 440,000 daltons. No other chemoattract^{ant} described has a molecular size greater than this, although fibronectin (mol.wt. 850,000 daltons), released from alveolar macrophages, has been found to be slightly chemotactic for neutrophils (Yamada et al., 1983). In addition, α_2 macroglobulin (molecular weight approximately 800,000 daltons) has been shown to stimulate random neutrophil locomotion (Hakansson et al., 1983). However, this macromolecule is only found in serum, and so even if not thoroughly removed by washing during lung fragment preparation, would be expected to appear in control as well as challenged supernatants, which is not the case.

Thus, with these reservations in mind, we have shown that the

NCA released in time and dose dependent fashion from stimulated lung fragments, in addition to sharing anion exchange properties with serum HMWNCA, is also of high molecular weight.

Although, as discussed in the previous section, the methods used were not free from criticism, they appeared to provide a fast screening method for the detection of high molecular weight NCA, and it is hoped that with the introduction of new gel filtration columns for the FPLC that these methods will be significantly improved within the near future.

(v) Summary

Human lung fragments, challenged with anti-IgE, released neutrophil chemotactic activity which was associated with proteins having a molecular size > 440,000 daltons (as assessed by HPLC) and with similar behaviour on anion exchange chromatography (FPLC) as serum HMWNCA.

This was not observed when fragments were incubated with a control immunoglobulin preparation. The generation of HMWNCA (which paralleled histamine release) was time-dependent and required an optimal concentration of anti-IgE.

Thus the combination of FPLC and HPLC provided a very rapid method of characterising HMWNCA when compared with conventional gel filtration and ion-exchange chromatography.

CHAPTER SIX

HMWNCA RELEASE FROM FREEZE-THAWED HUMAN LUNG FRAGMENTS

(i) INTRODUCTION.

Having elucidated that HMWNCA was released from lung fragments challenged immunologically with anti-IgE, it was necessary to determine whether HMWNCA was a preformed mediator stored in mast cell or basophil granules or whether it was a newly-formed mediator generated by the process of mast cell activation.

The time course of HMWNCA release from lung fragments was very similar to that of histamine, perhaps suggesting that it is a pre-formed mediator. However, the arachidonic acid metabolites PGD_2 and LTC_4 are generated rapidly on mast cell activation, and the time course of their release is also rather similar to that of histamine. The concentration of anti-IgE required for histamine and leukotriene release from human lung mast cells are also very similar (MacGlashan et al., 1983) and so this is not a plausible method of differentiating pre-formed from newly formed mediators.

Repeated rapid freezing and thawing lung fragments or cell suspensions disrupts cell membranes, releasing pre-formed, granule-associated mediators without activating the cell; this method has been used in the investigation of ECF-A (Kay et al., 1971) and is commonly used as a method of measuring the total histamine content of cells. Paterson et al. (1976) quantitated the total histamine content of dispersed human lung mast cells after disrupting them by freezing and thawing six times. Another widely-used method of disrupting dispersed lung mast cells is by suspending them in 5% trichloroacetic acid (Church et al., 1982). Other methods of determining the pre-formed

nature of mediators are by cellular subfractionation (Goetzl et al., 1974), a method used in the investigation of ECF_A , by histochemical localisation of mediators (Schwartz et al., 1981), using specific strains, or the immunochemical localisation using specific antibodies.

The aims of the experiments described in this chapter were to disrupt lung cell membranes by freezing and thawing and to determine whether HMWNCA was liberated by this process.

(ii) MATERIALS AND METHODS

Materials.

As for Chapter 5.

Liquid nitrogen, BOC.

Methods.

Lung fragments were prepared as described in Chapter 3. Fragments were suspended in ice-cold Tyrodes buffer, without Mg^{++} and Ca^{++} (1 gm lung tissue in 2 mls buffer). The lung tissue suspension was then "snap"-frozen in liquid nitrogen, followed by rapid thawing, to a point at which the buffer had just become liquid, in a 56°C water bath. The freezing and thawing process was repeated twice. The supernatant was removed with a pasteur pipette, and cell debris was removed by centrifugation (300 g, 10 min). Supernatants were then tested for neutrophil chemotactic activity. Dose response experiments, using from 0.5 - 6 gms lung tissue were performed, and the resulting NCA in the crude lung supernatants was tested.

The character of the NCA was analysed by anion exchange

chromatography on FPLC and by gel filtration on HPLC, as described in Chapter 5. Immunologically challenged lung-derived HMWNCA, and HMWNCA derived from asthmatic serum were also analysed under identical conditions for comparison. Eluting fractions were tested for NCA using the modified Boyden chamber technique. These experiments were repeated twice.

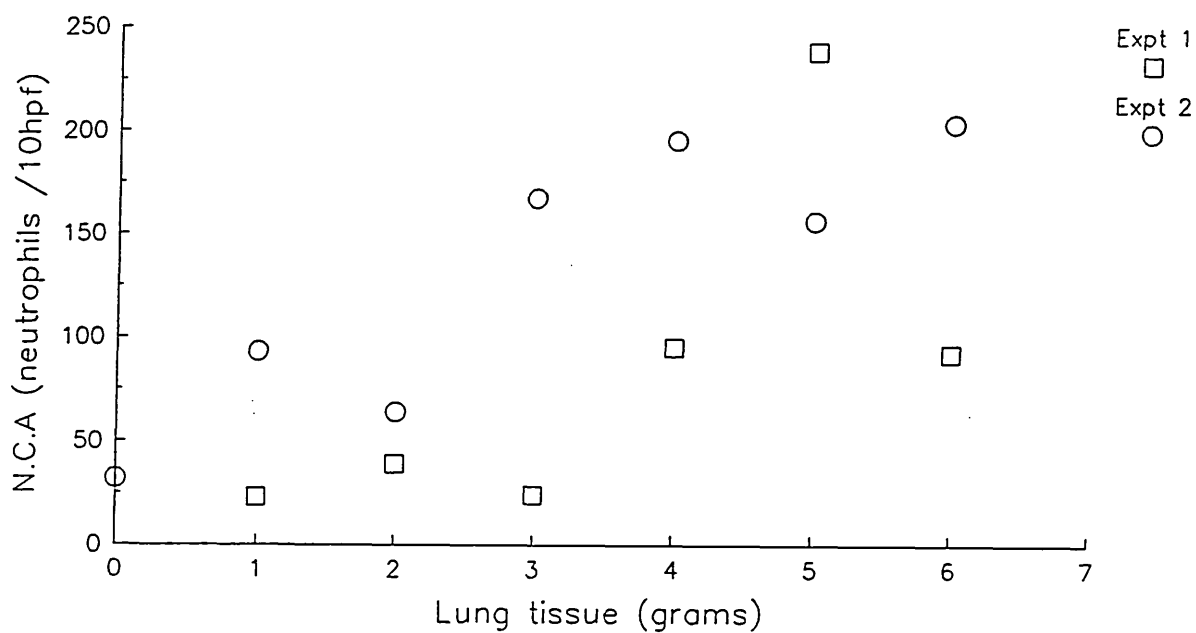
One time course experiment was performed. In this experiment, lung fragments were challenged with anti-IgE (1:200 concentration) and the supernatants were removed at various time points (from 0-30 minutes) after challenge. Fragments were then washed free of anti-IgE in Mg^{++} and Ca^{++} free Tyrodes and resuspended in the same buffer, to the same volume. Fragments were then frozen and thawed, as previously described, and the supernatants removed. NCA from both actively challenged, and freeze-thawed (F/T) lung fragments were then analysed by FPLC and HPLC techniques, as described above. Eluting fractions from all the samples were tested for NCA in the same chemotaxis assay, randomly coded, and read blind.

(iii) RESULTS.

Freezing and thawing of lung fragments released neutrophil chemotactic activity that survived heating for 30 minutes at $56^{\circ}C$. In two experiments, the chemotactic activity was dependent on the mass of lung fragments (Fig. 6.1). This chemotactic activity was analysed by anion-exchange chromatography on FPLC, as described in Chapter 5, and the elution profile is shown in Figure 6.2. Fig. 6.3 shows the anion exchange characteristics (using FPLC) of freeze-thawed

Figure 6.1

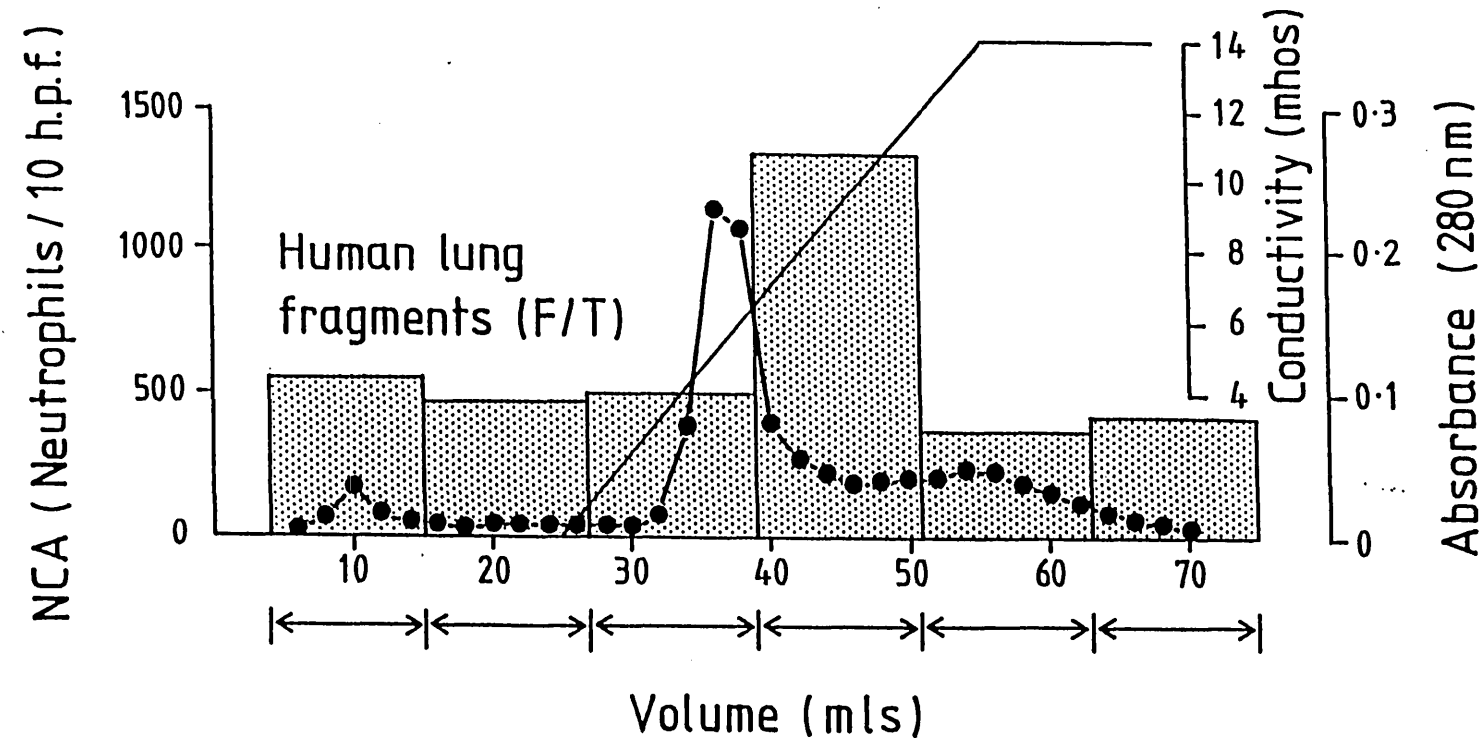
NCA release from freeze-thawed lung tissue; dose response
between mass of lung tissue and supernatant NCA.



Graph represents two experiments (open circles and open squares, in which increasing quantities of lung tissue (1-6 gms) was frozen and thawed three times and the supernatant analysed for neutrophil chemotactic activity (NCA).

Figure 6.2

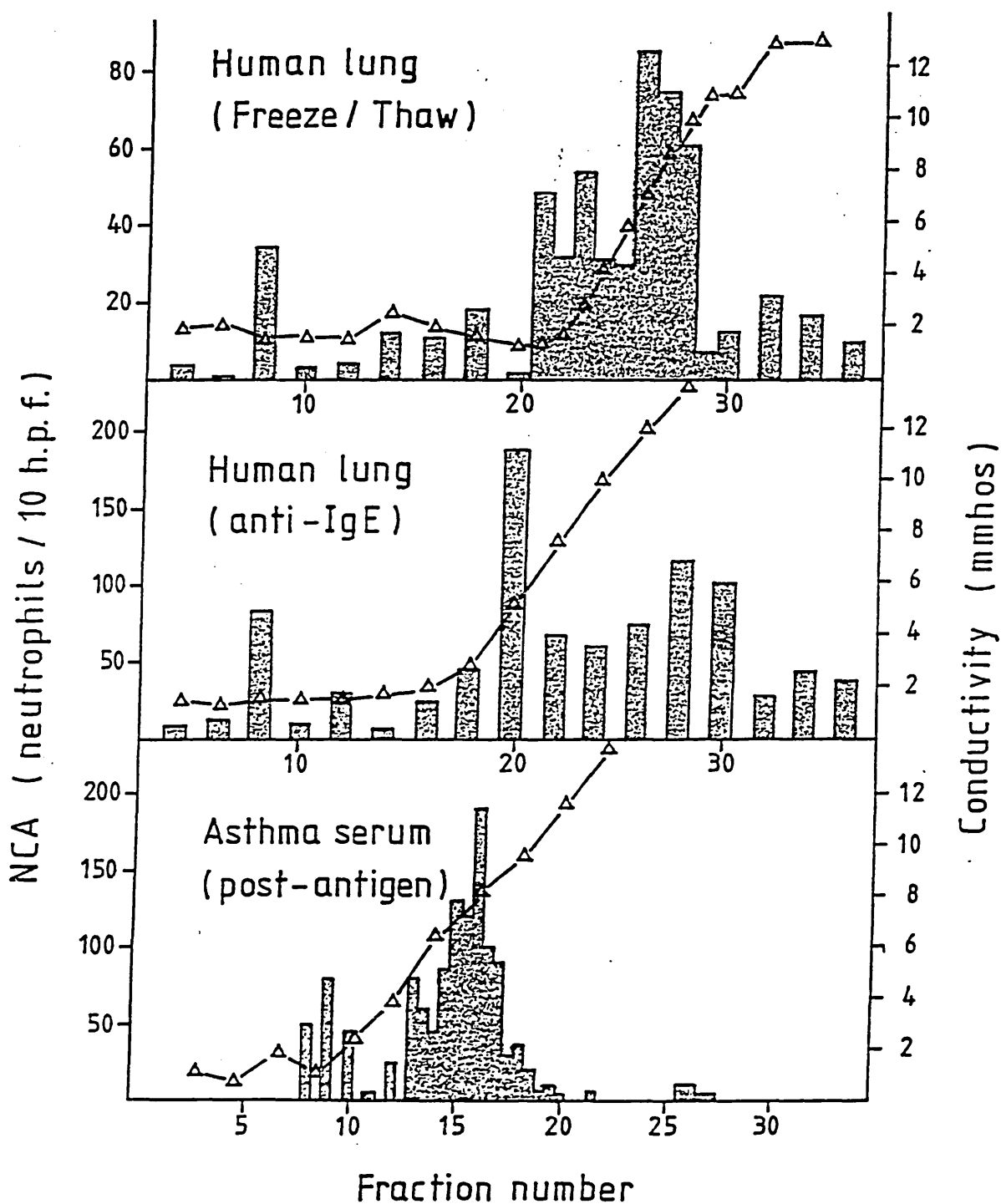
Anion exchange chromatography (FPLC) of supernatants of freeze-thawed lung fragments.



Supernatants from freeze-thawed lung fragments were analysed on Polyanion SI 17 columns on the FPLC. The closed circles represent the optical density of the eluent, whilst the salt gradient (0 - 0.5 M NaCl) (as measured by the eluent conductivity) is shown by the solid line. Individual fractions were pooled, as indicated by the arrows below the graph, and the neutrophil chemotactic activity (NCA) in the pooled fractions is represented by the shaded bars.

Figure 6.3

Anion exchange chromatography (FPLC) of supernatants of challenged lung fragments, freeze-thawed lung fragments and asthmatic serum.



Lung supernatants and asthmatic serum were analysed under identical conditions using Polyanion SI 17 columns on FPLC. The salt gradient (measured by the conductivity of the eluting fractions) from 0 - 0.5 M NaCl is represented in each case by open triangles. Neutrophil chemotactic activity (NCA, neutrophils/10 hpf) in the eluting fractions is shown by the shaded columns.

lung supernatants, in comparison with those of serum NCA and challenged lung NCA. Freeze-thawed (F/T) lung NCA eluted in a broad peak, indicating heterogeneity of the activity, over the first 2/3 of the salt gradient (0 - 0.35 M NaCl). The gel filtration profile of this partially purified NCA using microbondagel columns on HPLC is shown in Figure 6.4, in which F/T lung NCA is compared with serum and challenged lung NCA. The F/T lung NCA elutes in fraction 2, in common with challenged lung and serum NCA. Although this method does not accurately assess molecular weight, it can be reliably assumed that F/T lung NCA is of "high" molecular weight, i.e. with a molecular size of greater than 440,000 daltons.

In an experiment to assess changes in F/T NCA following challenge with anti IgE, supernatants from challenged NCA were compared with those from the lung fragments, frozen and thawed, over a time course, and were analysed by FPLC and HPLC. By these methods, it was shown that, as HMWNCA was released into supernatants following challenge with anti IgE, the amount of HMWNCA made available by freezing and thawing decreased. The results of HPLC analysis (following FPLC analysis) are shown in Figure 6.5. Unfortunately, owing to the time consuming nature of the experiment, it was only performed once.

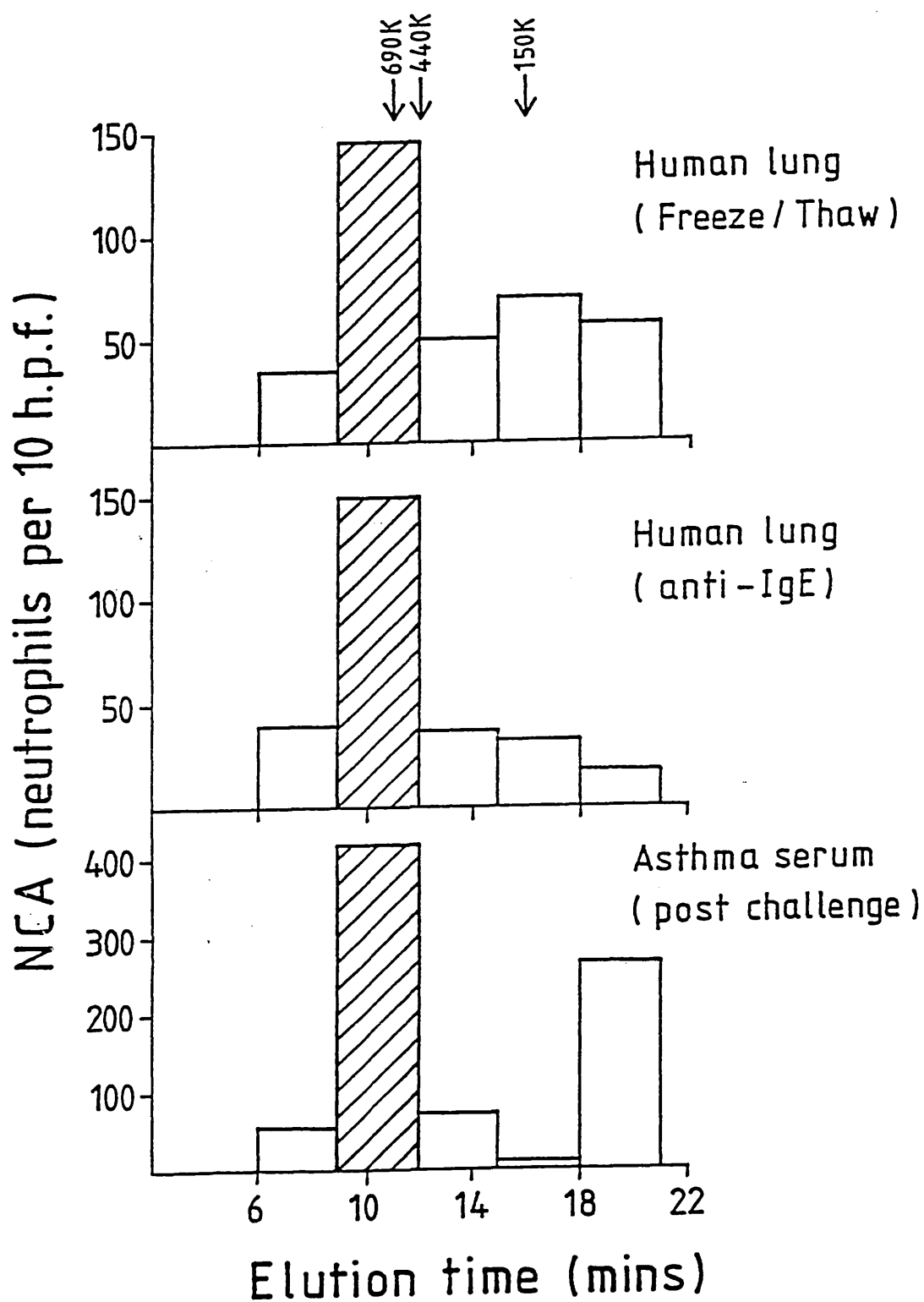
However, this single result suggests that HMWNCA may be stored as a fixed amount in lung tissue, and that these stores are depleted when HMWNCA is released following cell activation.

(iv) DISCUSSION.

A neutrophil chemotactic activity was released upon gentle

Figure 6.4

Gel filtration (HPLC) of supernatants from challenged lung fragments, freeze-thawed lung fragments and asthmatic serum.



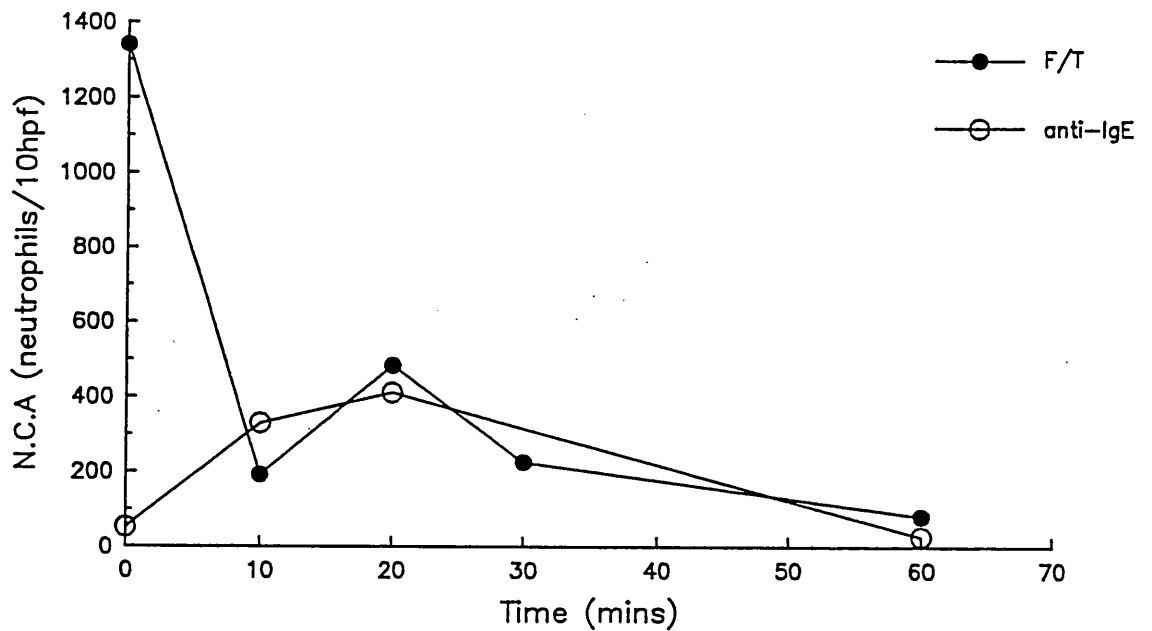
For legend, see over.

Legend to Figure 6.4.

Lung supernatants and serum had previously been partially purified by anion exchange chromatography on FPLC. Each sample was subjected to gel filtration (Microbondagel columns, HPLC) under identical running conditions. Fractions were pooled as discussed in Chapter 5. NCA of the pooled fractions is represented by vertical bars, the shaded bar being that in which HMWNCA elutes. The positions of the high molecular weight markers thyroglobulin, ferritin and aldolase are indicated above by arrows.

Figure 6.5.

Time course of anti-IgE stimulated release of HMWNCA versus residual (freeze-thawed) lung HMWNCA.



Lung fragments were stimulated with anti-IgE and supernatants collected at time points 0-60 mins. Residual fragments were resuspended in buffer, then frozen and thawed 3 times before the supernatant was removed. HMWNCA from anti-IgE stimulated fragments (0-0) and freeze-thawed residual fragments (●-●) was assessed following partial purification by FPLC and HPLC.

freezing and thawing of lung fragments which was similar in physicochemical properties to the HMWNCA which is released after immunological challenge. This could either be because HMWNCA is stored pre-formed, like histamine, in mast cell granules, or because freezing and thawing stimulates the lung cells. However, HMWNCA release is dependent on the mass of lung tissue present, and not on the number of freezing and thawing processes; this suggests that the explanation lies in HMWNCA being a pre-formed mediator. However, it is known that freezing and thawing does act as a minor stimulant to cell membranes; in 1975 Kay et al. described SRS-A as a preformed mediator because of its release from freeze-thawed lung fragments, and so any similar results should be interpreted with caution.

Better ways of assessing the preformed nature of HMWNCA would be by subcellular fractionation or by immunochemical detection. However, the methods used for subcellular fractionation would be likely to disrupt HMWNCA, and attempts at raising a specific antibody to HMWNCA were unsuccessful. The method of ultrasonically disrupting the lung tissue was also considered. However, it was felt that this might stimulate the cells in the same way as does freeze-thawing, and might in addition break up HMWNCA.

Another factor favouring HMWNCA as a pre-formed mediator is the time course results, in which it appears that the amount of HMWNCA available to be liberated by freezing and thawing decreases as HMWNCA is released following anti-IgE challenge.

This paints the picture of a finite quantity of HMWNCA, stored in granules, which is liberated into the lung supernatant after immunological challenge. Unfortunately, this experiment was performed only once, as the experiment was extremely time consuming. The main objective of these experiments was to determine whether large amounts of HMWNCA were stored pre-formed in lung tissue, so that freezing and thawing of lung tissue could be used as a source of larger quantities of HMWNCA, which could subsequently be used in the production of an antibody to the HMWNCA molecule.

It was not deemed to be of paramount importance to identify the precise source of HMWNCA; whether from mast cell or other inflammatory cell types, or whether granule-associated or not. Thus further studies, using dissociated lung cells, and ultrastructural filtration of those cells, to determine the exact source of HMWNCA, were not performed, as was the case for eosinophil chemotactic factor of anaphylaxis (Wasserman et al., 1977).

As has already been discussed in Chapter 3, the major problem with this work was that NCA could not easily be quantitated, and variations between NCA assays were so great (presumably because of differences in indicator neutrophils) that different experiments were not easily comparable. In retrospect, it would have been preferable to describe NCA in terms of units, one "unit" of NCA perhaps having the equivalent chemotactic potency of 10^{-8} M f-met-leu-phe. The problem with using f-met-leu-phe as an internal standard in a quantitative way, however, is that

different batches of f-met-leu-phe seemed to vary in their chemotactic potency.

In later work from the department (Buchanan et al., 1986), serum NCA was measured in such units, allowing more direct comparison between assays, and this uncovered differences in baseline serum NCA between mild asthmatics, severe asthmatics and normal controls, which had not been apparent before the use of NCA units was introduced. By using units, it may also have been possible to estimate the percentage recovery of NCA during the various purification procedures used.

The use of "pre-formed" HMWNCA in the search for a HMWNCA antibody will be described in Chapter 7.

(v) Summary

Attempts were made to determine whether HMWNCA existed in a pre-formed state in human lung tissue. Supernatants, free of particulate matter, were prepared from chopped human lung frozen and thawed three times. In a limited number of experiments, it was possible to demonstrate material with a similar molecular size and ion-exchange properties to that released by immunologically challenged lung fragments.

CHAPTER SEVEN

ATTEMPTS TO PRODUCE A MONOSPECIFIC

RABBIT ANTIBODY TO HMWNCA

(i) INTRODUCTION.

Many of the problems that occurred during the work for this thesis, were due to the fact that neutrophil chemotaxis assays, performed on different days and using indicator neutrophils from different donors, were not directly comparable, although, as has previously been mentioned, the use of "units" of NCA might have partially solved this problem. Moreover, with the methods used, the number of samples that could be assayed together, and were therefore directly comparable, was severely limited, as large assays required excessive removal of blood from the donor. When neutrophils from subjects with a neutrophilia were used, in order to perform a particularly large assay, it seemed that these neutrophils were generally unresponsive to chemotactic stimuli (perhaps suggesting that they had already been activated in vivo). The limitations of the assay size caused severe problems in screening column fractions, as was described in Chapters 5 and 6. The object of the work outlined in this chapter was to raise an antibody to HMWNCA, as a preliminary step to raising monospecific antibodies and then to the development of a radio-immunoassay for HMWNCA. It was hoped that this would allow HMWNCA to be measured quantitatively and reproducibly. Unfortunately, my efforts at raising an antibody were almost completely without success, and the one antibody that reacted on immunoelectrophoretically with HMWNCA, had no effect at all on its activity.

(ii) MATERIALS AND METHODS

Materials

Rabbits - Half-lop

Complete Freund's adjuvant (Difco, West Molesey, Surrey)

Incomplete Freund's adjuvant (Difco, West Molesey, Surrey)

Sonicator (Silverman, Chesham, Bucks)

Agarose (BDH, Poole, Dorset)

Glass plates (LKB, Selsdon, Croydon, Surrey)

Gelbond

Immuno-electrophoresis tank (LKB, Selsdon, Croydon, Surrey)

Power pack (LKB, Selsdon, Croydon, Surrey)

DEAE-Sephacel (Pharmacia, Uppsala, Sweden)

Sterile normal saline (0.9%)

Cyanogen-bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden)

Other chemicals by BDH (Analar grade)

METHODS

Rabbits.

Six half-lop rabbits were purchased at 6 days of age; they were left to settle in for a period of two weeks before any immunisation schedule was started. Prior to the first inoculation with antigen, 20 mls of blood was withdrawn from an ear vein via a 19 gauge needle. Serum was separated by centrifugation (300g, 15', 4°C) and stored at -20°C. This "pre-immunisation" serum was subsequently used as a negative control for serum taken after immunisation.

Preparation of Antigens.

Each rabbit was immunised against a different partially purified HMWNCA.

a) Rabbit I was immunised with serum NCA purified by Sephacryl S400 filtration followed by anion exchange chromatography on DEAE-Sephacel, followed by chromatofocusing on Polybuffer 94 exchanger.

b) Rabbit II was immunised with HMWNCA from anti-IgE challenged human lung fragments, purified by anion-exchange chromatography on FPLC followed by gel filtration on HPLC, as described in Chapter 3.

c) Rabbit III was immunised with HMWNCA from unchallenged lung fragments which had been repeatedly frozen and thawed. HMWNCA was purified by anion exchange chromatography on FPLC, followed by gel filtration on HPLC, as described in Chapter 4.

d) Rabbit IV was immunised with HMWNCA from nasal secretions following nasal antigen challenge of a patient with perennial rhinitis. HMWNCA had been partially purified from 6 M urea treated secretions by cation-exchange chromatography on SP-Sephadex gel HMWNCA was dialysed free of urea and salt prior to use.

Antigens were lyophilised, and protein content, as expressed as mgs. protein per mg. dried material, was assessed by the Bio-Rad protein assay. Lyophilised material was then re-dissolved in normal saline at a concentration of 1 mg protein per ml, and stored at -20°C.

Preparation of water-in-oil emulsions.

a) Primary inoculation.

An emulsion of antigen in complete Freunds adjuvant was made as follows:

Antigen protein (650 µgs) was dissolved in normal saline (2.5 mls), and was added to complete Freunds adjuvant (2.5 mls). The mixture was sonicated at in a glass container over ice, until an emulsion was formed. The emulsion was tested by letting a drop fall into a beaker of cold water; when the emulsifying process was complete, the drop of emulsion remained discrete and did not disperse in water.

b) Secondary inoculation.

Antigen protein (75 µgs) was dissolved in normal saline (1 ml) and added to incomplete Freunds adjuvant (1 ml). The mixture was sonicated over ice until an emulsion had formed, as described above.

Immunisation Schedules.

On day 1, antigen emulsified in complete Freunds adjuvant was injected intramuscularly in four sites, each site receiving 1 ml of emulsion.

On day 28, 20 mls blood was withdrawn from an ear vein and serum stored at -20°C.

On day 30, antigen emulsified in incomplete Freunds adjuvant (see above) was injected subcutaneously into two sites on the upper thigh, each site receiving 1 ml of emulsified material.

On day 44, blood (20 mls) was withdrawn and serum saved and stored at -20°C.

On day 46, antigen (75 µgms) in normal saline (1 ml) was injected intravenously via an ear vein.

On day 52, 20 mls blood was withdrawn.

Booster injections (75 µgms antigen in 1 ml normal saline) were then given every two weeks, and 20 mls blood withdrawn 5-7 days following each injection. This process of boosting was continued until it was felt that the rabbit had reached a satisfactory or maximal level of antibody production. The rabbit was then lightly anaesthetised with ether, and bled out by cardiac puncture.

Anamnⁿestic Response.

Following several intravenous boosting injections with purified antigen, which had not led to measurable antibody production, rabbits II and III were injected intravenously with a solution of cruder (less pure) material in normal saline.

These crude materials consisted of:

Rabbit II: HMWNCA from human lung fragments challenged with anti-IgE, that had been partially purified by anion exchange chromatography on FPLC only.

Rabbit III: HMWNCA from unchallenged but repeatedly frozen and thawed human lung fragments that had been partially purified by anion-exchange chromatography on FPLC only.

20 mls of blood was taken from each rabbit on successive days, starting from Day 5 following the injection of crude material. This was continued until detectable levels of

antibody against the antigenic starting material were achieved. At this point the rabbits were bled out as described above.

Purification of Rabbit IgG.

a) Ammonium sulphate precipitation.

Ammonium sulphate $[(\text{NH}_4)_2 \text{SO}_4]$, 500 gms] was heated at 60°C with stirring in approximately 500 mls of distilled water until most of the salt had dissolved (after approximately one hour). The saturated solution was allowed to stand overnight at room temperature, and adjusted to pH 7.0.

Rabbit serum was precipitated at 0.45 (45%) saturation. The volume of saturated ammonium sulphate solution (y mls) needed to precipitate a known volume of rabbit serum (X mls) was calculated as follows:

$$y = \frac{0.45}{0.55} \times X \text{ mls}$$

The known volume of salt solution (y) was added to the serum dropwise, very slowly, with constant stirring, to avoid localised higher concentrations of ammonium sulphate. After mixing, the precipitation was allowed to proceed for 1-2 hours. The precipitate was then washed three times in 45% saturated ammonium sulphate, and redissolved in X mls of distilled water.

b) Anion-exchange chromatography (DEAE Sephacel).

Column bed volume	-	35 mls
Sample size	-	20 mls
Flow rate	-	35 mls/hr
Running temperature	-	4°C

Starting buffer	-	0.1 M Tris/HCl, pH 7.8
Eluting buffer	-	As above, containing 0.5 M NaCl
Fractions collected	-	2 mls

Following application of the sample, the column was washed with 50 mls of starting buffer, before starting the gradient (100 mls, 0 to 0.5 M NaCl).

Fractions were assessed for protein concentration by measuring absorbance at 280 nm. The peak of protein eluting from the column before the start of the salt gradient was assumed to be IgG and was pooled.

Tests for Purity of Rabbit IgG.

Rabbit IgG, purified by ammonium sulphate precipitation followed by anion-exchange chromatography on DEAE Sephacel, was assessed for purity by immunoelectrophoresis against goat antiserum to normal rabbit serum. Normal rabbit serum was used as a positive control.

Agarose (1% in 25% Veronal buffer) was poured onto glass plates (8 cm X 8 cm), each plate receiving 8 mls of agarose.

Circular wells (diameter 1.2 mm) were cut out of the gel 3 cms from the left-hand edge using a steel cutter. These were separated by longitudinal troughs, running from left to right of the plate.

The test material (10 μ l) or normal rabbit serum control (5 μ l) was poured into the circular wells, and the material electrophoresed from left to right (i.e. parallel to the longitudinal troughs) for 90 minutes at 30 mV. The buffer used

for the electrophoresis was a 50% dilution of Veronal buffer, and this was connected to the agarose plates by means of wetted strips of filter paper. Following electrophoresis, anti-normal rabbit serum (20 μ l) was poured into each longitudinal trough. The plates were left overnight in a humidified incubator at room temperature to allow immunodiffusion to proceed. The plates were then dried, stained in Coomassie blue stain (1%) and destained in ethanol (45%) and acetic acid (10%).

Agarose (1% in 25% Veronal buffer) was poured onto clean, dry glass plates (8 cm X 8 cm) (8 mls per plate). A circular well (1.2 mms diameter) was cut in one corner using a steel cutter. Antigen (10 μ l) was poured into the well and was electrophoresed in a direction parallel to one edge of the plate at 30 ml for 90 minutes. After rotating the plate through 90°, a window (5 X 3.5 cms) is removed from the gel and replaced using 1% agarose (2.5 mls) containing rabbit antiserum being tested (approx. 250 μ l). A potential difference (10 mV) was then applied so that the antigen proteins were electrophoresed into the rabbit antibody overnight (18 hrs).

The plates were then dried, stained in 1% Coomassie blue stain for 15 minutes, and destained in ethanol (45%) and acetic acid (10%).

As a positive control, normal human serum (50%, dilution 2 μ l) was placed in the well, and rabbit anti-normal human serum (75 μ l) placed in the window.

Preparation of Gels for Affinity Chromatography.

IgG from rabbit III (which had mounted an IgG response to injected antigen protein) was purified by ammonium sulphate

precipitation followed by anion-exchange chromatography on DEAE Sephacel, as described above. As a negative control, IgG was purified in an identical way from serum saved before the communication schedule commenced (pre-immunisation serum).

Following anion-exchange chromatography, IgG containing fractions were pooled, lyophilised, and assayed for protein content by the Bio-Rad protein estimation technique.

Cyanogen-bromide activated Sepharose 4B (CNBr. Sepharose 4B) (2.5 gms) was swollen in 1 mM HCl for 30 minutes, then washed in 1 mM HCl (500 ml) followed by coupling buffer (0.1 M NaHCO_3 , 0.5 M NaCl, pH 8.3, 500 mls) on a siltered glass filter. The gel was then re-suspended in coupling buffer (12 mls) and mixed with a solution of IgG (100 mgs) in coupling buffer (8 mls), the total volume of gel/IgG suspension being 20 mls. After thorough mixing on an end-over-end mixer for 2 hours at room temperature, IgG-coupled gel was transferred to coupling buffer containing blocking agent (0.2 M glycine, pH 8.0) and mixed in end-over-end mixer for 2 hours at room temperature. The effect of the 0.2 M glycine is to block any remaining active groups on the CNBr Sepharose 4B. Excess, non-specifically adsorbed protein was removed by washing alternatively with acetate buffer (0.1 M, NaCH_3COO , 0.5 M NaCl, pH 4) and coupling buffer. Gel was then resuspended in coupling buffer containing sodium azide (0.1%) and stored at 4°C prior to use. Two affinity gels were made:

- (i) Using IgG purified from "post-immunisation" serum -
("post" IgG)
- (ii) Using IgG purified from "pre-immunisation" serum -
("pre" IgG)

(iii) RESULTS.

Heated serum from rabbit I-VI failed to inhibit chemotaxis induced by asthmatic serum NCA or human lung NCA, and had no effect on f-met-leu-phe-induced chemotaxis. IgG from each rabbit was extracted by ammonium sulphate precipitation and purified by gel filtration on Sephacryl S400 columns. Purity of the IgG was checked using immunoelectrophoresis, and in 1 case (rabbit IV), traces of contaminating albumin were removed by passage over a Blue Sepharose column.

The activity of each IgG against HMWNCA was tested using crossed immunoelectrophoresis. Rabbits I-IV and rabbit VI showed no activity against HMWNCA.

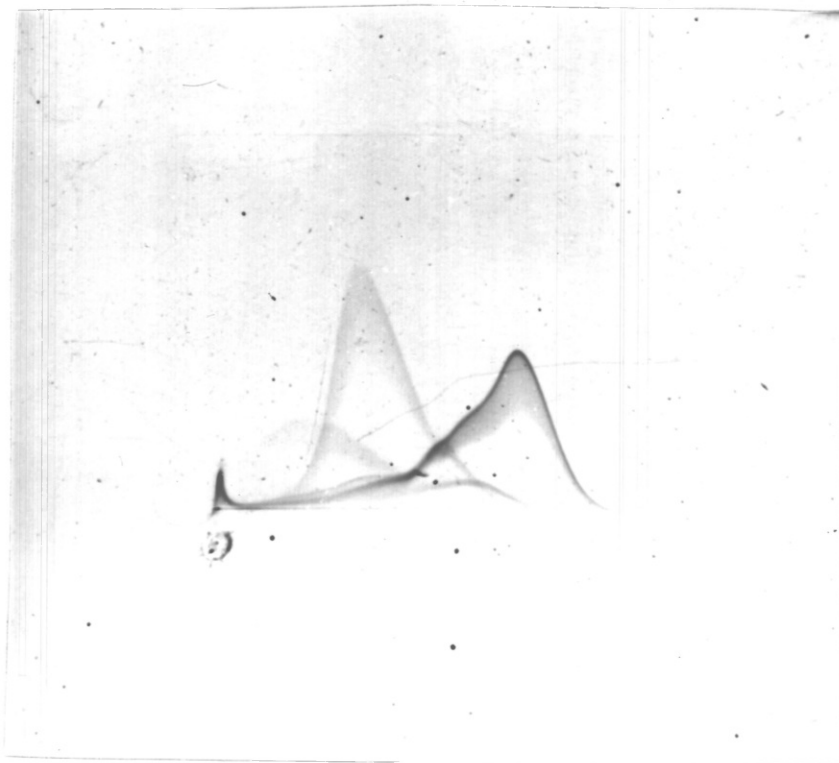
HMWNCA from nasal secretions, immunologically challenged human lung and freeze-thawed lung sources, formed at least 2 precipitin lines with anti-normal human serum (Figs. 7.1, 7.3 and 7.5). These corresponded to the albumin fraction and around the transferrin fraction of the immunoelectrophoresis pattern of normal serum.

The incorporation of rabbit V IgG into an intermediate gel brought down the precipitin lines, indicating that the IgG had immunoreactivity against both components of HMWNCA (Figs. 7.2, 7.4 and 7.6).

However, rabbit V IgG did not inhibit the chemotactic activity of serum or lung HMWNCA in vitro. Rabbit IgG was linked to an affinity gel as earlier described - the "active" column contained rabbit IgG after immunisation, whilst the "control" column contained IgG from serum collected prior to the immunisation schedule. The "active" column did not bind

Figure 7.1

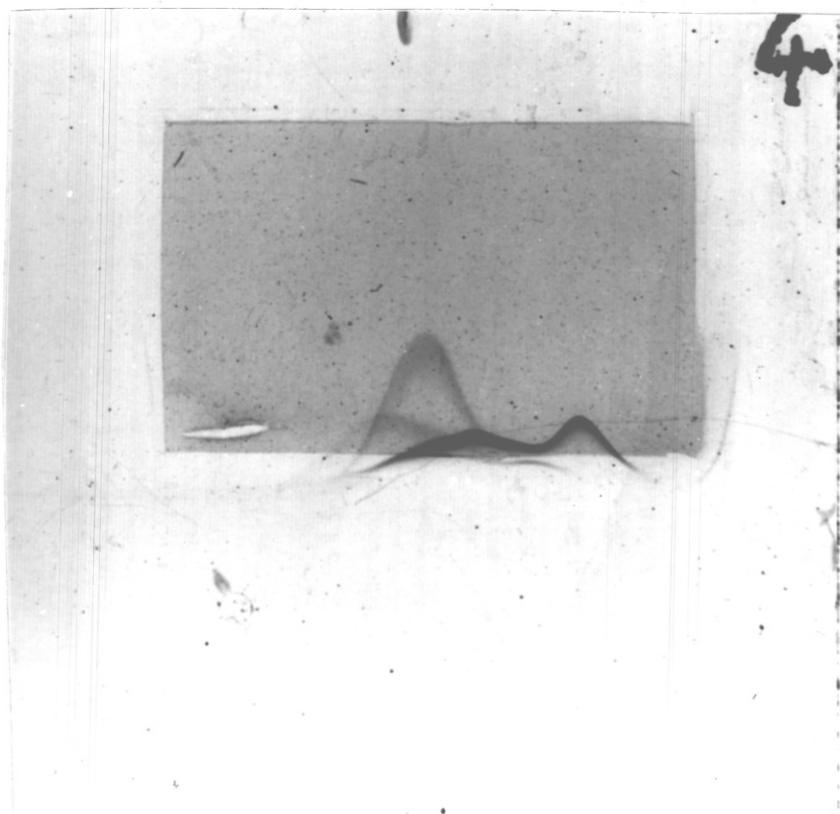
Crossed immunoelectrophoresis of nasal HMWNCA against anti-NHS.



HMWNCA from nasal secretions, post SP Sephadex and Sephacryl S400 (10 μ l) was electrophoresed in the first dimension (90 mins, 30 mV) and then in the second dimension (18 hrs, 10 mV) into a window containing 75 μ l of anti NHS.

Figure 7.2

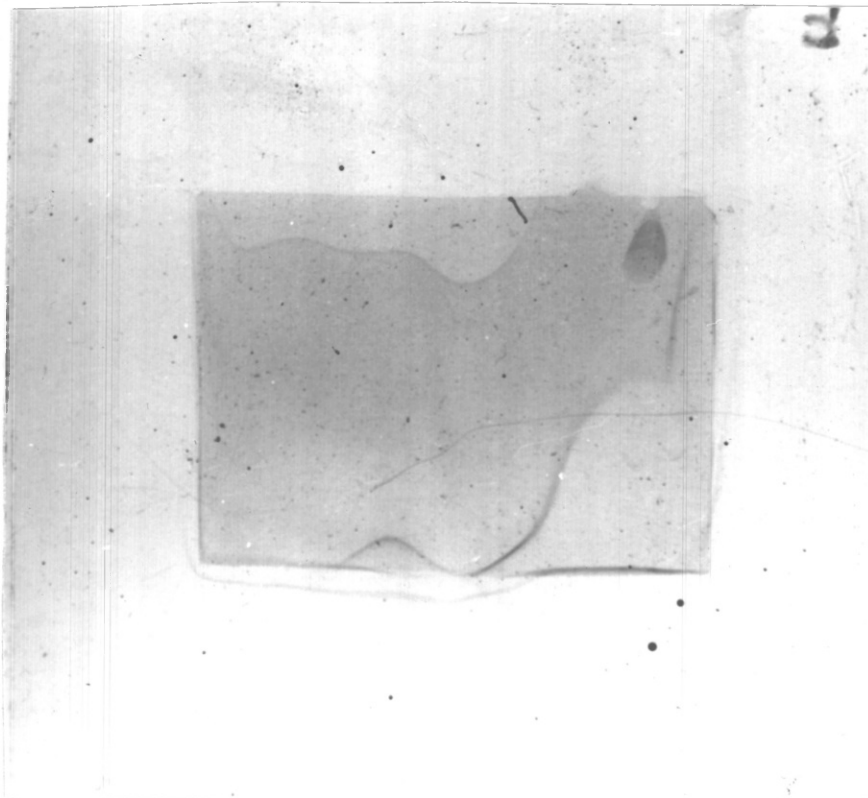
CIE of nasal HMWNCA against anti-NHS, incorporating an intermediate gel containing anti-HMWNCA.



HMWNCA from nasal secretions was electrophoresed in both directions as described in Fig. 7.1. In this case, an intermediate gel was incorporated which contained 20 μ l of rabbit IgG against HMWNCA (rabbit V).

Figure 7.3

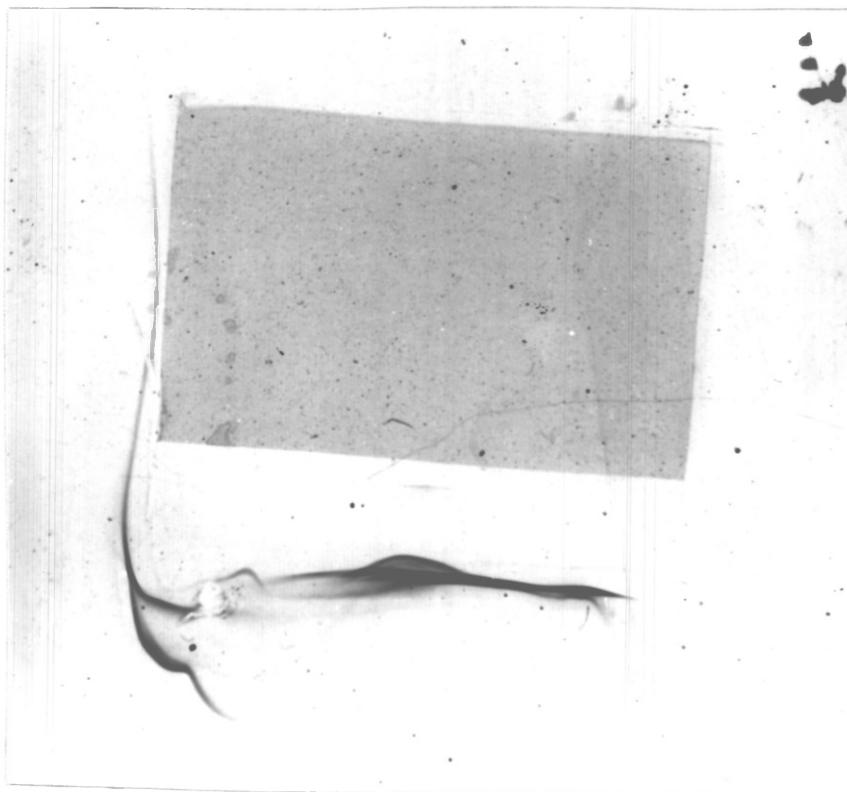
Crossed immunoelectrophoresis of HMWNCA from challenged lung fragments against anti-NHS.



HMWNCA from immunologically challenged human lung fragments, post-FPLC and HPLC (10 μ l) was electrophoresed in the first dimension (90 mins, 30 mV) and then in the second dimension (18 hrs, 10 mV) into a window containing 75 μ l of anti-NHS.

Figure 7.4

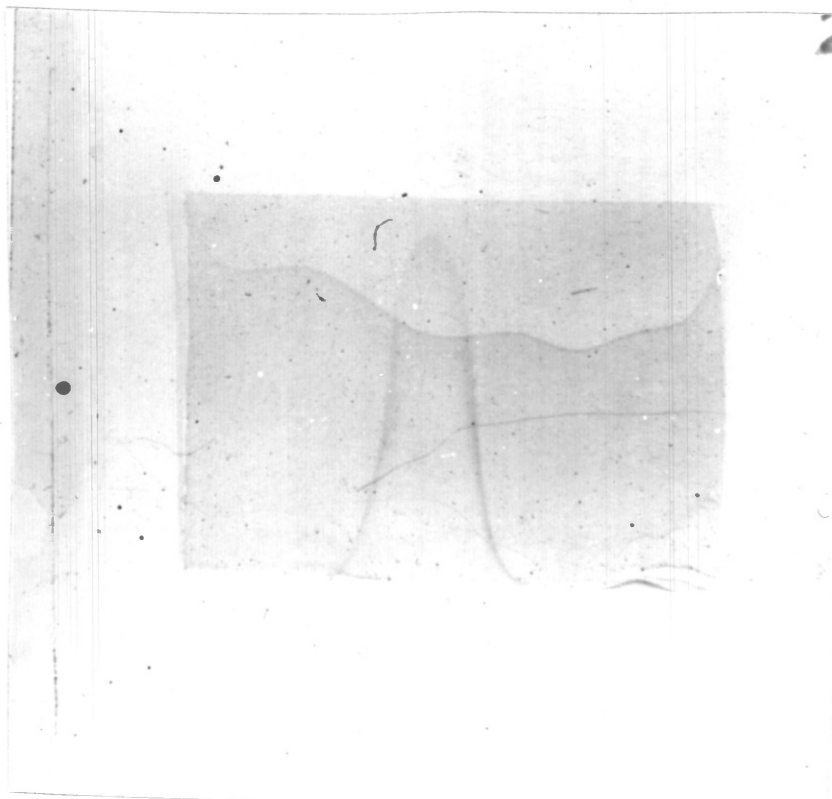
CIE of HMWNCA from challenged lung against anti-NHS,
incorporating an intermediate gel containing anti-HMWNCA.



CIE of HMWNCA from immunologically challenged lung fragments,
post-FPLC and HPLC, was performed as described for Figure 7.3.
An intermediate gel window, containing anti-serum to HMWNCA (20
 μ l) was incorporated.

Figure 7.5

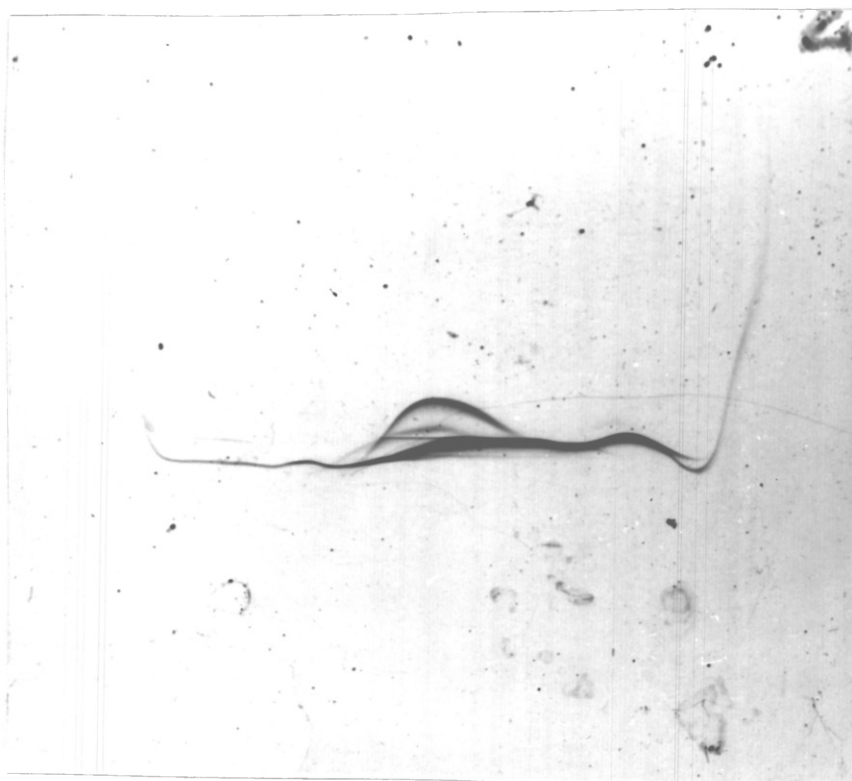
Crossed immunoelectrophoresis of freeze thawed lung HMWNCA
against anti-NHS



HMWNCA from freeze-thawed human lung fragments post-FPLC and HPLC (10 μ l) was electrophoresed in the first dimension (90 mins, 30 mV) and then in the second dimension (18 hrs, 10 mV) into a window containing anti-NHS (75 μ l).

Figure 7.6

CIE of freeze-thawed lung HMWNCA against anti-NHS, incorporating an intermediate gel containing anti-HMWNCA.



CIE of freeze-thawed lung HMWNCA was performed as described in Figure 7.5. An intermediate gel window containing antiserum to HMWNCA (20 μ l) was incorporated.

neutrophil chemotactic activity from either serum or freeze-thawed lung supernatants. It did, however, bind some activity from actively challenged human lung fragments, but there was no significant difference in this experiment between these active and control columns (Fig. 7.7).

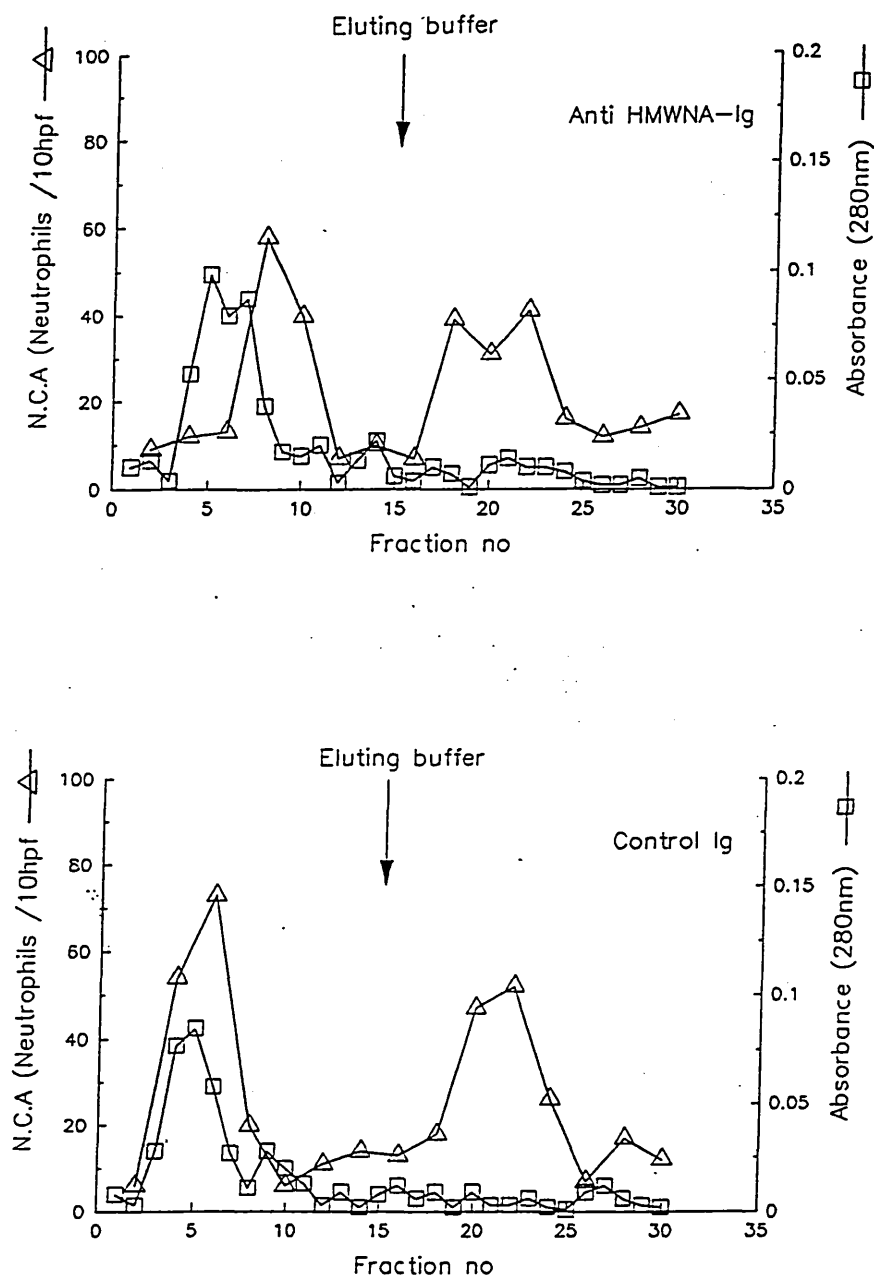
(iv) DISCUSSION.

It was disappointing that out of six rabbits, none produced an antibody which inhibited the action of HMWNCA, and only one produced any immunoreactive activity against HMWNCA. However, in retrospect, there may be a number of reasons for this and further work may produce a more specific antibody. The antibody response to a single antigen is not uniform; heterogeneous populations of antibody molecules are produced which vary in both their immunological reactivity and their physico-chemical properties (Krause, 1970). The HMWNCA antigens used in these experiments were not pure, so that antibody production is likely to be extremely heterogeneous. Antibodies of one class (for example IgM and IgA) can inhibit the production of other classes of antibodies, to the same antigen, by a complex feedback mechanism (Sahiar et al., 1964), and so it is possible that IgG production in these rabbits was suppressed by other antibody groups. IgA tends to be produced primarily in response to oral immunisation techniques, but it is possible that IgM production was stimulated in these experiments.

The immune response is very age-dependent. New born animals are virtually incapable of mounting an immune response, whilst old animals cannot mount a response as efficiently as animals

Figure 7.7

Effects of an affinity column containing anti-HMWNA on
NCA in lung supernatants.



Immunologically challenged human lung supernatants were subjected to affinity chromatography (CN-Br Sepharose AB) columns linked with rabbit IgG antibody to HMWNA (top section) or control rabbit IgG (lower section). Following the elution of the major peak of optical density, an eluting buffer was applied. Absorbance (280 nm) of eluting fractions is represented by open circles whilst NCA is represented by closed circles.

"in their prime" (Kay et al., 1981). Other factors adversely affecting antibody production to immunogens are poor nutrition, disease (especially carcinomatous change), dirty housing and stress (Keller et al., 1981). Certain antigens can elicit responses in one inbred strain, but not in another, and this ability or inability to respond to an antigen is in some cases under the control of the major histocompatibility gene (Hardy, 1986). If this applies to inbred strains, the variability in out-bred strains would be expected to be even higher. Some antigens provoke an immune response in only 10% animals immunised, and so it is feasible that no response was produced in these experiments by this explanation alone, i.e. poor "antigenicity" of HMWNCA.

Another possible explanation is that the dose of HMWNCA used was not suitable. Antibody production is related to the dose of antigen injected by a sigmoid dose response curve. The exact value of optimum antigen dose depends to a large extent on the site of injection (probably maximal in the hind foot pad) but also depends on the stability of the antigen in the immunised animal.

Immunisation schedules vary, and tend to be chosen arbitrarily, as there have been no controlled studies to favour one over another. Most schedules, however, make use of adjuvants of one kind or another. Adjuvants cause immunopotential by a variety of mechanisms including enhancement of post-antigen B cell proliferation, entrapment of lymphoid cells in local lymph nodes, alteration^o of T helper : T suppressor cell ratios, formation of a depot for prolonged

antigen release, and formation of an immunogen from a soluble antigen (Dresser, 1986). Complete Freund's adjuvant (CFA) is an oil-based adjuvant containing mycobacteria, and is probably the most commonly used adjuvant in immunisation techniques. The schedule used in these experiments had been successfully used to prepare antibodies to transferrin (Sass-Kuhn et al., 1983). However, priming injections were given in both thighs simultaneously, and it has been suggested (Dresser, 1986) that intramuscular injections of CFA and antigen should be spaced by at least 8 weeks to produce high titres of antibodies. Thus it is possible that modification of the schedule for immunisation, and possibly different timing for blood collections, would more successfully produce antibodies to HMWNCA.

It is also possible that the method for IgG purification were not ideal, although these methods are well described. Ammonium sulphate (45-50%) selectively precipitates IgG and the precipitate should contain > 95% IgG (Hardy, 1986). Although many other groups use gel filtration to purify IgG, this method may be more suitable for IgM purification and anion exchange chromatography may be a better technique to use for IgG.

In re-designing these experiments, I would use a larger number of rabbits, and alter the immunisation techniques and IgG purification techniques as outlined above. In addition I would immunise with purer antigen by injecting single bands from PAGE or immunoelectrophoresis, sonicated with CFA, as high antibody titres can be obtained in this way (Dr. Christine Harvey, personal communication).

(v) Summary

Attempts were made to raise a polyclonal antibody to partially purified HMWNCA. This was largely unsuccessful and the possible reasons are described.

CHAPTER EIGHT

CHARACTERISTICS OF TRANSFERRIN-INDUCED NEUTROPHIL
ADHESION TO POLLEN GRAINS

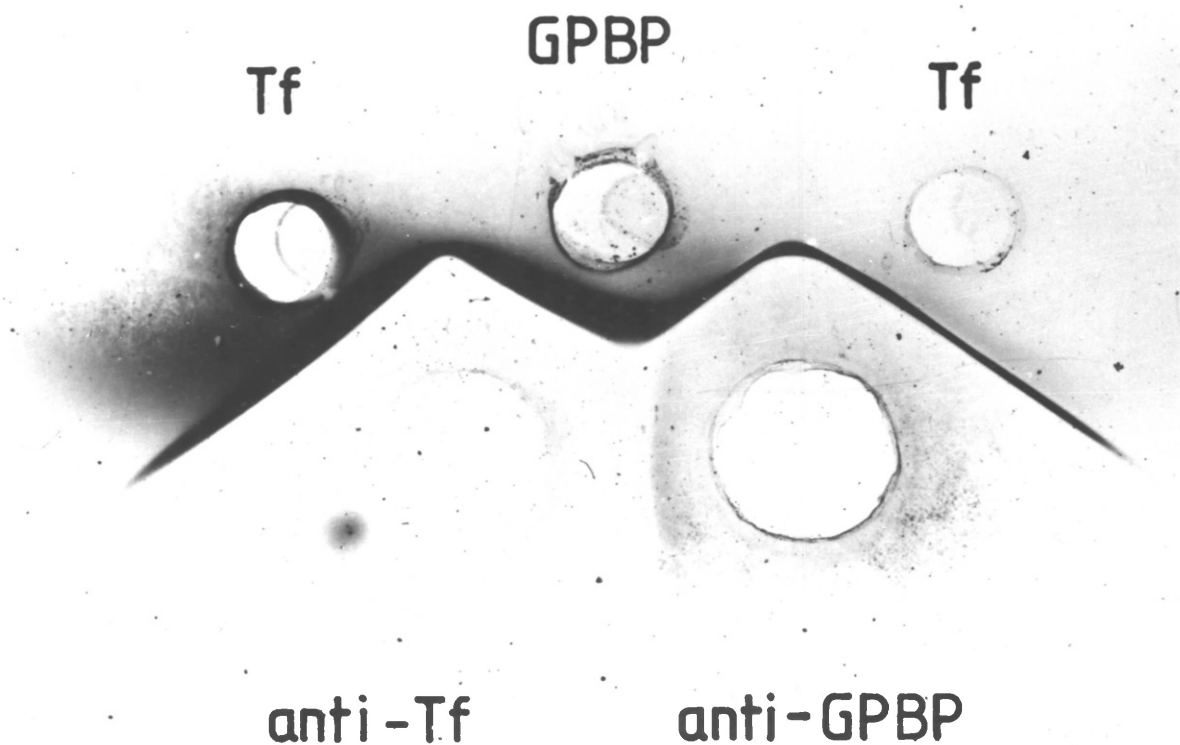
(i) INTRODUCTION.

The following chapter refers to the recognition of transferrin as a serum protein which promotes granulocyte binding to pollen grains in vitro, and to the characteristics of this granulocyte pollen binding reaction. Much of this work has been published (Sass-Kuhn et al., 1984; Mackay et al., 1986). The early work, leading to the identification of granulocyte/pollen binding protein (GPBP) as being transferrin, was performed by Suzanne Sass-Kuhn and Dr. Redwan Moqbel. The experiment described in this chapter was performed either by myself alone, or in conjunction with Suzanne Sass-Kuhn.

During the course of a series of experiments designed to investigate the activation of complement by common inhaled allergens, it was observed that both heated and unheated serum promoted the adherence of granulocytes to pollen grains. This adherence was prolonged (18 hours) and was not related to the atopic status of the serum donor. The factor producing this adherence, termed granulocyte-pollen binding protein, or GPBP, was purified using gel filtration (Sephadex G200), anion exchange chromatography (DEAE Sephacel) and affinity chromatography (Blue Sepharose and Protein A Sepharose). Purified GPBP was found to have the same properties as transferrin on crossed immuno-electrophoresis (CIE) against antibody to normal human serum. Addition of GPBP to human serum enhanced the transferrin peak on CIE. GPBP and transferrin gave lines of identity on immunodiffusion with antisera to both GPBP and transferrin (Fig. 8.1). Moreover, antiserum to transferrin removed the activity of GPBP. Thus it was concluded that GPBP

Figure 8.1

Immunodiffusion of GPBP and transferrin with anti-transferrin and anti-GPBP antisera; lines of identity between transferrin and GPBP.



GPBP, purified by anion exchange chromatography, gel filtration and affinity chromatography and commercially obtained human transferrin were allowed to diffuse overnight in agarose in the presence of wells containing rabbit antisera to transferrin and to GPBP.

an transferrin were identical.

This chapter deals with the characteristics of the granulocyte-pollen binding reaction.

(ii) MATERIALS AND METHODS.

Materials

Pollen grains:

Timothy grass (*Phleum pratense*)

Meadow grass (*Poa, pratensis*)

Rye grass (*Lolium perenne*)

False oat grass (*Arrhenatherum elatius*)

Giant ragweed (*Ambrosia trifida*)

Short ragweed (*Ambrosia elatior*)

Plantain (*Plantago lanceolata*)

Silver birch (*Betula pendula*)

Ash (*Fraxinus excelsior*)

- kindly provided by Mr. David Moran (Beecham
Pharmaceuticals, Epsom, Surrey, U.K.)

Iscoves medium, free of bovine serum albumin, human transferrin and soya bean lecithin - Flow Laboratories, Irvine, Scotland.

Anti-human transferrin - Dako, Denmark.

Rabbit anti-human albumin - Dako, Denmark.

Human serum albumin, human transferrin, ferric chloride, magnesium chloride, calcium chloride - Sigma, Poole, Dorset.

Lactoferrin - kindly provided by Dr. A. Segal, University College Hospital, London.

OKT9 antibodies, OKT6 antibodies - kindly provided by Dr. M. Greaves, Imperial Cancer Research Fund, London.

Dextran, Ficoll-Hypaque, Metrizamide, Microtitre plates.

Methods

Leucocyte/pollen binding assay.

Leucocyte-rich plasma, obtained by sedimentation of whole blood on 20% dextran in the presence of heparin (1 U/ml) was applied to a Ficoll-Hypaque density gradient in the ratio of 2.4 : 1, and centrifuged at 200g for 30 minutes to obtain a pellet containing granulocytes and some contaminating erythrocytes. These erythrocytes were lysed using ice-cold 0.83% ammonium chloride, and the remaining granulocytes were washed twice in Iscove's medium, and resuspended to a final concentration of 2×10^6 cells/ml. Timothy grass pollen (TGP) grains, having been washed twice in Iscove's medium, were resuspended in the same medium to a final concentration of 2000 TGP grains/ml. 50 μ l test solution were incubated with 50 μ l cell suspension and 50 μ l TGP grain suspension in flat-bottomed polypropylene microlitre plates, in an atmosphere of 95% air and 5% CO₂. The time and temperature of the incubation was varied as described in time-course and temperature-dependency experiments, but in all other experiments, incubation was for 18 hours at 37°C. Following incubation, cells and pollen grains were visualised with 0.1% aqueous toluidine blue dye; the percentage and degree of

adherence of cells to pollen grains was assessed using bright field contrast microscopy. The percentage of adherence represented the percentage of pollen grains with 4 or more adherent granulocytes, whilst the degree of adherence was defined as $\pm$ (4-7 adherent cells) + (one layer of cells completely surrounding the pollen grain), ++ (2-3 layers of cells), +++ (3-4 layers of cells) and ++++ (more than 4 layers of cells). All samples were performed in duplicate and the results in most experiments were recorded by two independent observers.

Time course experiments.

Using a fixed dose of transferrin, GPBP or control albumin (300 $\mu\text{g}/\text{ml}$), cells and pollen grains were incubated for various periods of time, from 30 minutes to 18 hours. Results were recorded immediately on removal from the incubator. These experiments were performed three times.

Dose-response experiments.

Granulocytes and pollen grains were incubated for 18 hours in the presence of increasing concentrations of transferrin, GPBP, lactoferrin or control albumin, from 1.25 $\mu\text{g}/\text{ml}$ to 300 $\mu\text{g}/\text{ml}$. The experiment was performed six times.

Temperature-dependency experiments.

Granulocytes and pollen grains were incubated with 300 μg transferrin or GPBP for 18 hours, at the following temperatures: 4°C, 22°C (room temperature) and 37°C, in each case in an atmosphere of 95% air and 5% CO_2 . The results shown are the means of 4 experiments.

Neutrophil/Eosinophil experiments.

The participation of eosinophils vs. neutrophils was assessed in six experiments, using cells from patients with elevated circulating eosinophil counts. The degree of eosinophilia in each patient was between 8 and 20%, and the diagnosis in 4 of the patients was bronchial asthma, whilst the remaining 2 patients had an unexplained eosinophilia. Eosinophils were separated from neutrophils using discontinuous Metrizamide gradients as described by Vadas et al. (1979). Eosinophils were obtained with 75-98% purity, and any contaminating cells were neutrophils. Dose response experiments were conducted, using 30-300 g/ml of GPBP, comparing the adherence of neutrophils (2×10^6 /ml) with that of eosinophils (2×10^6 /ml).

Mg⁺⁺ and Ca⁺⁺ requirements experiments.

Granulocytes and pollen grains were washed and resuspended in Iscove's medium containing varying concentrations of MgCl₂ (1.0 - 2.5 mM) and/or CaCl₂ (1.8 - 4.5 mM), with or without EDTA (to bind divalent cations). Cells and pollen were incubated with GPBP, transferrin or albumin, at a concentration of 300 µg/ml, also prepared in the same medium and adherence was compared with that produced by medium alone. Adherence was assessed after 18 hours incubation at 37°C. The results shown are the means of three experiments.

Differential adherence to pollen grains from different sources.

The adherence of neutrophils produced by GPBP, transferrin

or albumin (30 and 300 µg/ml) to a wide range of pollen grains was assessed. The pollen grains tested included grass pollens, (Meadow grass, False oat and Rye), weed pollens (Giant ragweed, short ragweed and Plantain) and tree pollens (Silver birch and Ash). For comparison, adherence of neutrophils to inert particles of a similar size, including Sephadex G-10, Sepharose 4B and agarose beads, was assessed.

Effect of transferrin receptor antibodies on adherence reaction.

The effects of a monoclonal antibody to the reticulocyte transferrin receptor (OKT9 antibody) was investigated; OKT9 antibody (100 µg/ml) was incubated with granulocytes at 1:2, 1:6 and 1:10 dilutions for 30 minutes before excess antibody was washed off. Controls used were monoclonal antibodies to thymic tissue (OKT6 antibody) and mouse ascites immunoglobulin. Treated granulocytes were then tested over a dose range of GPBP and transferrin (from 30-300 µg/ml) for adherence to TGP grains.

Uptake of ^{59}Fe -transferrin by neutrophils.

Commercially-acquired, iron-free, transferrin was incubated with ^{59}Fe until the transferrin was saturated and developed a salmon-pink colour. Excess ^{59}Fe was removed by gel filtration on Sephadex G75 columns. Neutrophils ($4 \times 10^6/\text{ml}$) were then incubated with 300 µg/ml ^{59}Fe -labelled transferrin for varying lengths of time from 30 minutes to 18 hours. Pollen grains, in the presence and absence of neutrophils were also incubated with ^{59}Fe -transferrin over a similar time-course. After incubation, cells were spun through oil in microfuge tubes (500 g, 2

minutes), and the radioactivity of the cell/pollen pellet was counted in a conventional gamma-counter. K562 cells (4×10^6 /ml), cultured from a haemopoietic cell line, were used as a positive control.

Effect of transferrin/GBPB on neutrophil granular enzyme release.

The neutrophil enzymes, myeloperoxidase and lysozyme, were assayed colorimetrically. The cytoplasmic enzyme, LDH, (also measured colorimetrically), was used as a marker of cell death and lysis.

Neutrophils, in the presence or absence of TGP grains, were incubated with GPBP, transferrin, albumin (300 μ g/ml) or medium alone for varying periods of time from 15 minutes up to 18 hours, and neutrophil enzymes were measured in the cell supernatants after removing the cells by centrifugation. Cytachalasin B and fMet-Leu-Phe stimulated cells were used as a positive control for the experiment.

In a single experiment a subject with perennial rhinitis was challenged intranasally with an aerosol solution of house dust mite (*Dermatophagoides pteronnyssinus*) antigen and nasal washings were collected 10 minutes following challenge (as described in Chapter 4). Washings were tested for transferrin content by rocket immunoelectrophoresis and compared with known concentrations of transferrin as described in Chapter 3.

All results are expressed as means and standard errors of the mean of at least three experiments. Where quoted, statistical analysis was performed using the Student's paired t test.

(iii) RESULTS.

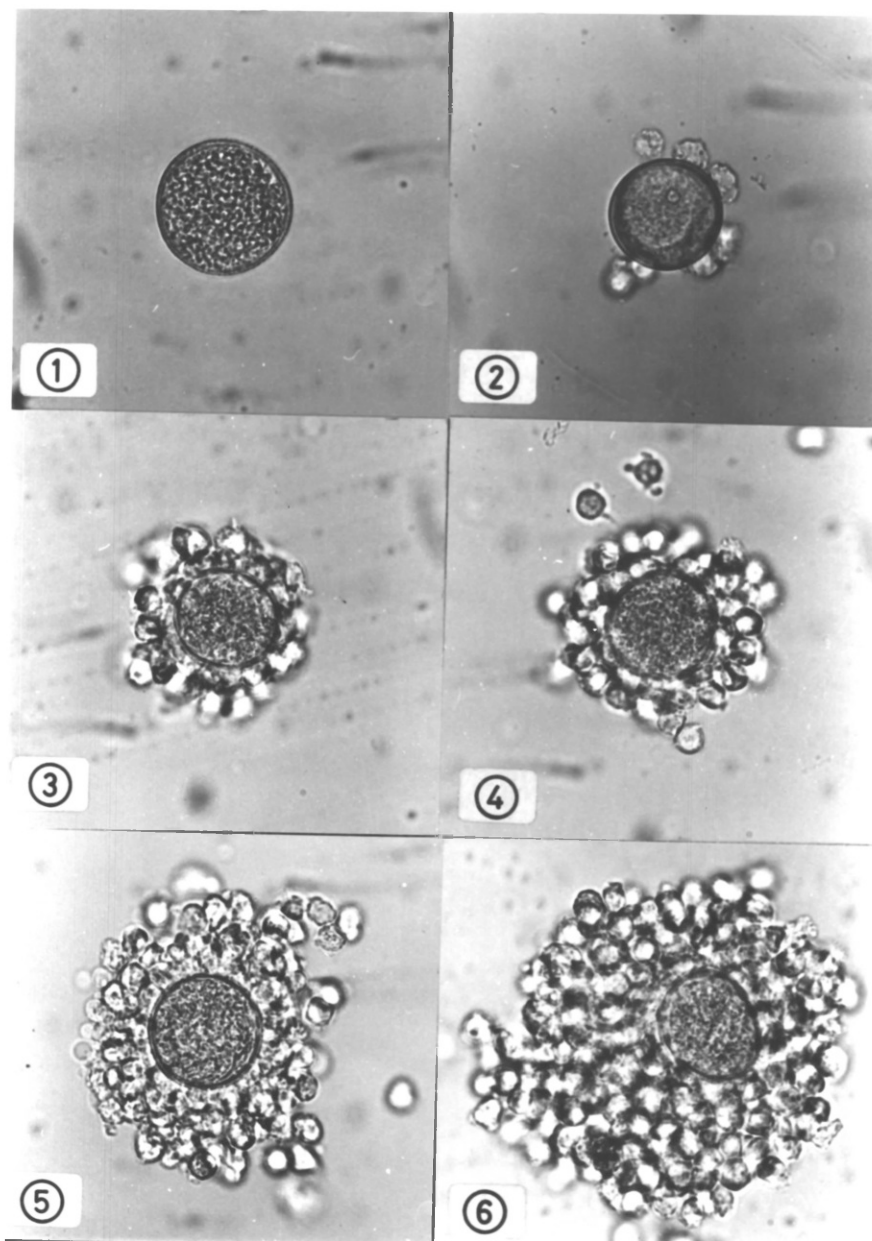
Photographs depicting the various degrees of adherence recorded are shown in Figure 8.2. Photographs 1-6 depict 0 - ++++ adherence respectively. Adherence was confined to the pollen grain surface and there was no evidence of penetration by cells of the pollen wall. At high degrees of adherence, when more than 1 layer of cells surrounded the pollen grains, granulocytes adhered to each other as well as to the pollen surface. However, neutrophils that were not in the vicinity of pollen grains did not form aggregates and neither did neutrophils exposed to GPBP or transferrin (3-300 µg/ml) in the absence of pollen.

The dose requirements of GPBP or transferrin for pollen/granulocyte adherence are shown in Figure 8.3. In these experiments, lactoferrin was used as an additional control protein, as it is another iron-bearing glycoprotein with similar physicochemical characteristics to those of transferrin. Binding induced by transferrin/GPBP was significantly greater than that produced by lactoferrin or albumin controls at doses as low as 1.25 µg/ml ($p < 0.02$ by paired student t test), and these differences became highly significant ($p < 0.001$) at doses of 30 µg/ml and above. No significant binding (i.e. greater than that produced by medium alone) was obtained with albumin or lactoferrin over the same dose range. As would be expected, the dose response curves for transferrin and GPBP are almost identical.

The time course of the adherence reaction is shown in Figure

Figure 8.2

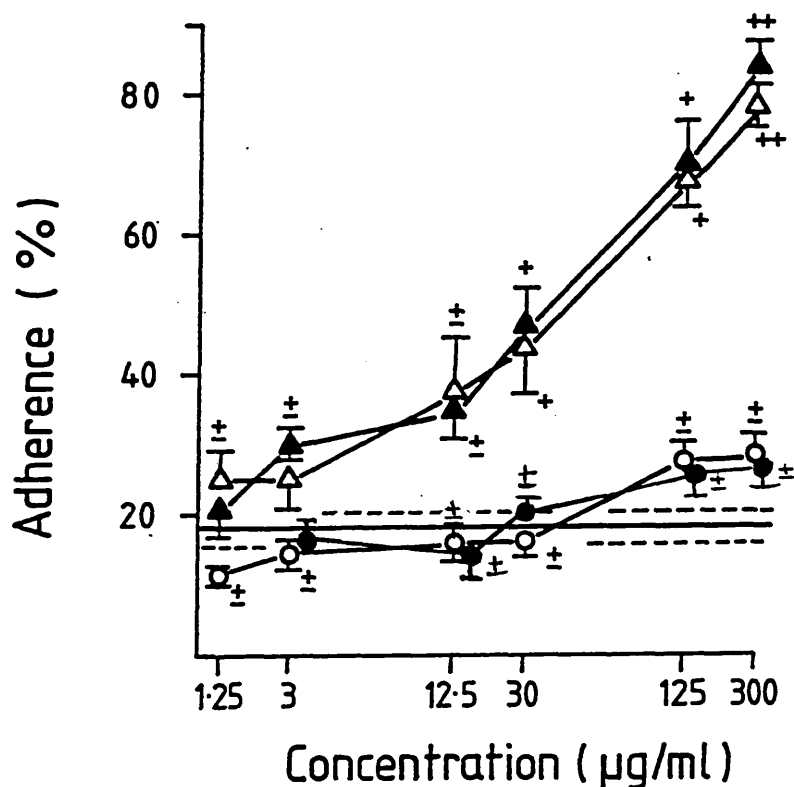
The granulocyte-pollen adherence assay.



Neutrophils, pollen grains and protein were incubated together for 18 hours as described in the text. The adherence was quantitated by calculating the percentage of pollen grains with adherent cells, and the degree of adherence was also noted as depicted above, 1 = 0, 2 = $\pm$, 3 = +, 4 = ++, 5 = +++ and 6 = ++++. Microphotographs were performed using direct interference contrast microscopy (magnification X500).

Figure 8.3

Dose-dependence of transferrin-induced neutrophil adherence to pollen grains.



Results are the percentage and degree of adherence produced by 1.25 - 300 µg/ml of GPBP (▲), transferrin (△), albumin (○) and lactoferrin (●) and represent the mean $\pm$ 1 SEM of six experiments. Adherence produced by medium alone is indicated by the solid line (mean) and interrupted lines (1 SEM). Statistics were performed using the Student's t test; GPBP produced significantly greater adherence than albumin ($p < 0.05$) at all concentrations tested, whilst transferrin promoted significantly greater adherence at concentrations of 3 µg/ml and above.

Plates were examined after an incubation period of 18h.

8.4, which compares binding produced by GPBP, transferrin and albumin (300 µg/ml) with that produced by medium alone. Even in the absence of binding protein, there is a high percentage adherence 2 hours following incubation, although the degree of adherence with medium alone (+) is less than with transferrin (++) or GPBP (+++).

However, after 2 hours, cells begin to disassociate from pollen grains in the absence of transferrin or GPBP, whilst these substances promoted prolonged adherence, still present after 18 hours of incubation. The significance of these findings will be discussed in the discussion section of this chapter.

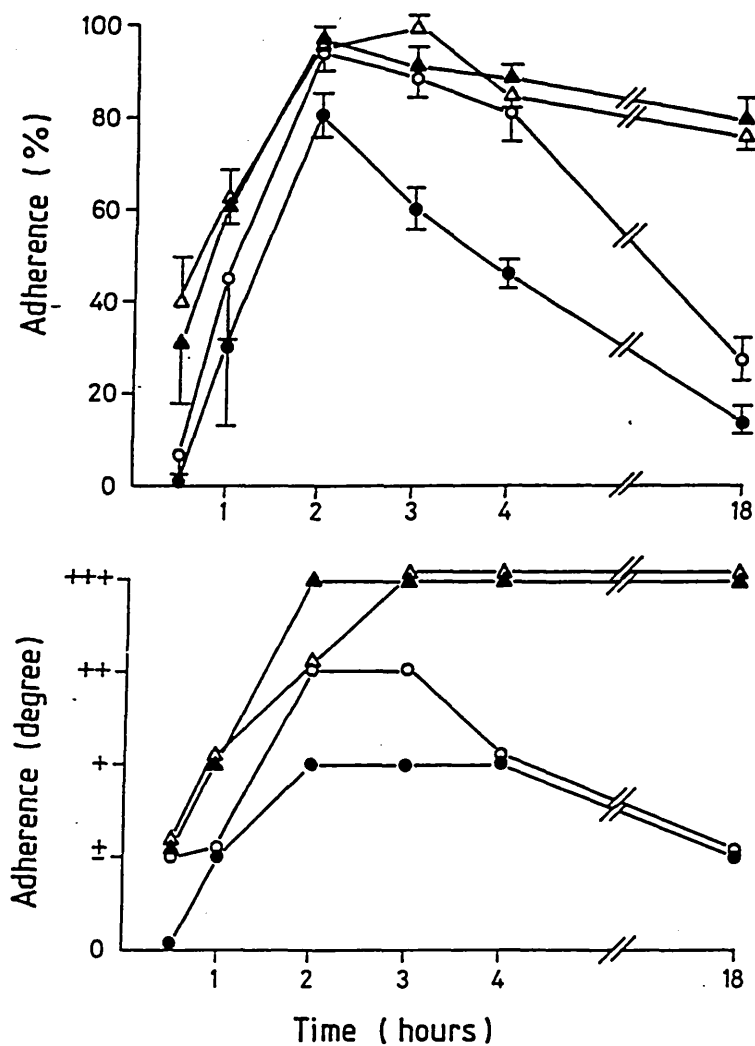
In a series of 4 experiments, granulocytes did not adhere to pollen grains at 4°C, whilst a moderate percentage and degree of adherence was achieved at room temperature (22°C). Full adherence was only observed at 37°C (Figure 8.5). This was not due to differences in atmosphere composition, as each experiment was performed in an atmosphere of 95% air and 5% CO₂.

Only neutrophils were shown to participate in the binding reaction; purified eosinophils from six patients with eosinophilia gave no measurable binding, whilst the patients' own neutrophils showed the same previously observed dose-dependent adherence in the presence of GPBP (Figure 8.6).

Pre-incubation of neutrophils with monoclonal antibody to the transferrin receptor (OKT9) had no effect on the adherence reaction when compared with control OKT6 antibody and ascitic IgG. In addition, neutrophils did not take up ⁵⁹Fe labelled transferrin either in the presence or absence of pollen

Figure 8.4

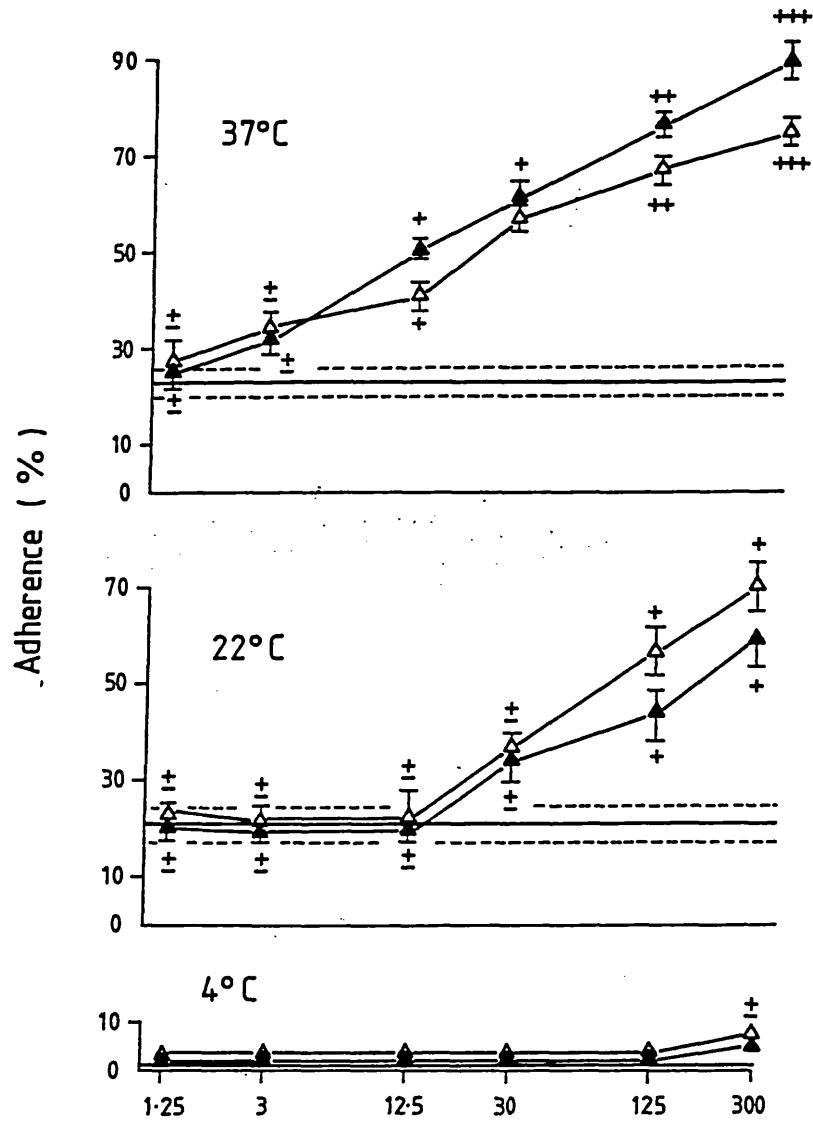
The time-course of the granulocyte-pollen adherence reaction.



GPBP (▲), transferrin (△), albumin (○) at concentrations of 300 µg/ml, and medium alone (●) were incubated with neutrophils and pollen grains for varying lengths of time, as described in the text. The top figure shows the percentage adherence (mean $\pm$ 1 SEM of three experiments) whilst the lower figure shows the average degree of adherence from three experiments.

Figure 8.5

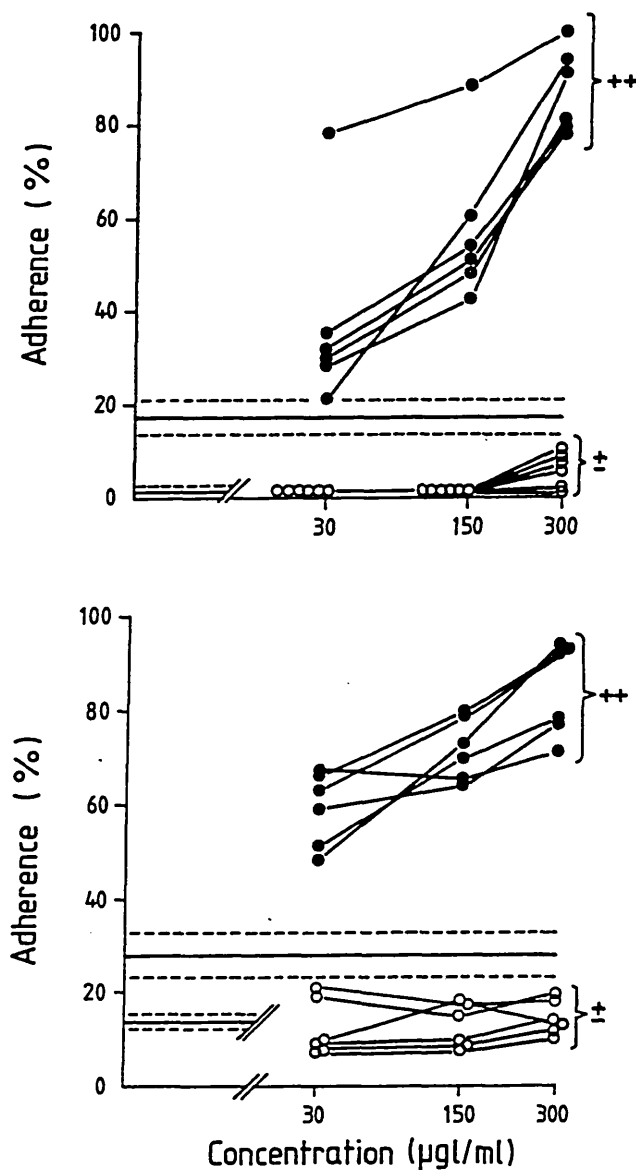
Temperature dependence of neutrophil adherence to pollen grains.



Cells, pollen grains and either GPBP ($\blacktriangle$), transferrin ($\triangle$) or medium alone (solid line) were incubated at 37°C (top section), 22°C (middle section) or 4°C (lower section) in a constant atmosphere of 95% O₂, 5% CO₂. The experiments were read at 18 hours, and the points represent the mean $\pm$ 1 SEM of four experiments.

Figure 8.6

Comparison of adherence of neutrophils, eosinophils and mononuclear cells to pollen grains.



Granulocytes, comprising > 95% neutrophils, were separated from mononuclear cells (lymphocytes with 10-20% monocytes) on Ficoll-Hipaque. Eosinophils were purified from granulocyte preparations using Metrizamide gradients, and the resulting purity varied between 75% and 98%. The results of six experiments are shown; the upper section compares the adherence of granulocytes (●) with mononuclear cells (○) produced by 30-300 µg/ml of GPBP, whilst the lower section compares neutrophils (●) and eosinophils (○) over the same concentration range. In each case the mean (± 1 SEM) adherence produced by medium alone is represented by a solid line and interrupted lines. Each experiment was read at 18 hours.

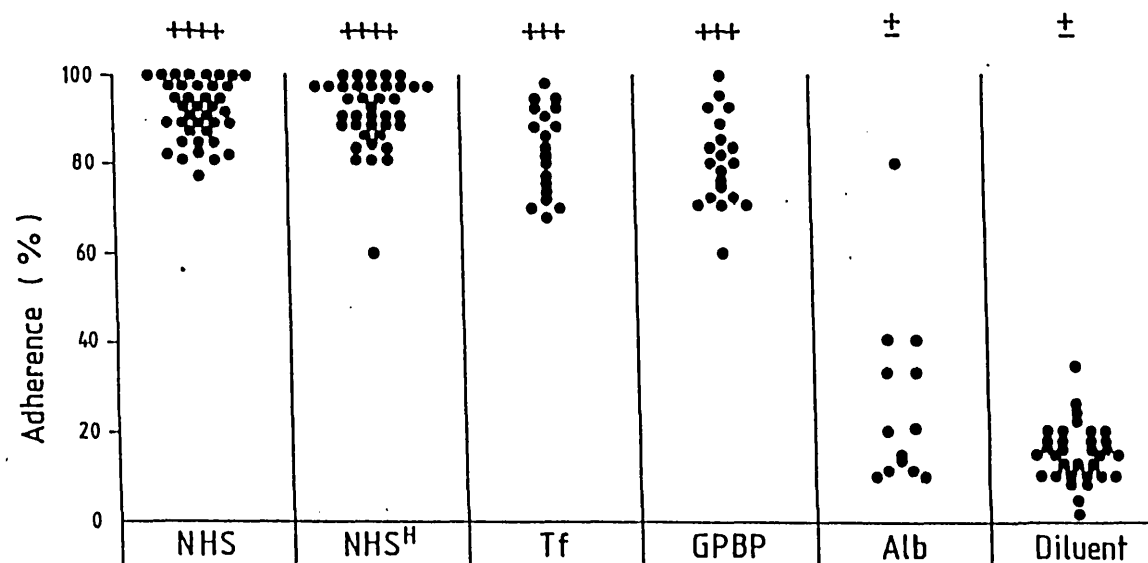
grains, over a prolonged time period, unlike the control K562 (haemopoietic cell line) cells which bound labelled-transferrin avidly (results not shown). Transferrin and GPBP had no effect on lysosomal enzyme release from neutrophils over an 18 hour period, either in the presence or absence of pollen grains, when compared with albumin control. In contrast, neutrophils incubated with cytochalasin B and subsequently stimulated with the formylated peptide, f-Met-Leu-Phe, released approx. 60% and approx. 80% of their total lysozyme and myeloperoxidase respectively (results not shown).

Figure 8.7 shows the results of three experiments designed to investigate the requirements for divalent cations of the adherence reaction. GPBP-adherence is represented by the shaded blocks and transferrin-adherence by the open blocks, whilst the medium control is represented by the lower blocks. In the absence of Mg^{++} ions, there was very little adherence (20-30%), irrespective of the presence or absence of Ca^{++} ions. Only the addition of Mg^{++} ions allowed adherence to take place, and this appeared to be independent of the addition of Ca^{++} ions, as the "dose-response" curve for Mg^{++} was identical to that for Mg^{++} and Ca^{++} . As expected, in the presence of EDTA, which binds any excess divalent cations, more Mg^{++} was required to produce optimal adherence. However, in the presence of EDTA, Ca^{++} ions seemed to play a more important role, as better adherence was achieved in the presence of both Ca^{++} and Mg^{++} , although Ca^{++} alone produced only minimal binding. Neither Ca^{++} nor Mg^{++} had any appreciable effect on binding promoted by medium control.

Figure 8.8 shows accumulated data comparing binding produced

Figure 8.8

Comparison of the effects of serum (heated and unheated), GPBP, transferrin, albumin and medium in promoting the adherence reaction.



GPBP, transferrin (TF) and albumin (Alb) were used at a concentration of 300 µg/ml and the normal human serum, both unheated (NHS) and heated for 1 hour at 56°C (NHS^H), were used at a final dilution of 1 in 3. The experiments were read at 18 hours. The degree of adherence (shown above the figure) represents the average degree of adherence observed in each experiment.

by heated and unheated human serum with that produced by GPBP, transferrin and albumin and medium controls. Serum, both heated and unheated, produces slightly stronger adherence, both in terms of percentage and adherence than do transferrin and GPBP, suggesting that there may be additional binding factors in serum. However, binding achieved with transferrin and GPBP are very similar, and significantly greater than that produced by albumin and diluent (although in 1 experiment, albumin produced nearly 80% adherence).

A wide range of grass, weed and tree pollens were tested in the binding reaction and the results of four experiments are shown in Table 8.1. The adherence to the different varieties of pollen was rather variable, but transferrin/GPBP promoted adherence to some extent in all pollen grains tested, the strongest adherence being to TGP, meadow grass pollen, giant ragweed and ash tree pollens. In contrast, no measurable binding was observed when neutrophils were incubated with a variety of inert beads of approximately the same magnitude as pollen grains, including Sephadex G-10, Sepharose 4B and agarose beads (results not shown). In one patient, a sufferer from seasonal rhinitis, who was challenged with grass pollen extract intranasally to simulate acute allergic rhinitis, nasal secretions collected after 10 mins were found to contain immunoreactive transferrin at a concentration of 30 g/ml (results not shown).

(iv) DISCUSSION.

The rosette assay for neutrophil-pollen grain adherence was easy to perform and highly reproducible. Inter- and intra-assay

TABLE 8.1 Transferrin induced neutrophil adherence to a variety of grass, weed and tree pollens

	GPBP ($\mu\text{g/ml}$)		Transferrin ($\mu\text{g/ml}$)		Albumin ($\mu\text{g/ml}$)		Diluent
	300	30	300	30	300	30	
Timothy	++ 71.0 (5.5)	+ 52.0 (3.0)	++ 70.0 (6.1)	$\pm$ 49.4 (5.8)	$\pm$ 23.0 (1.5)	17.7 (2.9)	$\pm$ 21.0 (2.0)
Meadow	++ 76.0 (4.1)	+ 53.7 (3.3)	++ 74.7 (4.3)	+ 56.3 (3.5)	$\pm$ 29.7 (3.5)	$\pm$ 24.0 (3.5)	$\pm$ 19.3 (4.3)
False oat	+ 47.0 (8.5)	$\pm$ 33.7 (10.7)	$\pm$ 52.7 (9.8)	$\pm$ 33.7 (11.2)	$\pm$ 19.0 (4.2)	$\pm$ 16.3 (3.2)	$\pm$ 13.0 (4.7)
Rye	+ 67.0 (4.8)	+ 58.0 (3.7)	+ 66.0 (3.8)	+ 51.0 (0.9)	$\pm$ 48.0 (2.6)	$\pm$ 21.0 (6.1)	$\pm$ 32.0 (1.8)
Giant ragweed	+ 69.0 (4.4)	+ 54.3 (2.0)	++ 75.3 (5.7)	+ 54.0 (5.0)	$\pm$ 19.7 (6.2)	$\pm$ 16.7 (2.6)	$\pm$ 14.3 (6.2)
Short ragweed	+ 64.3 (3.2)	+ 51.0 (1.5)	+ 64.3 (3.4)	$\pm$ 45.0 (3.8)	$\pm$ 17.0 (3.6)	$\pm$ 14.3 (2.3)	$\pm$ 18.7 (4.1)
Plantain	+ 59.3 (1.2)	$\pm$ 37.7 (3.5)	+ 56.0 (2.8)	$\pm$ 34.7 (8.3)	$\pm$ 38.3 (6.3)	$\pm$ 16.0 (6.8)	$\pm$ 17.6 (1.4)
Silver birch	+ 68.7 (2.7)	+ 45.3 (2.4)	+ 62.7 (4.0)	+ 43.0 (4.8)	$\pm$ 23.3 (3.6)	$\pm$ 14.7 (6.3)	$\pm$ 18.0 (2.2)
Ash	+ 61.0 (0.4)	+ 50.7 (1.8)	++ 72.3 (3.5)	+ 51.3 (2.9)	$\pm$ 23.0 (6.8)	$\pm$ 17.3 (4.8)	$\pm$ 24.7 (5.9)

GPBP, transferrin and albumin were tested at concentrations of 30 and 300 $\mu\text{g/ml}$. The ratio of pollen grains to granulocytes was constant. The experiments were read at 18 h and the numbers represent the mean ($\pm$ 1 SEM) of four experiments. The degree of adherence ($\pm$, + etc.) is also recorded.

variations were 10% and 5% respectively. Binding of leucocytes to pollen grains with rosette formation in vitro is not an entirely new observation. Siegel et al. (1970) described the morphological changes in neutrophils that took place following adherence to pollen grains. Since neutrophils have been shown not to adhere to inert particles with similar diameters (30-60 μ), this may be due to a property of the pollen wall itself.

Angiosperm pollen grains possess extremely complex walls, divided structurally into an outer wall, called the exine, and an inner wall, called the intine. The wall of the pollen grain plays a role in protecting the male plant gametophyte on its journey between the anther and the stigma, and hence needs to be strong and resilient. The intine is composed of cellulose and callose (Knox et al., 1970), whilst the principle exine material has been named sporopollenin (Brooks et al., 1971). It is the most resilient wall polymer produced by plants, being resistant to most physicochemical degrading processes, although it does undergo very slow auto-oxidation (Heslop-Harrison, 1968). Sporopollenin is a polymer of carotinoid and its esters (Brooks et al., 1971). The exine can be subdivided into an inner layer, the nexine, and a superficial sculptured layer, the sexine, which is composed of radially directed rods, or bacula (Heslop-Harrison, 1975). The sexine is perforated by large numbers of microspores, allowing communication between the pollen grain contents and the environment.

On moistening, pollen grains rapidly release a number of

enzymes, particularly acid hydrolases which arise from the intine (Knox et al., 1970) and they also release a variety of proteins derived from the exine material (Heslop-Harrison, 1975). Amongst these wall-associated proteins are the antigens responsible for allergic rhinitis; antigen E, one of the allergens responsible for ragweed-induced rhinitis in the U.S.A., has been found to be released within 2 seconds of pollen grain moistening and arises from two distinct sites, the cellulose intine layer and the cavities of the outermost sexine layer (Howlett et al., 1973).

In our experiments, pollen grains had been washed in aqueous medium prior to being presented to granulocytes; from previous experimental evidence, the soluble allergenic proteins would have been expected to leach out in the first wash, and thus it appears that the ability of neutrophils to adhere to pollen grains is not related to the allergenic nature of pollen.

Although transferrin has been shown to be chemically and immunologically identical to GPBP, both proteins were used in these experiments in order to show that they behaved identically in the adherence reaction.

Human transferrin has a molecular weight of 79,500 daltons; it is a single chain glycoprotein containing two homologous domains, each of which bears one iron-binding site. The binding of iron to these sites is strong but reversible (Marx, 1984). Iron is bound in its ferric (Fe^{3+}) state; the binding constants for iron binding to the C-terminal site is 6 times stronger than those for the N-terminal site (Aisen et al., 1978). Iron binding is also pH dependent; at lower pH, transferrin loses

its iron, although the C-terminal iron binding site is more acid-stable than the N-terminal.

Diferric transferrin has a much higher affinity than the monoferric form for the transferrin receptor (Huebers et al., 1983).

Transferrin is an essential requirement for the growth and proliferation of many cell types, including HeLa cells, and GH₃ and M2R cell lines (Hutchings et al., 1978).

The concept of the transferrin receptor on immature red cells was suggested by Handl et al. (1959), and rat reticulocyte membrane receptors for transferrin were identified using ¹²⁵I-labelled rat transferrin (Sullivan et al., 1978). We investigated the possible role of the transferrin receptor in the adherence reaction, using an OKT9 antibody. OKT6 antibody, a similarly prepared monoclonal antibody to a thymic cell surface antigen, not related to the transferrin receptor, was used as a control monoclonal.

OKT9 is a murine monoclonal antibody raised to human leukaemic cells. It was also shown to bind to various other cell types including other forms of leukaemia cells, a variety of leukaemia and tumour cell lines, foetal thymus and liver, and some immature haemopoietic cells and immature lymphocytes. Only actively proliferating cells appeared to possess OKT9-binding receptors and the antibody was also found to precipitate receptor-bound transferrin. As the receptor for OKT9 and that for transferrin are chemically very similar, it was suggested that OKT9 was a monoclonal antibody to the transferrin receptor (Sutherland et al., 1981).

K562, an erythroblastoid cell line, bears many surface transferrin receptors, as shown by avid binding to OKT9 antibody.

Neutrophils have not been shown to bear transferrin receptors on their cell membrane; this is not surprising as they are end-cells, and transferrin receptors tend to be associated with actively proliferating cells. In my own experiments, neutrophils were not shown to bind to ⁵⁹-Fe labelled transferrin, supporting these data, and the transferrin induced adherence reaction was not influenced by a monoclonal antibody to the conventional transferrin receptor (OKT9). There is some variability between the results of three experiments analysing the effects of the OKT9 antibody. The most likely reason for this is that cells may have been lost during the washing process following incubation with antibody; as incubation with the control antibodies, OKT6 and mouse ascites Ig also produced variable results.

The time course of the adherence reaction shows that, up to 2 hours, adherence produced by GPBP, transferrin, albumin and diluent is very similar. However, only GPBP/transferrin promoted prolonged, firm binding of leucocytes to pollen grains. This may play a role in the removal of residual pollen grain shells that have escaped removal by mucociliary mechanisms.

Binding was produced by minute quantities of transferrin (1.25 µg/ml) but was not achieved by lactoferrin, another iron-binding protein which is structurally similar to transferrin, over a wide dose range (1.25 - 300 µg/ml), which suggests that this a property special to transferrin, but which is not related

to its iron-carrying properties.

The fact that adherence only takes place optimally at higher, more physiological temperatures suggests that this is an active process by neutrophils and not purely a physical phenomenon, and which may suggest that it is of some physiological relevance.

The adherence reaction appeared to be dependent on magnesium ions but not calcium ions in the absence of EDTA. The results in the presence of EDTA are more complex; the incremental addition of Ca^{++} alone has no effect on pollen binding, whereas Ca^{++} and Mg^{++} ions together promote better binding than Mg^{++} alone, suggesting that the cations possibly act synergistically. I find it impossible to rationally explain the discrepancy between ion requirements in the presence and absence of EDTA. The relative lack of importance of Ca^{++} ions in the reaction is surprising. Phagocytosis of albumin-coated paraffin oil particles by human granulocytes and rabbit alveolar macrophages is dependent on the presence of divalent cations (Stossel, 1973) and is stimulated by Ca^{++} , Mg^{++} , Mn^{++} and Co^{++} ions, although at high concentrations ($> 20 \text{ mM}$), Mg^{++} and Ca^{++} ions have an inhibitory effect on phagocytosis.

It was also surprising that transferrin induced pollen grain binding did not promote neutrophil lysosomal enzyme release. Neutrophil adherence and ingestion of opsonised phagocytosable particles is known to be associated with enzyme release. In addition, Lindberg et al. (1982) found that pollen grains induced chemiluminescence from human leucocytes, which is another marker of white cell activation. It is possible that the colorimetric methods used for measuring myeloperoxidase and

lysozyme were simply not sensitive enough to pick up small changes in enzyme concentrations.

The lack of requirement for Ca^{++} ions and the lack of enzyme release suggest that neutrophils in adhering to the pollen grains are not performing the function of "frustrated phagocytosis", in which phagocytes vainly attempt to ingest particles much larger than themselves.

The adherence reaction was not confined to timothy grass pollen. Transferrin promoted granulocyte adherence to a wide range of grass-, tree- and weed-pollens, which had a variety of sizes and exine architectures.

Although the percentage of neutrophil/pollen binding produced by serum and transferrin were comparable, serum promoted a higher degree of binding (++++ vs. +++). This may be because serum, even when diluted 1:3 as in these experiments, contained a higher concentration of transferrin than the 300 $\mu\text{g/ml}$ tested. However, it also remains a possibility that there are additional factors in serum that promote the adherence reaction. These are unlikely to be related to the complement pathway (and opsonisation of pollen grains by C3b) as heated serum was just as effective as unheated serum as an adherence-inducer. However, it is possible that serum contains another, heat-stable factor with GPBP activity.

It is physiologically possible that pollen grains, neutrophils and transferrin should be present simultaneously in

the large airways during antigen-induced asthma and rhinitis. Transferrin has been identified in bronchoalveolar lavage fluid from smoking and non-smoking patients and volunteers (Reynold et al., 1974), along with other serum proteins such as albumin, IgA, IgG and α_1 -antitrypsin. Tracheal mucus (Diaz et al., 1984) and sputum (Boat et al., 1973) have also been shown to contain transferrin although whether levels are higher in asthmatic patients than normals has not been ascertained. My own experiments show that transferrin is released into nasal secretions following antigen-induced rhinitis along with albumin.

Neutrophils have been identified in nasal washings following nasal antigen challenge (Pelikan, 1983), and in bronchoalveolar lavage fluid from asthmatic subjects (Takayama, 1984; de Monchy et al., 1987) and in dogs with ozone-induced bronchial hyperreactivity (Fabbri et al., 1983).

Finally, pollen grains with diameters of 30-60 μ , are of a suitable size to be inhaled into the nasal airways, bronchi and larger bronchioles, and may not be removed immediately by the action of mucociliary clearance.

It is suggested that transferrin-induced neutrophil adherence to pollen grains may act as a "back-up" removal system for biological waste following inhalation of pollen grains.

(v) Summary

Human granulocyte/pollen binding protein (GPBP), previously identified as serum transferrin, promoted prolonged firm adherence of neutrophils to Timothy grass pollen. Some

characteristics of this adherence reaction are reported. GPBP-induced binding was time-, temperature- and concentration-dependent. Maximal adherence was observed by 2 h and was only slightly decreased at 18 h. The optimal temperature for adherence was 37°C. Concentrations of GPBP as low as 1.25 µg/ml gave significantly greater binding than the albumin or lactoferrin control. Eosinophils, monocytes and lymphocytes did not appear to participate in GPBP-induced pollen binding reactions at concentrations up to 300 µg/ml. In the presence of GPBP, neutrophils adhered to a range of grass, weed and tree pollens. These included timothy, meadow, false oat, rye, giant and short ragweed, plantain, silver birch and ash. GPBP did not facilitate the adherence of granulocytes to inert particles of similar size such as Sephadex beads and agarose. The adherence was Mg^{++} , but not Ca^{++} -dependent and was not inhibited by a monoclonal antibody to the transferrin receptor (OKT9). Transferrin /GPBP did not bind to either neutrophils or pollen grains. A purified commercial transferrin reacted in all respects like GPBP in these pollen binding studies. These observations indicate that GPBP/transferrin-induced adherence of granulocytes to pollen grains is a hitherto unrecognised property of transferrin which appears unrelated to iron transport or the conventional transferrin receptor.

CHAPTER NINE

GENERAL DISCUSSION

GENERAL DISCUSSION

In Chapter 4 to 6 of this thesis, I have described the measurement of HMWNCA in the supernatants of immunologically challenged human lung fragments and in nasal secretions from patients with allergic rhinitis.

There were many problems inherent in the methods used for both the partial purification of HMWNCA and for the measurement of neutrophil chemotactic activity. FPLC and HPLC techniques were used for partial purification because they provide rapid separation of proteins. Although Polyanion SI columns on FPLC provided reasonably reliable resolution by anion-exchange chromatography, and similarly, Mono P columns proved satisfactory for chromatofocusing, the gel filtration provided by Microbondagel columns on HPLC was generally unsatisfactory. It was extremely difficult to assess accurately the molecular size of NCA using this method; it was only possible to separate NCA of high molecular weight (i.e. greater than 440,000 daltons) from that of intermediate or low molecular weight. Most other chemotactic factors, for example complement derived factors and leukotriene B₄, are of low molecular weight. The only other known protein which is present in plasma and connective tissues and which displays some chemotactic activity for leucocytes including neutrophils is fibronectin (Yamada et al., 1984).

Since this work was completed, serum HMWNCA has been partially purified using gel filtration columns on FPLC (Buchanan et al., 1987). This provides better separation of high molecular weight proteins whilst being a relatively rapid

method of purification.

The modified Boyden chamber technique is a well-tried and trusted method of measuring chemotaxis. However, there are a number of faults and problems with this method. As the number of cells used in each chamber is high (2×10^6) and each sample is measured in duplicate or triplicate, a large number of cells ($4-6 \times 10^6$) is needed for each sample. As cells from different donors differ in their chemotactic responsiveness to the same stimulus, only one neutrophil donor is used per assay. This means that, to avoid excessive venesection, there is only a limited number of samples that can be measured in one assay.

Alternative methods of assessing chemotactic activity are available. Previous attempts to simplify the chemotaxis assay involving a multichamber apparatus (Swanson et al., 1977) were largely unsuccessful because the apparatus needed to undergo centrifugation prior to removal of the filters. More recently, a microchemotaxis assay, using a multichamber apparatus, has been used to detect monocyte chemotaxis (Falk et al., 1980). This reduces the necessary volume of chemoattractant to 25 μ l (compared with 800 μ l in the conventional Boyden chamber technique) and the number of target cells to 10,000 (compared with 2 million). Results using this micro-technique have compared well with those using standard blind well chambers.

It would also be possible to radioactively label neutrophils, using ^{125}I and then to measure the amount of radioactivity (and indirectly the number of neutrophils) to completely traverse a membrane in response to a chemoattractant.

The limited number of samples in each assay, and the wide variation of results between assays, depending on varying responsiveness of donor neutrophils, meant that it was very difficult to compare experiments. In retrospect, it would have been preferable to use standardised units for NCA. This would be possible if NCA were expressed in units, one unit being the degree of chemotaxis induced by a known concentration of a known chemoattractant, for example, f-Met-Leu-Phe or leukotriene B₄. In this way, different assays could be compared despite large differences in the activity of the target neutrophils. A similar method has been used to describe serum NCA, in order to compare the baseline serum NCA of large numbers of patients with mild, moderate or severe asthma with that of normal subjects (Buchanan et al., 1987).

An alternative way of solving the problem of comparison between assays would be to measure the activation of neutrophils rather than chemotaxis itself. It is known that chemotactic factors, including HMWNCA, activate their target cells as detected by an increase in the expression of cell surface receptors (Nagy, 1982). It might, therefore, be possible to measure the concentration of HMWNCA by the degree of enhancement of neutrophil C3b receptors. Tetteroo et al. (1986) have described a method of measuring neutrophil activation using monoclonal antibodies to neutrophil-associated membrane proteins, which do not seem to be related to the C3bi receptor.

It was, of course, hoped to be able to develop a specific radioimmunoassay for the detection of HMWNCA. However, attempts to produce a specific antibody to HMWNCA were unsuccessful. I

have outlined some reasons for this lack of success in Chapter 7. If I were repeating the work, I would use different methods of antigen presentation, including antigen partially purified by gel electrophoresis, and would use different methods of immunisation.

This thesis has described the release of HMWNCA from immunologically challenged human lung fragments and from the nasal mucosa of patients with allergic rhinitis. The time course of HMWNCA release from lung fragments parallels that of histamine. Following bronchial provocation testing in sensitive individuals, both histamine and HMWNCA have been identified in the serum. The release of both these mediators was blocked by the prior use of cromolyn sodium and salbutamol; this suggests that these mediators are released in conjunction with mast cell activation, as neither cromolyn nor albuterol has any effect on basophil histamine release (Holgate, 1987). The release of factors which are chemotactic for neutrophils may contribute to the accumulation of neutrophils at the inflammatory site in asthma (Cockroft, 1982).

Neutrophil chemotactic factors released in response to allergen challenge may not originate from lung mast cells, however. Mast cells in the airways (and in BAL fluid) are few in number when compared with alveolar macrophages. Alveolar macrophages have low affinity IgE receptors on their plasma membrane (Dessaint et al., 1979), and the cell can be activated by cross-bridging of these receptors. Activated human alveolar macrophages release a chemotactic activity for neutrophils (Hunninghake et al., 1980) which is heat stable, pH stable and

had two major isoelectric points, one of which was neutral (pI 7.6). However, this is unlikely to account for HMWNCA, as it was, at least partially, lipid in nature and had a low molecular weight (600 daltons and 10,000 daltons).

Following antigen and exercise induced asthma, activated leucocytes have been detected in the peripheral circulation (Papageorgiou et al., 1983; Carroll et al., 1980). Activated neutrophils are capable of generating other factors which are capable of recruiting and activating other cells. These factors include LTB_4 , PAF-acether and another platelet activator, named neutrophilin (Chignard et al., 1986).

Activated neutrophils may be responsible for the phagocytosis and removal of both particulate allergens and foreign waste material. Chapter 8 describes the adherence of neutrophils to pollen grains in vitro, stimulated by the presence of transferrin. As it is physiologically possible for neutrophils, transferrin and pollen grains to co-exist in the larger airways, this action of neutrophils may remove pollen grains and so prevent the prolonged presentation of allergen-bearing particles to the airway mucosa.

In summary, this thesis investigates one of the mechanisms by which neutrophils may be attracted to the site of an allergic reaction, either in the lung or the nasal mucosa, and also a possible action of neutrophils in the removal of allergenic particulate material.

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