Steroid Modulation of Neutrophil Function

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There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes. *Stimulate the phagocytes!*

Sir Ralph Bloomfield Bonnington

The Doctor's Dilemma

George Bernard Shaw

TO MY PARENTS

TABLE OF CONTENTS

CHAPTER TWO: MATERIALS AND METHODS

CHAPTER THREE: RESULTS

List of Figures

LIST OF ABBREVIATIONS

BAE	Bovine Aorta Endothelium
BSA	Bovine Serum Albumin
BSS	Balanced Salt Solution
Ca2+	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
CFDA	Carboxyfluorescene Di-acetate
CMS	Control Monocyte Supernatant
ECM	Extracellular Matrix
F10	Ham's F10 Culture Medium
fMLP	formyl Methionyl-Leucyl-Phenylalanine
GTP	Guanosine Triphosphate
H2O2	Hydrogen Peroxide
HETE	Hydroxyeicosatetraeinoic Acid
HH	Hank's HEPES
HO	HEPES Water
HPLC	High Pressure Liquid Chromatography
HS	HEPES Saline
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Intercellular Adhesion Molecule
IL	Interleukin
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MARCKS	Myristoylated Alanine-Rich C-Kinase Substrate
MPO	Myeloperoxidase
NaCr	Sodium Chromate
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NSAID	Non Steroidal Anti-Inflammatory Drug
PBS	Phosphate Buffered Saline
PHOX	Phagosome Oxidase
PI3K	Phosphatidyl Inositol 3 Kinase
PIP2	Phosphatidyl Inositol bis Phosphate
PLA	Phospholipase A
PLC	Phospholipase C
PKA	Protein Kinase A
PMA	Phorbol Myristate Acetate
PMN	Polymorphonuclear Leukocyte

PMS	Polymorph Migration Stimulator
O2-	Superoxide Ion
SEM	Scanning Electron Microscope
STMS	Steroid Treated Monocyte Supernatant
TNF	Tumour Promoter Factor
µg	micro gram
µl	micro litre
µm	micron
µM	micro molar
v/v	Volume by volume
w/v	Weight by volume

SUMMARY

Dexamethasone, a glucocorticoid, is used extensively in clinical medicine in the treatment of respiratory diseases, notably asthma. This medical effect is probably due in part to a down-regulation of many cytokines e.g.. IL-8. However, the side effects of steroids often may outweigh the beneficial effects, especially if the disease in question is of a chronic nature. This combined with steroid resistance has led to an increase in the use of non-steroidal anti-inflammatory drugs. These tend to be aspirin derivatives, and many have side-effects. I have investigated a factor that is naturally produced by the body which may alleviate the symptoms of inflammation without many of the side-effects, with an aim to providing a viable alternative.

A novel steroid induced monocyte derived factor was described twenty years ago by Stevenson as part of an MD thesis submitted to this university. This remains an unusual phenomenon, as most of the effects of steroids are due to a down-regulation of release; here is one of the few examples of the reverse. Although it raised many interesting points about dispersive motility of populations of polymorphonuclear leukocytes (PMN) it did not look at individual cells, nor at many of the interesting properties of neutrophils.

One of the known effects of some steroids (though not Dexamethasone), and many NSAIDs, is a reduction of adhesion of PMN to venous endothelium. Human umbilical vein endothelial cells (and bovine aorta endothelium) were used as an *ex vivo* model for this, and my results show there is a marked decrease in this adhesion. This effect was observed not only on resting endothelium, but also when the endothelial cells had been pre-treated with IL1β or thrombin. This decreased adhesion was due to an interaction with the PMN and the factor, as shown by adhesion being reduced when protein coated coverslips were used as the substrate for adhesion. It is suspected that the reasons for this may be due to an inactivation of integrins such as Mac-1, although a

role for selectins cannot be ruled out. Recent work by Diaz-Gonzalez suggests that some NSAIDs act by inducing PMN to shed L-selectin.

Other effects of this steroid induced monocyte derived factor on human PMN were determined using biological and biochemical techniques.

Previous work has shown a novel dispersive effect of this factor on PMN when used in a uniform concentration, and so I decided to look at the morphology of treated PMN. Using scanning electron microscopy I observed a polarisation of the PMN, but without any ruffling of the membrane, a feature that is normally observed with polarisation. This morphology was not observed with control supernatants taken from monocytes cultured in the absence of Dexamethasone. This morphology was conserved using cells in suspension and adhered to bovine aorta endothelium. The underlying actin cytoskeleton was examined using confocal microscopy, and the microfilamentous array was noted as being devoid of spikes, observed with activation, for example with fMLP.

Late cytoskeletal controlled effects, the release of granule contents, were also investigated, and it was noted that the release of primary granule contents could be inhibited by this factor in a dose dependent manner. This fusion of granules with the plasma membrane is controlled by the activation of numerous tyrosine kinases, and follows a strict order. Secondary granule release was shown to be inhibited also, as assayed by the cleavage of type I collagen, and analysis of SDS gels.

The effects on the metabolic burst showed conflicting results with inhibition being present, again in a dose dependent manner, but much of this activity was removed with increasing purification, leaving only very slight inhibition.

INTRODUCTION

1.1.1. The role of the innate immune response in inflammation

Innate immune responses are defined as the protective responses of the body to pathogens which do not require immunological memory and can therefore be mounted as immediate defence mechanisms (Roitt). In higher animals many of the innate response mechanisms depend on the properties of the complement proteins, but the major contribution is made by the cells now termed as professional phagocytes.
Corticosteroids are recognised as the major physiological modulators of neutrophil function. The work in this thesis stems from the fact that steroid treated monocytes secrete a factor that modulates neutrophil behaviour (Stevenson, 1974, Chettibi et al., 1993). The aims were to characterise the factor in terms of the known properties of neutrophils

1.1.2. Professional phagocytes

Granulocytic cells of the myeloid lineage are recognised as being major effectors of the innate immune response. They have been traditionally classified in terms of histochemical properties as neutrophils, eosinophils and basophils (Murphy). Of these, neutrophils are both the most abundant and the most active phagocytic cells (Roitt). Mammalian neutrophils, eosinophils and basophils have a highly characteristic morphology in that the largely inactive nucleus is multilobed and this has led to the widespread use of the nomenclature polymorphonuclear leukocyte abbreviated to PMN. The granules which characterised neutrophils eosinophils and basophils are responsible for the histochemical properties of these cells and therefore differ markedly between the cell types. The cell types are also characterised by surface antigens using the CD nomenclature with CD14 being characteristic of human PMNs.

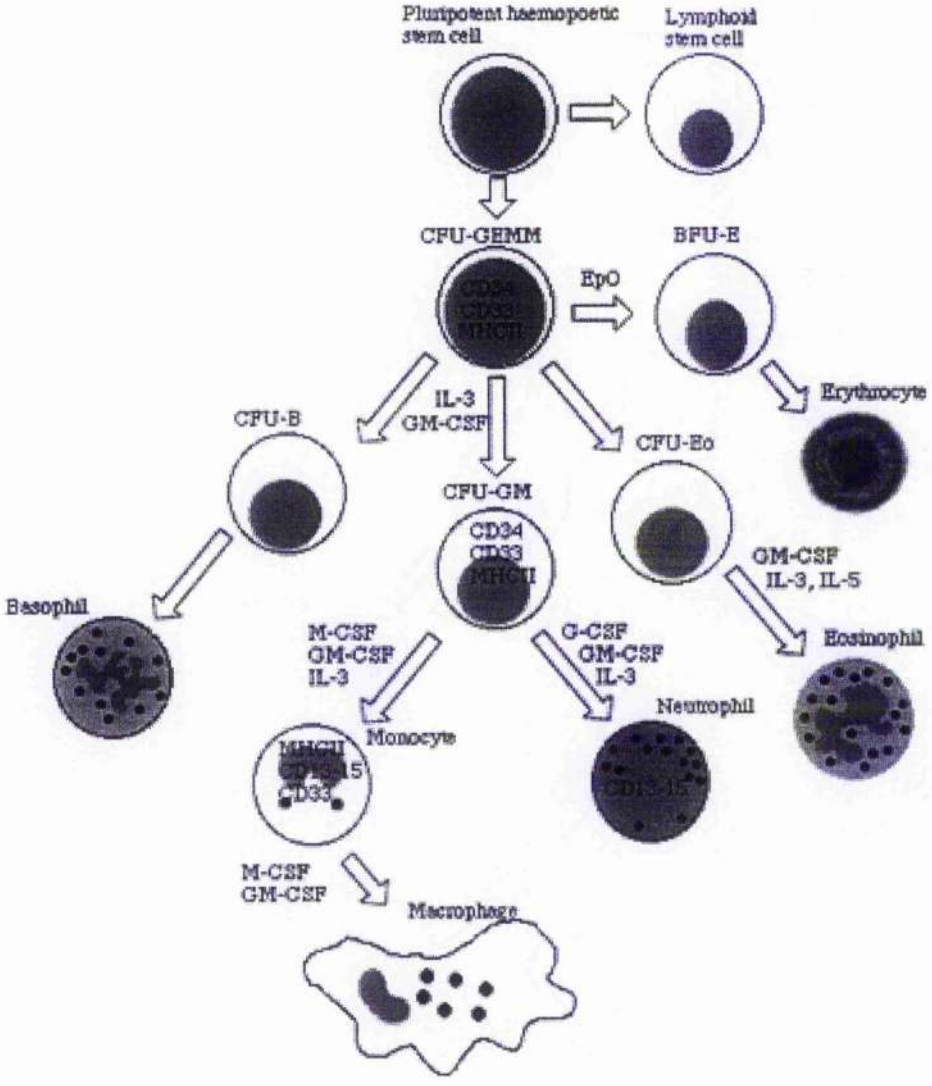
At any one time the average adult human has around 10^{11} PMNs with a half life in circulation of approximately 8 hours (Abbas). PMNs develop from pluripotent stems

cells by pre-programmed cell division within the bone marrow and continue to differentiate over a period of 5-7 days, figure 1. Commitment to each stage of this developmental pathway, which is irreversible, is regulated by a variety of signalling peptides. The biosynthetic activity involved in maintaining the PMN population is calculated to be almost as great as that involved in maintaining the far more abundant erythrocyte population.

The second class of professional phagocytes are monocyte derived, consisting of circulating mononuclear cells (monocytes) which are weakly phagocytic and leave the blood stream after a short residence period, to become highly phagocytic tissue macrophages. Macrophages are antigen presenting cells of crucial importance to the acquired immune response (Abbas). Both monocytes and macrophages are active producers of cytokines which regulate the activities of neutrophils, among other cells. In principle, macrophages should regulate local activities, whilst monocytes could act as global regulators. The best characterised signalling functions of these cells is the release of proinflammatory cytokines which up-regulate neutrophil activity (Roitt). However they are also known to be the primary targets for glucocorticoids (corticosteroids). Because glucocorticoids are among the most powerful known anti-inflammatory agents they are believed to be the major physiological down regulators of the innate immune response. Within the circulation they are ideally placed to function as global regulators and to this end the monocytes (and endothelial cells) could be seen as the primary targets.

Finally it should be noted that the enormous task of clearing senescent neutrophils from the blood stream is probably carried out by macrophages, although the details of this are obscure.

Fig 1. Haematopoesis. Differentiation of granulocytes



Different cytokines produce the different granulocytes in an irreversible process whereby once a cell fate is determined it cannot be changed. CD antigens present on the cell surface are indicated within the body of the cells. Not to scale. (After Roitt).

1.2. Neutrophil activation in inflammation

1.2.1. Adhesion

Neutrophils in the bloodstream are divided into the non-marginating and marginating pools; the former being in free suspension in the plasma and the latter being more or less tightly associated with blood vessel walls. Thomas Wharton-Jones (1842) made the initial observations which suggested that leukocytes roll along the endothelium of veins, but the reasons for this remained confused for some time (including that specific gravity was forcing the heavier cells to the outside (Krogh)). More recently it has been postulated that there is a basal level of rolling of PMNs, but that adhesion is only triggered by specific mechanisms (Lorant et al., 1995; Kubes, 1993).

Signals for the innate immune response include, the recognition of non-self (non-protected) surfaces by complement components, specific bacterial toxins, especially LPS and by chemotactic factors. These signals are recognised by mononuclear cells and endothelial cells which respond by sending out secondary signals, e.g. histamine, IL-1β, TNFα (Roitt). The abluminal surface of the endothelium has receptors for such molecules and responds by releasing Weibel-Palade bodies from the cytoplasm to the luminal surface. These bodies contain integral membrane proteins that are required for the adhesion of leukocytes, especially E-selectin - a ligand for the neutrophil adhesion receptor L-selectin, and also a quantity of P-selectin, which also has role in inflammation (Lorant et al., 1993).

It is now known that adhesion of neutrophils to the endothelium has two distinct components mediated by selectins and integrins (Bevilaqua, 1993; Pardi et al., 1992). Selectins are carbohydrate binding proteins on the external surface of the plasma membrane which are analogous to plant lectins. They have high specificity for sialyl residues and they themselves are sialyl glycoproteins. Their proposed role is to retard the PMNs as an essential preliminary to stronger adhesion. Neutrophils constitutively

express L-selectin on the cell surface, and ligands for this (including E-selectin) are released to the PM of the endothelial cells; i.e. there is selectin mediated adhesion on both surfaces. The implied hypothesis is that selectin based rolling adhesion forms the basis for margination, but in the absence of an inflammatory signal this remains reversible. Upregulation of ligands for L-selectin causes PMNs to be retarded sufficiently to enable strong binding to occur between β2 integrins on the PMN (Spertini et al., 1991), (especially Mac-1), with ICAMs in the endothelial PM (Smith et al., 1988). This control mechanism, whereby the endothelium only releases the ligands in response to injury or insult, prevents activated PMN from adhering non-specifically to non-activated endothelium. A second control step is initiated when the L-selectin is shed from PMNs on activation (Gearing & Newman, 1993). Thus PMN that become detached cannot readily adhere to other areas of resting or activated endothelium. Soluble L-selectin may be capable of adhering to E-selectin bound to endothelial cells, and so prevent neutrophil adhesion to anywhere other than localised areas of endothelium downstream from the site of adhesion (Gearing & Newman, 1993).

1.2.2. Extravasation / diapedesis

Resting endothelium forms a tight permeability barrier to blood cells and to serum proteins >40 kD. Activation of endothelium by IL-1β/TNFα permits gap formation between cells in response to PMN adhering to them (Kaplanski et al., 1994)), and depends on the translocation of cytosolic endothelial vesicles containing E-selectin (Weibel-Palade bodies) to the PM (Asako et al., 1992). Integrin based adhesion of PMNs to the endothelium is a precondition for the migration of the PMN across the endothelial junction into the tissue, a process normally called diapedesis. The evidence suggests that interaction of integrin with its cellular ligand activates locomotion which enables the PMN to find a gap between cells of the endothelial sheet.

1.2.3. Migration / chemotaxis

Diapedesis leads to PMN migration through the basement membrane into other tissues. This process has been studied in vitro using endothelial cell sheets and in vivo using the rabbit ear models. PMN deform during passage between endothelial cells, and this has been attributed to rearrangement of the actin cytoskeleton. Downey and colleagues (1991) observed reduction of actin cross linking whilst the cells were passing through the endothelium and basement membrane. They proposed that phosphorylation of actin by PKA reduced its ability to polymerise, and also that prostaglandins released by the endothelial cells could limit cell stiffening, thus allowing PMNs to deform and facilitate emigration. The granules within PMNs contain nascent receptors for ECM components e.g. laminin, fibronectin, and also degradative enzymes such as collagenase and elastase which may be used for migration purposes (Borregaard et al., 1993). These proteases which are specific for connective tissue together with the non-specific cathepsins are known to damage to the extra-cellular matrix, although this is believed to be reversible. It would be of interest to study the effects of e.g. elastase gene knock-out, but at this time there are no such mutants. PMNs are capable of migrating through relatively loose collagen gels without necessarily degrading them (Crockett, K.V., PhD thesis, 1987). Directional migration to the injury site is brought about by concentration gradients of chemotactic agents such as IL-1, IL-8, derived from macrophages, complement component C5b or formylated peptides etc. derived from pathogens.

1.2.4. Phagocytosis

When the PMN reaches its final destination, defined by direct contact with the pathogen, phagocytosis is initiated and the organism is destroyed within the phagolysosome by a battery of mechanisms. Most attention has been focused on the generation of active oxygen species which requires the assembly of the phagosome NADPH oxidase (PHOX) and the activation of the hexose monophosphate shunt,

which provides the energy supply for this system. The activity of PHOX is controlled by its regulatory subunits (Segal & Abo, 1993). The membrane components of the oxidase are found in more than one class of granule, all of which fuse with the phagolysosome during the killing process. Concomitant with membrane fusion is the release of soluble peptides (e.g. defensins) and enzymes into the phagolysosome, many of which are maintained in an inactive form due to a low pH which is maintained within the granules (Ganz & Lehrer, 1994). The activation of the oxidase and osmotic flux both aid the increase of the pH and so enzymes such as lysozyme can attack the bacteria (Segal & Abo, 1993).

It is of interest here to outline the role of these phenomena in determining the four classic signs of inflammation turgor (swelling), rubor (redness), calor (increase in body temperature) and dolor (pain). Rubor is due to increased blood supply ultimately controlled by the arterioles of the capillary bed. Turgor would be a consequence of gap formation in the endothelial sheet together with increased capillary pressure. [One of the important consequences of enhanced permeability of the capillary wall should be to increase the local concentration of complement components in the tissue.] Calor is mediated by actions of the pro-inflammatory cytokines IL-8 and TNFα and dolor is due to the activation of bradykinin, synergised by prostaglandins.

1.3. Neutrophil granules

The secretory granules which are abundant components of the cytosol of PMNs have very important roles in function. It is now recognised that four major classes of granule can be described, based on the contents and the order of release of contents. All granule types appear to be released by elevation of cytosolic calcium and granules can therefore be ordered in terms of the concentration of calcium (or the increase in calcium concentration) required to release their specific contents (Sengeløv et al., 1993). Granule secretion is proposed to serve two purposes, the translocation of membrane

proteins to the cell surface (the PM) and the mobilisation of luminal proteins. Some of these may be destined for the extracellular medium, but others may be required to act primarily in the phagolysosome. Smaller, less dense, granules would be more efficient for moving membrane components to the cell surface, whereas larger, denser, granules would be more appropriate for transporting soluble proteins for release into the phagolysosome (Sengeløv et al., 1993). Phagocytosis produces an increase in cellular volume, with osmotic flux accounting for much of the observed swelling (Grinstein et al., 1986).

1.4. Degranulation

PMNs have at least four types of granules, classified as Secretory, Gelatinase, Specific and Azurophil in order of release (Sengeløv et al., 1993). [Previously these had been classified as primary, secondary and tertiary, corresponding to azurophil, specific and secretory respectively]. Degranulation (the visible disappearance of granules from the cell) may involve movement of granules to the surface, but massive degranulation probably involves the fusion of granules with each other in addition to fusion with the plasma membrane. Degranulation is always associated with the release of components into the extracellular medium (Weissmann et al., 1972). For the early vesicles, incorporation of components into the plasma membrane and release of enzymes into the extracellular *milieu* appear to be important for function, but for the later vesicles such leakage is now thought to be secondary to the process of phagocytosis.

Secretory vesicles contain many soluble plasma proteins normally found outside the cell, but on the membrane itself there are numerous proteins important for later activation of the cell, e.g. fMLP receptors. These proteins, by being released (possibly nearest the side of the cell exposed to the highest concentration of agonist) may contribute significantly to the overall shape change observed in the first few seconds of activation, and facilitate chemotaxis (as described in Watts, et al., 1991). Kaplanski et

al. (1994) have produced evidence to show that binding of PMNs to an activated endothelial cell sheet causes the individual cells to contract and thicken thus generating gaps between them. Surprisingly, changes in calcium concentrations do not play a major role in the extravasation ability of neutrophils (Zaffran et al., 1993), although endothelial calcium concentrations ([Ca]i) can modulate PMN migration (Huang et al., 1993). The secretory vesicle membrane contains the major cellular pool of the integrin Mac-1, primarily responsible for PMN adhesion with the endothelium, hence fusion of the secretory vesicle with the PM is a powerful mechanism for regulating adhesion, but to be effective it requires to be triggered very rapidly as a result of selectin-mediated interactions (Lorant et al., 1995). The receptors for chemotactic agents are present at low level in these vesicles, and are thus upregulated by the events in margination. Release of these vesicles to the contact surface could be a mechanism for polarisation. CD45, the classic tyrosine phosphatase, is present in high concentrations (relative to the other granule types). This may allow activation of many proteins inside the cell, especially small GTPases such as Fyn allowing activation of PLCγ, PI_3K etc., which may be important for all subsequent processes (Gaudry et al., 1992). These vesicles are noted as having an unusually low proportion of phosphatidyl ethanolamine, and high in phosphatidyl choline in the membrane compared with other granule types (Borregaard et al., 1993). The levels are closer to those observed in the PM (Tauber 1987).

Gelatinase granules were the most recently described, with very few characterised contents. Mac-1 and the near ubiquitous fMLP receptor are found on the membrane (although at apparently lower levels than with specific granules) with acetyl transferase and gelatinase in the lumen (Petrequin et al., 1987). Gelatinase is defined as being types IV and V collagen specific (along with denatured collagen) (Michaelis et al., 1992), whilst collagenase, as found in secondary granules, has greater specificity for types I, II and III collagen. CD45 is also present on the membrane of these vesicles (Lacal et al., 1988).

Specific granules have been the most intensively studied of all the PMN granule types. Their major membrane components are Mac-1 and cytochrome b558 as well as receptors for vitronectin, fibronectin and laminin. These granules have the largest pool of fMLP receptors together with receptors for IL-8 and C5b, and are clearly important for the late stages of chemotaxis. Receptors for ECM components present on the surface of these granules could provide positional information important in signalling. Many lytic enzymes are present within the matrix of the granules, such as lysozyme, collagenase and enzymes that would be capable of dissolving glysocaminoglycans e.g., heparanase, sialidase. These enzymes could be used to facilitate extravasation through the basement membrane and ECM. These granules also contain gelatinase, although it appears that the enzyme is complexed with a 25 kD subunit termed NGAL (neutrophil gelatinase associated lipocalin) (Kjeldsen et al., 1994), which is exclusive to specific granules. NGAL has a low binding affinity with fMLP and could be the low affinity receptor of Rossi (1986).

Primary granules store most of the proteolytic and bactericidal enzymes and peptides of the cells, but they have no receptors for chemotactic agents or adhesion molecules, and this distinguishes them from the other types. It is now understood that these vesicles differ from lysosomes in that their internal enzymes are active at neutral rather than acidic pH. In the pre-released state, the contents of the primary granule are maintained at low pH, and are only activated when the pH rises to neutrality. It is therefore essential to prevent tissue damage that these enzymes are contained within the phagolysosome and not released into the tissue space.

To a limited degree, different granules are activated by different mechanisms: PMA activates specific granules; opsonised zymosan activates O_2^- specific granules and azurophil granules and formation of phagolysosome; fMLP activates O_2^- and some lysosomal enzymes (Takahashi et al., 1991). The overall control of degranulation requires a co-ordinated series of signals, involving a rise in cytosolic calcium

concentration (Niessen et al., 1991) and at least one other signal, possibly involving transport of other ions e.g. chloride (Fittschen & Henson, 1994) or perhaps lactic acid which has been proposed to buffer the intracellular pH during the release of proteases into the phagolysosome (Simchowitz & Textor, 1992). Whatever signals are required for the initial stages of the signal transduction pathway leading to degranulation, disassembly of microfilaments is almost certainly required (Muallem et al., 1995).

1.5. Signalling in the neutrophil

Human neutrophils respond to three major types of signalling molecule, the chemokine IL-8, chemotactic peptides such as fMLP, and complement components, especially C5b. They also respond to lipid derived agents, e.g., LTB4. Signalling triggers shape changes (polarisation), enhanced mobility, modification of adhesion, chemotaxis and phagocytosis. Despite the diversity of types of molecule, the known receptors for chemotactic stimuli are remarkably similar, being of the class of trimeric G-protein activating serpentine receptors and then activating the classic phosphatidyl inositol bisphosphate (PIP_2) signalling pathway. Binding of the ligand triggers a conformation change in the receptor, allowing the Gα subunit to exchange its resident GDP molecule for GTP and then dissociating from the GβGγ complex. It then activates a cytosolic phospholipase C enzyme (PLCβ) to increase the hydrolysis of phosphatidyl inositol bis phosphate to produce inositol tris phosphate (InsP3) and diglyceride (Janmey, 1994). Binding of InsP3 to its tetrameric receptor on the endoplasmic reticulum can raise [Ca2+]i from 100nM to 1mM (Clapham 1995). [Ca2+]i gradients have been proposed to initiate cell migration, but other evidence suggests that cells that are already migrating do not have changes in calcium concentration during chemotaxis (Laffafian & Hallet, 1995). Diglyceride (DG) recruits cytosolic protein kinase C to the membrane in a calcium dependent fashion, thereby activating it against a range of substrates of which the best characterised is the Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) in the PMN and pleckstrin in the platelet. Many of the actions of the

signalling molecules can be mimicked by phorbol esters which activate protein kinase C directly without changing cytoplasmic calcium concentration. The neutrophil can respond to any one signalling molecule over a very wide concentration range, and because the responses at high concentrations may differ from those at low concentrations our interpretation requires either the presence of several different receptors or else the modulation of receptor concentration which could be achieved by storing the receptors in pools within the granule membranes. Nevertheless because signalling pathways share many common features it is hard to see how, for example, responses to IL-8, fMLP or C5b could differ in any substantial way. Some light may be shed on this by an analysis which shows that elevated calcium can produce a wide spectrum of cellular responses depending on the precise way in which the concentration changes with time (Berridge, 1997).

1.5.1. Diversity of signalling pathways

Much attention has been focused on the role of the alternative pathway involving the activation of the phospholipase C isoform PLCγ, which is recruited from the cytosol by phosphorylation of tyrosine residues in an integral membrane receptor protein (reviewed by Clapham, 1995). This isoform is able to hydrolyse a specific pool of PIP_2 which is normally sequestered by proximity to profilin, a protein that was believed to control the ratio of polymerised to unpolymerised actin in the cytosol. Phosphatidyl inositol 3-kinase (PI3K) which like PLCγ is activated by recruitment to tyrosine phosphorylated integral membrane proteins is also known to be activated by fMLP and thrombin (Wennstrom et al., 1994), and this is claimed to be essential for PDGF-stimulated re-arrangement of actin filaments in fibroblasts and forms the basis for membrane ruffling and activation of the small G-protein rac.

1.5.2. Priming

Priming is a process that has stimulated much debate in recent years. The release of superoxide by neutrophils is a response to chemotactic agents, providing they are present at sufficiently high concentrations. If the cells are initially exposed to a low concentration of the agent, then the response pattern to the high concentration is modified and this modification is termed 'priming'. However there are two interpretations of the nature of priming. One is that priming increases the sensitivity of all of the cells of the population to the agonist and the second is that priming sensitises a large population which do not respond to a single exposure of a high concentration of agonist. One possible cellular mechanism is that sequential release of granules increases the concentration of agonist receptors and PHOX enzymes in the PM. It is possible that priming is achieved by the release of secretory vesicles to the surface of PMN, thereby activating the cellular machinery to produce superoxide, without actually stimulating the enzyme directly (Manara et al., 1991). Interestingly, cytochalasin B (which depolymerises actin filaments) is the most effective known priming agent, but its mechanism of action is not fully understood. The role of primary granules is central to the study of priming because unlike the other granules, they are not released in an unprimed cell. This suggests that components released to the cell surface by the early granules play a dominant role in priming for late granules. Ackerman and colleagues (1993) have shown that integrins arrive at the surface of adherent cells in discrete clusters, consistent with local vesicle fusion, One possible mechanism is that early vesicles which carry integrins also transport to the surface a component that is essential for the degranulation of primary vesicles. Because CD45 is not present on untreated cells, or is present at very low levels, and has a large number of potential targets, priming may be associated with its release to the plasma membrane.

Before degranulation of primary vesicles, there is an increase in the concentration of cGMP (Wyatt et al., 1993), and an activation of a kinase which phosphorylates

vimentin, facilitating the degranulation. It is of interest that this phosphorylation and the change in cGMP concentration does not happen to cells in suspension, and conversely cytochalasin B is not required for degranulation of adherent cells. As both cytochalasin B and the phosphorylation of vimentin are likely to cause cytoskeletal re-organisation, one or the other is probably required for degranulation.

1.5.3. Signalling by arachidonate metabolites

The neutrophil (and other myeloid cells) reinforces the initial signalling mechanisms by generating powerful signalling species derived from arachidonate. The most important of these are the prostaglandins and the leukotrienes which are derived from arachidonate by the actions of the enzymes cyclooxygenase and lipoxygenase respectively. Both types of molecules have very diverse and largely proinflammatory actions. Some prostaglandins are known to be hyperalgesic (dolor) and one of the leukotrienes, LTB_4, is a very potent activator and chemotactic agent for neutrophils. Thus generation of these lipid signals is one of the positive feedback loops in inflammation. Because the production of all of these species requires arachidonate to be mobilised from membrane phospholipid, much attention has been paid to the enzymes involved in this process. Most of the early evidence indicated that release was due to phospholipase A_2 (PLA_2) activity, but other pathways exist and there is evidence that mobilisation may require an initial cleavage by either PLC or phospholipase D (Zhou et al., 1993). The demonstration that lipocortin 1, produced in response to glucocorticoid, strongly inhibited arachidonate mobilisation suggested a firm link between steroid action and the production of arachidonate metabolites.

1.5.4. Summary

In summary there is a cascade of co-ordinated responses to a single signalling mechanism which produces cell polarisation, diapedesis, chemotaxis and phagocytosis

which involves a co-ordinated degranulation process in which this process itself contributes to the upregulation of signal receptors.

1.6. Morphology

1.6.1. Shape changes (polarisation)

In suspension PMNs are spherical with slight membrane ruffling but, when stimulated by chemotactic agents they elongate and develop a polar shape similar to that of attached cells (Coates et al., 1992). This is accompanied by an increase in volume of around 20% (Grinstein, 1986), around 2/3 of which is due to sodium influx. The degree of stimulation of motor activity is such that PMN become the fastest moving of all mammalian cell types.

1.6.1.1. Involvement of Actin

The earliest observed response is the uncapping of barbed ends of filaments which leads to a burst of nucleation activity near to stimulated receptors (Howard et al., 1990, Watts et al., 1991, Coates et al., 1992). The initial increase of F-actin is a symmetrical accumulation at the cell periphery, followed by development of asymmetry and finally by a change from spherical to polar (Watts et al., 1991). Stimulation of cells causes 60-80% of G-actin to polymerise, effectively doubling the quantity of F-actin (Cassimeris et al., 1992). It is quite clear that these changes involve stimulation of motor activity, but the organisation of motors within complex actin meshworks is poorly understood.

1.6.1.2. Bundling of Filaments

Elongating actin filaments are crosslinked to form networks and bundles. Condeelis (1993) has argued that the random nature of these arrays would prevent myosin mediated sliding causing the filaments to expand in a mode consistent with pseudopod

formation. Unphosphorylated MARCKS is known to crosslink actin networks at the membrane, but this is inhibited either by phosphorylation or by calmodulin binding (Aderem, 1992). This would provide a credible mechanism for modulating actin dynamics provided that the signal itself was periodic e.g. if cytosolic calcium levels oscillated in response to a persistent signal (Berridge, 1997).

1.6.2. Time course of responses

Up to 45 sec after stimulation with fMLP there are no observed changes in PMN morphology or in their distribution of actin; however by 90 sec the actin distribution is asymmetrical in 95% of the cells and by 120 sec most of the cells have developed an actin rich pseudopod which is much thinner than the cell body (Coates et al., 1992). The duration and extent of actin polymerisation is the major determinant of the degree of polarisation of PMNs (Watts et al., 1991). Initially the F-actin concentration is lowest in the pseudopod (suggesting that microtubules may be important for the early stages of pseudopod formation), but by 5 minutes polymerised actin is more concentrated in the pseudopod than in the cell body.

Colocalisation of F-actin and α-actinin at the front of a migrating neutrophil may favour the extension of pseudopods, the combination of F-actin and myosin at the tail may be the basis for contraction towards the tail and for forward cytoplasmic streaming; tubulin is all but absent at the tail of migrating PMN (Keller and Niggli, 1993).

1.6.3. Signal transduction affecting actin dynamics

Recent work has shown that rac and rho G-proteins play a vital role in the determination of morphology of actin polymerisation and hence of cell shape (Allen et al., 1997), although little of this work has been carried out with PMN.

PI3-kinase activation is essential for PDGF-stimulated re-arrangement of actin filaments that causes membrane ruffling (in fibroblasts); this enzyme has an SH2 domain which binds to phosphotyrosine in target proteins and thereby causes its activation (Wennstrom et al., 1994). Wortmannin inhibits both PI3-kinase activity and it also inhibits PDGF stimulated membrane ruffling in fibroblasts and the fMLP induced metabolic burst in PMN. rac1 has been implicated as a downstream event in this process (Wennstrom et al., 1994). Hence it seems plausible that the synthesis of PIP3 (and other PI3K products) may activate rac either directly or indirectly in these cells. Because rac is a small G-protein this regulation could be mediated via the guanine nucleotide exchange proteins (Wennstrom et al., 1994); Ridley & Hall (1992) have suggested that rac and rho may be involved in modulating PI turnover and, in this respect, it is interesting that the p85 subunit of PI3-kinase has a rhoGAP-like domain (stimulates hydrolysis of GTP).

1.6.4. Role of microtubules

Microtubules are not easily studied in the neutrophil, due to the small size and convoluted shape of the nucleus. However, a number of workers have produced evidence that microtubules have a role in the control of cell shape in PMNs.

Selective stabilisation of microtubules can generate microtubule-based cell asymmetry (Gunderson & Bulinski, 1988). Microtubules parallel to a chemotactic gradient elongate, with those perpendicular shorten (Anderson et al., 1982). Those microtubules which point to the leading edge may have been recently stabilised, consistent with an increasing number of microtubules being de-tyrosinated with time (Gunderson & Bulinski, 1988). However, there are still dynamic tyr-microtubules at leading edge, and this is consistent with a second messenger only effecting a small number of stabilising factors. Disruption of microtubules leads to a loss of directionality of movement, demonstrated by wide angles of turn. Selective stabilisation of microtubules in the

direction of migration would be important in maintaining directionality. Microtubules do not provide the vector of locomotion but rather stabilise and orient the 'baggage' (Malawista & De Boisfleury Chevance, 1982). Microtubules are important for accurate turning and for maintenance of cell shape and polarity of moving cell (Gunderson & Bulinski, 1988).

1.6.5. Interactions between Cytoskeletal Components

Microtubules can interact with actin and intermediate filaments, communicating polarity and asymmetry information to the rest of the cytoskeleton. Equally, actin networks can influence microtubules (Euteneur & Schliwa, 1985). Changes in the axis of cell polarity and direction of movement would have to involve highly orchestrated conditions which polymerise and depolymerise microtubules simultaneously within the same cell (Anderson et al., 1982). Microtubules do not set up polarity, but reinforce polar organisation set up by the cell cortex. Once the centrosome is set up behind the leading lamella it tends to exert a constraining influence on the cell's ability to turn (Euteneur & Schliwa, 1992). Cortical tension generated by the contraction of microfilaments might be opposed by microtubules and might maintain cell shape (Keller et al., 1984). Reorganisation of microtubules following fMLP may be a weak signal for activation of motile apparatus; co-ordinated interactions between actin, microtubules and intermediate filaments (Keller et al., 1984).

1.7. Cell motility

Because of the random nature of arrays, it is unlikely that myosin will be able to mediate sliding of filaments to expand the volume of networks to form a pseudopod (Condeelis, 1993). Myosin light chain kinase is phosphorylated when neutrophils are stimulated by fMLP. If there is a focal depolymerising of cortical actin then contraction of MLCK / F-actin would increase core pressure and squeeze actomysin dependent

flow of cytoplasm towards the weakened area of cortex (Anderson et al., 1982). The pseudopod therefore will form at the zone of least actin initially, followed by polymerisation in the end of the developing pseudopod.

A transient cAMP elevation may be indicative of microtubule reorganisation in general associated with chemotaxis but not in the pathway of signal transduction (Keller et al., 1984), as increasing cAMP inhibits chemotaxis (Downey et al., 1991). Activation of PKA is essential for changes in shape and motility, with a threshold level of cAMP required for activation of PKA (Downey et al., 1991). PKA phosphorylates MLCK, reducing its interaction with calmodulin and thereby interfering with the phosphorylation of myosin P-light chain which is essential for the activation of myosin (Downey et al., 1991). Inhibition of MLCK by phosphorylation, with a consequent loss of myosin activity destabilises the microfilament lattice and cause it to disassemble (Downey et al., 1991).

1.8. Phagocytic killing mechanisms and tissue injury

Killing by neutrophils is largely believed to occur in the phagolysosome. There is good evidence however that small quantities of the killing agents are released and can damage host tissues, over and above the damage produced by protease activity involved in neutrophil migration (Weissmann et al., 1972). The majority of tissue injury is caused by the release of oxidising agents (O_2^-, hydrogen peroxide, etc.) and digestive enzymes. The process of phagocytosis requires that internal granules fuse with the PM to form a phagosome which is then rapidly internalised. Thus exposure of the granule membrane and granule contents to the external medium is unavoidable.

1.9. The respiratory burst

The phagocytic killing mechanism which is most accessible to study is the generation of active oxygen species. This has largely come about as a consequence of the development of luminometry in which the reaction of active oxygen species with detector molecules is assayed by light emission, This is an extremely sensitive method which can be applied to intact cells. Although it might be advantageous for neutrophils to produce superoxide ions for some considerable length of time (probably hours rather then minutes), luminometry assays show that the response of neutrophils (in suspension) is characterised by a short burst of activity which decreases quite rapidly and production of superoxide which stops within a matter of minutes (Black et al., 1991). A more prolonged period of oxidative output to inactivate plasma components and to activate secreted enzymes would increase the destruction / killing of bacteria (Nathan, 1987).

1.9.1. The phagosome oxidase (PHOX)

The enzyme responsible for the respiratory burst is the NADPH oxidase located in the plasma membrane. The oxidase is formed by components arranged in an electron transport chain where the flavoprotein acts as NADPH dehydrogenase-cytochrome b reductase. Cytochrome b_{558} would finally reduce O_2 with formation of O_2^- (Rossi, 1986). Cytochrome b_{558} is the only membrane protein required for activity (Segal & Abo, 1993), most cytochromes only contain a single subunit (p21 phox). The β-subunit is a heavily glycosylated FAD-containing flavoprotein dehydrogenase (gp91phox).

During phagocytosis, only the enzyme on the portion of the membrane which is invaginated and forms the phagosome is activated. This allows the formation of oxygen compounds in a strategic position for killing of ingested organisms. Soluble stimuli

activate the oxidase throughout the plasma membrane. The activation is associated with a redistribution of the enzyme in and to the plasma membrane (Rossi, 1986), with superoxide only being produced by the membrane in contact with the article being phagocytosed. A lag period allows for the vacuole to be closed before the O_2^- is produced, and so prevents release of reduced oxygen to the exterior (Segal & Abo, 1993).

The first intermediate of oxygen produced is the superoxide ion which is released outside the cell or inside the phagosome. Superoxide is highly reactive, but unstable and undergoes spontaneous dismutation to form hydrogen peroxide. Eukaryotic cells and aerobic bacteria contain the enzyme superoxide which catalyses this reaction. However hydrogen peroxide is also damaging to cells and is itself removed by catalase or peroxidatic mechanisms (Rossi 1986). The killing action of superoxide is also greatly enhanced by a secondary mechanism. Neutrophil granules contain the enzyme myeloperoxidase (MPO) which catalyses the formation of hypochlorite (HOCl) from superoxide and chloride ions, (Vilim & Wilhelm, 1989). HOCl is one of the most powerful bactericidal agents known.

Respiratory burst termination factors limit PMNs ability to produce superoxide ions and so protect normal tissues from neutrophil derived oxy-radicals. Bacterial destruction would be enhanced and normal tissue necrosis be controlled by prolonging the oxidative burst (Black et al., 1991) providing that the burst is confined to a limited area.

Nathan and colleagues (1987) showed that the metabolic burst was greatly enhanced both in duration and magnitude of response when the neutrophils were adherent to surfaces coated with extracellular matrix components.

1.9.2. Effects of drugs on NADPH oxidase

Some NSAIDs decrease luminol dependent luminescence via direct action on MPO reactions which is reduced possibly by their anti-oxidant properties (Vilim & Wilhelm, 1989). Aspirin inhibits the production of prostaglandins (Vane, 1971), as do over 40 other NSAIDs in use. Aspirin inhibition of prostaglandin H2 synthesis (coxII) produces 15 hydroxyeicosatetraeinoic acid (HETE) which is metabolised to 14,15diHETE - a potent inhibitor of the metabolic burst (Cronstein & Weissmann, 1995). Proximity to prostaglandins released from endothelial cells may limit sequestration of PMNs and subsequent tissue injury away from the site of inflammation (Downey et al., 1991). All NSAIDs inhibit aggregation via CD11b/CD18. Some inhibit enzyme release /O_2^- (Cronstein & Weissmann, 1995). Sodium salicylate and piroxicam block pertussis toxin effects on aggregation / O_2^- inhibition, suggesting that the drug interferes with binding of toxins to the α-subunit of heterotrimeric G-proteins thereby blocking the formation of DAG that follows cell activation (Cronstein & Weissmann, 1995).

1.9.3. Cytoskeletal involvement

rac may serve to co-ordinate the NADPH oxidase with phagocytosis (in vivo) (Hall et al., 1993) involving changes in polymerised actin at the plasma membrane (not dissimilar to the observed ruffles in fibroblasts). Activation may involve the movement of: the cytochrome from specific granules, PKC, or flavoprotein from cytosol to PM (Rossi 1986), although the proportion of cytochrome b_{558} associated with the cytoskeleton does not alter with activation (remaining at 15%) (Woodman et al., 1991); cytochrome b_{558} is also located in specific granules which fuse with the plasma membrane replenishing stocks of this and other molecules when required (Segal & Abo, 1993).

p47phox is phosphorylated on a serine residue allowing activation (Hallett & Lloyds, 1995). and transportation to the plasma membrane, where it binds to cytochrome b_{558} (Segal & Abo, 1993), activating the oxidase. O_2^- production can take place in the absence of p47phox, and so therefore is not a component of oxidase as such (Woodman et al., 1991), but it is required for initial activation.

Extracellular factors that induce O_2^- also induce membrane ruffling and it is possible that rac proteins play a co-ordinating role in regulating both processes with different actin binding proteins being specifically recruited by rac and rho to the sites of actin polymerisation thereby dictating the localisation of the NADPH oxidase (Ridley & Hall, 1992). With three quarters of the oxidase components associated with the cytoskeleton, this would allow local activation of the oxidase in the cell rather than a stimulus producing a total cell response (Woodman et al., 1991). Activation of the oxidase is associated with movement of rac, unaccompanied by rhoGDI to the membranes. A complex of rho, p47phox and p67phox docks with the flavocytochrome inducing a conformational change favourable to electron transport (Segal & Abo, 1993).

More than 95% of the detectable O_2^- activity is associated with triton insoluble fractions and so most of O_2^- forming activity in PMA activated is associated with the cytoskeleton (Woodman et al., 1991).

1.9.4. Activation of the oxidase

The respiratory burst begins when 10-20% of fMLP receptors are occupied, the magnitude of the response depending on the rate of binding; continuous new interactions are required between receptor and agonist for maintaining the level of response (Rossi, 1986). PKC has a high affinity for PS containing membranes, and there is a marked increase in negatively charged phospholipids on the cell surface during the first three minutes of PMA stimulated activation (Tauber, 1987). As well as

NADPH being generated by HMP shunt, there is also one proton generated for each electron required to reduce oxygen. This proton is removed either via a sodium - proton antiporter (Tauber, 1987), or by a proton - lactic acid cotransporter (Simchowitz & Textor, 1992) which controls pH intravacuolarly.

1.10. Defensins

Defensins constitute more than 5% of the total PMN protein, and are antimicrobial and cytotoxic peptides that contain 29-35 amino acids, including 6 highly conserved cysteine residues that form 3 intramolecular disulphide bonds (Tamura et al., 1994). The abundance of defensins in specialised host defence cells of the blood and lungs suggest that defensins could play a role in innate immunity to infection (Kagan et al., 1994), perhaps being responsible for oxygen independent microbial killing (Yomogida et al., 1996), by forming voltage-regulated channels in the susceptible cell's membrane (Kagan et al., 1994; Wimley et al., 1994). Phagocytosed bacteria would encounter high concentrations of defensins (Ganz & Lehrer, 1994). At high concentration, defensin can remove the inhibitory effects of serum proteins on elastase and cathepsin G (Panyutich et al., 1995), as well as inactivating protease inhibitors such as $\alpha 1$ antitrypsin.

Defensins increase the expression of CD11b, CD11c and ICAM-1 on PMNs (Panyutich et al., 1995), but inhibit superoxide release during phagocytosis of opsonised zymosan, without altering the ability to bind the opsonised particle (Yomogida et al., 1996).

There is a leakage of defensins during phagocytosis (10-20% of total defensins) suggesting that there may be a modulatory function of PMNs behaviour at sites of inflammation (Yomogida et al., 1996). Lack of defensin transcription can be a cause of

CGD (more correctly referred to in this case as SGD - specific granule deficiency). (Tamura et al, 1994)

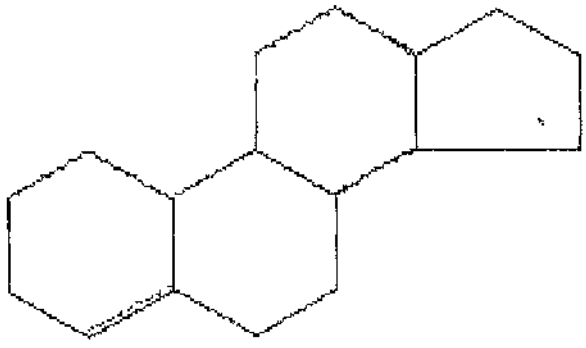
1.11. Chronic inflammation

Acute inflammation is almost always beneficial (as seen by the morbidity of inherited leucocyte adhesion molecule deficiency diseases and chronic granulomatous disease (where phagosome oxidase cannot be activated). However in many conditions inflammation does not stop once the injury has been resolved and inflammation is then said to be chronic. A positive feedback state is initiated whereby more PMNs / monocytes / macrophages are recruited and hyperactivity of these cells causes severe damage to connective tissue (Woodman et al., 1993), and may initiate a new immune response in its own right.

1.11.1. Control of inflammation

1.11.1.1. The role of steroids (corticosteroids / glucocorticoids)

Steroids have a four-ring core structure, the last of which is generally a five carbon ring.



Side-groups, and double bonds within the rings produce different effects, but as a rule, there is no relation between structure and function in steroids (Stryer).

Steroids fall into two main categories, sex steroids and cortico-steroids. Both can be produced by the adrenal cortex, but only cortico-steroids have been shown to produce PMS (Stevenson, 1976). The primary effects of steroids is on gene expression rather than enzyme activation or transport processes, with the site of action being the cell nucleus rather than the PM. Dexamethasone has a receptor in the cytosol which migrates to the nucleus after activation by dexamethasone binding, where it binds to a limited number of bases of DNA (Stryer).

Abundant evidence has accumulated to show that steroids of the class of glucocorticoids are major physiological down-regulators of inflammatory processes. First to be studied was cortisol itself, but many synthetic glucocorticoids have been synthesised and among these, hydrocortisone, dexamethasone and prednisolone have gained especial prominence. Arthritis and asthma are the best known examples of debilitating chronic inflammatory states. The initial discovery of the anti-inflammatory effects of cortisol lead to its widespread use in the treatment of arthritis. However the spectacular symptomatic relief produced by initial treatment was followed by horrendous side effects. Although steroidal drugs are of great value as topical inhaled drugs, systemic steroid treatment is fraught with hazardous side effects. One of the major efforts in inflammation research has been to identify the pathways of steroid action with the hope that the beneficial and harmful responses to steroids may be mediated by different intermediate molecular species. If this were the case then drugs might be devised that would mimic selected actions of steroids. However in the case of inflammation, steroids appeared to act by down regulating a battery of proinflammatory cytokines and the molecular control mechanisms do not indicate that these processes are separable. Thus the main thrust of drug development has been in the search for non-steroidal anti-inflammatory drugs, NSAIDs (reviewed below).

1.11.1.2. Steroid control of gene activity

Glucocorticoids have direct actions on gene expression mediated by a cytoplasmic receptor complex. A wide variety of transcription regulating mechanisms have been described, the best characterised involve the steroid receptor complex interacting with a regulatory domain (the GRE) or else acting by means of the transcription factors AP-1 and NF-κB. The outcome is that mechanisms exist for the up-regulation or down regulation of gene expression by glucocorticoids. In a given cell type however, it may not be possible to select for up-regulation or down-regulation by choice of different corticosteroid agonists. In many tissues a major effect of glucocorticoids is to upregulate the production of specific proteins. In the immune system however, the predominant action of steroids is to down-regulate protein synthesis. Thus the expression of the genes for IL-1, IL-2, IL-8 in monocytes and macrophages are all negatively regulated by dexamethasone.

1.11.1.3. Possible upregulation of gene activity by steroids

The possibility that steroids upregulate the activity of some genes and that the gene products might be positive anti-inflammatory mediators has been raised in several studies, but most notably by the work of Flower and co-workers. Following an early observation that serum from steroid treated rats inhibited the mobilisation of arachidonic acid in test animals, this group was able to isolate a polypeptide inhibitor of phospholipase A2 action. The protein, initially called lipomodulin or macrocortin (later termed lipocortin) was isolated from monocytes or macrophages (Flower & Blackwell, 1979; Russo-Marie et al., 1979). With the discovery of a family of related proteins this putative anti-inflammatory mediator was termed lipocortin 1. Eventually this family of proteins was termed the annexin family, based on their ability to bind to both actin and membranes in a calcium dependent manner, and so lipocortin 1 became annexin 1. In a paper that was seminal to the present study it was demonstrated that a nonapeptide

derived from annexin 1 contained the phospholipase A2 inhibitory activity of the molecule and was also anti-inflammatory in an animal model (Perretti et al., 1995). The status of annexin 1 as an in vivo phospholipase inhibitor was later called into question and it is now held that this is a consequence of non specific binding of the protein to phospholipid surfaces which acts as a steric block to a variety of enzymes, but only at non-physiologically high concentrations (Davidson et al., 1987). Nevertheless it is of interest that annexin 1, the only member of this family that has been reported to have anti-inflammatory activity is the only member to be unregulated by glucocorticoids and also to be secreted from a cytoplasmic pool. It is of further interest that secretion is by a non exocytotic route. The emphasis on the anti-inflammatory actions of annexin 1 has now shifted to studies of their effect on neutrophil / endothelial cell interactions and will be discussed below (Cronstein et al., 1992).

At about the same time that annexin 1 was first characterised as a putative anti-inflammatory product produced by myeloid cells, Stevenson showed that a factor secreted by monocytes and lymphocytes in the presence of glucocorticoids had a profound effect on neutrophil migration (Stevenson, 1974). and was initially termed Polymorph Migration Stimulator. Production of this factor required protein synthesis and the activity was sensitive to protease action. There was some difficulty in ascribing anti-inflammatory properties to a factor that stimulated neutrophil locomotion. This work was later resumed by Chettibi et al in an attempt to see whether or not the activities of this preparation could be attributed to the presence of annexin 1. The factor, now referred to as STMS (steroid treated monocyte supernatant) (Chettibi et al., 1993) was shown to confer dispersive motility to the PMNs which appear to migrate across protein coated glass in an unusual and highly distinctive manner. The cells (when exposed to a uniform concentration of STMS) travel in apparently extended pathways. This is in contrast to the random walk induced by fMLP where the intervals (distances) between turning are very much shorter, but resemble the migratory pattern seen when PMNs migrate in a concentration gradient of fMLP. There has been some difficulty in

carrying out simultaneous studies to see whether the effects of STMS can be reproduced by pure annexin 1, but the balance of current evidence indicates that these two molecules are unrelated. Young et al. (1997) have shown that STMS can inhibit the migration of neutrophils in a gradient of fMLP (Boyden chamber assays). This is of potential therapeutic importance as the cells may not be able to respond to other environmental cues and so be incapable of participating in inflammation. The potential side-effect of losing some immunity would have to be monitored in a clinical situation.

Induction of proteins and peptides by glucocorticoids is due to the interaction of glucocorticoid receptors with c-Jun and c-Fos instead of those with cytokine inducing promoters. The upregulatory mechanisms (AP1, NF-kB) are distinct from those which down-regulate cytokine production. This facilitates anti-inflammatory transcription, but not pro-inflammatory (Cato & Wade, 1996). One such protein whose synthesis is inhibited by the presence of steroids is macrophage chemotactic activating factor. This 7 kD protein is normally produced in response to IL-1 or TNFα, but incubation with dexamethasone inhibits its transcription (Mukaida et al., 1991). Flower has suggested that the anti-inflammatory effects of steroids are mediated in monocytes and macrophages primarily through the induction of the synthesis of lipocortin I (annexin I). This protein has been sequenced and cloned, but recombinant lipocortin is not effective against oedema formation. The anti-inflammatory effects of dexamethasone can be suppressed by inhibitors of mRNA synthesis and/or steroid receptors; as visualised by lack of induction of lipocortin synthesis (Peers & Flower, 1990).

It is known that steroids produce changes in the behaviour of monocytes, macrophages and PMN *in vivo*, but these changes all but disappear when the effects of steroids are investigated *in vitro*. For example steroids inhibit the adhesion of neutrophils to endothelium in vivo, but this effect is lost when in vitro experiments are performed (Chettibi et al., 1994). Stevenson (1974) postulated that this effect is mediated by

monocytes / macrophages, and that there is a substance produced by monocytes in the presence of steroid that alters the behaviour of neutrophils (which may be lipocortin).

Since hydrocortisone was first used by Hench et al. in clinical medicine (1948) there has been considerable research into the biochemistry and biology of this class of drugs. In the field of inflammation, the glucocorticoids especially have come under close investigation.

1.11.1.4. Investigation of the anti-inflammatory effects of steroids

In vivo experiments studying effects of steroids on inflammation have, in many cases, used animal models. These follow the traditional physicians' "Rule of Thumb" by investigating the four rumors; calor, rubor, dolor & turgor. The assays for these involve rectal temperature monitoring, the analysis of the rabbit ear patch assay, and the effect the patients report, i.e. pain. More commonly, however, assays involve the inhibition of inducible oedema. This involves the injection of a known antigen under the skin of the animal and harvesting those cells induced to migrate into the peritoneum later. Initially (after 3 hours) most cells observed in the induced peritoneum are neutrophils but by 24 hours this has become predominantly macrophages, i.e. there is an influx of neutrophils before macrophages (Davies & Thomson, 1975). Addition of steroid 24 hours before the addition of antigen, inhibits the number of cells and fluid that extravasate (by about 50%), but differential cell counts do not alter; the proportion of each cell type remains constant. It is possible that if neutrophil extravasation could be completely inhibited then there would be no secondary wave of macrophages.

When steroid is administered before the agonist, it has been observed that small dyes do not accumulate in the induced œdema (Davies & Thompson, 1975). This was initially thought to be due to hindering the transfer of cells from the blood to the extra-cellular space, but the model was discounted because cell migration is inhibited more

slowly than dye leakage, the opposite of expectation if 'holes' in the endothelium were to be closed over a period of time. However, if the endothelium was to remain pro-adhesive for blood cells, then they would continue to attempt to extravasate, even in the presence of steroid. Cronstein (1992) showed that steroid treatment inhibits endothelial transcription of ICAM-1 and E-selectin, thus reducing the adhesiveness of the endothelium. However, Youssef et al. have more recently shown (1996) that treatment with steroid, although reducing ICAM-1 and E-selectin have no effect on the levels of PECAM-1, a cell adhesion molecule required for transendothelial migration (Vaparciyan et al., 1993, Muller et al., 1993). NSAID treatment also has no effect on the levels of PECAM-1 expression, nor on PMN adhesion to endothelium (Gonzalez-Alvaro, et al., 1996, Pellegatta, et al., 1996).

There is a decisive role for PECAM (CD31) during the inflammatory response, with the distribution of PECAM on the endothelium altered. Endothelial cells grown in culture have a uniform level of PECAM over most of the cell surface. However, when confluency is reached there is a redistribution of PECAM to the cell borders where it is thought to participate in the tight junctions between endothelial cells (Bevilaqua, 1993). As described above (1.2.2) pro-inflammatory mediators can induce retraction of endothelial monolayers (Kaplanski et al., 1994, Bradley et al., 1995), and binding of neutrophils to the endothelium will also cause changes in endothelial conformation (Delmaschio et al., 1996). This change in conformation will release unbound PECAM to the surface of the endothelium, which can then be bound by leukocytes (both neutrophils and monocytes bind to PECAM through homotypic adhesion (Luscinskas & Lawler, 1994)). Indeed, this binding to PECAM will increase the binding of CD11b/CD18 (Berman & Muller, 1995), possibly by phosphorylation of the integrin (Pardi et al., 1992).

In vitro experiments examining the permeability barrier of the endothelium often utilise Boyden Chambers, but normally only single cell types are used. These experiments

mirror the in vivo experimental results, i.e. steroid therapy inhibits the movement of neutrophils through an endothelial monolayer grown on filters (Ward 1966 - inhibition of chemotaxis by 50%); Mowat & Baum (1971) and Rinehart et al (1974) say there is no effect in vitro. Merkow et al (1971) showed that steroid treatment in vivo does not inhibit the phagocytosis of bacteria, but the subsequent degranulation into the phagolysosome is inhibited.

1.11.1.5. Possible effect of steroids on vasculature

In 1956 Germuth postulated that all the effects of steroids could be explained by effects on the vasculature (Germuth, 1956), in accord with the vasoconstrictive effects of steroids on the blood vessels (Wyman et al., 1954), and inhibition of vasodilation in inflamed veins (Ebert & Wissler, 1951; Allison et al., 1955). There is also tightening of the junctions between endothelial cells when treated with steroids.

1.11.1.6. Side-effects of steroids

Side effects of steroids include retention of water and sodium, excretion of potassium, loss of mineral from bone leading to osteoporosis and fractures, hypertension, personality changes, gastric ulcers and thinning of the skin. (Cato & Wade, 1996). Also, patients with chronic rheumatoid arthritis and systemic lupus erythomatosus have antibodies (IgG and IgM) to annexin 1, especially if oral steroid has been administered. Oral administration of steroid may lead to steroid resistance, and there are also groups of people who possess innate resistance to steroids. It would appear that ingesting steroid orally is not the best long term solution to a problem, and as many people have an aversion to needles, other drugs have been developed.

1.12. Non-steroidal anti-inflammatory drugs (NSAIDs)

1.12.1. Aspirin

NSAIDs became an analgesic / anti-pyretic therapy in the early 1900s (40 years before steroids were first used clinically). The best known and most widely used NSAID is aspirin (acetylsalicyclic acid), first synthesised by Felix Hoffman in 1897. John Vane in 1971 showed that the anti-inflammatory effect of aspirin was due to inhibition of production of prostaglandins and thromboxanes. Aspirin has been described as being as close to a wonder drug as possible (Medical Sciences Bulletin, published by the pharmaceutical information associates limited), and yet it is probable that this compound would not be allowed to be released as a drug today because of its many and often dangerous side-effects (J. Lackie, personal communication). Vane's work (1971), showing that NSAIDs (with the exception of salicylates, (Cronstein & Weissman, 1995) inhibit prostaglandin synthesis, explains some of the side effects as prostaglandin synthesis by the endothelium inhibits neutrophil adhesion; inhibiting the release of prostaglandin by the endothelium induces neutrophil adhesion. Aspirin (and its derivatives) destroy Vitamin C, which is required to form strong collagen bands between cells, including those in blood vessels. It is possible that long term use of even low dose aspirin could cause subarachnoid haemorrhage by causing the formation of weak blood vessel walls. This results in increased incidence of strokes in patients who use aspirin for pain relief or blood thinning. At low doses aspirin inhibits platelet aggregation and interferes with blood clotting; in relatively large doses, aspirin reduces pain and inflammation in rheumatoid arthritis and several related diseases. (Medical Sciences Bulletin)

Although aspirin is good for the relief of pain, the analgesic effects must be weighed up against the potential risks. Some possible side-effects associated with aspirin are stomach irritation, gastrointestinal bleeding and reduced ability to form blood clots (hence its use as a blood thinning agent). Therefore, aspirin is "not recommended" for people with stomach ulcers (although applying a coating to the aspirin can reduce stomach irritation), certain liver problems, gout or bleeding disorders. Aspirin has been

associated with Reye's Syndrome in children using aspirin as an anti-pyretic. It is mainly because of these side-effects that derivatives of aspirin have had to be developed.

1.12.2. Aspirin derivatives / alternatives

Acetaminophen is an aspirin alternative which has a comparable analgesic value to aspirin but lacks the anti-inflammatory effect. The lack of an anti-inflammatory component removes some of the more serious side-effects associated with aspirin such as gastrointestinal bleeding and decreased ability to form blood clots and certain liver diseases. Therefore, acetaminophen can be used by persons with ulcers and bleeding disorders, but is of no long term benefit if the source of discomfort is inflammation (treating the symptom - pain, rather than the source of the symptom). Acetaminophen and its brand names was the leading drug sold in the US in 1995.

Ibuprofen is an aspirin alternative which has both analgesic and anti-inflammatory effects. As a result the side effects associated with Ibuprofen are similar to those associated with aspirin: stomach irritation, gastrointestinal bleeding and reduced ability to form blood clots. Like aspirin, it is not recommenced that people with stomach ulcers, gout or bleeding disorders take Ibuprofen.

Known side-effects of anti-inflammatory NSAIDs indicate aspirin-like problems, even for non-aspirin derivatives. This is a major problem, as many people are intolerant of aspirin, and asthma sufferers (among the largest users of steroids) are "not advised" to take steroids by the US Food & Drug Administration. As prolonged use of steroids can lead to resistance, and NSAIDs can cause gastro-intestinal ulcers and bleeding safer, more natural products could present an answer, although these may have their own side-effects.

1.12.3. Effects of NSAIDs on PMNs

Indomethacin and diclofenac have been shown to induce gastric ulcers, possibly due to an elevation of TNFα causing increased neutrophil adhesion. (Appleyard et al., 1996; Diaz-Gonzalez et al., 1995) although steroids prevent this damage. Indeed other NSAIDs (e.g. piroxicam) activate circulating neutrophils (Avila et al., 1996) and induce release of primary granule contents (as assayed by MPO activity) in vivo, although they induce lower levels of L-selectin shedding, an indicator of cellular activation (Diaz-Gonzalez et al., 1995). Wallace et al. (1993) showed that most of this damage could be prevented by blocking adhesion receptors on the endothelium, and postulated that a therapy to prevent ulceration could include a blocking of the interaction of neutrophils with the endothelium. Cronstein et al. (1994) showed that sodium salicylate inhibits induced adhesion of neutrophils to endothelium without altering basal levels (due to oxidative phosphorylation of ATP to adenosine and its subsequent release). This also showed that different NSAIDs operate in different manners, as indomethacin and piroxicam induced decreases in adhesion could not be reversed by removing adenosine.

Most NSAIDs tested have no effect on the release of secondary granule contents, however there are exceptions, e.g. Tenidap (Blackburn et al., 1991). More common are inhibitors of the primary vesicle degranulation.

1.12.4. Natural alternatives to steroids

It has been shown by Flower's group that one of the effects of steroids on monocytes is the induction of the synthesis and subsequent release of a class of compounds referred to as lipocortins (annexins) (Flower & Blackwell, 1979). These have many of the anti-inflammatory effects of steroids, e.g. the suppression of oedema formation under certain circumstances (e.g. carrageenin induced) but not others (e.g. dextran induced). Therefore it is probable that lipocortin cannot be the only mediator of steroid

action, especially as lipocortin has no effect on the adhesion of neutrophils to cytokine activated endothelium.

1.12.4.1. Lipocortin

The initial proposal that lipocortin-1 mediates the anti-inflammatory actions of corticosteroids by inhibition of arachidonate mobilisation has never been satisfactorily substantiated or refuted. However there is some evidence that lipocortin-1 mediates the effects of steroids on eicosanoid synthesis rather than release. Steroids and lipocortin (at mg/ml quantities in vitro) both inhibit the release of thromboxane A_2 when stimulated with fMLP or LTC4, but not with AA nor bradykinin (due to the site of lipocortin action being PLA_2 (Peers & Flower, 1990)). It is thought however that steroids act directly on the cyclooxygenase / lipoxygenase enzymes and so inhibit the synthesis of AA metabolites more directly (Masferrer et al., 1990), by inhibiting the expression (at the mRNA level) of cyclooxygenase but possibly not lipoxygenase (Cato & Wade, 1996). Davidson & Dennis (1989) gave evidence that lipocortin may act by sequestering the substrate rather than inhibiting the enzyme. PLA2 is capable of removing arachidonic acid from membrane phospholipids and as cyclooxygenase requires this as a substrate then there can be no activity of this enzyme. Recent papers from Flower's group have concentrated on an apparently unrelated action of lipocortin-1 as an inhibitor of diapedesis (Getting et al., 1997; Tailor et al., 1997). In vivo studies of neutrophil dynamics in the air pouch system show that steroids do not inhibit neutrophil rolling or strong adhesion, but prevent diapedesis. This activity is abolished by the administration of antibodies to lipocortin-I.

1.12.4.2. Other natural factors

A 2 kD peptide was described by Koltai that was produced by macrophages in the presence of steroids, but very little has been published on this since. Small peptides

with anti-inflammatory properties are increasingly being referred to as antiflammins (Lloret & Moreno, 1994). Also Hamann et al. (1995) published an account of a factor released from macrophages derived from dexamethasone treated monocytes. This 78 kD protein has similar heat labile and proteolysis susceptible properties as PMS, but is many times larger.

Indomethacin, another NSAID, has similar properties to lipocortin (both inhibit eicosanoid release and oedema by about 2/3). Both are ineffective against PAF, serotonin and dextran. Koltai et al (1987) partially purified a steroid induced 2 kD factor from macrophages which inhibited dextran induced oedema but not carageenin induced (lipocortin is effective against carageenin induced oedema). Steroids are effective against both of these agonists. At present to mimic the effects of steroids a number of drugs / factors would have to be administered simultaneously, with unknown side-effects from cross reactivity. Indeed, it is recommended that no more than one NSAID be taken at any one time. As no individual NSAID gives the range of anti-inflammatory and anti-pyretic effects as steroids, then the patient is still going to be in some discomfort.

1.12.4.3. Adenosine

Cronstein has reviewed (1994) the role of adenosine in the inhibition of phagocytosis, adhesion and generation of oxygen metabolites, but not of degranulation or chemotaxis. That superoxide production is inhibited only if stimulated by a chemotactic factor (e.g. fMLP, C5a) but not by PMA suggests that adenosine acts by extracellular blockade, possibly involving an inhibition of priming. Firestein et al. (1995) show that inhibition of endothelial adenosine kinase inhibits selectin-based adhesion (important in priming). Adenosine may act by interfering with the interaction of E and L-selectin (as demonstrated by removal of sialic acid from the endothelium and pre-shedding of L-selectin from PMN).

It has been suggested that adenosine acts via decrease of CD11b induction (Cronstein & Weissman 1995), but this may be unlikely as initialisation of adhesion is due to selectins (Kansas et al., 1993), not the release of integrins to the surface. Possibly of greater effect is that LPS induces an increase in the level of cyclo-oxygenase enzyme, but dexamethasone inhibits this increase (Masferrer et al, 1990).

Endothelial cells have been shown to be less adhesive for PMN when cultured in the presence of steroids (Cronstein et al, 1992), with the effect being on the transcriptional control of E-selectin. Therefore when endothelial cells are challenged with a pro-inflammatory stimulus there is less E-selectin on the surface for PMN to adhere to.

1.12.4.4. Nitric oxide

Nitric Oxide is produced by a variety of cell types, with PMN and platelets being among the cell types with the enzyme responsible (NOS) being constitutively activated (reviewed by Laskin & Pendino, 1995). Conversely macrophage NOS requires to be activated, and has been intimated as being involved in certain inducible oedemas, notably carageenin. NO reacts with O_2^- to produce peroxynitrite which is a longer lived cytotoxic oxidant. If the NADPH oxidase could be switched off, then it would be considerably more difficult to produce, and so tissue injury could be reduced. As O_2^- levels are reduced further by NO (forming the longer lived, but less cytotoxic peroxynitrite) it becomes apparent that control of the NADPH oxidase is of paramount importance. If there is little NADPH, then little NO can be produced, which cannot then react with O_2^-; the O_2^- having already been produced by removing NADPH. Therefore a middle ground has to be reached: as NO requires NADPH as does O_2^-, then making one molecule - peroxynitrite - on a molar basis must be a good way to reduce tissue oxidants (Laskin & Pendino, 1995).

1.13. STMS

STMS (Steroid Treated Monocyte Supernatant) was first described by Stevenson in 1974, who called it Polymorph Migration Stimulator (PMS). At this stage, the major pro-inflammatory cytokines had not been characterised, but later work revealed that the major effect of corticosteroids was to inhibit the production of proinflammatory cytokines, especially the chemokine IL-8. In addition, the logic of an anti-inflammatory agent that stimulated neutrophil locomotion was not clear. This work was subsequently resumed by Stevenson and collaborators who were able to show that the effects of STMS on neutrophil migration could not be attributed to the absence of proinflammatory agents found in control monocyte supernatant medium (CMS) (Chettibi et al. 1993). Because the culture conditions used for production of STMS closely resembled those used for the production of lipocortin-I, tests were undertaken to determine whether or not STMS activity was an attribute of lipocortin-1. Antibody blocking experiments proved to be negative, but in the absence of a pure sample of lipocortin-1 no conclusive demonstration of similarity or difference has been possible.

Stevenson (1974) observed that neutrophils contained in a small open-ended capillary tube placed in a uniform concentration of this factor moved outwards to form a broad, weakly cohesive migration 'slug'. In contrast, migration in CMS or in control culture medium was far less extensive and the migration 'slug' was highly cohesive. These capillary migration assays required a large number of neutrophils and were both labour intensive and time-consuming. Chettibi et al, (1995) used the cell tracking procedure of Dow et al. (1987) in an attempt to simulate the results of the capillary migration assay from locomotion data for individual cells. The initial results, using a frame interval of 20 seconds, confirmed that STMS stimulated apparent cell speed in comparison with CMS or control medium. However it was unable to distinguish between elevated migration speed and elevated cell persistence. This issue was resolved by the development of a high speed tracking system able to follow 80 cells at 5 second frame

intervals. The results showed very clearly that fMLP, IL-8, PAF, STMS and CMS all enhanced neutrophil locomotion, but with very different results depending on the frame interval. Thus concentrations which gave the same migration speed at 5 second intervals gave very different values for larger time intervals, clearly relating to the persistence of locomotion, as indicated by track linearity. Thus it became clear that in a uniform concentration of these agents, long-term migration in response to STMS far exceeded that in response to the other factors. These results were subjected to the classical analysis procedure of Gail and Boone (1971) to derive the parameters of speed and persistence for each cell and the parameter analogous to the diffusion coefficient which described the spreading of the cell pellet; the action of STMS was characterised as the induction of highly dispersive neutrophil locomotion. Paradoxically, the effects of a uniform concentration of STMS on neutrophil locomotion closely resemble those of fMLP acting in a concentration gradient.

1.13.1. The function of STMS

One of the perceived weaknesses of the capillary locomotion assay was that it failed to distinguish between the effects of enhanced locomotion and enhanced retardation by increased adhesion of the cells (J Lackie, personal communication). Exactly the same criticism applied to the modified tracking assay, but in an attempt to minimise population drift which could readily dominate the determination of an apparent population diffusion coefficient Chettibi et al. (1994) perceived that STMS enhanced locomotion was extremely sensitive to the tilting of the migration chamber, showing that the cells were very weakly adhesive. Preliminary experiments also showed that STMS lowered the adhesiveness of neutrophils to albumin-coated glass. STMS therefore acts as a direct, but not particularly powerful, stimulator of neutrophil locomotion, and an apparently non-specific inhibitor of cell adhesion. No model for a mechanism whereby reduced adhesion induces a highly dispersive type of locomotion has been presented.

Although stimulation of locomotion is not an anticipated anti-inflammatory response, non-specific lowering of adhesion would be a powerful inhibitor of neutrophil margination. In addition STMS could cause neutrophils to move away from a tissue site and thus act as an agent for termination of localised neutrophil activity.

1.13.2. Effects of STMS on the cytoskeleton

Stevenson (Thesis, 1978) argued that many of the effects of STMS could be attributed to disruption of microtubules (MTs) mediated by elevation of cAMP concentration. In adherent cells MFs extend from the periphery to a zone of MTs near the centre (Reaven & Axline, 1973) with MFs prominent near the focal adhesions. Bhisey & Freed (1971) showed that MT disrupting agents such as colchicine caused a change in motility from a smooth gliding to a more amoeboid, jerky motility. The tilt assay confirmed that STMS caused loss of adhesion (Chettibi et al., 1994a), but, uniquely, of the substances tested, caused PMNs to have a high diffusion coefficient, albeit always travelling down the slope. MT inhibitors reduce PMN adhesion (Lackie, 1974), and Gail & Boone (1971) observed neutrophils rounding up in the presence of colchicine. In contrast to this, Conry and co-workers (1992) found that disruption of microtubules induces polarisation of neutrophils in suspension. Throughout these assays, the STMS-treated neutrophils have a characteristic morphology when viewed with phase-contrast optics, being elongated and predominantly phase bright (essential for the tracking assay of Dow et al., 1987). This compares with the appearance of the control supernatant treatment where many of the cells are phase dark, and so not capable of being tracked. This does not affect the results unduly, because those cells that are phase dark are virtually non-motile.

1.13.3. The structure of STMS

The chemical nature of STMS has not been elucidated. Stevenson's data indicated that STMS was a peptide and possibly a cleavage product of a larger protein. He showed that the production of STMS could be inhibited using inhibitors of protein synthesis (cyclohexamide) and also that the activity was sensitive to protease attack. Gel filtration data suggested an MW >10kD. In contrast the active material was included by small-pore gel filtration media, indicating an MW of around 1-2 kD (Chettibi, personal communication). Observations of the behaviour on reverse phase HPLC indicated that the active particle was not especially hydrophobic and it was not absorbed by phenyl sepharose at high salt concentration. Similarly the activity was not absorbed by concanavalin-A derivatised sepharose, suggesting that it was not heavily glycosylated.

With the possible exception of heparin, which has been shown to have small levels of activity (heparin size 910, 1540 mw approx.), biologically active oligosaccharide containing molecules tend to be high MW species, (e.g. 150,000 kD for glycosaminoglycans) and are also, on the whole, pro-inflammatory. Because STMS reduces cell adhesion (the changes in actin being due to a reduced number of functional focal contacts) a short oligosaccharide could act by a simple blockade mechanism. For example it could interact with the L-selectin receptor in which case it could produce similar anti-inflammatory effects to those observed in the LAD_2 mutants (absence of functional L-selectin -Etzioni et al., 1993) or with antibodies to selectins (Kaplanski et al., 1994) or short peptides (Li et al., 1993, 1995).

Aminosugars have also been recorded to have potential anti-inflammatory effects on neutrophils. Kamel et al. (1991) reported that N-acetyl-galactosamine and N-acetyl-glucosamine inhibit the release of elastase, and inhibit the respiratory burst. However, little research has been carried out since then in this area, possibly due to the effects being due to a quenching of enzymes released during degranulation, notably N-acetyl-

glucosaminidase. This may explain why this aminosugar was less potent at inhibiting the activation of neutrophils.

Because STMS is not hydrophobic it seems improbable that it will be a lipid derivative. Nevertheless it is known that 12,15 (S)-diHETE is an inhibitor of neutrophil adhesion to IL-1β activated endothelium. This anti-inflammatory lipid also reduces actin polymerisation within the cell (Takata et al., 1994). HETE is produced by the action of cyclooxygenase and lipoxygenase, which also enhances production of many pro-inflammatory mediators such as leukotriencs, and also of prostaglandins most of which are pro-inflammatory, e.g. prostaglandin E2 (Talpain et al., 1995). HETE functions by becoming incorporated into the cell membrane by esterification, and so should be removable from the medium by adding cells to it. There is a considerable body of evidence pointing towards LPA being a modulator of neutrophil function (Chettibi et al., 1994b, Eichholtz et al., 1993), but motility assays of this lipid indicate that in contrast to STMS it does not induce dispersive motility. Moolenaar's review (1994) indicates that most of the effects of LPA are pro-inflammatory.

1.13.4. Purification of STMS

That very little was known of the chemistry of STMS meant the only reliable assay for the presence of the molecule in extracts was the determination of a diffusion coefficient by using the modified cell tracking program. Various techniques were attempted in the purification STMS including filtration and ion exchange. Stevenson had previously shown that PMS was a protein actively synthesised (production is inhibited by adding cyclohexamide) by monocytes and macrophages in the presence of steroid, with an apparent molecular weight of 12kD to 15kD. The biological properties of PMS could be removed by heat treatment, and also by proteolysis (Stevenson, M.D. Thesis, 1978).

Very few assays had been attempted using partially purified material to determine the breadth of biological activity of STMS as the purification procedure was still evolving. Reverse phase HPLC (SMART System, Pharmacia) and subsequent size based separation (Pharmacia peptide column) allowed purification to a previously unsurpassed level. Problems still existed with the purification, especially getting high enough quantities to be able to determine the amino acid composition and sequence. One pint of fresh human blood provides less than 0.1µg of sample. The cell tracking assay does not follow a 'classic' dose-response curve, and so quantification of sample at early stages was virtually impossible. Stevenson referred (Thesis, 1978) to high density mixed leukocyte cultures as producing PMS with 'high potency', but even these samples lose activity after diluting by any more than four fold (personal communication).

MATERIALS AND METHODS

2.1. Materials

A variety of salines were used throughout the course of this work (PBS, BSS, IIS, HH), depending on the cell type, the assay being used, and the anticoagulant in the blood.

2.1.1. PBS (Phosphate Buffered Saline)

8g Sodium Chloride, 0.2g Potassium Chloride, 1.15g Disodium Hydrogen Orthophospate and 0.2g Potassium Dihydrogen Orthophosphate were dissolved in water, the pH adjusted to 7.4 and made up to 1L before sterile filtering into aliquots.

2.1.2. BSS (Balanced Salt Solution)

8g Sodium Chloride, 0.4g Potassium Chloride, 1g Glucose, 0.2g Magnesium Chloride ($6H_2O$), 0.2g Magnesium Sulphate ($7H_2O$), 0.14g Calcium Chloride, 0.06g Potassium Dihydrogen Orthophosphate and 0.24g Disodium Hydrogen Orthophosphate were dissolved in water, the pH adjusted to 7.4 and sterile filtered into aliquots before storing at 4°C.

2.1.2. HS (HEPES Saline)

2.38g HEPES, 1g Glucose, 8g Sodium Chloride and 0.4g Potassium Chloride were dissolved in water, the pH adjusted to 7.4 and sterile filtered into aliquots before storing at 4°C.

2.1.3. HH (Hanks' HEPES)

As HS but containing 1mM Calcium Chloride and Magnesium Chloride.

2.1.4. HO (HEPES Water)

5.25g HEPES were dissolved in water, the pH adjusted to 7.4 and dispensed into aliquots before autoclaving and storing at 4°C.

2.1.5. Antibiotics

A solution of L-Glutamine (29.2g/L) was made up in water, and sterile filtered. A solution of Streptomycin Sulphate (76.2mg/L) was also made up in water. 150ml (200mM) Glutamine solution, 100ml Streptomycin Sulphate and 12.5ml Amphoterisin B were mixed, aliquoted asceptically and kept frozen until use.

2.1.6. Ham's F10 Culture Medium

180 ml HO, 1ml 7.5% Bicarbonate solution, 5ml Antibiotic, 20µl Steroid (10^{-2}M Dexamethasone), 20ml Foetal Calf Serum asceptically added to a bottle and mixed gently. For monocyte culture, Ham's F10 was also made up without serum for final stage of production of STMS.

2.1.6.1. Trypsin / Versene

Versene was made up essentially as for HS (2.1.2) except that 0.2g / L EDTA was added before adjusting the pH to 7.5. The versene was then dispensed into 20ml aliquots prior to autoclaving and storage at 4°C. Immediately before use, 0.5ml of sterile 2.5% solution of trypsin (Gibco) was added to the versene and mixed.

2.1.7. PAGE

Electrophoresis Buffers

2.1.7.1. Upper Tank Buffer x5

31.6g Tris Base (Tris[Hydroxymethyl]aminomethane), 20g Glycine and 5g Sodium Dodecylsulphate (SDS) were dissolved in water, and made up to 1 litre with distilled water.

2.1.7.2. Lower Tank Buffer

60.5g Tris Base and 5g SDS were dissolved in water, the pH adjusted to 8.1 and made up to 1 litre with distilled water.

2.1.7.3. Running gel buffer

18.15g Tris Base and 0.4g SDS were dissolved in water, the pH adjusted to 8.9 and made up to 100ml with distilled water.

2.1.7.4. Stacking Gel Buffer

5.9g Tris and 0.4g SDS were dissolved in water, the pH adjusted to 6.7 and made up to 100ml with distilled water.

Electrophoresis

7.5% polyacrylamide gels (12ml of 28.5% acrylamide solution / 1.5% bis-acrylamide, 12ml running gel buffer, 24ml distilled water, 20µl TEMED, 150µl 1% ammonium persulphate) were freshly prepared with a 5% stacking gel (4ml 28.5% acrylamide / 1.5% bis-acrylamide solution, 6ml stacking gel buffer, 14ml distilled water, 20µl TEMED, 200µl 1% ammonium persulphate). The samples were prepared in boiling mix (2.5ml stacking gel buffer, 0.5ml Mercaptoethanol, 1g Lauryl Sulphate, 0.5ml Glycerol, 1% Bromophenol Blue made up to 10ml with distilled water) , applied to the gels and run at 25 mA. The gels were then prepared for silver staining, by washing in destain (5% methanol, 10% acetic acid in distilled water) for 30 minutes before fixing in 10% Gluteraldehyde (Aldrich) for a further 30 minutes. The gels were then washed overnight in distilled water (3 changes of water) and stained (19% silver nitrate (Sigma), 4ml Ammonia, 21ml 0.36% NaOH, 46ml distilled water). Three washes of distilled water over a ten minute period were applied and the gel developed using formaldehyde, citric acid. The developing was stopped using Amfix (diluted 1:3 v/v with water) for 1 minute and the gel washed thoroughly before photographing.

2.2. Cell Isolation & Culture

2.2.1. Preparation of Polymorphonuclear Leukocytes

Venous blood was collected over heparin (25 units /ml blood), and red blood cells were sedimented with 20% v/v of a 5% w/v solution of dextran 500 (Sigma) for 30 minutes.

The leucocyte-rich upper layer was aspirated off and carefully applied over an equal volume of Nycoprep 1.077 (Nycomed) and centrifuged at 800g for 15 minutes. The upper plasma, mononuclear cell and Nycoprep layers were removed and discarded. The pellet was then subjected to brief hypotonic lysis to remove contaminating erythrocytes. The yield from this procedure was 5 x 10^5 cells per ml of blood and the purity of the population, checked by microscopy was > 90%. Viability was routinely assayed by trypan blue exclusion, with viability generally greater than 95%.

2.2.2. Culture of Monocytes. The production of STMS and CMS

2.2.2.1. Preparation of monocytes from whole blood

Whole blood was sedimented with 5% dextran solution as per PMN purification, with the monocyte layer (fig. 2) being removed and washed twice. To remove contaminating lymphocytes and platelets the cells were cultured in Ham's F10 supplemented with 5% foetal calf serum (Both Gibco) for 1 hour at 37°C to allow monocytes to adhere to the surface of the culture vessel. The non-adherent lymphocytes and platelets were removed by three washes with HS. Initially, the adherent monocytes were then cultured in F10 medium containing 5% FCS for a further 24 hours. Subsequently, the culturing was carried out in serum free medium to facilitate purification of the active factor. In a recent modification this process was taken one step further with the monocytes being cultured in a serum free HH medium, thus removing a large number of potential contaminants, notably vitamin B12, from the STMS. The supernatant was drained off and filtered through 0.22µm Millipore filters before use / purification.

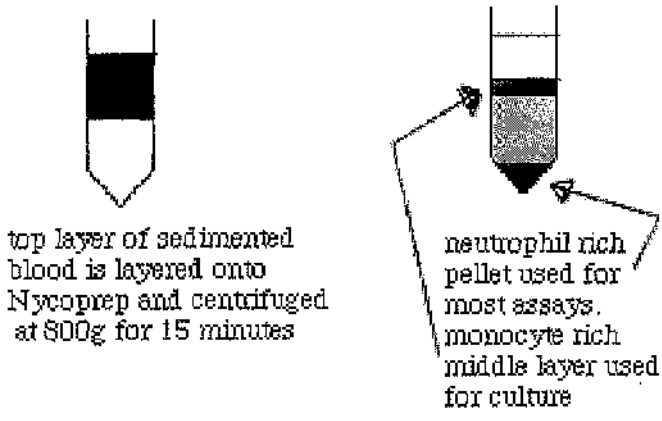


Fig. 2 Granulocyte preparation

2.2.2.2. Preparation from Buffy Coats

1. Standard Buffy Coats. Buffy coats were layered directly on to an equal volume of Nycoprep 1.077 and centrifuged at 800g for 20 minutes before aspirating the monocyte layer, then followed by the procedure described above. This procedure was hampered by the fact that many of the cells in the buffy coat clumped either before or during the purification resulting in poor yields of monocytes and STMS.

2. Buffy coats prepared by a modified procedure involving lower speed centrifugation did not suffer from the same clumping problem and thus produced a much higher yield of monocytes. The purification of monocytes from these buffy coats was exactly the same as above. When pints of blood (or buffy coats) were used for the culture of monocytes the anticoagulant was normally acid citrate dextrose. In consequence the blood clotted if the media used to wash the cells contained divalent cations Hence the first two washes were always carried out PBS or HS.

2.2.3. Culture of Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated essentially according to the method of Jaffé (1973). Fresh umbilical cords were collected from the Labour suite of the Queen Mother's Maternity Hospital, Glasgow and stored in BSS until ready for use. The cords were flushed gently to remove blood, and filled with 1mg/ml collagenase type IV(Gibco) in DMEM and clamped at either end. After 15-20 minutes incubation the collagenase was collected, the cord re-clamped and gently massaged to remove the endothelial cells. The cells were then flushed out with DMEM 5% FCS and washed three times before plating. After 24 hours the cells were washed with HS pre-warmed to 37°C and cultured in endothelial cell growth medium (ICN Clonetics). This culture medium contains dexamethasone, 1% FCS, undefined growth factors, and is suplemented with a further 4% FCS (Gibco). The cells were subcultured 2-4 times (using Trypsin / Versene) before use. Where steroid treatment of the HUVEC was not required, the cells were maintained in steroid free F10 for 2-3 days before use with 2 changes of medium.

2.3. Labelling Cells

2.3.1. Radiolabelling of PMNs

PMN were suspended at 10 million per ml and 100 μCi $Na^{51}Cr$ (Amersham) was added and incubated for 45 minutes at 37°C. The cells were shaken 3-4 times during the incubation period. The cells were washed at least twice with warm BSS 0.5% BSA (Sigma), or until supernatant counts were essentially at background, before assaying.

2.3.2. Fluorescent Labelling of PMNs

An alternative to ^{51}Cr was to use Carboxyfluorescene di-Acetate (CFDA) (Sigma) (4μg/ml final concentration). The procedure was essentially the same as radiolabelling, except the cells were physically counted using fluorescence optics on a Leitz microscope. Neutrophils were incubated in the presence of 4μg/ml CFDA for 30 minutes at 37°C with constant stirring. The cells were centrifuged at 2,000 rpm for 5

minutes and washed three times before use with Hanks HEPES containing 0.1% BSA (Goodall & Johnson, 1982; Li et al., 1993).

2.3.3. Staining of F-Actin

PMNs were allowed to adhere to BSA (1%) coated coverslips for 20 minutes before treating with agonist for 20 minutes and fixing with 1% para-formaldehyde for 60 minutes. The cells were washed twice with PBS 1% BSA before permeabilising with 1% Triton (Sigma) in PBS. The cells were washed a further twice before staining with TRITC-Phalloidin (Sigma) 0.1μg/ml for 15 minutes (in the dark) and washed thoroughly with PBS before mounting. Cells were analysed using confocal laser scanning microscopy with computer imaging of cells using the Metamorph program. Z-series composite images were used to visualise total F-actin content within the cells.

2.4. Adhesion Assays

Radiolabelled neutrophils were added to coverslips or multiwells coated with either BSA, BAE or HUVEC. The coverslips / multiwells were then incubated at 37°C for 30 minutes before gently rinsing to remove unbound PMN. The agonist was then gently added to the cells and returned to the incubator for a further 15-20 minutes. Wells / coverslips were washed gently three times to remove PMNs that were unbound. The specific radioactivity from each coverslip was then counted using a WILJ gamma counter 2001. If multiwells were used, then the cells were lysed with 100mM NaOH for 20 minutes before aspirating the supernatant and measuring the specific activity as above. This method has the advantage of not scratching any cells off of the surface of the coverslip when removing the coverslip from the dish. As an alternative to radiolabelling cells were labelled using CFDA as described in section 2.3.2 and counted manually.

2.5. Electron Microscopy

For scanning electron microscopy, cells were fixed in 2.5% gluteraldehyde (Sigma) in isotonic sucrose for 1 hour before washing twice in isotonic sucrose. The cells were

then post-fixed in 1% w/v osmium tetroxide (Sigma) in isotonic sucrose buffer, washed and stained with 0.25% w/v aqueous uranyl acetate for 1 hour in the dark. The cells were washed with water then dried either through a graded series of alcohols or acetones and critical point dried. When the PMN were treated in suspension, the acetone drying was performed with the cells sandwiched between 2 μm filters (Millipore) in a Swinney adapter. In some cases these filters were shaken on to double sided tape to ensure cells were not lost during the later stages of drying. The cells were mounted on aluminium stubs and sputter coated with a layer of gold using a modified Polaron E5000 sputter coater prior to viewing in a Phillips SEM (PSEM 500). Commonly a gold film of between 200 and 500 Angstroms was used. Images were captured using a camera mounted directly to the microscope or with a computer connected. It was noted that during the drying stages a large amount of shrinkage can occur (e.g.. up to 40% in some lymphocytes), and some proteins are dissolved during critical point drying, including microfilaments.

2.6. Analysis of the Respiratory Burst

The respiratory burst was recorded by incubating neutrophils at concentrations of 10^6 per ml at 37°C for 30 minutes in LKB Wallac 1251 Luminometer before the addition of Luminol and PMA 0.1μg/ml or fMLP 10^{-5}M (all Sigma). The results were continuously recorded utilising a custom computer program. The addition of 1μg/ml w/v cytochalasin B (Sigma) in HH increased the response to fMLP by around twenty fold, and so this was added prior to stimulation; i.e. the PMN were primed before activation (1.5.2.). Where several antagonists were to be assayed simultaneously PMA was used as the agonist as the respiratory burst is prolonged, and so the computer has a chance to record all the data. Where fMLP was used as the agonist, the response was too rapid to use more than three antagonists simultaneously.

2.7. Degranulation Assays

For these assays, neutrophils incubated at 37°C in F10 were exposed to agonist for twenty minutes before the cells were primed with 1 µg/ml w/v cytochalasin B (Sigma) in HH for a further five minutes. The cells were then challenged for ten minutes with 10^{-6} M fMLP (Sigma) before centrifugation (5 minutes at 2000 rpm). The supernatant was aspirated and stored on ice until use.

2.7.1. Primary Vesicles

Elastase assays were essentially as described by Janoff (1969). 6mg N-t-BOC- l-alanine p-nitrophenol ester (Sigma) was dissolved in 1ml acetonitrile and made up to 100ml in 50mM sodium phosphate buffer, pH 6.5. The change in absorbence at 347.5 nm was recorded using a Shimadzu UV-180 spectrophotometer over a three minute period. The results were plotted and the gradient of the line was used to approximate the relative amount of active elastase released. This was calibrated using porcine pancreatic elastase (ICN Flow).

As a control, myeloperoxidase (MPO) was assayed as per McRipley and Sbarra (1967). Guaiacol (Sigma) 20mM was freshly made up in water and added to sodium phosphate buffer (10mM, pH 7.0). 100µl of supernatant was added to a 3ml cuvette and activated with the addition of 20µl 1M H_2O_2. The method for quantitating release of active MPO was the same as for elastase, however the wavelength used was 470nm.

2.7.2. Secondary Vesicles

Collagenase activity was measured using gel electrophoresis of soluble type I collagen (2mg / ml) (Fluka) products following a fifteen hour incubation with the cell supernatant (described in section 2.7.) in the presence of Tris buffer (50mM Tris/ HCl pH 7.5, 5mM $CaCl_2$, 0.2M NaCl) essentially as per Aaku et al., 1990 and Michaelis et al., 1992. 0.5mM Aminophenyl mercuric acetate (Sigma) was added to activate any collagenase present in the supernatant, and PMSF and anti-trypsin were used to

inactivate serine proteases. Samples were mixed 1:1 with boiling mix and run on 7.5% polyacrylamide gels. Gels were analysed visually for differences in bands.

Plasminogen activator release (essentially as per Leigh, 1993) from specific granules was used as a back-up for collagenase release, and relies on the clearing of a casein gel (powdered milk). Gels used were 1cm thick, comprising 1% agarose (Gibco) and 1% skimmed milk powder in 50mM Tris-HCl pH 8.1, 150 mM NaCl. The gels were allowed to set and wells of 1/8 inch diameter were cut. Cell supernatants were added to the wells and incubated at 37°C overnight. The diameters of the cleared area around the wells were measured, with each assay being performed in triplicate.

2.7.3. Secretory Vesicles

Alkaline phosphatase was assayed as per DeChatelet & Cooper (1970), with the conversion of p-nitrophenyl phosphate being recorded at 410nm. PMN (10^7/ ml) were incubated as described in 2.7. with the exception that instead of centrifuging the sample, three volumes of ice cold F10 was added to stop the reaction. 0.5ml PMN suspension was mixed with 0.5ml p-Nitrophenyl Phosphate (2mg/ml aqueous) and 0.5ml aminomethyl propanol (0.1M, 1mM MgCl2, pH 10). This was incubated at 37°C for 30 minutes and terminated with 10ml 20mM NaOH. The absorbence at 410nm was recorded using a sample containing a pre-terminated blank (i.e. NaOH was added before the PMN).

2.8. Viability Testing

Release of the cytosolic enzyme Lactate Dehydrogenase (LDH) has been used as a sensitive method for testing cell integrity (Pesce, 1964). Cells to be tested were incubated in medium, removed by centrifugation, and the supernatant tested in a spectrophotometric LDH assay system. Each cuvette contained 3ml sodium phosphate buffer (100mM) pH 7.0, 1m mole sodium pyruvate (BDH) and 0.35m mole reduced

nicotinamide adenine di-nucleotide (NADH) (Sigma). 100µl supernatant was added to each cuvette and mixed. The rate of decrease in absorbence at 340nm recorded over a three minute period was used as a measure of the cell integrity.

2.9. Use of HPLC

2.9.1. Calibration of SMART System

Standards (Sigma) were used to produce a calibration curve of different prostaglandins / leukotrienes. The areas under the curves were plotted against the concentration. Lipids were extracted and analysed essentially as per Chilton (1989) and Rosenthal & Franson (1994) with the exception that the wavelength could not be adjusted to 270 / 234nm. Therefore 280nm was used. The standards from Sigma appeared to be monitored correctly; i.e. all of the peaks were in the correct relationship to each other.

2.9.2. Prostaglandin Release

The release of arachidonic acid metabolites was assayed using a methanol gradient on a SMART system HPLC (Pharmacia). 5µg Prostaglandin B_2 (Sigma) was added to each supernatant and acidified with 10% formic acid. Lipids were extracted into ethyl acetate. This was vacuum dried and dissolved in buffer A (55% methanol, 44.5% H_2O, 0.5% phosphoric acid). The column was allowed to equilibrate for 6 minutes before the buffer was changed to 100% buffer B (100% methanol) over 35 minutes (Chilton, 1989).

2.9.3. Purification of STMS

The purification strategies for STMS are summarised in fig 3.

Starting material was force dialysed using type I dialysis tubing, sterility being maintained by autoclaving all glassware and tubing before starting. The retained material was applied to a monoQ column and eluted using a salt gradient. The fractions that were active for dispersive motility were pooled and applied to a Pharmacia peptide column (gel filtration 0.1 to 7kD range). The large molecules not inhibited by the

column were pHed to 2 and applied to reverse phase where any small molecules held by ionic bonds would be released. It was noted that one such molecule was released. This molecule coincided with a small molecule from the peptide column which was applied to reverse phase chromatography also.

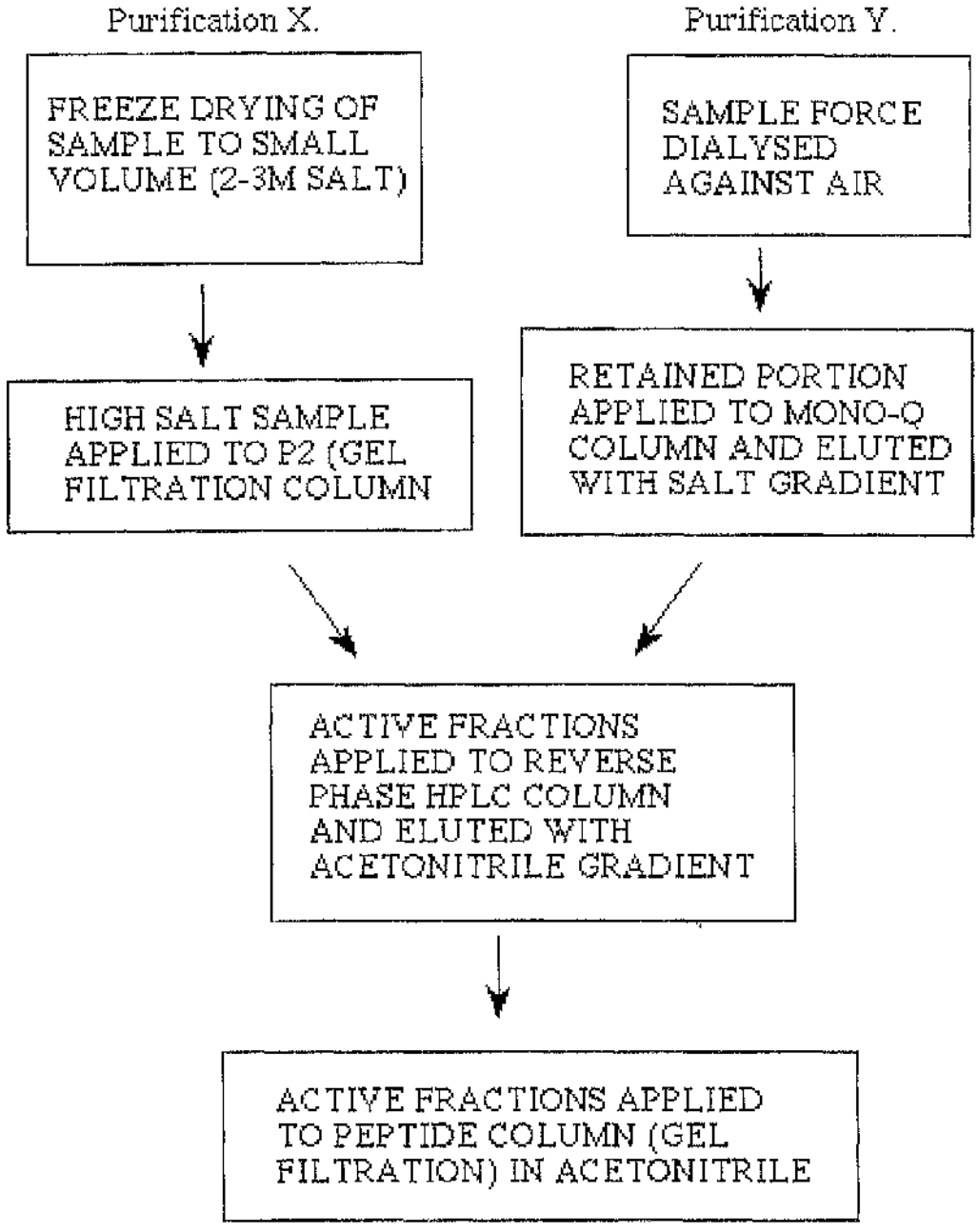
The material that passed through the dialysis tubing was freeze dried and fractionated with P2 (Bio-Rad) using 150mM NaCl as eluent. The fractions in the size range of 1kD-2kD (including vitamin B12) were pooled and applied to reverse phase column. The activity eluted at approximately 55% buffer B (50% acetonitrile, 0.12% TFA), with the active fractions being subjected to a second reverse phase separation.

Previously, STMS had been purified using other techniques. These included the freeze drying of the starting material and applying the concentrated sample to P2 (Bio-Rad) using 150mM NaCl. The fractions that produced dispersive motility were pooled, freeze dried again and applied to reverse phase HPLC. The active fractions were pooled, and applied to gel filtration (Pharmacia peptide column) in an attempt to separate the active molecule from the contaminating material.

2.10. Inhibition of Phagocytosis

To determine if there were any effects on the ability of neutrophils to phagocytose, latex beads (Sigma) at a final dilution of 1:10,000 were added to the neutrophils for 30 minutes, before fixing with 1% paraformaldehyde (Sigma), washing and counting the number of beads phagocytosed per neutrophil. Wojiak and Crossan (1993) used fluorescent latex beads for this assay counting the beads under fluorescence optics but, surprisingly, this was not found to be necessary, although using fluorescent optics did make the beads more immediately visible.

Fig. 3 Schematic diagram of purification strategies employed with STMS



2.11. Tracking assay

The assay used chambers as designed by Dow et al., (1987) where a coverslip was fastened to the bottom of a chamber by wax, and the resulting well coated with 1% BSA solution for 20 minutes before washing with saline and addition of PMN at a concentration of 10^6 per ml. These were allowed to adhere for 15-20 minutes before washing with saline. The negative control was always checked at this stage to ensure that the quantity of cells was adequate for tracking. If there were too many cells, then they chambers required to be washed more vigorously before use. If there were too few PMN, then the chambers were left for a further 5 minutes before washing (and the above procedure used to determine the cell number. If at that point there were too few cells, then the washing stage was reduced). The well was filled with substrate (around 400µl) and the chamber closed with a second coverslip and sealed with wax.

The microscope used was a Leitz inverted phase contrast microscope fitted with a stage micrometer and thermostated chamber. This allowed a stable temperature to be maintained throughout the experiments, and for the stage incubation chamber to be open for the shortest time possible whilst appropriate fields of cells were selected.

For experiments with a tilted stage, the microscope was tilted to an angle of 30° , where is was held throughout the course of the experiment.

2.11.1. Tracking procedure

The assay chamber was put on to the stage, and the field displayed on the real time monitor. When the tracking program was started, a frame was taken and the cells displayed in 16 grey levels, the threshold was pre-set at a level where most motile cells (phase bright) could be tracked. Islands above this level were outlined in red, but those below a threshold area were discarded. In the first frame, the computer stored the centre co-ordinates of all of the selected cells. In subsequent frames, this procedure was repeated but the centre positions of the cells were joined to the previously determined

centres thus displaying the continuous track for each cell. This meant that the observer could compare the real time behaviour of the cells with the tracking procedure to check whether the cells were being correctly identified.

The tracking data was displayed as:

1) instantaneous speed, i.e. the average distance covered by all cells in one frame,

2) the number of cells still being tracked,

3) the mean displacement of the cell population,

4) the net displacement of the cell population from the origin (drift),

5) the difference between the mean displacement of the population and the drift,

6) the square of the mean displacement of the cell population,

7) the square of the drift,

8) the difference between the square of the mean displacement of the cell population and the square of the drift.

2.11.2. Data processing

The slope of the linear portion of the plot of the difference between the square of the mean displacement of the cell population and the square of the drift was measured by an on-screen line drawing program. the slope of this line was used as the diffusion co-efficient, and the intercept on the time axis was the persistence of locomotion.

RESULTS

3.1. Remit of work done

Despite work carried out on this project to date a number of important points have yet to be resolved. These include answers to the questions:

Is STMS a single factor, or more than one factor acting in synergy?

Is the observed activity independent of the ionic composition of the extract?

Is the synthesis of STMS stimulated by steroids

Is the release stimulated by steroids?

Is the release simply a result of cell death

Is STMS a mediator of steroid anti-inflammatory action.

3.2. STMS - Overview

This work was carried out as part of a joint project where the main aim was the purification and chemical characterisation of the factor(s) responsible for STMS activity. The first phase was to identify the spectrum of activities present in the starting culture supernatant and to determine which of these activities co-purified with the dispersive motility (Chettibi et al, 1994b). The initial problem with handling STMS concerned the apparent size of the molecule. Techniques available for measuring size included gel filtration and force dialysis. When STMS was handled at low salt concentration, the active species appeared to be partially retained by a dialysis membrane. In consequence, dialysis appeared to split the activity into two roughly equal portions. When it was partially purified, and handled at low salt concentration, the activity was eluted with the electrolyte peak on gel filtration. This gave the model that the active species was a small molecule normally bound to a protein, but when released from this protein, interacted strongly with the gel filtration matrix. Hence there was no easy way to desalt STMS, or to change the ionic composition of the initial extract. To a large extent this problem was resolved by the observation of J. Young that purified STMS was active at much higher dilution than crude STMS (personal

communication), from this he deduced that the activity passing through the dialysis membrane was very much less in real terms than the retained activity, although the measured activity appeared to be very similar.

The stages of purification of STMS are given in Fig.3 Most of the work was performed using the purification strategy X.

Other components of STMS

The most noticeable action of steroids on cells of the immune system is inhibition of protein synthesis. This was borne out by comparison of STMS and CMS on SDS-PAGE. The former has very few bands, whilst the latter has a complex band pattern (A. Lawrence, personal communication). One of the problems of the incubation procedure is that proteolytic activity cannot be effectively inhibited because protease inhibitors tend to be toxic to cells. Thus degradation is certain to occur because these cells secrete proteolytic enzymes. However it is also known that steroids inhibit the release of proteolytic enzymes (see below) and therefore proteolytic activity should be much less prevalent in STMS compared with CMS. The best evidence for the presence of proteases in these extracts comes from characterisation of some of the molecular species found in STMS / CMS. IL-8 and thymosin β4 are present in STMS indicating that trypsin-like protease activity is minimal. However, one small peptide purified from STMS was unambiguously characterised by Edman degradation as a proteolytic fragment from actin generated by cleavage of an V-P bond and a Q-G bond, leaving PSIVGRPRHQ (G. Kemp, personal communication). It is of interest that this fragment is one of the postulated thymosin β4 binding sites on actin (Safer et al., 1997). Throughout the purification by strategy X vitamin B12 co-purified with the active fractions. The reverse phase step succeeded in separating the activity from native vitamin B12 but not from (a very minor component of vitamin B12) acyano Vitamin B12 (D. Pappin, personal communication).

Physico-chemical properties of STMS

STMS does not appear to be highly hydrophobic, as it elutes early from reverse phase HPLC and it does not absorb on to phenyl sepharose. The activity is not retained by concanavalin A sepharose indicating that it is unlikely to contain oligosaccharide. It is clearly small with a molecular weight between 1 and 5 kD. The activity will be absorbed strongly by gel filtration media, indicating a high degree of polarity.

STMS differs from all other known factors which stimulate neutrophil migration in that it gives persistent locomotion at a uniform concentration. One aim of this work was to obtain a model for persistence which could apply to loosely adherent cells in which locomotion was clearly discontinuous, and this required a detailed determination of the shapes of the cells, and the mode of attachment to the substrate.

STMS from monocytes

As stated above, monocytes were allowed to adhere to tissue culture plastic in the presence of serum, and then cultured in serum free medium. This facilitated the purification of the supernatant which would otherwise be an extremely protein rich solution. Previous work had shown that the yield of STMS activity was comparable in the presence or absence of serum. Monocytes cultured in this way for 24 hours produced supernatant with STMS properties, but no detectable activity was produced after the culture supernatant was replaced with fresh serum free media for a further 24 hours. Those monocytes that remained adhered to the plastic looked no different after this second incubation, but many of the monocytes had become detached from the plastic (approx. 30%). To test if the STMS-like activity could be produced continuously, these experiments should be performed in a complete medium with aliquots removed at intervals of perhaps 12 hours. This would be a useful diagnostic

test of the ability of monocytes to produce the factor indefinitely, but would not have been particularly helpful as the purification of media containing serum would increase the difficulties presented several fold. This would also be the case for purification strategy Y, as serum contains many molecules that would not be removed by dialysis, and could prevent efficient running of columns. Equally, purification strategy X would retain the small molecules present in serum in the P2 column.

STMS from other cell sources

Culture of Endothelial cells or neutrophils with dexamethasone do not produce STMS-like activity in the supernatant. Platelets have been lysed, but again the supernatant did not show any STMS-like activity (results not shown). Macrophage-like cell lines did produce some activity when cultured in the presence of dexamethasone, but to a lesser extent than monocytes in culture. These cells were cultured further in the presence of serum to allow growth to continue, and the culture process repeated. Considerably less STMS-like activity was produced in this subsequent culture (as assayed by tracking experiments). This indicated that STMS-like activity was something that cells produced once, but were incapable of continuing to produce (result not shown). The reasons for this remain unexplained, but are consistent with the earlier work, where STMS-like activity was only produced once from monocytes (J. Young, personal communication).

3.3. Observations of the properties of STMS treated cells

When cells were added to the tracking chambers, they were initially rounded. During the next five minutes they became increasingly blackened (phase dark) as if they were spreading out and becoming very much thinner. However, over the next twenty or so minutes they tended to return from this phase black state to a phase bright condition where they were highly motile. During the first hour of tracking, the speed and the persistence increased. This was in contrast to the CMS treated cells which also turned

phase black initially, but did not return readily from this state, and as such were difficult to track. There was no persistence from CMS treated cells. It was noted that other agonists commonly used to stimulate PMN motility, e.g. fMLP also induced the cells to blacken initially, return from this condition to be tracked, but did not maintain their speed of locomotion for more than half an hour under these conditions. STMS treated PMN moved in an unusual manner, that was distinctive of STMS treatment. The migration of an individual cell appeared to be a discontinuous process. For most of the time, the cell appeared to be stuck by a tail to the substrate and appeared to undergo a tethered exploration in any direction. The cell body would extend out from the anchor and eventually the tail would follow the leading edge. Translocation of the tail tended to occur as a rather sudden movement, leading to a suggestion that the motility is based on a looping model (somewhat similar to how a caterpillar travels). An individual cell appeared to migrate in a series of jumps across the screen, inconsistent with the model that direction was determined by inertia. Cells migrated in a stochastic manner where once extending a leading edge, they continued to do so until the tail became detached. The tail of the cell either retracted back into the cell body, or was propelled over the top of the PMN body, but normally at a shallow angle and the locomotory step repeated. It has not yet been resolved if the motility is by the caterpillar walk, or by a tumbling end over end. The fast step was the movement of the tail up to the cell body; the rate limiting step therefore appears to be the exploratory movement of the body of the cell.

3.3.2. Tilt assays

Because the locomotion of these cells is highly dispersive, the calculation of the apparent diffusion coefficient depends strongly on population displacement. If all of the cells have a component of movement in a single direction, this will give it a disproportional effect in calculating net displacement. Thus it was very important to allow for any component of drift in the system. The initial tracking assay displays drift per frame as a vector and it was frequently noticed that the drift vector tended to point in

the same direction. Possible explanations for this were temperature gradients being formed by the heater, or gravitational force caused by an unlevel stage. To check whether this last factor made a significant contribution, tracking assays were carried out with the microscope tilted at approximately 30 degrees. The results of this were quite dramatic. For fMLP treated cells, there was virtually no difference between the migration of cells on a flat or on a tilted stage. For STMS treated cells, in contrast, the migration was extremely strongly polarised down the slope. Nevertheless, our observations showed that the cells were not free floating. The tracking assay was set a search limitation of 10μm radius per 5 sec frame. Cells moving faster than this were lost. Free floating cells were observed and in all cases, they were not tracked, but the majority of cells migrated at a speed considerably more than cells on the untilted stage, but clearly not the speed of cells in free fall (fig. 4.). In these cells, the rate limiting step was the detachment of the cell tail, with the exploratory phase apparently being very much faster than in experiments where the stage was level. This highly polar morphology was particularly distinctive in starting material, perhaps less so in later purifications.

3.4. Electron Microscopy

3.4.1. PMN Treated in Suspension

PMN when viewed under SEM have a characteristic morphology. Untreated (control) cells are commonly spherical with very few if any protrusions, although there are obvious folds of membrane on the surface (fig. 5). These folds may be capable of allowing the cells to increase their surface to volume ratio dramatically in the absence of any translocation of new membrane to the surface. As noted above (2.5) there can be a noticeable shrinkage of the cells during preparation. However, as all treatments were prepared in the same manner, no appreciable differences in size should occur, and so can be discounted from being a source of error within these assays. Treatment of PMNs in suspension with fMLP (10^{-7}M) showed that cells retained a rounded body

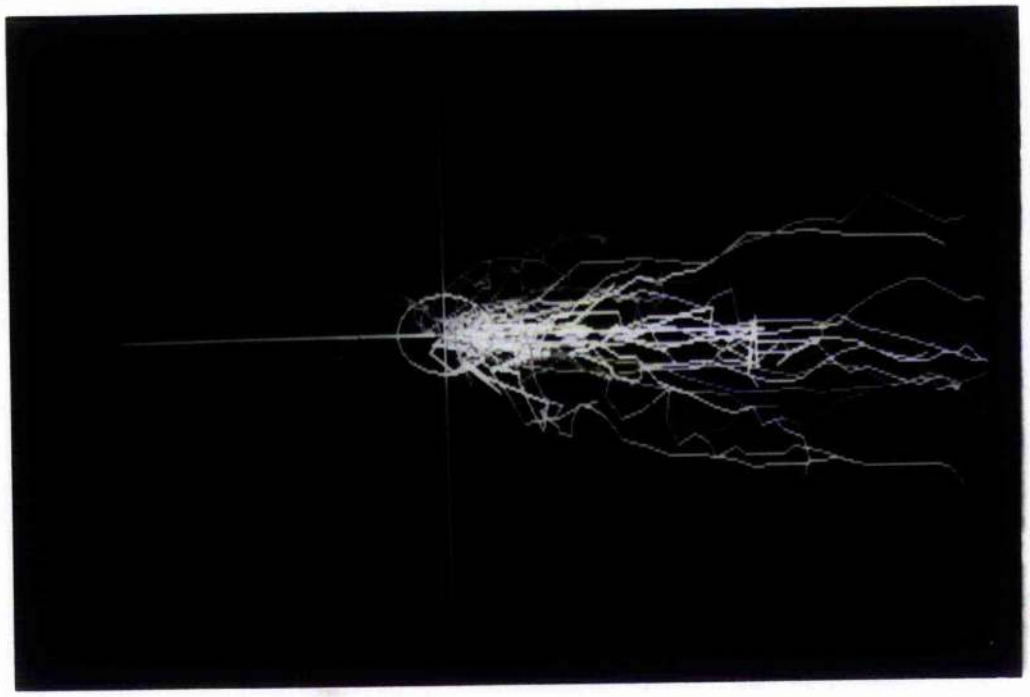
consistent with phase brightness, with some of the ridges observed on the control cell extending well beyond the cell body, with an appearance expected for actively ruffled membrane (fig. 6). Similarly, treatment with IL-8 gave an extremely irregular and rough surface morphology with numerous, highly distorted projections (fig. 7).

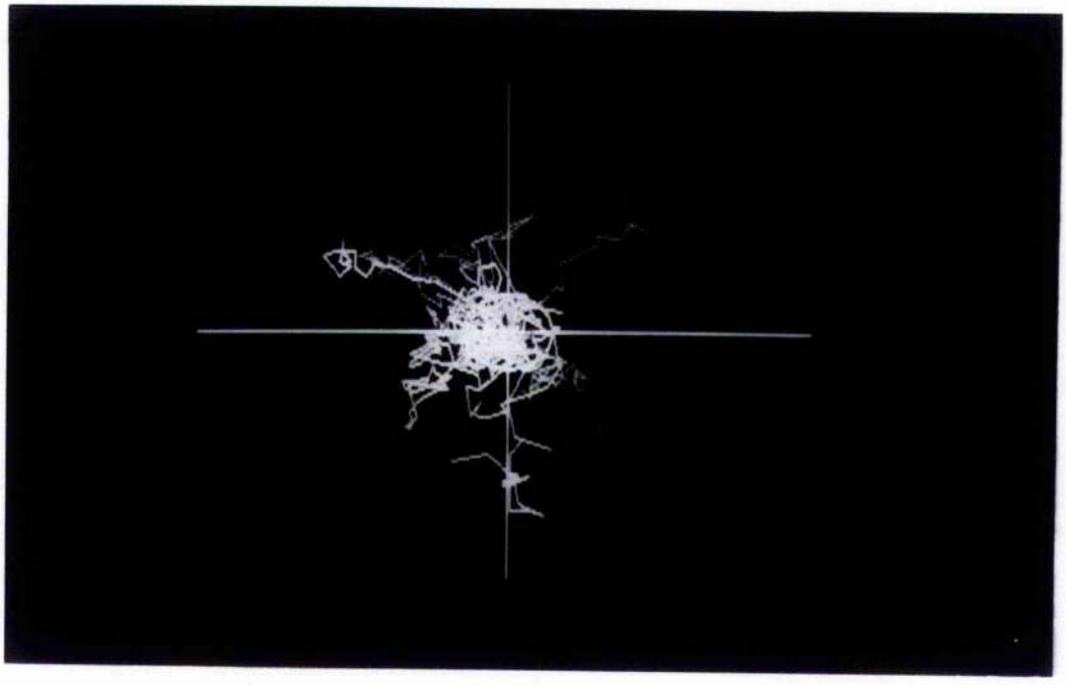
Neutrophils were allowed to adhere to BSA coated coverslips in wells as described in materials and methods. The wells were washed, filled with test substrate and sealed. The chambers were incubated at 37°C for twenty minutes before tracking was commenced. fMLP cells were observed to continue to track as normal, with very little diffusion away from the starting point. STMS treated cells however, were observed to migrate down-stage.

Upper image, STMS treatment

Lower image, fMLP treatment

Fig. 4a. Screen images from tracking experiments with a tilted stage, showing tracks of fMLP and STMS treated neutrophils.





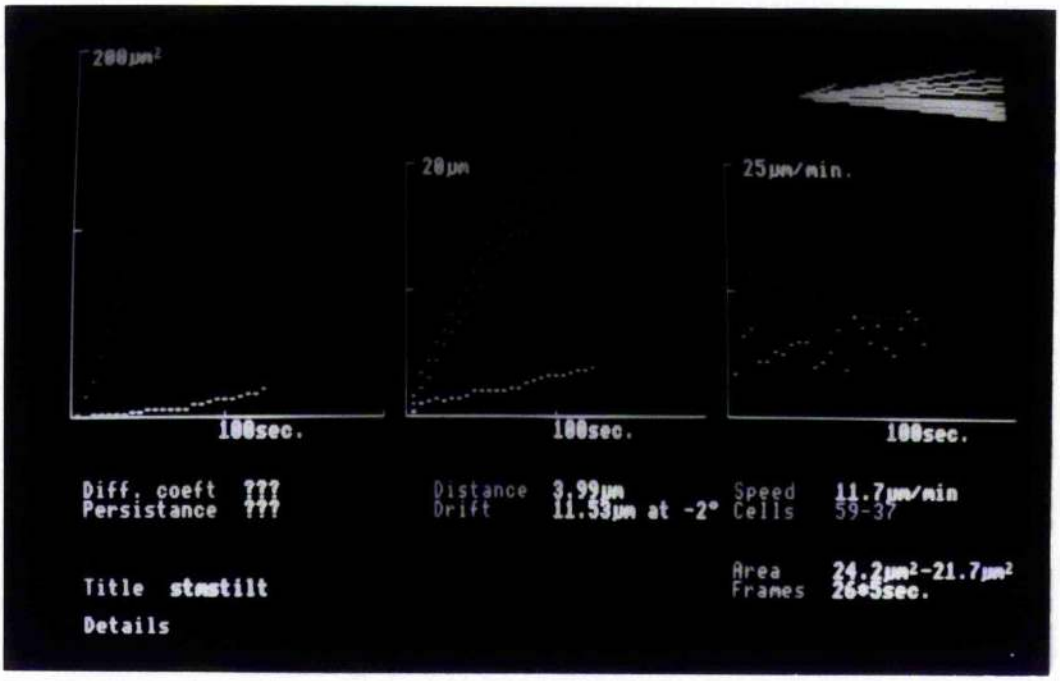
The data compiled by the computer shows that fMLP treated PMNs are not effected by drift, whereas almost the entire motility induced by STMS treatment is due to drift. As the overall drift is subtracted from the speed of the PMNs, then the figure measured by the computer is erroneous. The speed has to be corrected to allow for this drift (5.8μm per minute), giving a speed comparable to fMLP treatment.

The microscope stage was measured to be at an angle of 30^{o}.

Upper image, STMS treatment

Lower image, fMLP treatment

Fig. 4b. Screen images from tracking experiments with a tilted stage showing data gathered from STMS and fMLP treated neutrophils.



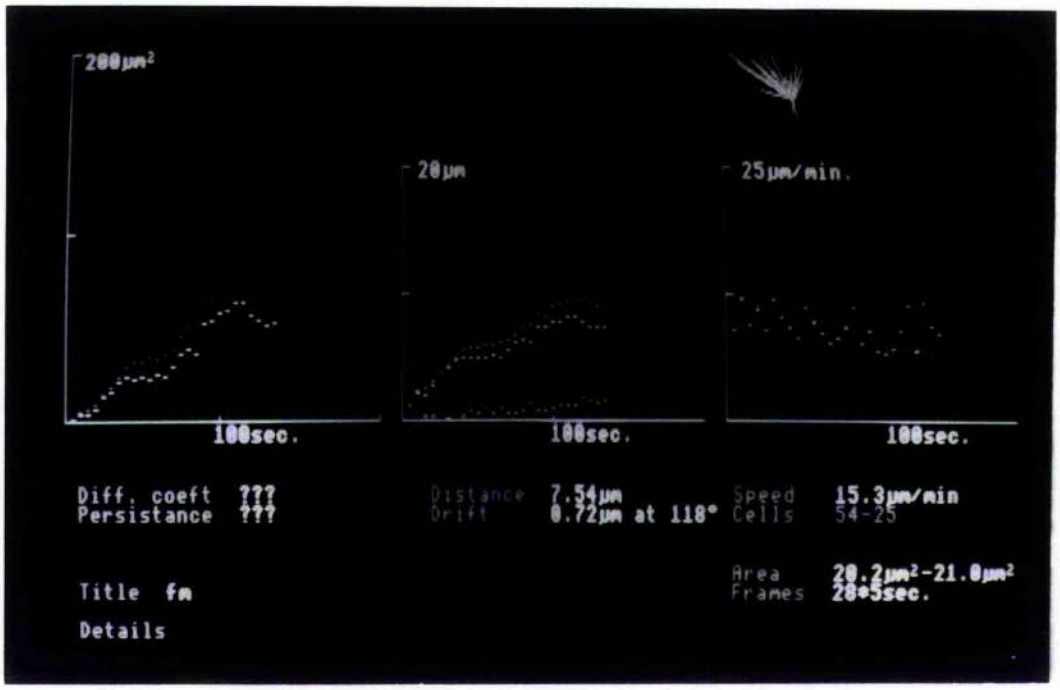
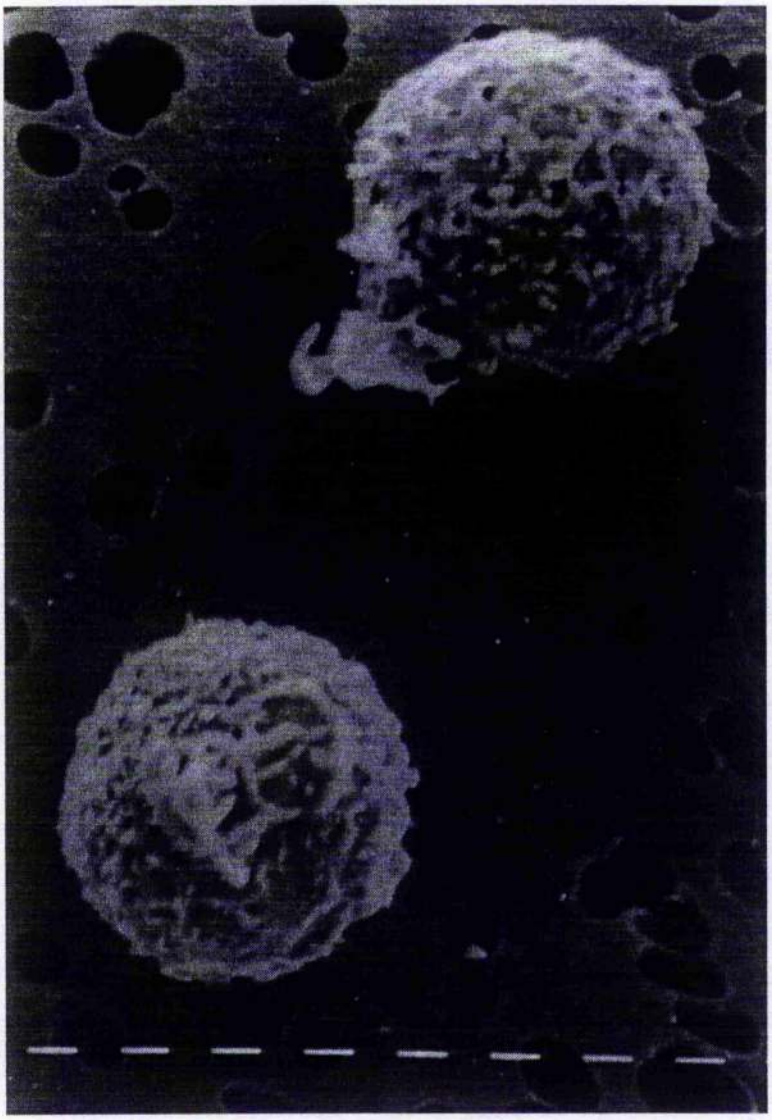
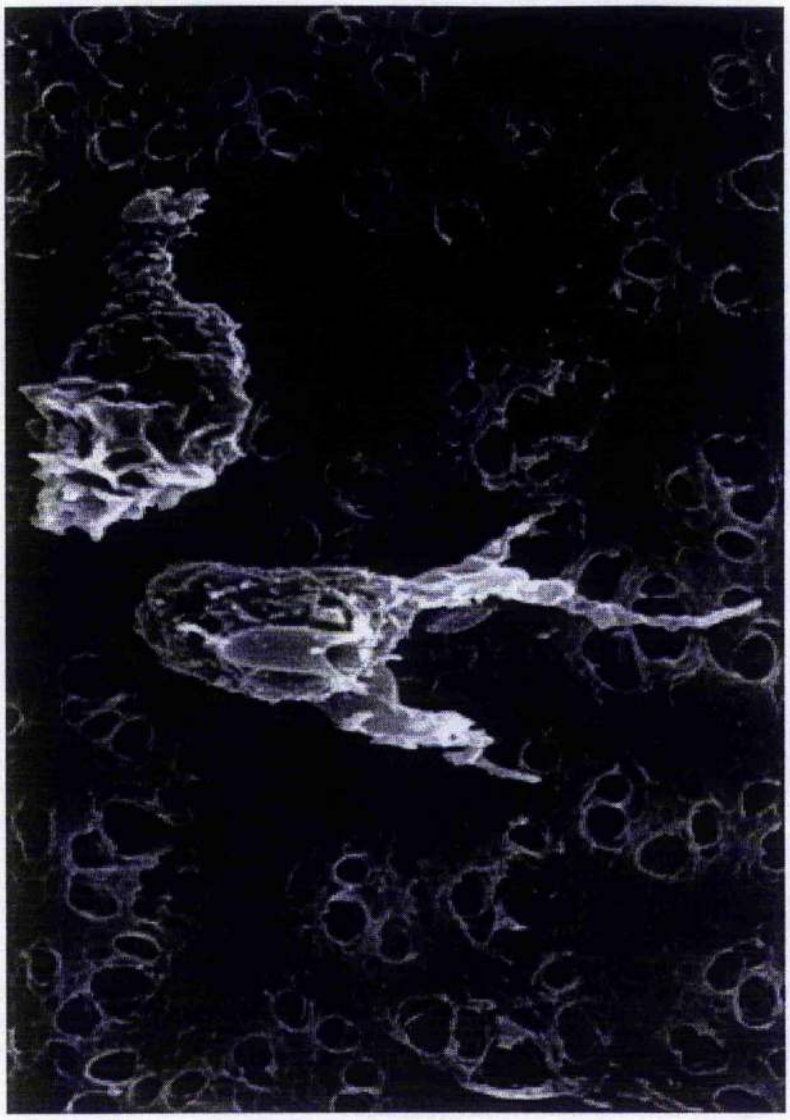


Fig 5. Scanning Electron Microscope image of Hams F10 treated neutrophils



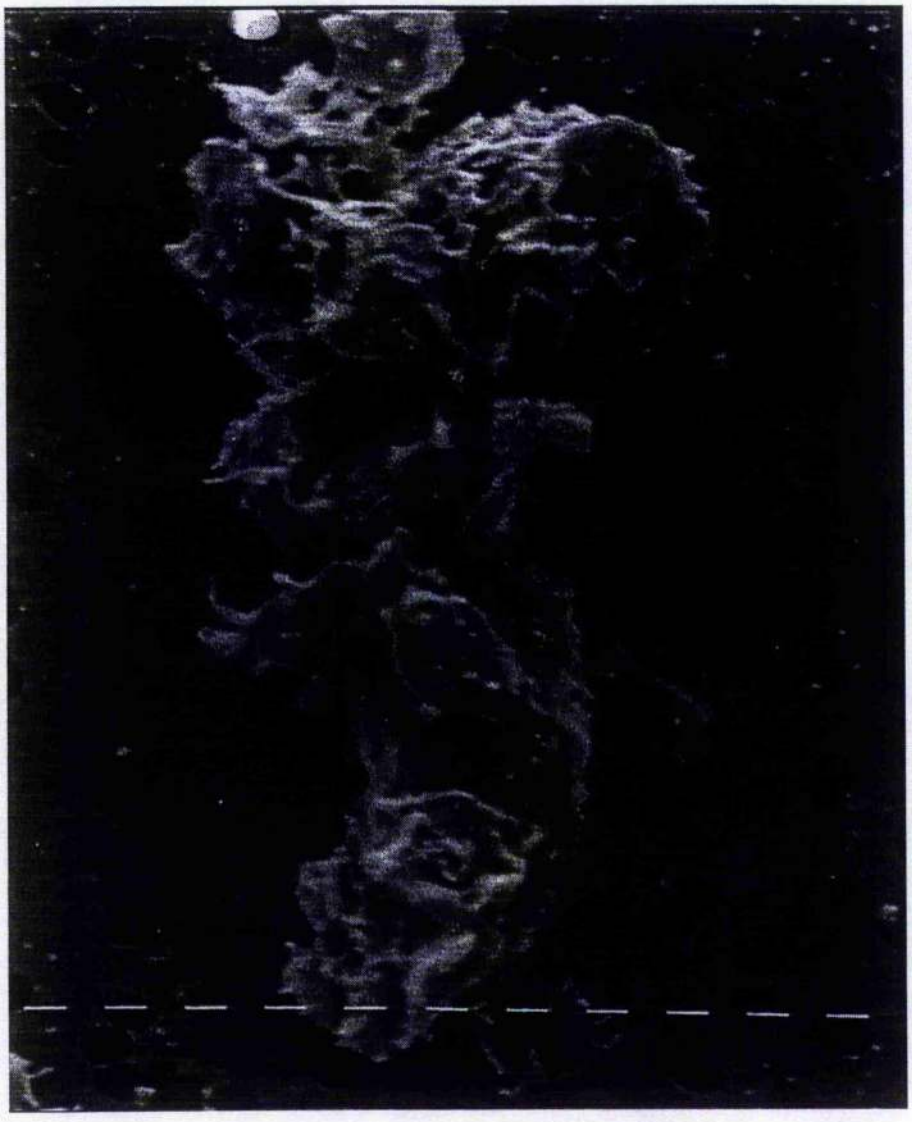
Neutrophils were treated in suspension with Hams F10 for thirty minutes and prepared for scanning electron microscopy. Magnification 6,400; scale bars are 1μm.

fig 6 Scanning Electron Microscope image of fMLP treated neutrophils



Neutrophils were treated in suspension with 10^{-7}M fMLP for thirty minutes and prepared for scanning electron microscopy as described in materials and methods. (Magnification 6,400)

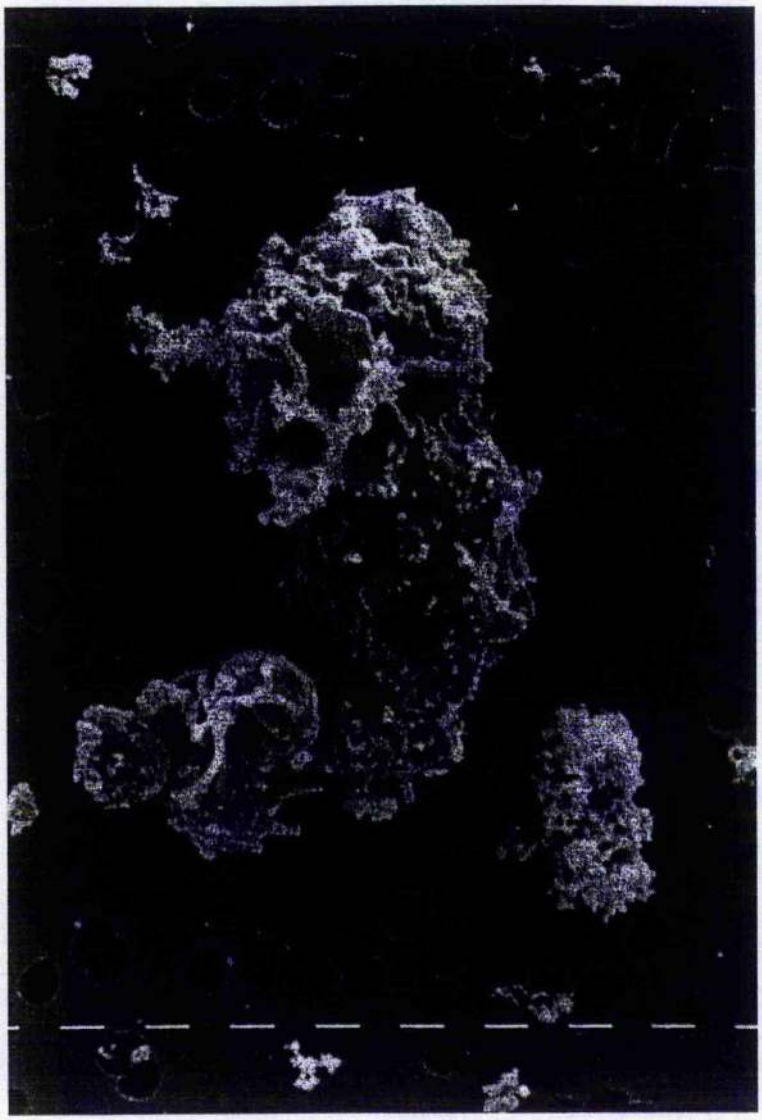
Fig. 7 Scanning electron microscope image of an IL-8 treated neutrophil



Neutrophils were incubated with IL-8 (1 μg/ml) for thirty minutes before preparation for scanning electron microscopy. Magnification 6,400; scale bars 1μm.

Cells treated with CMS were very flattened consistent with the phase black projections (fig. 8). In marked contrast, cells treated with STMS were elongated. Although membrane folds were present, the surfaces were extremely smooth. The appearance was sufficiently characteristic to allow STMS treated cells to be identified from the SEM alone. The cells have a prominent tail, which is connected to the elongated cell body by a thinner region. The end distal from the tail appear to have weak lamella-forming activity (fig. 9). The inference from these results is that cells are bipolar with one end being dominant, and morphologically different from the rest of the cell body. The narrow neck of the cell could well have acted act somewhat like a hinge in the tilt assays, with the tail being the point of firm adhesion. dexamethasone treated control PMNs were virtually indistinguishable from control PMN treated with F10 medium. Stevenson observed that colchicine caused PMN to disperse from a cell pellet in a manner similar to PMS (STMS), and so it was of interest to examine the effects of colchicine (10^{-5}M) treatment of PMN to determine if migration pattern could be linked to cell shape. However, PMNs in suspension treated with colchicine were radically different in shape from PMN treated with STMS (crude) under the same conditions, producing drastic changes in the appearance of cells when treated in suspension. Copious ruffles were observed on the surface (fig. 10), though these were not as deep nor as prominent as those produced in response to fMLP treatment. Furthermore, widespread projections over part of the surface, give it a roughened appearance not produced by STMS. This was evident from comparing STMS treated samples with the smooth surface, uniform bipolarity with only occasional projection(s), whereas with colchicine samples the most common feature was a single projection from a rounded cell body with copious ruffles over other parts of the surface. This contrasts with the effects of colchicine on fibroblasts, where the cells become rounded, and considerably smaller in size (Gail & Boone, 1971). There is no evidence to suggest that this single projection is a persistent feature that could account for persistent locomotion of the cell.

Fig 8 Scanning Electron Microscope image of a CMS treated neutrophil

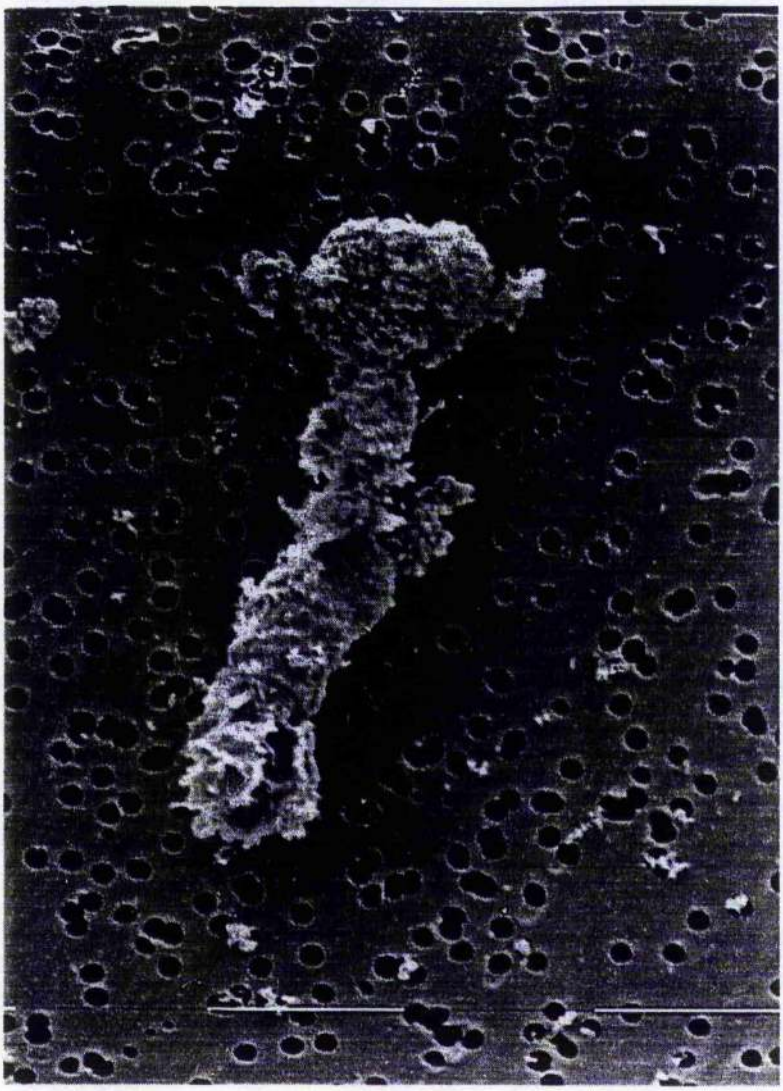


Neutrophils were treated in suspension with CMS for thirty minutes and prepared for scanning electron microscopy. Scale bars are 1μm; magnification 6,400.

Fig. 9 Scanning electron microscope image of STMS (crude) treated neutrophils

Neutrophils were treated with STMS (crude) in suspension for thirty minutes before preparation for scanning electron microscopy. The dried filters were shaken on to double sided tape coated stubs and coated with gold as described in materials and methods. Magnification 6,400

Fig. 10 Scanning electron microscope image of Colchicine treated neutrophil



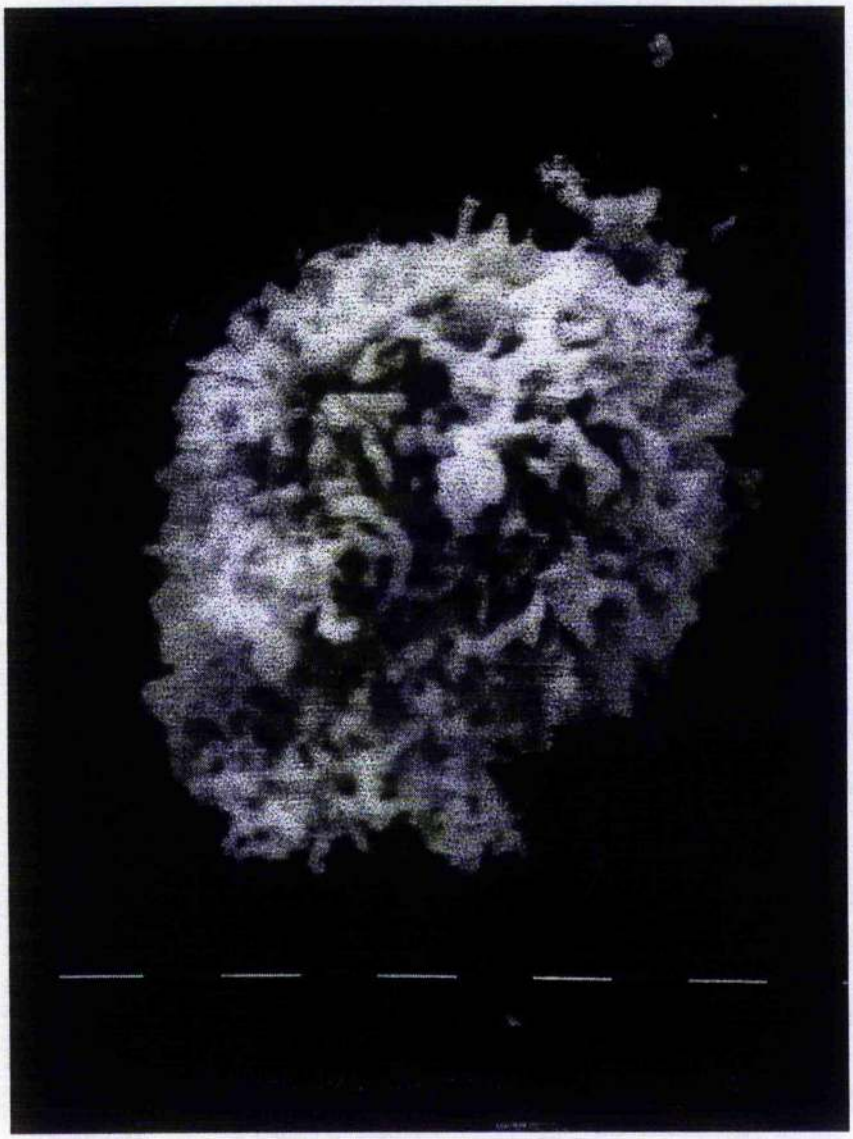
Neutrophils were treated with colchicine (10^{-5}M) in suspension before preparing for scanning electron microscopy.

A possible interpretation of the morphology of STMS treated cells is that polarisation of cells by STMS is not accompanied by degranulation, so that no excess membrane is brought to the surface. This would account for the unparalleled smooth appearance of the membranes.

3.4.2. PMN Seeded on to Endothelial Monolayers

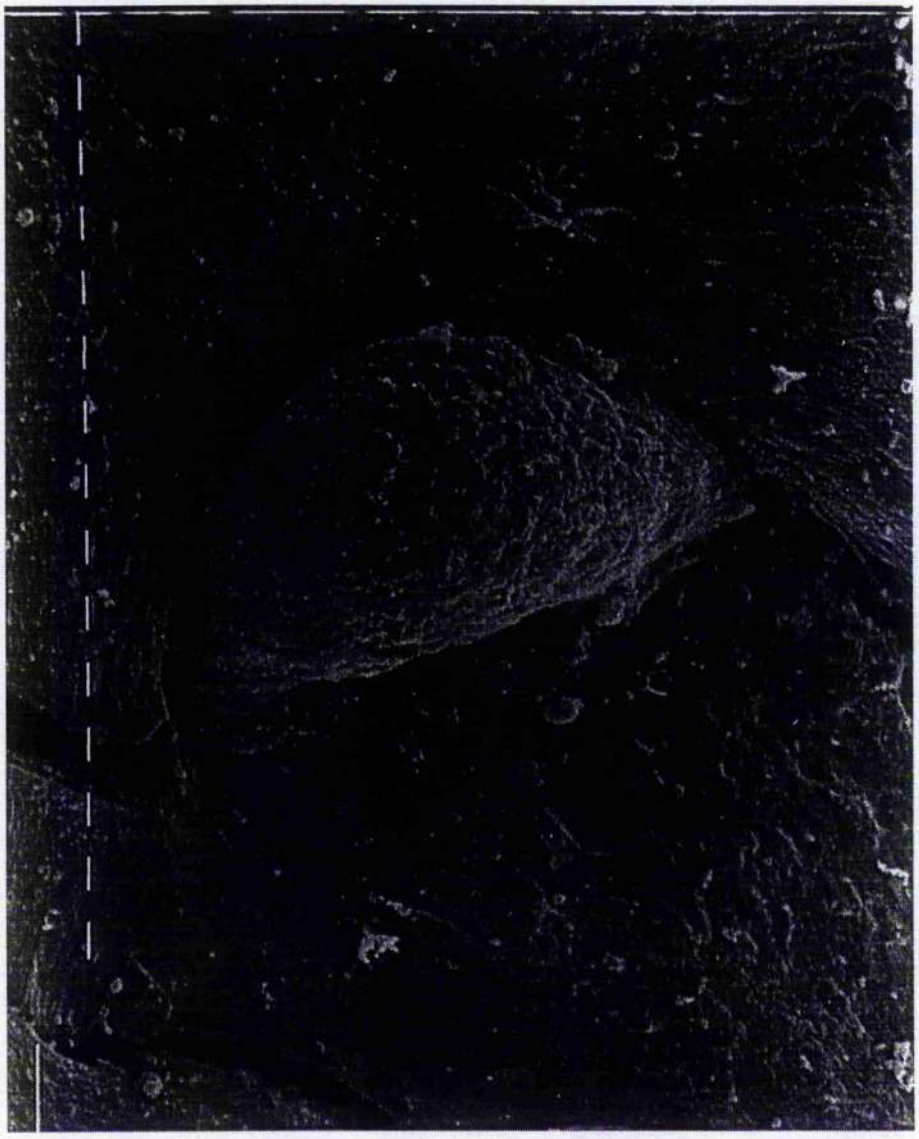
Endothelial cell sheets (cultured without steroid for 1-2 days) normally show small but perceptible gaps between individual cells. When these sheets were seeded with untreated PMNs (fig. 10) the PMNs remained rounded, but were normally found in association with endothelial cell junctions. When PMNs seeded on to the endothelial cell sheet were subsequently treated with fMLP (10^{-7}M) the appearance of the surface was virtually unchanged, although fewer cells were found in non-junction regions. The reduced visible bulk of the cell indicated that active transmigration was taking place, and these cells had a relatively smooth upper surface. fMLP treatment of PMNs in association with the endothelial cell sheet caused a visible increase in the number of gaps between the endothelial cells, with many of the PMNs migrating through these gaps (fig. 12). The morphologies of the PMNs not associated with endothelial cell junctions were characterised by a relatively smooth surface and a definite leading front, this being very similar to that described by Bakowski and Tschesche (1992). STMS treatment of PMNs already seeded on to endothelial monolayers produced the same general effect, but PMNs had a noticeably smoother surface, and there was very little evidence that any of them were trans-migrating through the endothelial cell gap. Conversely STMS cells appear to continue to migrate across the surface of the endothelium (fig. 13). PMNs treated with STMS and then seeded on to the endothelial surface were found predominantly over the gaps between the endothelial cells, but there was no evidence that they were engaged in migration through the gap (result not shown). These gaps between cells are not anticipated to be due to the drying stages, as freeze-drying was used. This method of drying does not induce as high a level of

Fig. 11 Scanning electron microscope image of a F10 treated neutrophil adherent to endothelium.



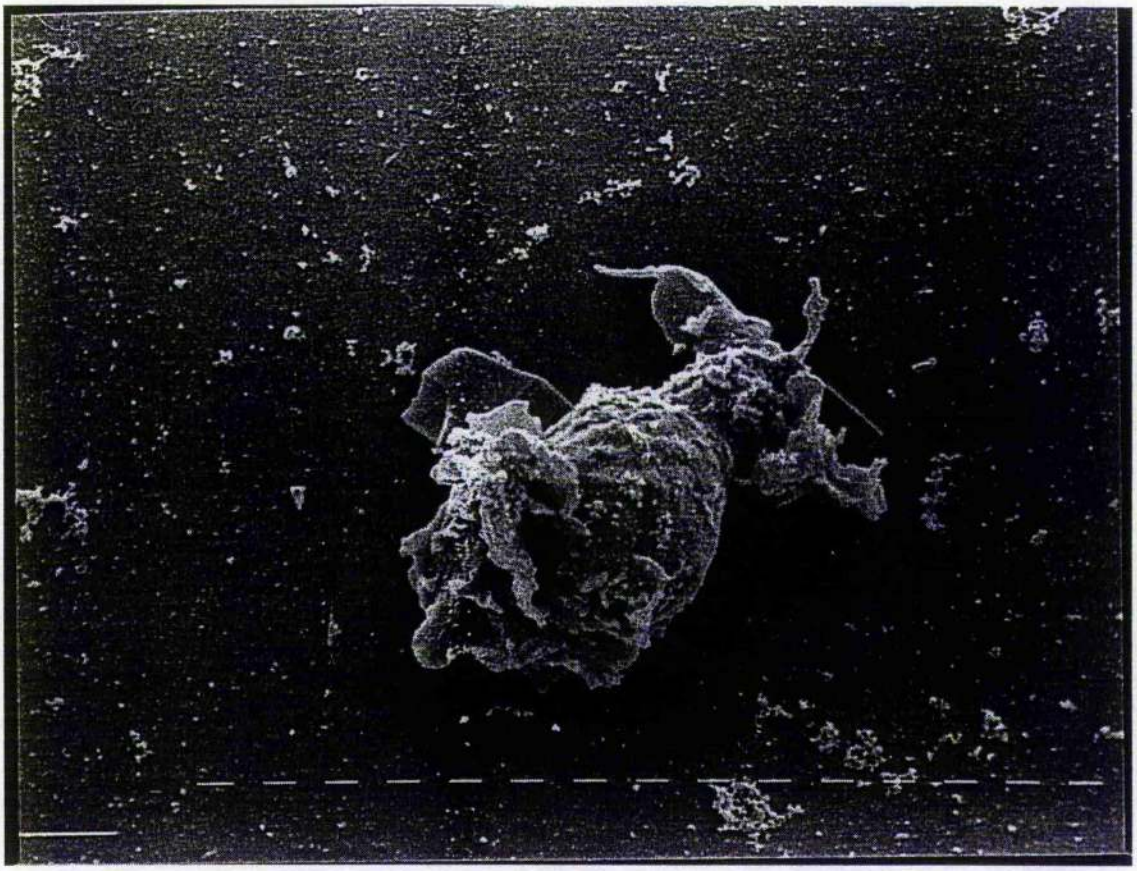
Bovine aorta endothelium was grown to confluency in the absence of dexamethasone. The monolayer was washed and seeded with neutrophils which were allowed to adhere at 37°C for twenty minutes before washing and incubating in F10 at 37°C for thirty minutes before preparation for scanning electron microscopy. Magnification 6,400; scale bars are 1μm.

Fig. 12. Scanning electron microscope image of an fMLP treated neutrophil seeded on to bovine aorta endothelium.



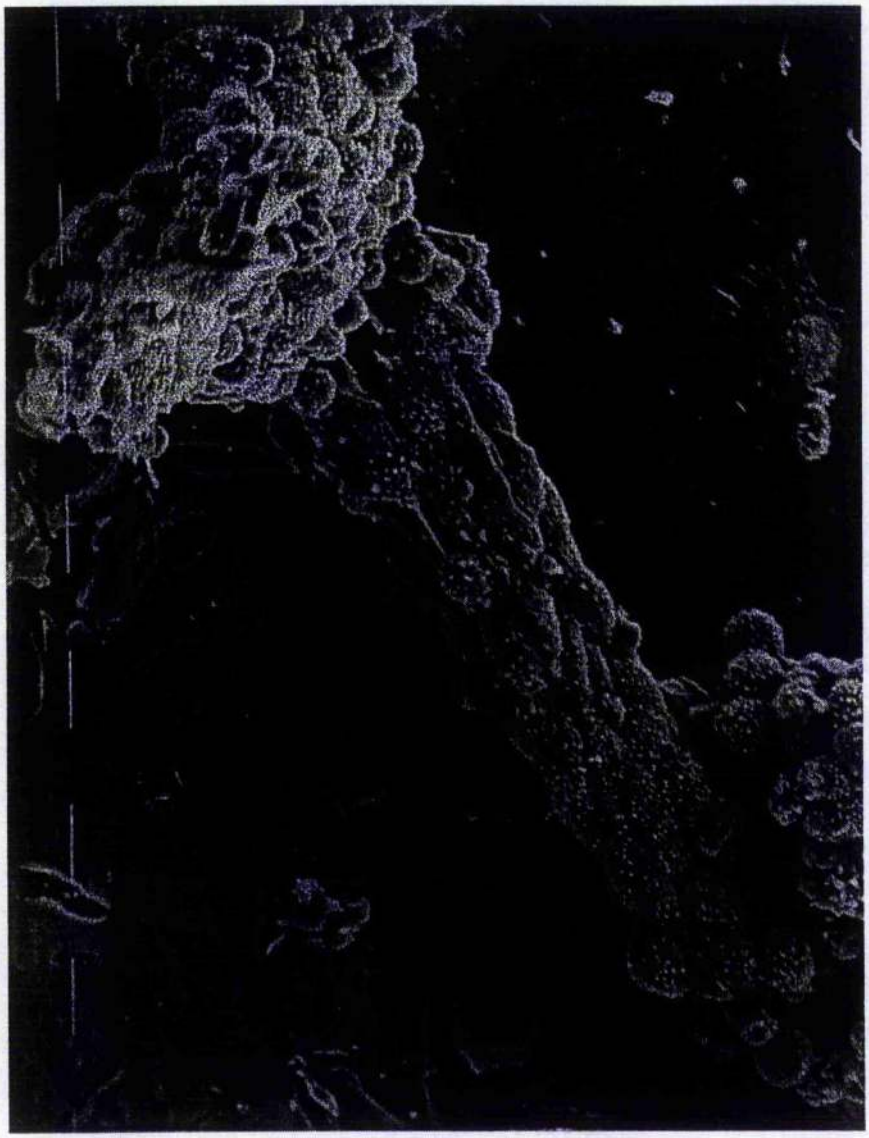
Neutrophils were seeded on to bovine aorta endothelial cells (which had been grown in the absence of dexamethasone) for twenty minutes and then washed. fMLP (10^{-7}M) was then added and the cells incubated at 37°C for a further thirty minutes before preparation for scanning electron microscopy as described in materials and methods. Magnification 3,200, scale bars are 1μm.

Fig. 13 Scanning electron microscope image of an STMS treated neutrophil on bovine aorta endothelium.



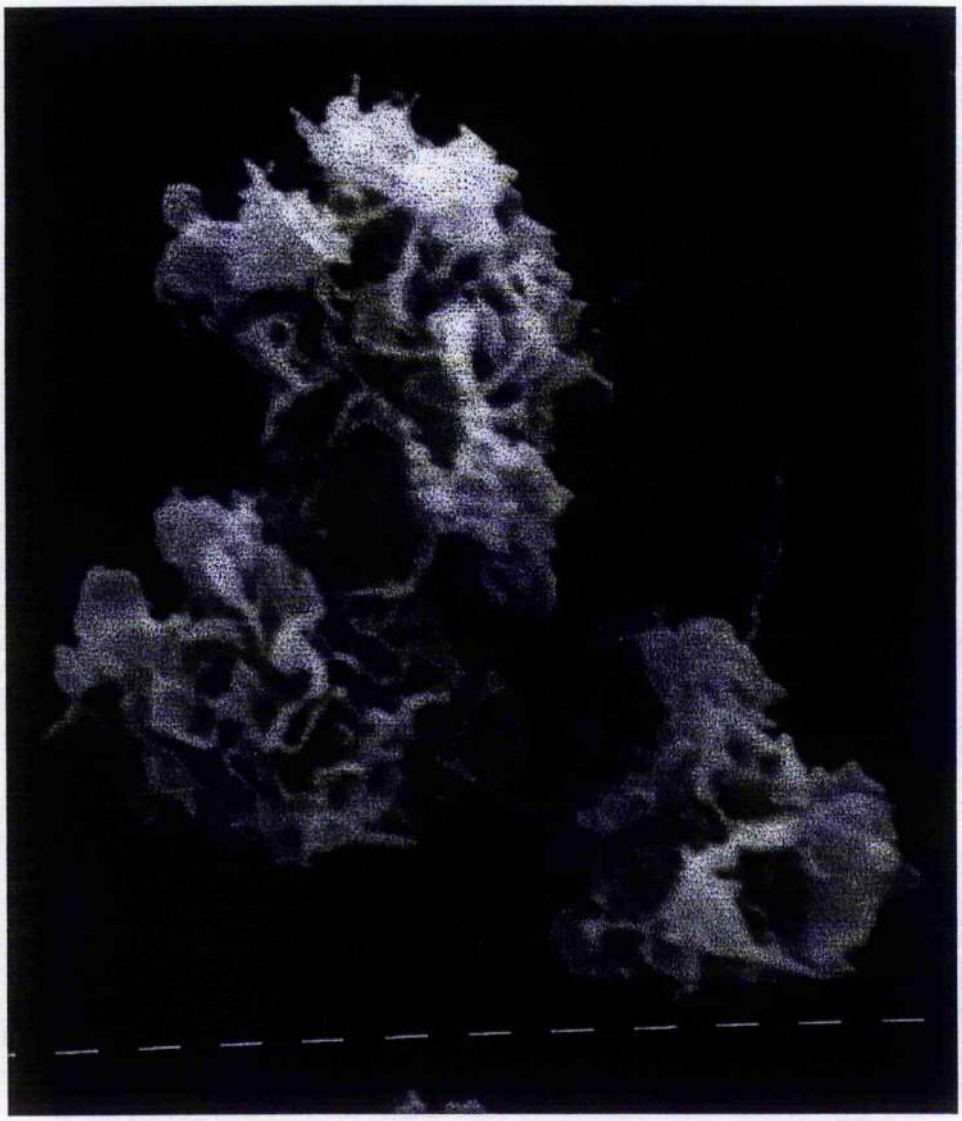
Neutrophils were seeded on to endothelial monolayers which had been grown in the absence of dexamethasone and allowed to adhere for twenty minutes before washing and incubated in STMS for a further thirty minutes at 37°C before preparation for scanning electron microscopy as described in materials and methods. Magnification 3,200, scale bars are 1μm.

Fig. 14 Scanning electron microscope image of neutrophils seeded on to bovine aorta endothelium before treatment with IL-8.



Neutrophils were seeded on to bovine aorta endothelial cells (which had been cultured to confluency in the absence of dexamethasone) and allowed to adhere for twenty minutes at 37°C before washing and then incubated in the presence of IL-8 (1 μg/ml) for a further thirty minutes before preparation for scanning electron microscopy. Magnification 1,600, scale bars 5μm.

Fig. 15 Scanning electron microscope image of colchicine treated neutrophils adherent to bovine aorta endothelium.



Neutrophils were seeded on to bovine aorta endothelium (which had been cultured in the absence of dexamethasone) for twenty minutes before washing. Colchicine (10^{-5}M) was then added to the cells and the incubation was resumed for a further thirty minutes before the coverslips were prepared for scanning electron microscopy as described in materials and methods. Magnification 6,400, scale bars 1μm.

shrinkage (compared with critical phase drying) and so the size of the gaps is less likely to be exaggerated during the drying stage.

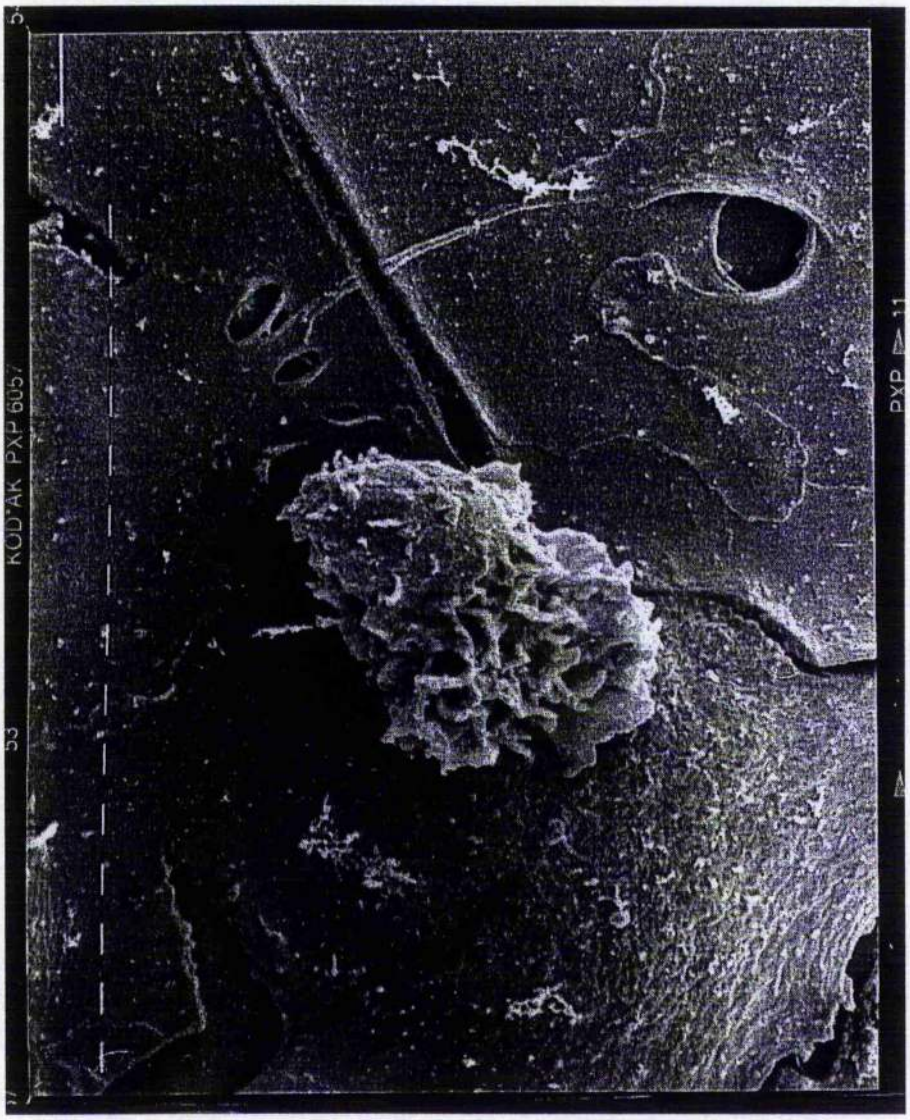
Neutrophils treated with IL-8 when adhered to endothelial cells, have a very high degree of homotypic aggregation (fig. 14), with those cells not aggregated appearing very similar to fMLP treatment. Colchicine treatment of PMNs produced very deep ruffles over much of the surface of the cell (fig. 15), and also apparently a slight decrease in the size of the cell (not shown), in line with reports by Gail and Boone (1971), but conflicting with Lackie (1974).

3.4.2.1. Steroid Treatment of Endothelium

Endothelial cell sheets (cultured for 1-2 days) in the presence of steroids normally show considerably fewer gaps between the individual endothelial cells. PMN seeded on to monolayers and then treated with fMLP were predominantly found in the endothelial junction regions, and these PMNs did not possess a leading edge (fig. 16). The PMNs however did not transmigrate to the same degree as they did on non-steroid treated endothelium. This was observed also with IL-8 treatment of the neutrophils (fig. 17). As expected from results with non-steroid treated endothelia, STMS PMNs showed virtually undetectable transmigration activity (fig. 18).

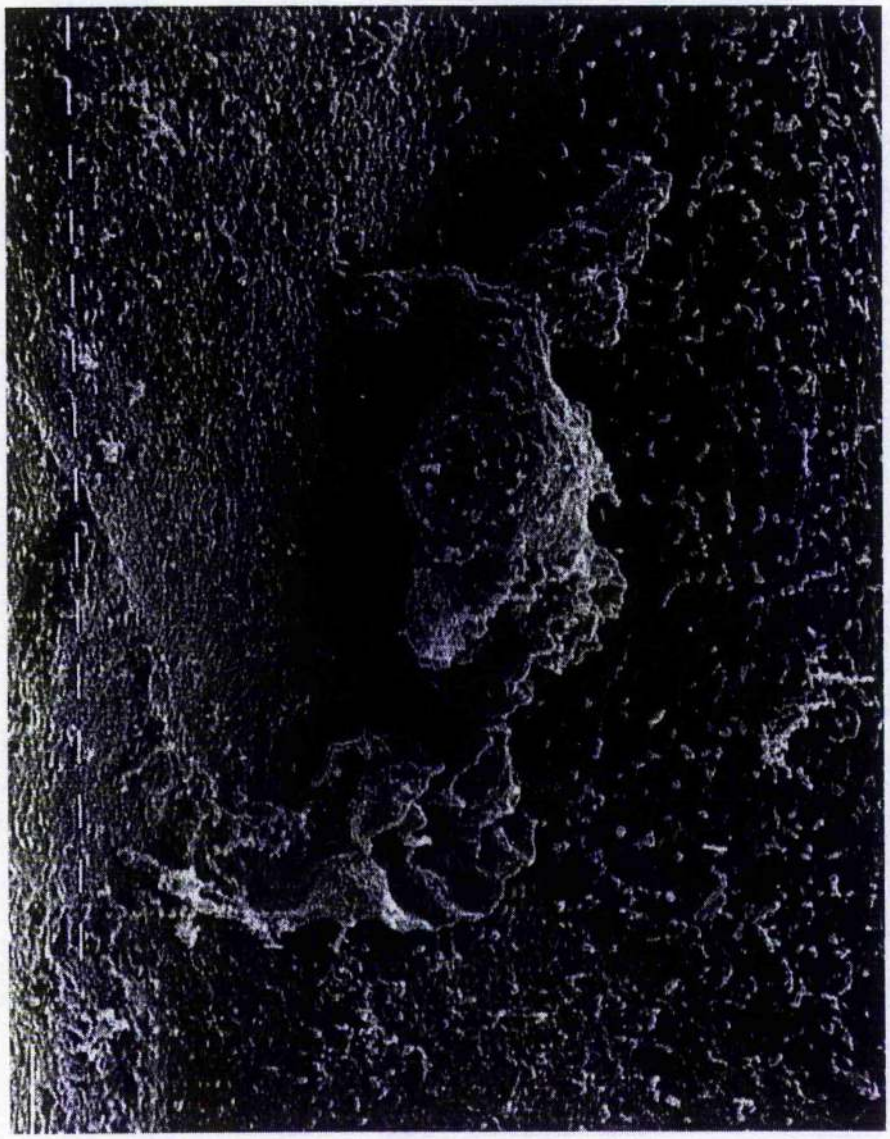
The size of neutrophils as observed by SEM has to be used as a guide between treatments, and not as a measurement of the size of cells. This is because all cells shrink when dried by critical point drying, and it is known that microtubule based structures are more resistant to shrinkage than microfilament based structures (L. Tetley, personal communication). It was therefore of interest that the extreme morphologies displayed by colchicine treated PMNs were maintained in the virtual absence of microtubules, where susceptibility to collapse should be greatest.

Fig. 16 Scanning electron microscope image of an fMLP treated neutrophil on dexamethasone treated endothelium.



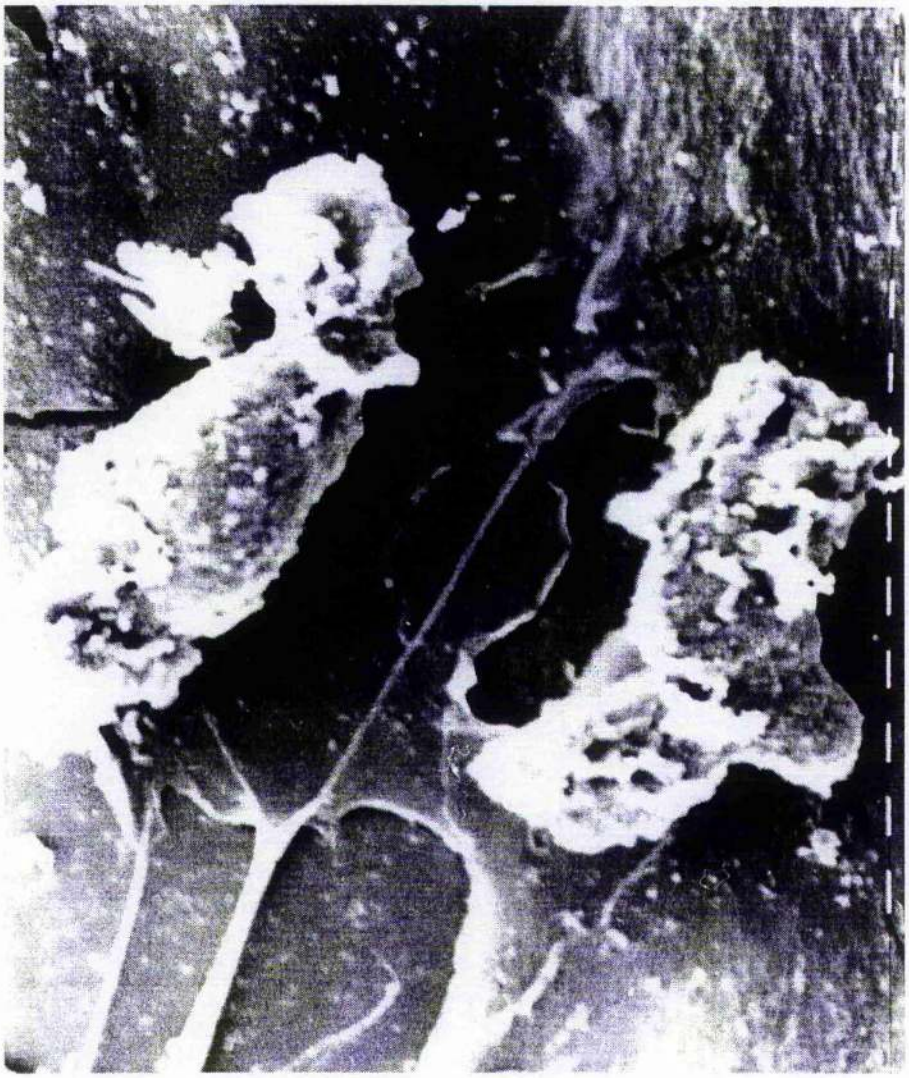
Neutrophils were seeded on to bovine aorta endothelium (which had been cultured to confluency in the presence of 10^{-6}M dexamethasone) and allowed to adhere for twenty minutes before washing. The coverslips were then incubated in fMLP (10^{-7}M) at 37°C for a further thirty minutes and prepared for scanning electron microscopy as described in materials and methods. Magnification 3,200; scale bars 1μm.

Fig. 17 Scanning electron microscope image of an IL-8 treated neutrophil adhered to dexamethasone treated bovine aorta endothelium.



Neutrophils were seeded on to bovine aorta endothelial monolayer (which had been grown to confluency in the presence of 10^{-6}M dexamethasone) and allowed to adhere for twenty minutes before washing and IL-8 (1 μg/ml) added. The cells were incubated at 37°C for a further thirty minutes before preparation for scanning electron microscopy as described in materials and methods.

Fig. 18 Scanning electron microscope image of STMS treated neutrophils adherent to bovine endothelium



Bovine aorta endothelial cells were grown to confluency in the presence of 10^{-6}M dexamethasone. The monolayers were incubated overnight in medium which had not been supplemented with dexamethasone and washed. Neutrophils were seeded on to coverslips and allowed to adhere for twenty minutes before washing. The coverslips were then incubated with STMS for thirty minutes at 37°C before preparation for scanning electron microscopy as described in materials and methods. Magnification 3200, scale bars 1μm.

3.5. Confocal Microscopy

3.5.1. Staining of polymerised actin

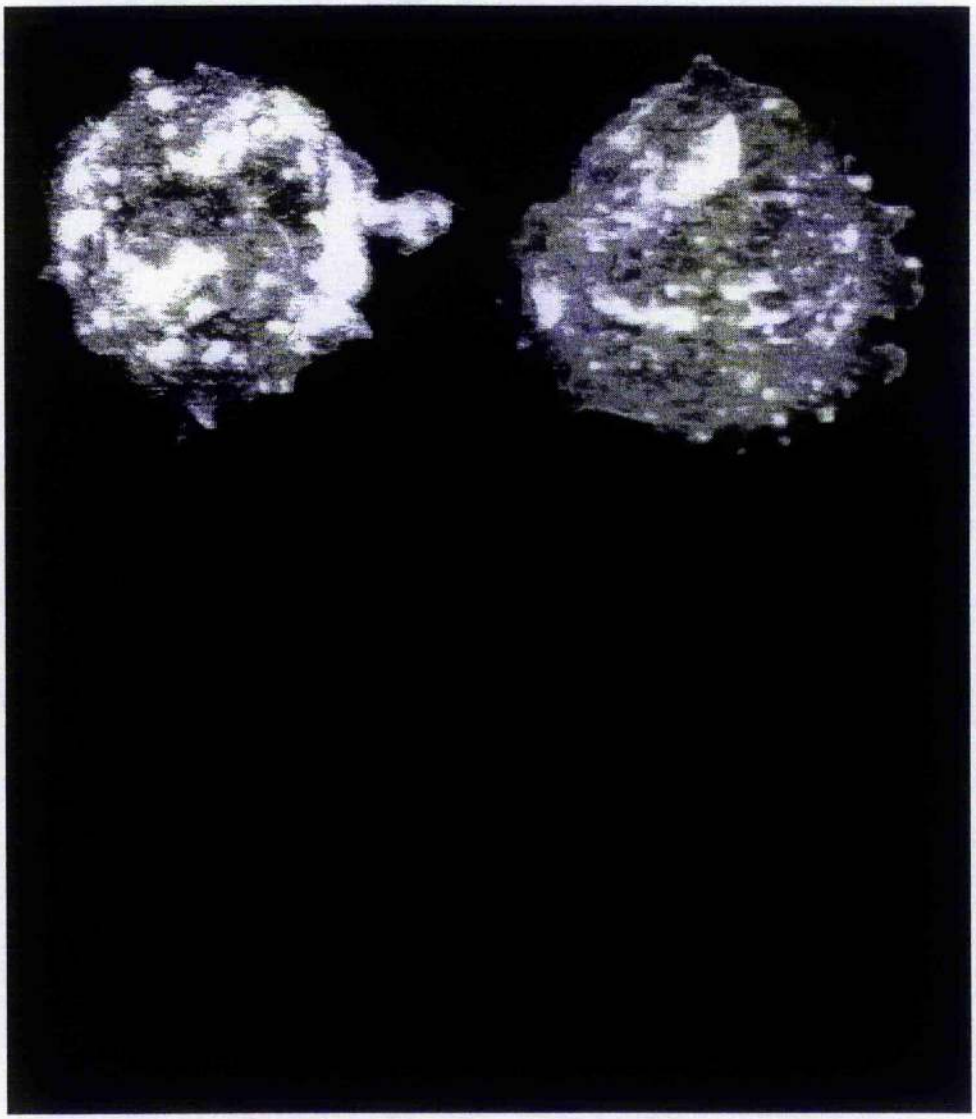
Z-series taken from confocal laser microscopy of PMN on BSA coated coverslips reveals very distinct differences between cells treated with various agonists, but in particular it revealed a highly unusual distribution in STMS treated cells.

Control (Ham's F-10) treated cells are rounded and have a patchy distribution of polymerised actin throughout the cell, the patches being randomly distributed and varying in size, but in most cases are close to the cell surface (fig. 19). There is a tendency for cells to have 2-3 larger patches of denser staining. Watts and colleagues (1991) have proposed that denser foci of polymerised actin act as sites where pseudopod formation may be initiated, and here the PMN on the left of the field appears to be initiating a pseudopod from such an actin concentration.

fMLP treated PMN (fig. 20) were characterised by a much greater density of polymerised actin which was often concentrated into regions within the cell body, and by large, branched pseudopods containing more diffuse polymerised actin.

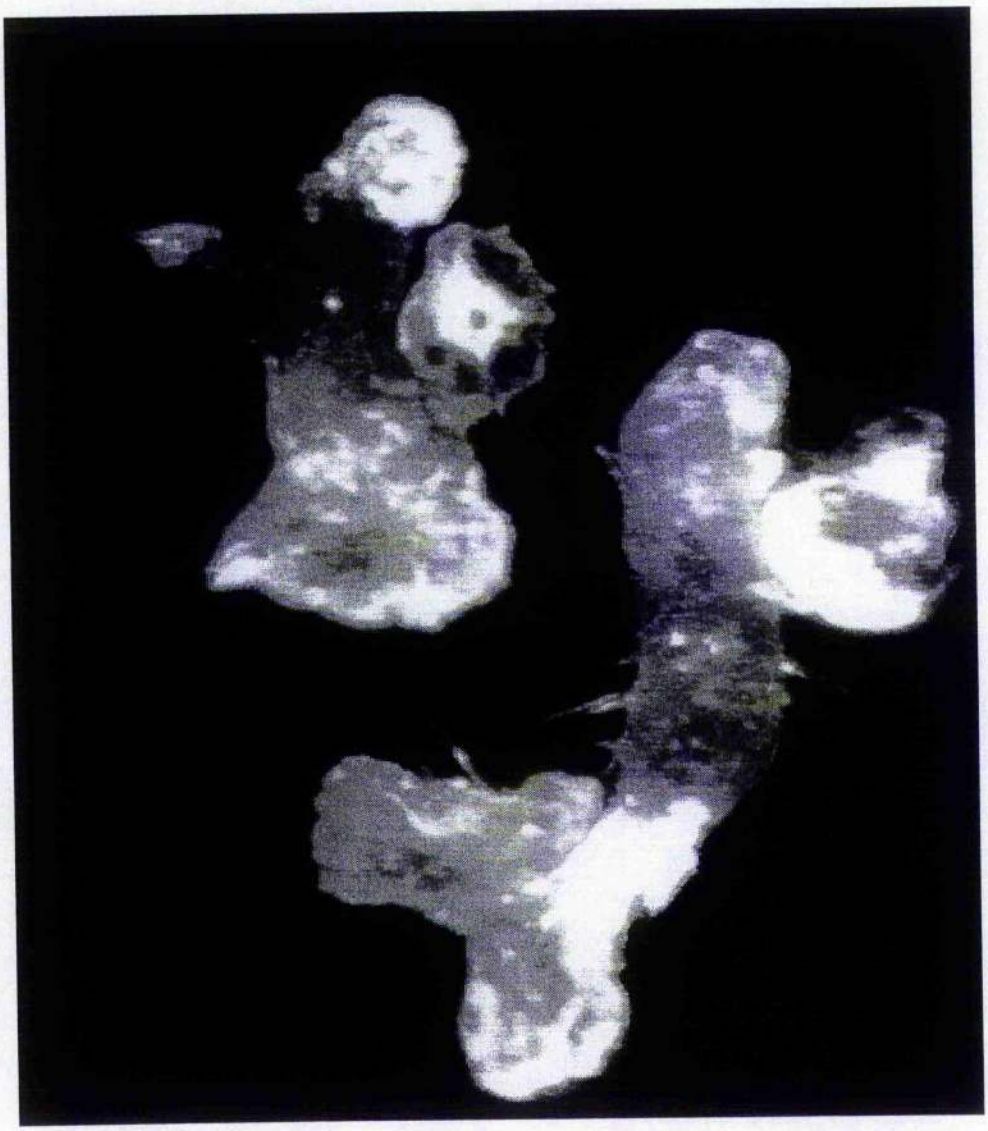
IL-8 treated PMNs commonly had very dense areas of polymerised actin associated with psuedopodia (fig. 21), with a definite polarity set up. However, this polarity is clearly different from that induced by STMS treatments. The extension and branching of the pseudopodia indicate a large degree of polymerised actin in both of the opposing poles of the PMNs.

Fig. 19 Confocal Microscopy image of neutrophils treated with F10, stained with TRITC Phalloidin



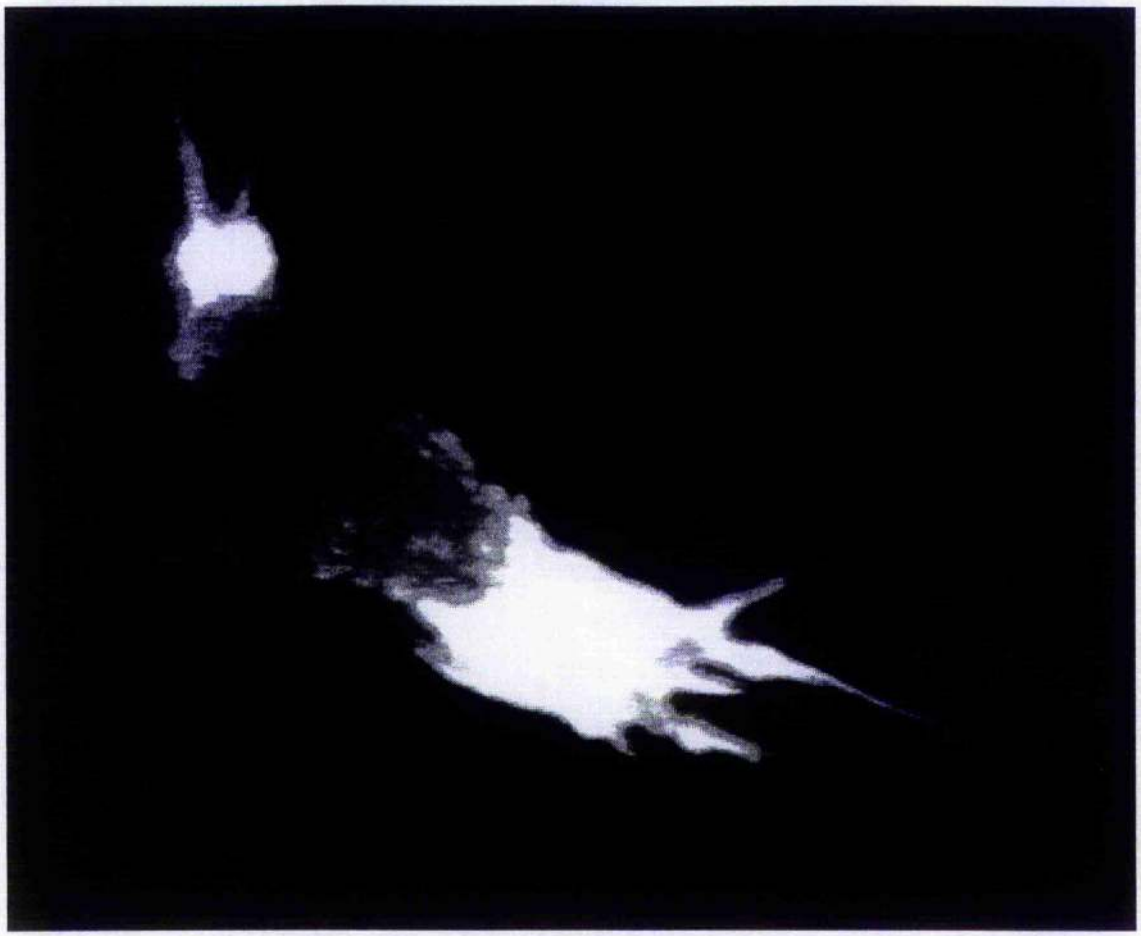
Neutrophils were allowed to adhere to BSA coated coverslips for twenty minutes before washing and incubation in Hams F10 for a further 30 minutes at 37°C before fixation with 1% paraformaldehyde and preparation for fluorescence microscopy as described in materials and methods. Magnification x100.

Fig. 20 Confocal microscopy image of neutrophils treated with fMLP (10^{-7}M), stained with TRITC Phalloidin.



Neutrophils were allowed to adhere to BSA coated coverslips for twenty minutes and washed before incubation with 10^{-7}M fMLP at 37°C for a further thirty minutes before fixation in 1% paraformaldehyde and preparation for fluorescence microscopy as described in materials and methods. Magnification x100.

Fig. 21 Confocal microscopy image of adherent neutrophil treated with IL-8, stained with TRITC Phalloidin.



Neutrophils were allowed to adhere to BSA coated coverslips for twenty minutes before washing and incubation with IL-8 (1 μg/ml) for thirty minutes at 37°C. The cells were fixed with 1% paraformaldehyde and stained for F-actin as described in materials and methods. Magnification x100.

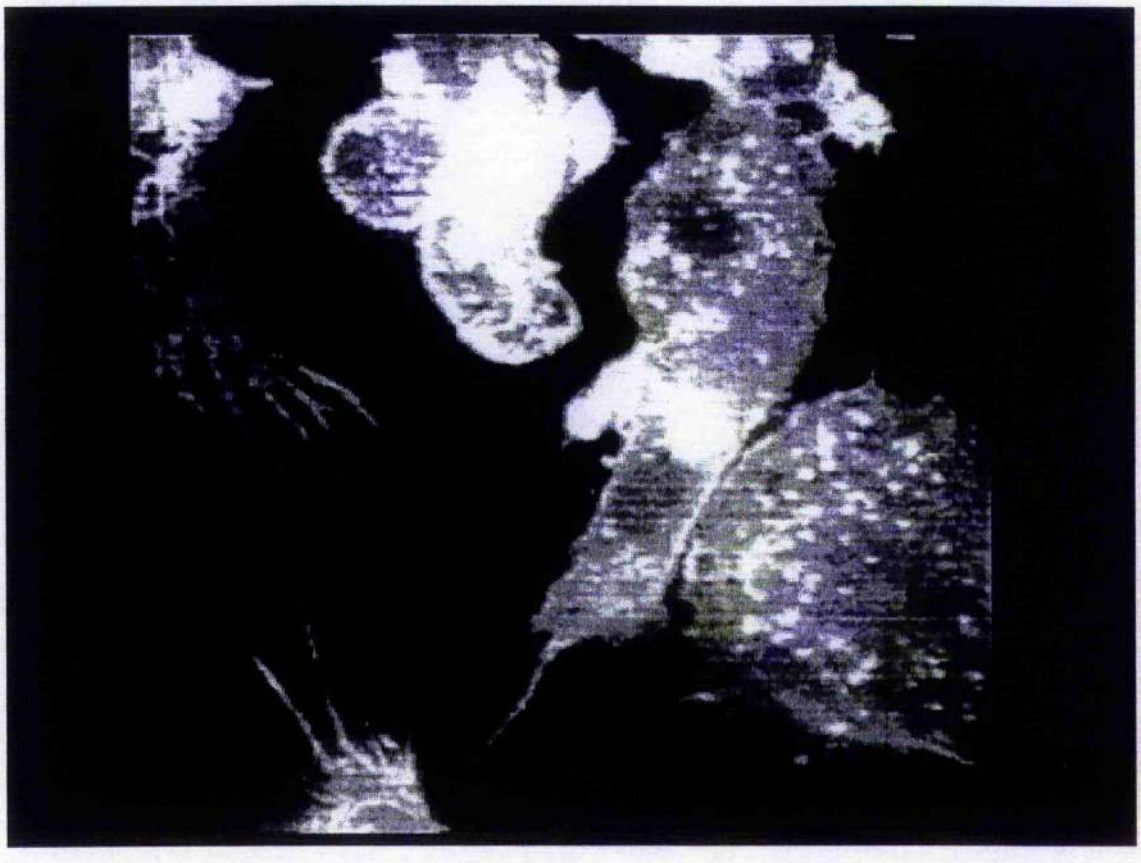
CMS treated PMNs tended to have more foci of condensed actin (fig. 22), but not as strongly associated with pseudopodia which were themselves smaller and less branched than those on fMLP treated PMN.

TNF-α treated PMNs were characterised by having numerous foci of relatively diffuse actin staining, with very few pseudopodia observed (fig. 23). There also tended to be a larger focus of actin towards the periphery of the PMN.

STMS treatment of PMN gave a visibly distinct image from any other agonist tested. The bipolar cells displayed one major actin concentration at the narrower of the two poles with a much lower concentration at the opposite pole, and little found anywhere else (fig. 24). The closest resemblance was to IL-8 treated PMNs, but other aspects of the morphology were quite distinct and in particular the long spikes apparent in IL-8 treated cells were absent in STMS treated PMN.

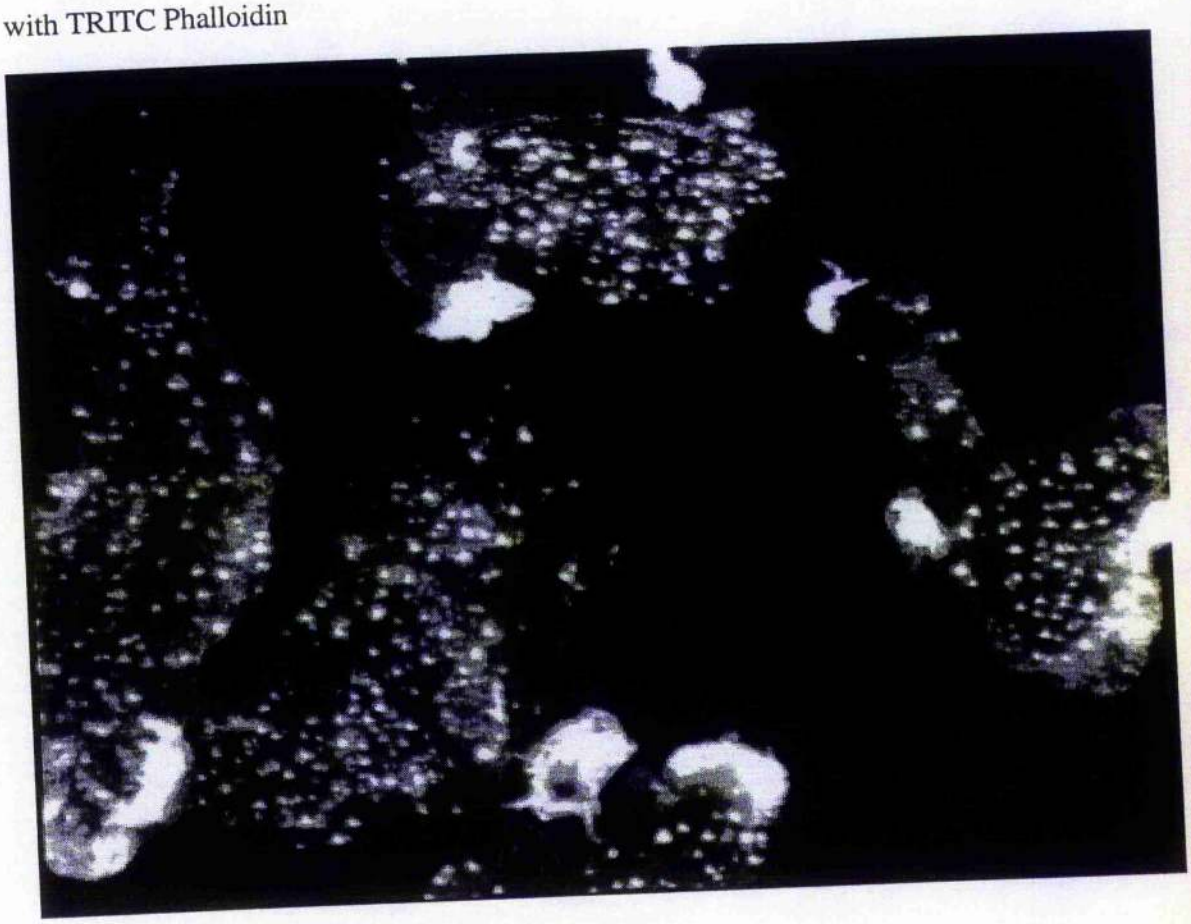
Regions of polymerised actin are characteristic of focal adhesions in other cell types, and so it is not unrealistic to assume that these represent adhesion sites in the present case. The results are consistent with the visual observation that PMNs treated with IL-8, TNF-α, fMLP etc. have multiple adhesion sites, whilst cells treated with STMS behave as if they had only one point of attachment.

Fig. 22 Confocal microscopy image of adherent neutrophils treated with CMS, stained with TRITC Phalloidin.



Neutrophils were seeded on to BSA coated coverslips and allowed to adhere for twenty minutes before washing and incubation with crude CMS for thirty minutes at 37°C before fixation with 1% paraformaldehyde and staining of F-actin using TRITC Phalloidin as described in materials and methods. Magnification x100.

Fig. 23 Confocal microscopy image of TNF-α treated adherent neutrophils, stained with TRITC Phalloidin



Neutrophils were allowed to adhere to BSA coated coverslips for twenty minutes and washed with saline before incubation with TNFα (8×10^3 units/ ml) for thirty minutes. The cells were fixed with 1% paraformaldehyde and stained for F-actin as described in materials and methods. Magnification x100.

Fig. 24 Confocal microscopy image of adherent neutrophils treated with STMS , stained with TRITC Phalloidin.

Neutrophils were allowed to adhere to BSA coated coverslips for twenty minutes before washing and incubation with crude STMS at 37°C for thirty minutes before fixation with 1% paraformaldehyde and preparation for fluorescence microscopy as described in materials and methods. Magnification x100.

Fig. 24 Confocal microscopy image of adherent neutrophils treated with STMS , stained with TRITC Phalloidin.

Neutrophils were allowed to adhere to BSA coated coverslips for twenty minutes before washing and incubation with crude STMS at 37°C for thirty minutes before fixation with 1% paraformaldehyde and preparation for fluorescence microscopy as described in materials and methods. Magnification x100.

3.6. The effect of STMS on Adhesion of PMNs

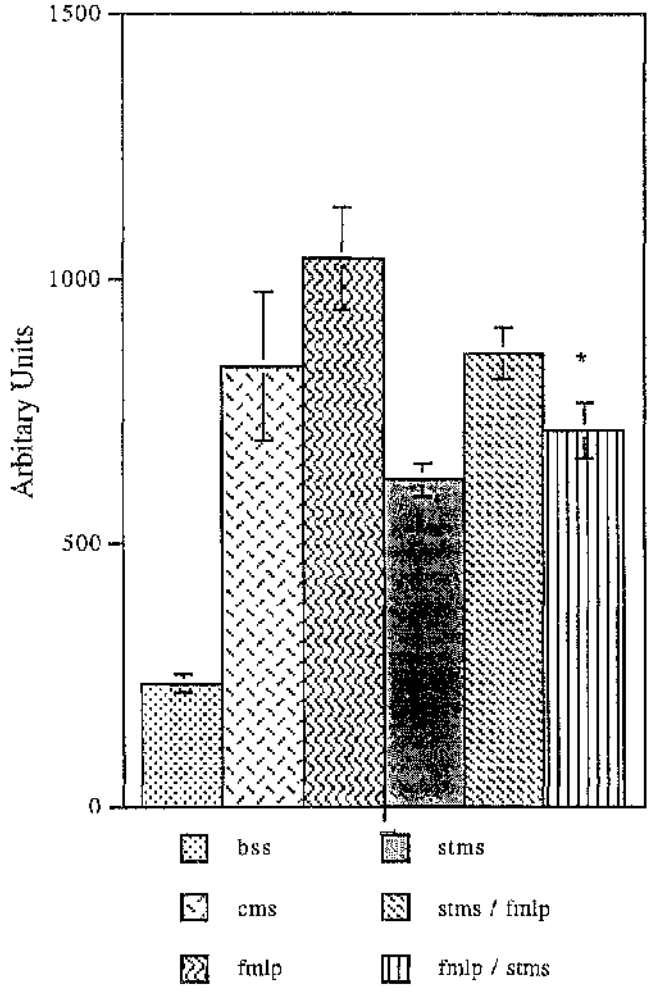
3.6.1. Adhesion to Protein Coated Glass

Crude STMS was reported to reduce the adhesion of PMNs on protein coated glass (Chettibi et al., 1993). However, this work was repeated several times and it became clear that STMS treatment of PMNs increased the basal levels of adhesion to BSA coated glass, but not as much as fMLP (10^{-6}M) nor CMS treatment. It is possible that the presence of IL-8 in crude STMS may account for this increase. In contrast, partially purified STMS (fig 2. stage 2 and stage 3) decreased adhesion by around 40% (not shown). The true effects of STMS are perhaps better shown when it is used to counter fMLP induced adhesion, reducing the adhesion by 20% (fig. 25).

3.6.2. Adhesion to endothelial cells

Preliminary tests showed that the adhesives of PMNs to untreated endothelial cells was very low, and in order to test the effect of STMS in this system the endothelial cell sheet was pre-activated by a number of agonists known to increase its adhesives (Gamble et al., 1985; Lorant et al., 1991). These included various cytokines (IL-1β, TNF-α, Thrombin) and in the procedure adopted, the endothelial cells were incubated with an optimal level of IL-1β for either 4 or 24 hours before addition of PMN. This pre-incubation increased the basal level of adhesion by 600% (fig 26).

Fig. 25 STMS reduction of fMLP induced adhesion of neutrophils to protein coated glass



Neutrophils were seeded on to BSA coated glass, and allowed to adhere for twenty minutes before washing and incubating at 37°C for thirty minutes. The coverslips were washed and the specific radioactivity quantitated as described in materials and methods. Statistical analysis (Student's T-test) showed that fMLP treatment followed by STMS treatment significantly lowered the adhesion of the PMN to the BSA coated glass. Also, fMLP treatment after STMS treatment significantly increased the adhesion of PMNs.

Fig. 26 Increase in basal adhesion of neutrophils to HUVEC pre-treated with cytokines

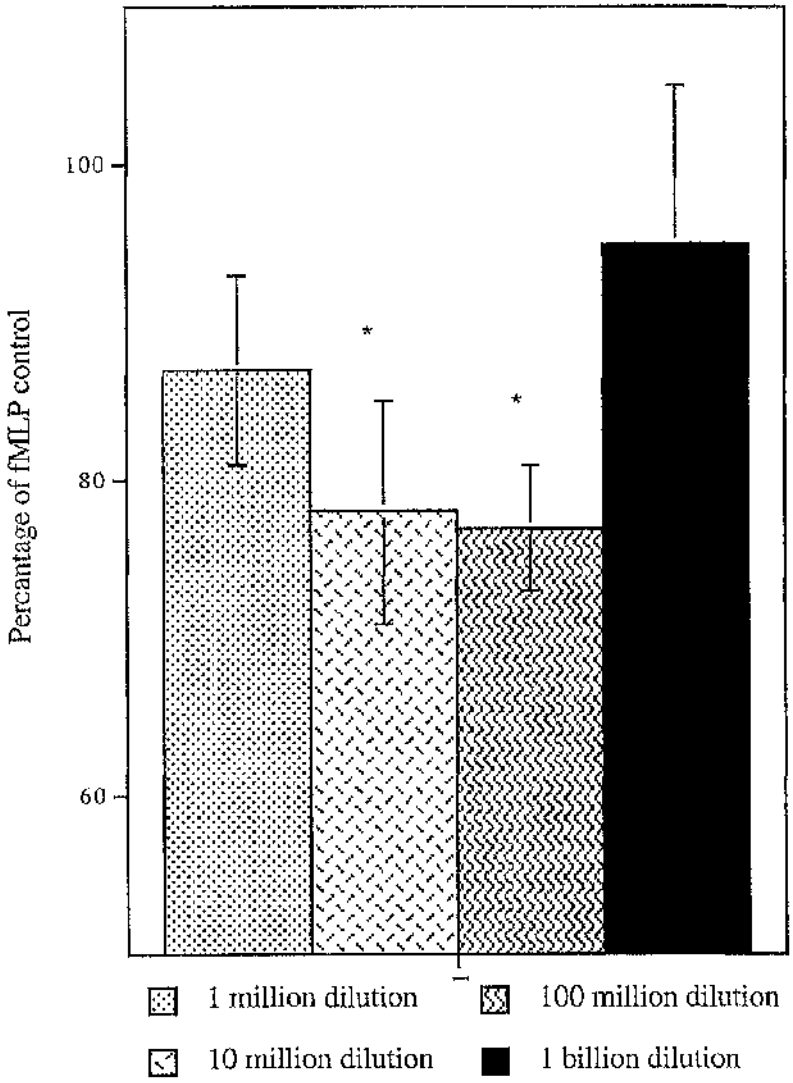
Treatment	Average	Percentage of F10 control
F10	33	100
IL1-β	60	182
TNFα	82	248
IL1-β and TNFα	92	279
Thrombin	60	183

HUVEC were treated for 6 hours with cytokines; IL-1β (10 units / ml) TNF ($8x10^3$ units / ml) (except thrombin (2 units / ml), which requires ten minutes treatment), before washing and seeding of neutrophils. The coverslips were incubated for thirty minutes before careful washing and counting. Neutrophils were loaded with CFDA as described in materials in methods, and counted using fluorescence objectives (x10).

3.6.3. Inhibition of Adhesion to Cytokine Activated Endothelium

Neutrophils that had been allowed to adhere for 30 minutes to endothelium pre-treated with IL1-β were then treated with different concentrations of highly purified STMS, or controls for a further 30 minutes. The effects of different treatments are shown in fig. 27. Increasing concentrations of STMS clearly show a dose response when compared against the fMLP control with 10^7 and 10^8 dilutions having significant inhibition of adhesion. The quantitation of STMS was based on the UV absorption at 214nm. Using the relationship that 1mg/ml of protein has an A_{214} of 15 then it is possible to calculate from the UV trace the maximum concentration of peptide that could be present, making the assumption that STMS is a peptide, then its threshold concentration for inhibition of adhesion of PMNs to endothelium is about 10ng/ml. During all of these assays, care was taken to wash all wells evenly, and with the same volume of liquid to eradicate as many sources of error as possible. However, large error margins are inherent with this type of experiment, and so a number of experiments were performed using visual counting of cells, either under the SEM (as an after thought when visualising the images for 3.4.2.) or by using CFDA to visualise the cells on BSA coated coverslips. In all cases, ppSTMS was shown to inhibit adhesion by at least twenty percent, although sometimes the preparation of STMS could produce inhibition of up to almost one half of the F10 control.

Fig. 27 Inhibition of adhesion by of neutrophils to IL-1β pre-treated HUVEC ppSTMS



Neutrophils were seeded on to Human Umbilical Vein Endothelial Cells which had been grown to confluency on coverslips and pre-treated with IL-1β. After 30 minutes, the coverslips were washed and different concentrations of STMS were added, and incubated at 37°C for thirty minutes. fMLP (10^{-7}M final concentration) was then added to the coverslips, and incubated at 37°C for a further twenty minutes. The specific radioactivity was recorded as described in the materials and methods.

* indicates statistically significant reduction in adhesion compared with fMLP control.

3.7. Degranulation Assays

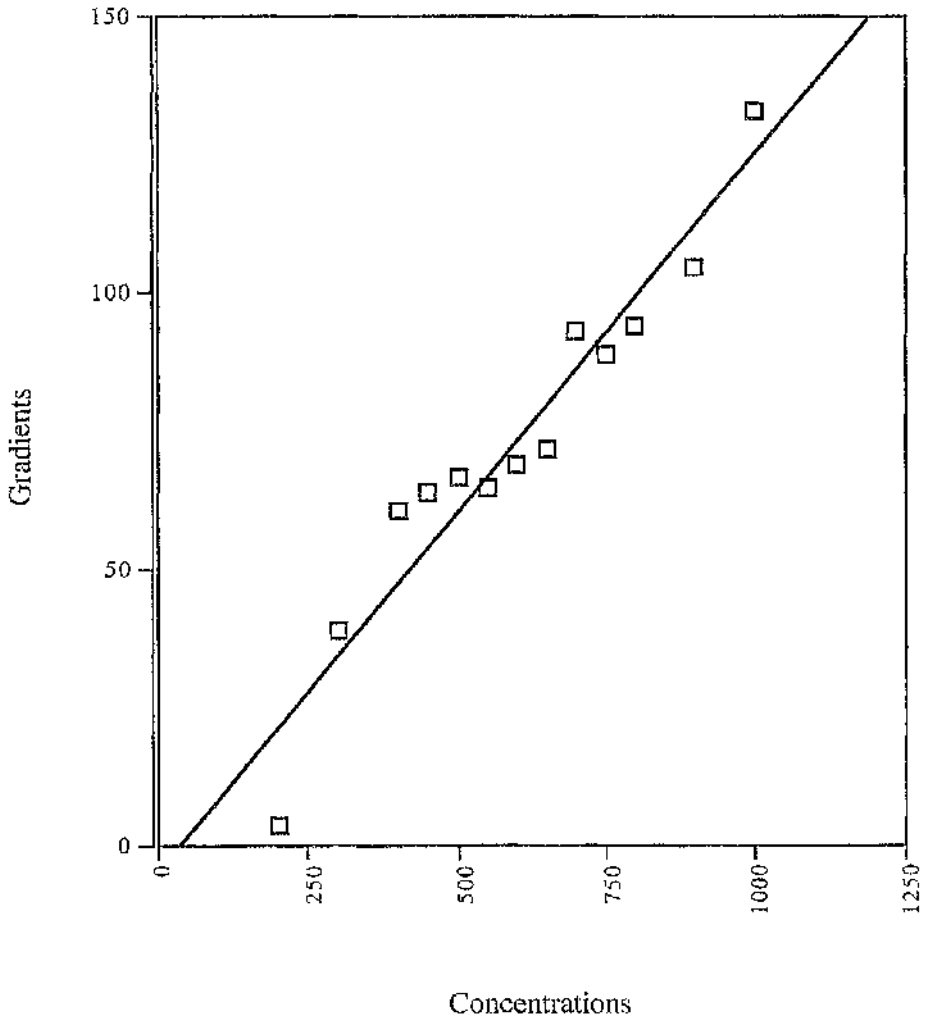
3.7.1. Calibration of Elastase Assays

Porcine pancreatic elastase (ICN Flow) was used to calibrate the system used to determine concentrations of elastase in supernatants. Fig. 28 shows a typical reaction using increasing concentrations of elastase. The straight line portion of the reaction was used to determine the gradient of the slope. Four readings were taken for each concentration, and the mean gradients plotted. Due to fairly rapid deterioration of the substrate (over the course of 3-4 hours), it was not always possible to perform full calibrations with every experiment.

3.7.2. Release of Elastase from Primary Vesicles

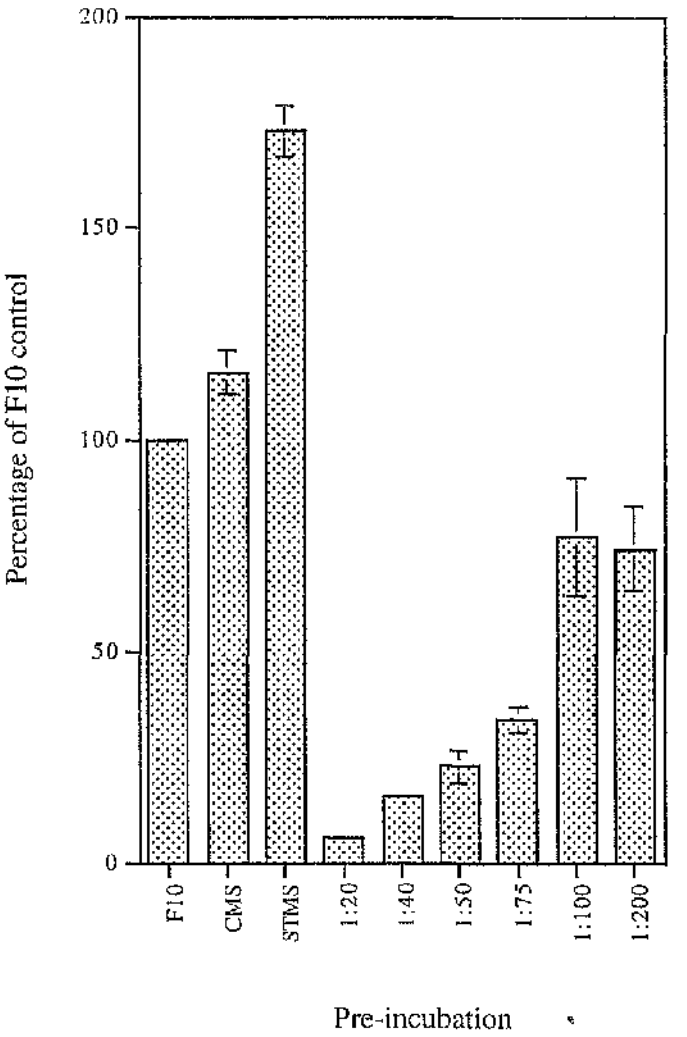
STMS had a dramatic effect on the inhibition of elastase release in response to fMLP and this was conserved throughout the purification including the high resolution reverse phase procedure, but has not been assayed using more highly purified material. The activity of the factor might therefore be followed using the inhibition of elastase release as an assay method. Supernatants of neutrophils primed with cytochalasin B (1μg/ml)were activated with fMLP (10^{-6}M) having been pre-treated with varying concentrations of STMS. Crude STMS activated the release of primary vesicle contents, by at least ten percent, but as purification proceeded, the inhibitory power increased (fig. 29). Results were quite variable, but with certain preparations of STMS it was possible to inhibit the release of elastase by in excess of 90%.

Fig. 28. Calibration of elastase using porcine pancreatic elastase



Porcine pancreatic elastase was dissolved in PBS. The enzymatic activity was determined by adding appropriate quantities to a cuvette containing N-tBOC-l-Alanine p-nitrophenyl phosphate and the change in absorption at 347nm was recorded. The gradients were calculated and plotted.

Fig. 29 Inhibition of release of elastase from human neutrophils pre-incubated with increasing concentrations of partially purified STMS



Neutrophils were pre-incubated with concentrations of STMS indicated, with F10 (positive control) or crude cell supernatant for thirty minutes before activation of primary granule release. Gradients of the conversion of N-tBOC l-alanine p-nitrophenyl phosphate were calculated and plotted. Significance was calculated using Student's T-test.

3.7.3. Release of Myeloperoxidase

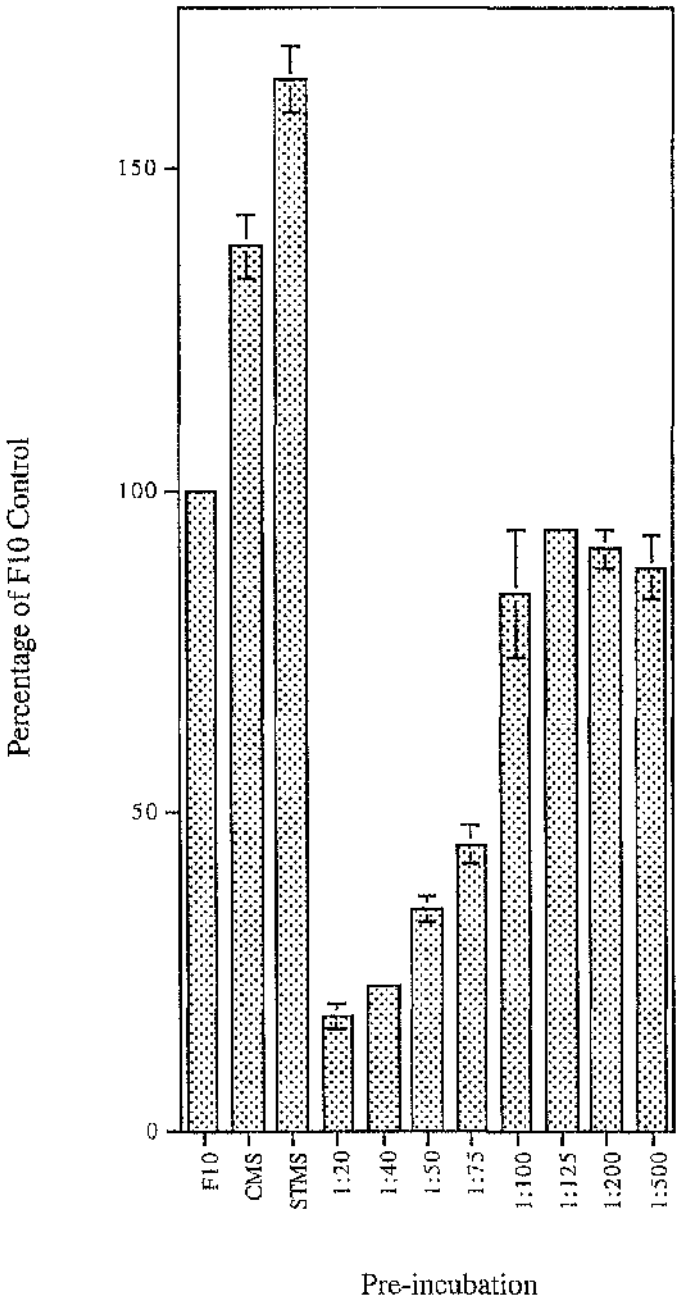
To confirm that the effect above was due to inhibition of the release of primary vesicles to the surface of PMNs, the enzyme myeloperoxidase (MPO) was also assayed (fig. 30). Both assays are equally sensitive, and myeloperoxidase release closely mirrored that of elastase. (The lack of perfect correlation could reflect further heterogeniety of the vesicle population) At this stage the activity, as measured by inhibition of primary granule release, correlated well with the activity in the dispersive motility assays and the adhesion assays.

More recent purifications have produced a material which gives bell-shaped dose response curve when assayed for dispersive locomotion. These results were also observed for release of primary vesicle contents (fig. 31), where higher dilutions of highly purified STMS inhibited more than lower dilutions.

3.7.4. Release of Secondary Vesicles

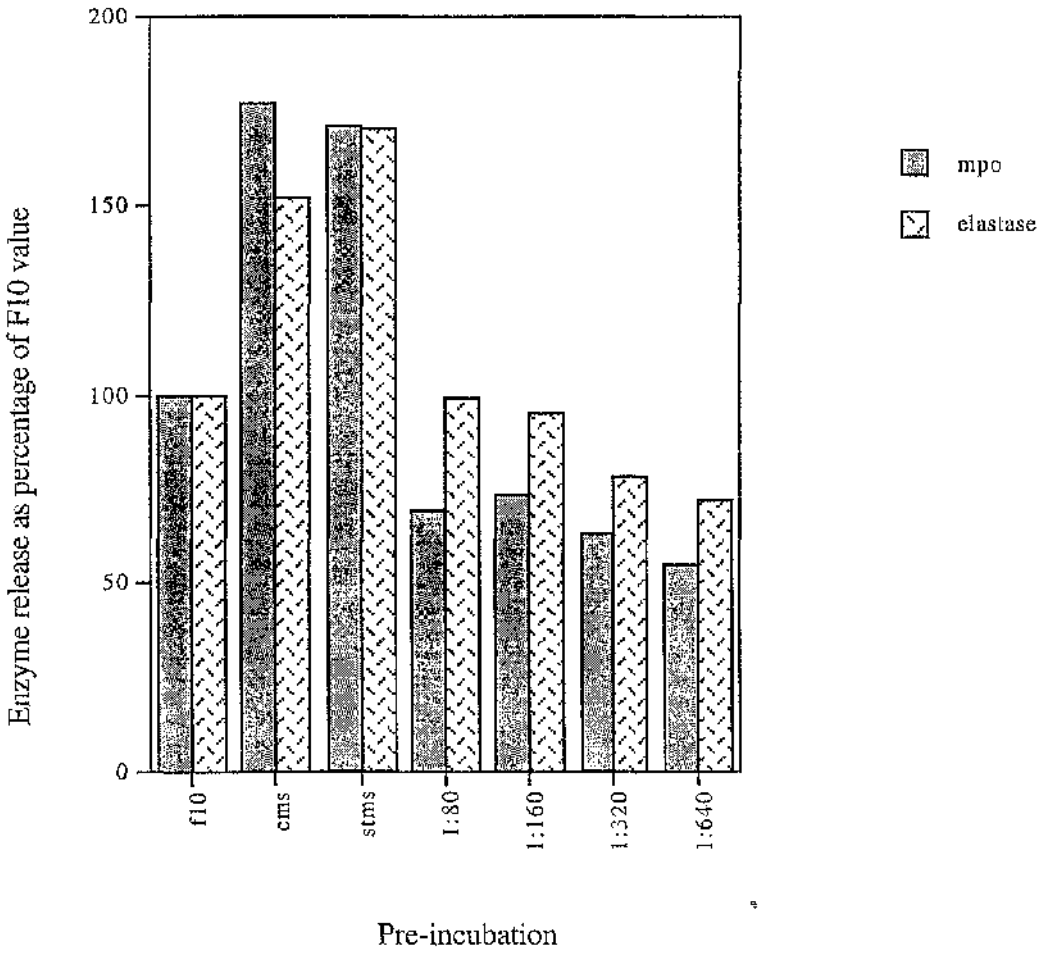
The use of collagenase as a marker for release of secondary vesicles proved to be exceptionally difficult. Unlike the assays for primary vesicle enzymes, this assay requires quantification of the cleavage of type I collagen polypeptides by SDS-PAGE. The collagen bands appeared to be similar to those reported by Ohlsson (1980) and Sorsa et al (1985), with α and β bands visible, together with low mw degradation products. In the assay loss of the native collagen bands is correlated with the appearance of the specific cleavage products, nevertheless there is considerable uncertainty in the identification of the bands. Despite these problems there was a clear indication that release of the enzyme was inhibited by STMS. However the dose response curve was completely unexpected in that inhibition decreased with increasing STMS concentration. The problem with the assay apart

Fig. 30 Inhibition of release of Myeloperoxidase from neutrophils pre-incubated with increasing concentrations of partially purified STMS



Neutrophils were pre-incubated with concentrations of STMS indicated, or with F10 (positive control) for thirty minutes before activation of primary granule release. Gradients of the conversion of N-tBOC l-alanine p-nitrophenyl phosphate were calculated and plotted. Significance was calculated using Student's T-test.

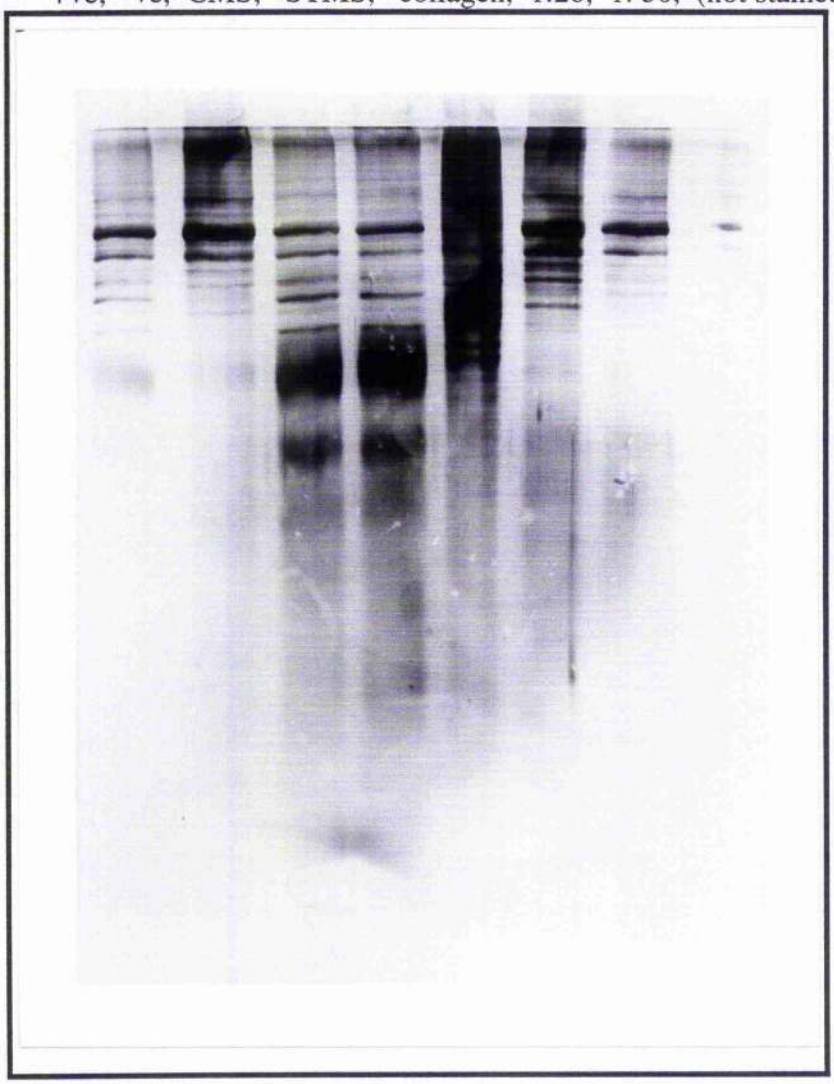
Fig. 31 Inhibition of release of primary granule contents using highly purified STMS



Neutrophils were incubated with highly purified STMS for thirty minutes prior to stimulation of primary granule release. Only one assay was possible with this sample, and therefore statistical errors were not calculated.

Fig. 32 Inhibition of collagenase release by STMS pre-treatment.

+ve, -ve, CMS, STMS, collagen, 1:20, 1: 50, (not stained)



Neutrophils were incubated at 37°C in the presence of highly purified STMS for thirty minutes before activation of release of granule contents as described in materials and methods. The supernatants were assayed for release of collagenase (Aaku et al., 1990). The gel was stained by silver staining as described in materials and methods.

The unstained lane was 1:100 dilution

from its inherent slowness was the preliminary activation of the enzyme. Native collagenase is inactive, and activation requires oxidation catalysed by MPO. In vitro, the oxidation is carried out with amino phenyl mercuric acetate. This appeared to be quite variable in its effect. It is possible that success required the presence of MPO, hence inhibition of primary granule secretion could effect the subsequent assay of collagenase. Therefore the results are at best semi-quantitative.

This result was the first indication that the action of STMS on PMNs might decrease at high concentrations giving a classic bell-shaped dose response curve (fig. 32), however at this stage the effect was considered to be due to some artefact of the assay or to its inherent irreproducibility. Because of this the experiments were stopped after a few attempts.

3.7.5. Release of Plasminogen Activator

The assay requires measurement of areas of cleared casein in agarose gels. Untreated cells gave no measurable increase in the area of cleared gel. There was no statistical difference between different treatments of PMNs, and so it has to be stated that STMS did not have any effect on the release of plasminogen activator from PMN activated with fMLP (fig. 33).

Fig. 33 Release of Alkaline phosphatase and Plasminogen factor from neutrophils pre-treated with STMS

Treatment	Alkaline Phosphatase (units)	Plasminogen Activator (mm cleared)
F10	2.6	11.22
BSS	2.78	11
CMS	2.43	10.11
1:100 dilution ppSTMS	2.85	11.44
1:400 dilution ppSTMS	2.63	11
1:800 dilution ppSTMS	2.56	11.78
STMS	2.42	10.67

Neutrophils were pre-incubated with indicated solutions for twenty minutes before addition of cytochalasin B (1μg/ml) and fMLP (10^{-6}M. The supernatants were aspirated and used for degranulation assays. The cell pellets were re-suspended in F10 and assayed for alkaline phosphatase as described in materials and methods. The supernatants were assayed for plasminogen activator as described in materials and methods. The remainder of the supernatants were used for elastase / MPO and collagenase assays. All of these other assays produced results indicating an inhibition of release of enzymes.

3.7.6. Release of Alkaline Phosphatase

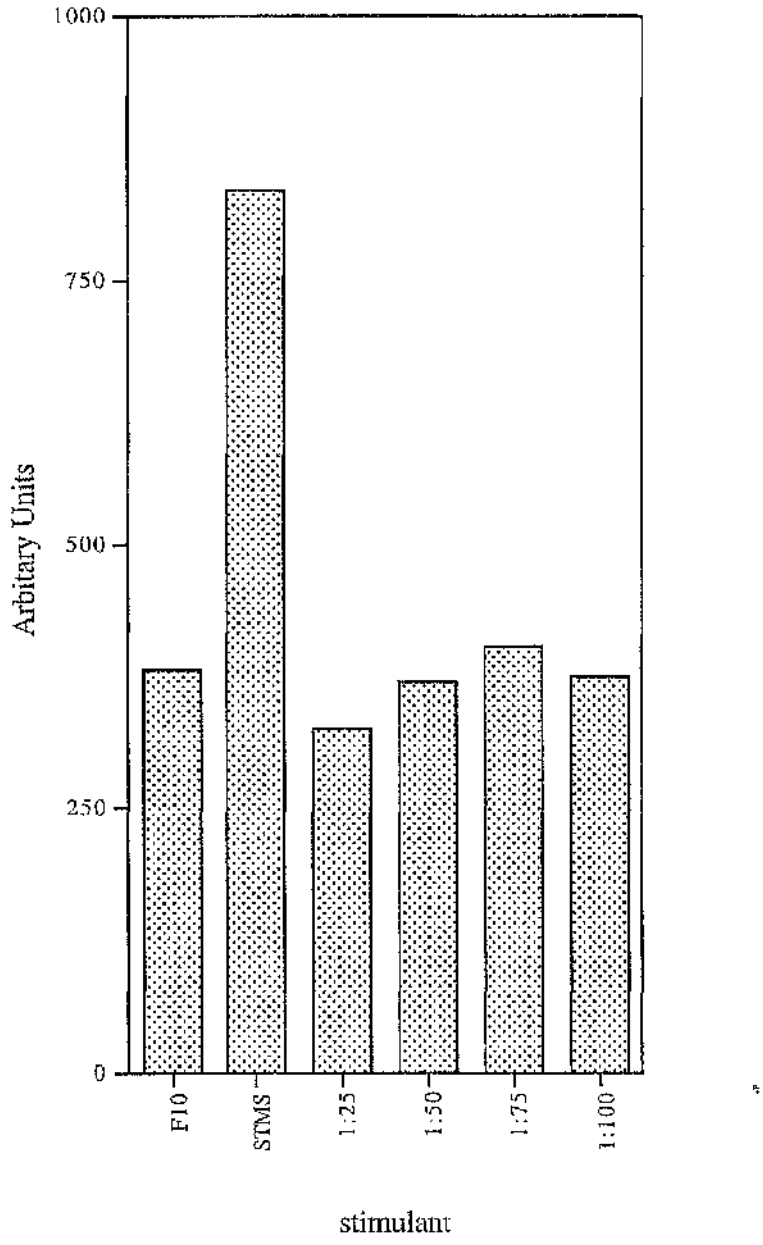
Alkaline phosphatase is an integral membrane protein found in the membranes of secretory vesicles, and as such can be used as a marker for release of vesicle contents. This is a highly specific assay, but also an exceptionally tedious one, with numerous stages. As the stimulation of the cells is stopped in a manner unlike the others (addition of three volumes ice cold buffer) this makes the assay incompatible with other degranulation assays. Also the numerous incubation steps, and volumes used prevent any other experiments being carried out concomitantly with this one. It does have the advantage, however, of being the easiest assay to quantitate. The amount of alkaline phosphatase present can be calculated from the extinction co-efficient for pNPP of 18.6 per mM per cm path length. As can be seen from fig. 33, there were no detectable differences between the treatments used.

3.7.7. Cell viability / secretory vesicles

The release of lactate dehydrogenase (LDH) has been used as a standard marker of non-apoptotic cell death. This enzyme was investigated for two reasons. The first was to see whether release of STMS from monocytes was due to cell lysis rather than secretion. The hypothesis being that steroids might promote lysis of monocytes in culture. The levels of LDH found in STMS was consistently higher than that found in CMS (about 15%), showing that dexamethasone treatment did induce a higher level of cell lysis than the control. The contaminating levels of LDH was removed from STMS during the first two stages of purification (fig. 3 strategy X).

Treatment of PMNs with purified STMS consistently decreased the levels of LDH in the supernatant released in response to fMLP. In fact STMS treatment gave less release of LDH than the negative controls. Fig. 34 shows a typical result for such an assay.

Fig. 34 Release of LDH from neutrophils treated with STMS.



Neutrophils were pre-treated with increasing concentrations of STMS, before stimulation with cytochalasin B (1µg / ml) and fMLP (10^{-6}M). Supernatant was assayed for the presence of LDH as described in materials and methods.

3.7.8. Phagocytosis of Latex Beads

In view of the inhibition of the release of primary granules by STMS it was anticipated that STMS would also inhibit phagocytosis. This was assayed using the latex bead assay (Wojiak & Crossan, 1993) and the results were consistently negative (not shown).

3.8.1. Metabolic Burst

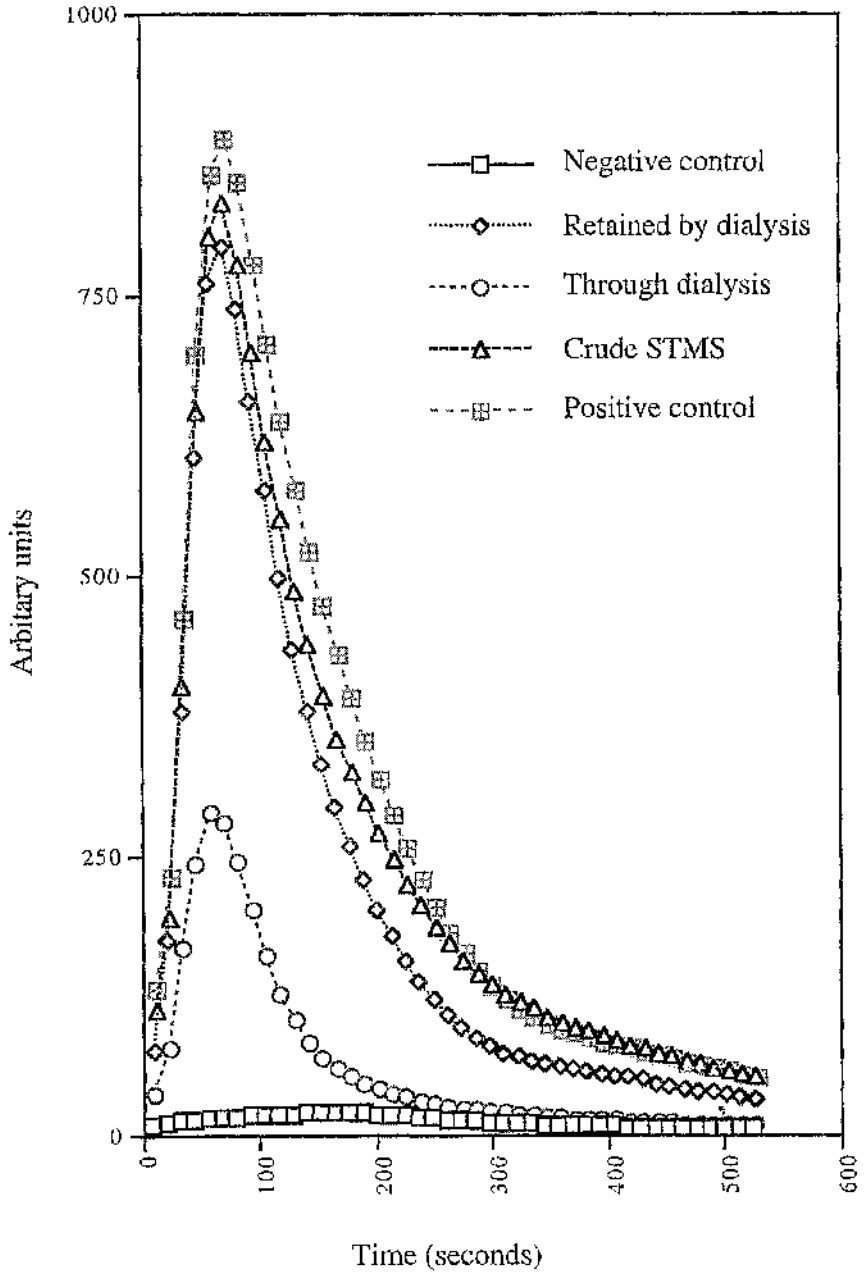
Of all the aspects of PMN function assayed, the analysis of the metabolic burst is the least technically challenging to perform, and as a result is the most extensively studied. The activation is by translocation of the regulatory subunits to the cytosolic surface of vesicle membranes or the PM. The process is activated by fMLP mediated by PKC and therefore PMA is an efficient agonist, and the agonist of choice in the assays, because the response is delayed and prolonged. Investigation of the effect of STMS on the PMA-stimulated respiratory burst was carried out at all stages of the purification (fig. 35). Starting material inhibited in a dose-dependent fashion (fig. 36), and this effect co-purified with the stimulation of the dispersive locomotion until the reverse phase step (fig. 37). After reverse phase, however highly purified STMS failed to retain the ability to inhibit the respiratory burst. The fractions containing the inhibitory activity have not yet been identified. This work was repeated using cytochalasin B primed PMN and fMLP as the stimulant. Because responses to these agents are much faster than to PMA, the results are less accurate, but the conclusion that pure STMS was inactive in this assay was verified.

3.8.2. Metabolic Burst Induced by Latex Beads

Phagocytosis of particles is an alternative (closely physiogical) method for initiating the metabolic burst of neutrophils. It was of interest to determine whether or not STMS had

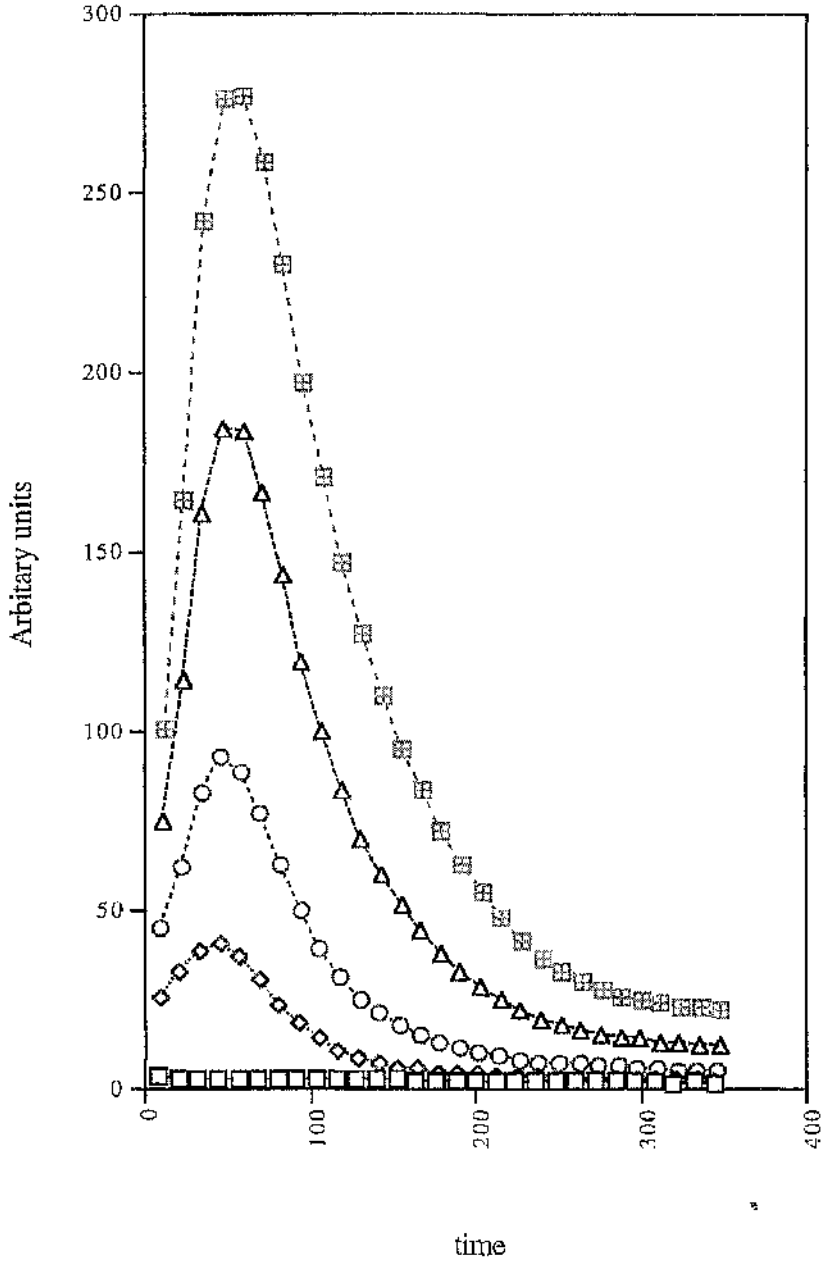
any effect on the phagocytosis induced metabolic burst. Latex beads at a final dilution of 100,000 were added as a stimulus to each tube, and the response was measured using the program described above. It was very clear that the response induced by beads differed from that induced by fMLP in several respects. It was much slower in onset and the peak response was much lower; however to compensate, it continued for a much longer time so that the net release of active oxygen species was in excess of that induced by fMLP (fig. 38a). If cytochalasin B is added to the cells immediately before the beads, then there is a small response, but this very quickly returns to baseline. However, if the cytochalasin is added one minute earlier, then there is no initial response by the cells when the beads are added (fig. 38a). Studies of the effect of STMS were carried out with material purified by protocol Y in which the major initial step is vacuum dialysis. Here the dialysate contains all of the small molecular components whilst the dialysand contains the small molecular components at their original concentration but is greatly enriched in the high MW components.

Fig. 35 Response of neutrophils incubated in different purities of STMS to fMLP stimulation as visualised by chemiluminescence



Neutrophils were incubated in dilutions of STMS for twenty minutes before stimulation with fMLP (10^{-5}M final concentration). The cells were primed with cytochalasin B (1µg/ml) immediately before addition of the stimulant. Dialysed STMS was diluted with HS to approximately the equivalent starting volume before addition to PMNs.

Fig 36 Inhibition of chemiliuminescence of neutrophils to STMS showing dose response

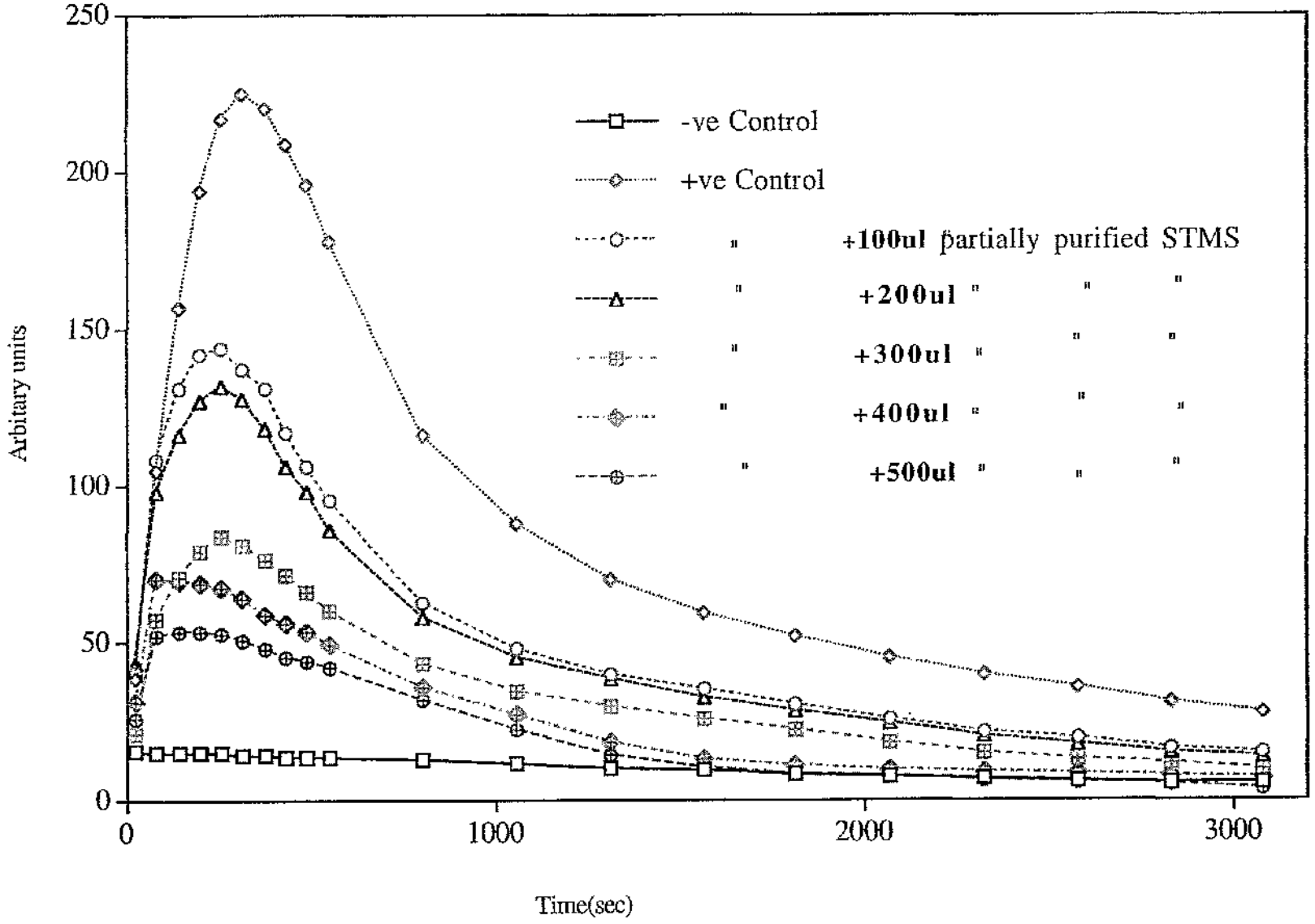


Neutrophils were incubated at 37°C for 30 minutes in the presence of increasing doses of crude STMS, 200µl, 500µl and 700µl. The cells were stimulated with cytochalasin B (1µg/ml) and fMLP (10^{-6}M).

p	-ve control	m	500 µl		
Δ	200µl	G	700 µl	⊕	+ve control

Fig. 37 Inhibition of chemiluminescence of neutrophils incubated in the presence of partially purified STMS.

Neutrophils were pre-incubated with dilutions of ppSTMS for twenty minutes before stimulation with PMA (1 μg/ml).


250
200
150
100
50
0
Arbitary units
0
1000
2000
3000
Time(sec)
-ve Control
+ve Control
" +100ul partially purified STMS
" +200ul " " "
" +300ul " " "
" +400ul " " "
" +500ul " " "

If the STMS is added to the cells immediately before the beads, then there is a marked decrease in the overall quantity of O_2^- produced. (fig 38b) Here 400 µl or 200µl of STMS that had passed through dialysis membrane (apparent cut-off 10kD) inhibited the production of O_2^- by 44% and 19% respectively. These amounts of STMS correspond to 44% and 22% of the total volume of the tube, and so it is possible that the effect may be due to a scavenging effect by the factor. To check if this is the case, it was decided to incubate the cells in the presence of the factor for 30 minutes prior to the addition of luminol and beads. In this case, 400µl and 200µl of STMS (again the dialysate) inhibited the response by 42% and 53% respectively (fig 38c). This must then exclude the STMS inhibition of latex beads induced metabolic burst from being a purely scavenging effect, but does tie in with the results of sections 3.8.2. The inference from these results is that the factor which inhibits the latex bead induced metabolic burst is not the same as the factor which induces dispersive locomotion.

Fig. 38 Inhibition of latex bead induced chemiluminescence of neutrophils by STMS

Fig. 38a

Treatment	Arbitrary Units	Percentage of Positive Control
Cytochalasin B + fMLP	58, 571	100
Cytochalasin B + Beads	0	0
Beads	94, 802	162

Fig. 38b

Treatment	Arbitrary Units	Percentage of Positive Control
Positive Control	59, 913	100
200µl Dialysate	48, 655	81
400µl Dialysate	33, 322	56

Fig. 38c

Treatment	Arbitrary Units	Percentage of Positive Control
Positive Control	44, 399	100
200µl Dialysate	20, 799	47
400µl Dialysate	16, 828	38

Neutrophils were incubated in the appropriate treatment for twenty minutes before stimulation with either fMLP (fig. 38a only) or with latex beads. The areas under the graphs, indicating the total luminescence produced) were calculated automatically by the computer and tabulated as above.

Summarised Table of Results

	Crude STMS	Through Dialysis	Freeze Dried Then P2	Through Mono Q	Reverse Phase HPLC	"Pure Material"
Dispersive Motility	√	√	√	√	√√	√√√
Decreased Adhesion	⇑	?	√	√	⇔	⇔
Inhibition of fMLP-Induced Adhesion	√	√	√	√	√√	√√
Inhibition of fMLP Induced Metabolic Burst	√	√	√	√	⇔	⇔
Inhibition of Latex Beads Induced Metabolic Burst	√	√	√	√	⇔	⇔
Inhibition of Primary Granule Release	⇑	?	√	√√	√√	?
Inhibition of Secretory Vesicles	⇔	?	⇔	⇔	⇔	?
Inhibition of Secondary Granule Release	⇑	?	√	√√	√√	?
Polarisation (S.E.M.)	√	√	√	√	√	√
Actin Redistrib-ution	√	?	√	√	√	√

√ **Noticeable Effect**
√√ **Strong Effect**
√√√ **Very strong Effect**
⇔ No **Noticeable Effect**
⇑ **Increase in Release**
? **Not Assayed**

DISCUSSION

4.1. Overview

Despite the intensive work on this factor the active component of STMS has not yet been identified and until this is achieved, questions will remain. The most important being is the activity due to a single molecule or a combination of events and circumstances. Secondary questions concern its role as a possible anti-inflammatory agent and mediator of steroid action. Little is currently known about the biogenesis of the molecule. There are several possible hypotheses:

1 synthesis and secretion in response to a steroid signal,

2 release of a cytosolic component due to steroid induced cell lysis,

3 proteolysis of a released protein/peptide, or failure of complete proteolyis resulting in an artefact.

Of these only the first possibility suggests that the factor could be a physiological mediator of steroid action. The initial work of Stevenson backed up to some extent by Chettibi indicated that the generation of STMS in response to dexamethasone was blocked by protein synthesis inhibitors. If this was substantiated, then STMS could well be the product of a steroid up-regulated gene. The molecular nature of STMS is again uncertain. Incubation with proteases has been used to abolish the activity, although in the light of the unusual dose response curve these experiments would require to be done with great precision. Information that has arisen during the purification procedure suggests the following;

1) STMS is produced as a high molecular weight complex which is dissociated only at extremely high salt concentration or very low pH and is therefore held together by ionic interactions. In this form it is highly active at the concentration found in the cell supernatant, but if diluted 10 fold the activity is too weak to detect.

2) When the complex is dissociated, the activity is entirely associated with the low molecular weight component, and the activity is increased dramatically. However the

activity is auto-inhibitory. It has a concentration optimum corresponding to a 1000 fold dilution of the starting material. However, at higher concentrations it is without apparent effect on the cells.

While the present work was being carried out, these features were being elucidated, and they impinge on the present results of the study. In terms of its biological action the production of dispersive locomotion is not unique, but the fact that it gives dispersive locomotion in a uniform concentration is apparently unprecedented. Despite these unusual features, it had to be borne in mind that STMS could be an already characterised molecule with another range of activities in addition to those described here.

4.2. Dispersive locomotion induced by STMS

The initial thrust of this investigation was to find out how dispersive locomotion worked. The problem is that dispersive locomotion requires either inertia or polarisation of the locomotory machinery. Inertia can be ruled out on two grounds: firstly low Reynold's number hydrodynamics applies to objects of cellular size and inertia is not possible. The second is that the cells quite clearly stop between 'steps'. Thus the problem of persistence must be due to polarisation of the motor itself. We therefore required to know how the cells move. Visual observation suggests that the motion is either looping or somersaulting. This point could be resolved by the use of side-view microscopy (G. Dunn, personal communication), but this has not yet been undertaken and so therefore the conclusions have to be indirect. In this respect the morphology of polymerised actin in STMS treated PMNs provides the main source of information.

For these two models of PMN locomotion, there would require to be either a gross movement of polymerised actin along the length of the PMN, or else there has to be a rapid polymerisation / depolymerisation of microfilaments at opposite poles of the migrating PMN. This would involve a number of actin sequestering proteins to interact

and to release their sequestered G-actin simultaneously in response to localised cues. One such cue could be a highly localised calcium concentration change as described by Berridge (1997).

Video footage (not shown) of PMNs migrating across a flat BSA coated glass surface shows a slow steady stretching of the leading edge of the cell, pulling the cell body until the cell body detaches from the substrate propelling the body forward. It is normally immediately after this recovery of a degree of roundness that any changes in direction of motility occur. The exception to this change in direction was when the tilted stage tracking assays were performed. Here the STMS treated PMNs migrated downhill, and perhaps these would make fascinating viewing with the Dunn side-view microscope.

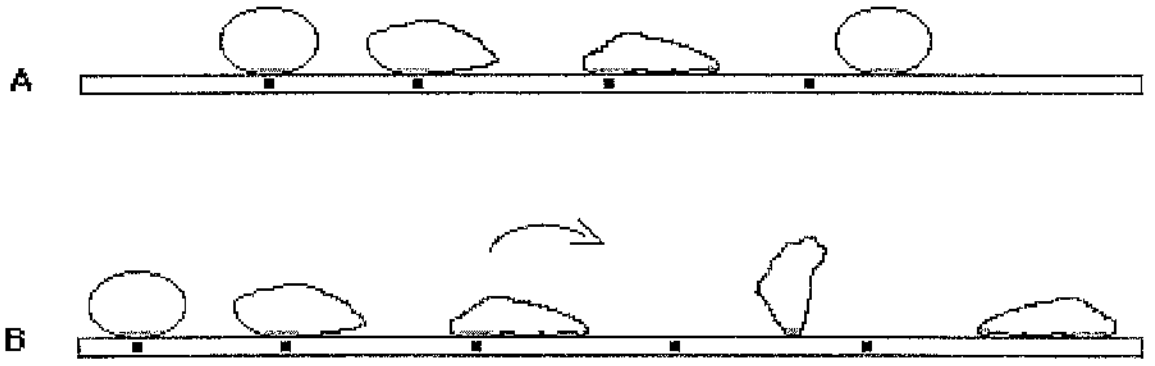


Fig. 39. Schematic Representation of Neutrophil Locomotion.

Visual observations are similar to the looping model proposed for locomotion of STMS treated cells, but bears no resemblance to the somersaulting model with its continuous reversals of polarity. However, STMS-treated PMN appear to have a broader polymerised actin-poor pole and a narrow polymerised actin-rich pole (Fig. 24). Again by analogy the narrow end would be taken as the tail. Our observations are consistent with the narrow end being the adhesive (adhered) end whilst the broad end would be the exploring (head) end. This model requires a transient switch of role such that the

head becomes adhesive, followed by breakage of tail adhesion and retraction of the body. At this point the tail forms a strong adhesion to the substrate and the head loses its adhesiveness. This might require translocation of actin within the cell or else it could largely involve local perturbation of the equilibrium between polymerised and unpolymerised actin.

In order to bring about persistent locomotion this model requires that the adhesion site made by the tail is polarised and tends to act as a hinge giving a preferred direction to the apparently flexible cell body. Such polarity could easily be provided by parallel actin bundles.

It has been mentioned earlier that colchicine induces motility that has been similarly described to STMS (Stevenson's original assays showing the migration of cells away from a cell pellet), and it has been documented that colchicine reduces adhesion of PMN (Lackie, 1974). It is therefore possible that STMS mediates it's dispersive effects in a similar manner to colchicine, i.e. by reducing adhesion and so allowing rolling of the cells. Rolling, however, does not adequately describe the motility observed when adherent PMN are stimulated with STMS.

4.2.2. Does STMS have receptors?

For some agonists the receptors have not been identified. (This is currently the case with lipocortin 1.) In the case of STMS, the signalling system has the property that at high or low dose levels there is virtually no response, whilst there is an optimum response over a range of concentration of 10^2-10^3 fold. Such optimum responses are well known. fMLP, for example, gives a peak locomotory response at about 10^{-9}M, however there is a big difference between this and STMS because fMLP has a spectrum of high dose responses that do not include locomotion. For a ligand to have no action whatsoever at high concentration, there are very few mechanistic possibilities. Of these the major one is that the ligand signals by cross-linking the receptor. At low receptor

occupation, cross-linking is an efficient process, but at high receptor occupation cross linking becomes less and less probable (auto competitive inhibition). This mechanism has one special requirement, that the ligand is bidentate. One molecule that has appropriate characteristics is lipocortin 1, which has a fourfold repeat of a calcium binding/ lipid binding motif.

4.3. Adhesion

Neutrophil adhesion is well understood in terms of the selectins and the integrin/ cam molecules. However, these adhesive mechanisms do not necessarily apply to the behaviour on BSA coated glass. One of the interesting and perhaps relevant observations concerning selectin-based adhesion is that the active selectins / integrins are clustered on to microvilli (Pavalko et al., 1995; Erlandsen et al., 1993)) and that dispersed selectin / integrin is relatively ineffective in the adhesion process (Lorant et al., 1995). This mechanism for reduced adhesion fits nicely with the observations of the smoothness of the STMS treated PMN membrane. The smoothness was described by Dr. A. Ridley as 'quite remarkable' when presented to her on a visit to the laboratory, however, she could cast no light on to the possible signalling pathways likely to lead to such a phenomenon. The need for clusters of integrins might well apply to all adhesion molecules, including the unknown molecules that interact with protein-coated glass. Nevertheless because the number of surface adhesion molecules is not altered by this mechanism there may be a distinction to be made between static and dynamic adhesion (Ackerman et al., 1993; Lorant et al., 1995). Thus a moving cell may only be able to make an adhesive contact if it has a compact cluster of adhesion molecules exposed on a projection. However once it makes extensive contact with another surface this mechanism should be unimportant. Thus the dynamic adhesiveness of a smooth cell could be low whilst its static adhesiveness could be normal. This argument could be taken further to suggest that clustered adhesion molecules act cooperatively as a strong adhesion site, but isolated adhesion molecules, whilst

contributing equally to the overall adhesion energy, nevertheless make contacts that are easily broken. This suggests a basis for the results observed in the tilt assay.

4.3.1. STMS mediated adhesion

It was noted during the tilted tracking assays that STMS does not actually cause many cells to detach from the BSA coated glass, but instead acts in a more subtle manner. The motion of the neutrophils was observed to resemble that of the cells on a horizontal surface with the sole difference that they all moved in roughly the same direction. From the appearance it is highly unlikely that the cells were rolling or even sliding down the slope. This suggests that the cells were still attached to BSA coated glass, at a single attachment point and that the driving force for locomotion was still largely the intrinsic motor activity of the cell. Considering the effect of persistence on perceived cell speed, if the step time is much less than the frame-sampling time then the instantaneous speed measurement will be a gross underestimate. As the frame sampling time falls below the step-time, then the measured instantaneous speed will asymptote to its true value. Tilting the surface polarised all of the steps down the slope, and so the instantaneous speed of the cells was then measured correctly regardless of frame time. Thus on the tilt assay an increase in speed would be expected if the frame time was much greater than the persistence time. Because we measure a persistence of about 30 seconds with a frame time of 5 seconds, any increase in the measured speed in the tilt assay can only be due to a very small extent to the increased persistence. In the present case the speed is approximately doubled and this means that the locomotory process itself must be faster. One model for this might be that the trigger for locomotion is extension of the cell beyond a critical length; when this happens the cycle of formation of a head/ substrate adhesion begins. If this were the trigger for locomotion then tethered down slope locomotion would occur at a faster rate than locomotion on the flat.

4.3.2. Adhesion mediated by other factors

Treatment with other agonists such as fMLP and CMS not only did not induce the PMN to migrate exclusively downhill, and PMNs appeared to be unaffected by the application of a lateral gravitational force on the plane of migration. This could be primarily due to the PMNs treated with fMLP or CMS not having a loosely adherent head, and a more tightly adherent tail, but instead being more uniform in the locations of adhesions. This coupled with a more rounded cell shape would prevent these cells from setting up a gravitationally induced polarity, and so their migration would continue to be non-dispersive. Thus PMNs treated with an agent that does not induce this highly specific form of bipolarity (without decreasing adhesion) would be unlikely to migrate down the slope. IL-8 has been shown to produce a bipolar morphology which bears a superficial resemblance to that induced by STMS, but this would not be expected to produce a dispersive motility down slope because there is an increase in cell adhesion. Perhaps the presence of IL-8 in the starting material is in part responsible for the adhesive properties of crude STMS, and its removal during purification allows the true nature of STMS effects on adhesion to be elucidated.

It is possible that the loss of adhesion observed with ppSTMS treatments may be in part or wholly due to the unusual morphology of motile neutrophils in an STMS environment. With only a small part of the cell suspected to be in contact with the substratum (endothelial cells or protein coated glass) then when the cells were washed, several cells could be washed away.

4.3.3. Persistent motility is not due to locking of rho activation state

It has been noted that the induced dispersive motility by STMS caused an increase in the persistence of migration, and so although the cell is dynamic, and moves at high speed, the overall conformation of the cell did not alter significantly. This was in contrast to work by Ehrengruber et al. (1995) who showed that by blocking activity of the small G-protein rho by ADP-ribosylation, the rate of change of PMN shape was

increased. This locking of the activation state of the G-protein led to very small persistence (Takaishi et al., 1993) as shown by the clearing of gold particles from areas of migration (phagokinesis).

4.4. Degranulation

All of the degranulation and respiratory burst assays were performed with neutrophils in suspension, and so all of the results will be skewed towards lower values, especially the positive controls. Nathan (1987) showed that these responses were increased in magnitude and duration by using adherent neutrophils. Lack of sensitivity of the assay prevented this from being a suitable assay in my experiments. The results from the adhesion experiments would also have made the results from these assays open to question, especially any correction of the results for the number of cells likely to be present in the assay. The reduced degranulation response is conducive with the possibility of reduced MT content. Were there to be a reduced microtubule content, then it may be expected that degranulation may be inhibitable. As shown in 3.7.2-3.7.4, STMS in its partially purified state inhibits the release of primary granules and secretory vesicles, along with the respiratory / metabolic burst (3.8.). These effects may be due to inhibition of the motility of microtubules, but it is also possible (especially in the case of the respiratory burst) that the re-organisation of the microfilament network may have a direct bearing on the effects observed. Rossi (1986) summarised findings that microfilaments are required to move several components of the oxidase to the cell surface allowing activation. If the majority of microfilaments are sequestered to one end of a polymerised cell, then it is conceivable that the reduction in metabolic burst is due to the microfilaments not being capable of mobilising these components. That there is an increase in the level of metabolic burst in response to fMLP if the cells have been pre-treated with cytochalasin B, may indicate that a disruption of microfilaments is necessary for optimum respiratory burst activity, or for easier assembly of the oxidase components. However, if STMS were to make this disruption almost complete, or were to induce sequestering of F-actin to a localised area, then the oxidase could not be

assembled effectively. The apparent discrepancy arises when the differences between complete microfilament collapse and peripheral microfilaments are ignored. If cytochalasin D is used at low concentrations and for only a very short period of time, then only the peripheral microfilaments are apparently effected. STMS appears to effect all the microfilaments in neutrophils, and yet have no effect on endothelial cells at concentrations used to treat PMNs (not shown). It should though be noted that the addition of a concentration of cytochalasin B that increases fMLP induced metabolic burst, inhibits the metabolic burst induced by beads (3.8.2). STMS that has passed through dialysis tubing inhibits the response in a manner suggesting initially a scavenging role. However, in line with the inhibition of azurophil granule release this suggestion was reduced by the effect being increased dramatically by pre-incubating the cells with STMS. The increase in inhibition by a factor of 1.5 fold must be due to a signalling event taking place inside the cells.

4.4.1. Inhibition of primary granule release

The amount of elastase released from PMNs treated with STMS was of the order of 2500ng/ml supernatant. This compared with 4600ng/ml with the fMLP control (approximately 10^7 cells per ml). These values were consistently obtained with ppSTMS, although as has been noted earlier, different purifications and different methods of purification gave different activities. [These elastase levels are in equivalent units as calibration assays were performed with porcine pancreatic elastase (ICN Flow).]

The inhibition of late granules (azurophil) is a notably unusual effect for an anti-inflammatory drug. As noted above, many such drugs knock out the cyclooxygenase / lipoxygenase systems. Others, such as Tenidap (Blackburn et al, 1991) may interfere with the activation of key enzymes, such as myeloperoxidase. This enzyme, as well as having effects on the metabolic burst pathway is also required for the full activation of collagenase. With both of these pathways being inhibited, probably to a high degree,

then the neutrophil will have great difficulty in responding in an inflammatory manner. In much the same way therefore STMS should be a potent anti-inflammatory agent, even without any of the other effects. There is a possible precedent for the type of activity observed with STMS, in that there is inactivation of azurophil granule product release (Blackburn et al., 1991) and as a result there is inactivation of collagenase. Blackburn claims that Tenidap inhibits collagenase activity, and yet surely the real effect is in the inactivation of MPO release. My results show that there is also inactivation of NADPH oxidase even in response to PMA challenge.

4.4.2. Effects of STMS on secondary granule release

It should be noted that there was no noticeable changes in the levels of the fragments of collagen at 20-30kD as suggested by Bode et al. (1994). This may be because the gel front was generally allowed to run off the end of the gel to allow differentiation between the larger fragments and the α and β fragments (Sorsa et al., 1985). Johansson et al. have recently (1997) discussed the possibility that neutrophils use MPO for adhesive purposes. If neutrophils are inhibited from releasing MPO, this could decrease their ability to adhere to a substrate. This could explain the anti-adhesive function of STMS reported by Chettibi et al. (1993) using protein coated glass, but the loss of adhesion to cytokine activated endothelium is more likely to involve an inhibition of integrin / selectin function / recognition on the neutrophils.

4.5. The anti-inflammatory role of STMS

As STMS-like activity has only been shown to be derived from monocytes grown in culture (or recently removed from a spleen) then there is a possibility that STMS may have an anti-inflammatory role in the serum. This would therefore imply that the anti-inflammatory effect most likely to be noticed would be as an anti-adhesive. However, with the concomitant release of IL-8, it is not likely that STMS would have much of an effect, except in the reduction of fMLP-induced adhesion, which is after all, the agonist-induced adhesion that PMN respond to most quickly. Were STMS to be used

clinically, then it would of course be free from IL-8 and other contaminants, such as the molecule that inhibits the metabolic burst. Therefore the pure STMS as a drug would primarily act in an anti-adhesive capacity in the blood stream acting on the earliest of stages i.e.. the interaction between the PMN and the endothelium. Any PMN that manage to adhere and extravasate could be anticipated to be incapacitated by incubation in STMS for a prolonged period, and as a result be incapable of releasing the majority of the lytic enzymes (primarily in the azurophil granules). That inflammatory events such as the release of secondary vesicles and the activation of the respiratory burst are not totally inhibited by STMS would be of lessened concern as the PMNs would not be capable of causing any serious damage due to the inactivation of the release of MPO and therefore knocking out the collagenase damage and to a lesser extent damage caused by HOCl and its metabolites.

I have shown that the machinery to engulf beads or bacteria is not effected by incubation with STMS. But, with the release of myeloperoxidase effectively 'knocked-out' by this treatment, then killing of the bacteria may be reduced somewhat. This, of course, relies on there being incomplete internal degranulation, which may involve a different pathway from degranulation to the surface induced by soluble stimuli. Ideally experiments should be performed with opsonised particles to determine if the release of primary vesicles can be inhibited to these stimuli as well as with soluble stimuli.

4.6. What is STMS?

As has been stated above, STMS could be a fragment of another molecule, such as Lipocortin I, or be a small protein / peptide such as Thymosin β4. Indeed, because of the uncertainty over the size of STMS, it could be Lipocortin I. If STMS is a fragment of a larger protein, then there are two possibilities for its existence. The first is that it is an artefact produced almost at random with no real physiological role, as the crude supernatant is not in itself anti-inflammatory (as shown by increasing basal levels of adhesion and stimulating the release of granules). The second possibility is that STMS

is a specific cleavage product which is produced on demand by the monocytes and as such would have specific anti-inflammatory effects. It would seem more than coincidence that the only cell types that produce STMS-like activity are monocytes and macrophages, only produce it in response to corticosteroids and are the only cell types that would be in both the blood stream and sites of inflammation where production of such a factor would be beneficial.

If STMS is a product of a larger anti-inflammatory molecule, such as lipocortin I, then there is evidence that smaller fragments of lipocortin can produce anti-inflammatory effects (Lloret & Moreno, 1994), although the effectiveness of these has been disputed by other investigators (van Binsbergen et al., 1989).

If, as very recent work may indicate, STMS is oxidised Thymosin β4 (J. Young, personal communication), then it is possible that the decrease in elastase activity may be due to leukocyte elastase inhibitor, a factor that co-purifies with Thymosin β4 (Dubin et al., 1992). However, as MPO is also inhibited to a similar degree, it is unlikely that the decrease in measurable activity of primary vesicle contents would be caused by this factor. Also, this factor has sequence homology to plasminogen activator inhibitors, and as I have found no evidence of such inhibitory behaviour from ppSTMS then it is concluded that this leukocyte elastase inhibitor is unlikely to be responsible for the inhibition of primary granule release. Instead it is therefore probable that the inhibition is due to STMS.

4.7. Assays with ppSTMS

Partially purified factors are very difficult to quantitate, and concentrations are likely to change from purification to purification. With this in mind, the concentrations were calculated from the size of the fraction peak. As this often had a distinct shoulder, then the absolute concentration was impossible to determine, although the final concentration would be of the order of nanograms per ml. As has been stated above, the methodology

of purification was in a state of evolution during these assays, and therefore it follows that the concentrations and purity of samples produced were highly changeable. Most of the individual groups of experiments were performed with one particular sample, and whereas the results from assay to assay would not be expected to vary, the results between experiments would. This is especially true of tracking assays which vary on a daily basis.

Further work

Utilising the latest purifications of STMS, all of the above assays should be repeated, paying particular attention to the oxidation state of the factor. That oxidation of methionine residues can alter the ability of a peptide to affect PMN behaviour is not a new phenomenon, and so it was not entirely unexpected that the factor was present in isoforms indistinguishable by gel filtration or Mono-Q chromatography. Indeed, the two fractions are sufficiently close on reverse phase HPLC that they often appear together. Also, the non-oxidised form of the factor does not produce dispersive locomotion, and may even inhibit the oxidised form from being as effective.

Recently, Lawrence and co-workers have developed a conductimetric assay system that is sufficiently sensitive to assay many of the enzymes released by PMN when stimulated. The only requirement for this assay is that either the natural substrate produces a change in the ionic strength of the solution, or a synthetic analogue can be produced to be specific for the enzyme. This method would allow very rapid analysis of data, and with each chamber being independent of the others, it is possible that measurements could be made at very short time intervals, and so give the kind of time course information lacking from these results.

It would also be of interest to investigate the morphological studies in greater detail, including the use of fluorescent latex beads for phagocytosis assays. These beads would have the advantage in that they could be visualised in living tissue, and so the effects of STMS could be observed in real time on the phagocytosis of particles.

To continue the morphological assays using electron microscopy would be advantageous in determining vesicle transport within the cell. With the release of primary granules being inhibitable with STMS, and with abundant supplies of the

factor available to perform such assays, it would be of interest to determine if these vesicles are sequestered within the STMS treated PMNs.

The factor now appears to be an oxidised derivative of Thymosin β4, one of the major actin sequestering proteins in PMNs, and as this can be easily purified from other cell types, then the number of assays is almost limitless. It would be ideal to perform a series of toxicological screens on the factor (unlikely to be toxic at the levels required for dispersive motility) and perhaps even animal models of inflammation; oedema formation being an obvious experiment to attempt (bearing in mind the differences in inhibition of lipocortin I with different stimuli).

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