A thesis submitted for the degree of Master of Science to the

University of Glasgow

STUDIES ON THE ADHESION AND LOCOMOTION OF NEUTROPHIL LEUCOCYTES

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March 1990

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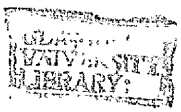

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Preface

This dissertation is submitted to the University of Glasgow, in accordance with the requirements for the degree of Master of Science in Cell Biology.

The work presented here has been carried out under the supervision of Dr. J. M. Lackie. I would like to express here my deepest gratitude to him for his guidance, constant interest and encouragement throughout the period of this research. In addition, I wish to thank Professor A. S. G. Curtis for providing me with every possible help in the department. My appreciation is also expressed to all members of the department for helping me throughout the period of my study. I should also thank the Saudi government for financial support. Finally I am very grateful to my husband for his patience and encouragement.

Abbreviation List

BSA : Bovine Serum Albumin.

C5a : Complement fragment (Anaphylatoxin).

CAMs : Cell Adhesion Molecules.

CD2 : Cluster Determinant-2.

CR1 : Receptor for C3b.

CR2 : Receptor for C3bi and C3d.

CR3 : Receptor for C3bi.

EAC : Erythrocytes Antibody Complement.

FCS : Foetal Calf Serum.

FHA : Filamentous Haemagglutinin.

FMLP : N-formyl-Methionyl-Leucyl-Phenylalanine.

H2 : Hanks Hepes (buffered balanced salts solution).

HS : Hepes Saline (Ca^{2+} and Mg^{2+} free salts solution).

HSA : Human Serum Albumin.

I-CAM : Intercellular Adhesion Molecule.

L-CAM : Liver Cell Adhesion Molecule.

LFA-1 : Lymphocyte Function-Associated Antigen.

LFA-3 : Lymphocyte Function Associated Antigen-3.

LTB4 : Leukotriene B4.

N-CAM : Neural Cell Adhesion Molecule.

Ng-CAM : Neural-glial Cell Adhesion Molecule.

PMN : Polymorphonuclear (neutrophil) leucocytes.

PT : Pertussis Toxin.

VLA : Very Late Antigen.

mAb : monoclonal Antibody.

Summary

Summary

1. The locomotion and the adhesion of neutrophil leucocytes in response to Bordetella pertussis products have been studied using automated tracking and flow chamber systems.

   a. The treatment of neutrophil leucocytes with 100 ng/ml pertussis toxin (PT) significantly inhibits the neutrophil adhesion and movement which are enhanced by the chemotactic factor FMLP. This inhibition was noticed after 30 minutes of pre-incubation of the cells with PT at 37°C.

   b. Pre-coating the flow chamber with filamentous haemagglutinin (FHA) has no effect on neutrophil adhesion, whereas the movement was inhibited. The inhibition of neutrophil movement by FHA was observed after 30 minutes incubation period with cells.

2. Neutrophil adhesion from flow has been investigated using a parallel-plate chamber. Coating the chamber with a stripe of zymosan-agarose significantly enhanced the adhesion of neutrophils suspended in 10% fresh rabbit serum. When the cells were suspended in heat-treated serum, zymosan activated serum , or heat-treated zymosan activated serum, the adhesion of neutrophil leucocytes to the zymosan-agarose stripe was reduced. The enhancement effect seems to suggest that C3b is the important component in the activated complement system, and that it is inducing an extremely rapid response from the cells.

3. Activation of the complement system through the alternative pathway significantly inhibits the locomotion of neutrophil leucocytes.

4. By using a variant of the flow assay the rapid response of neutrophils to changes in the suspension medium was tested. The response of neutrophils to chemotactic factor FMLP was slightly increased with $2x10^{-7}$ M and $2x10^{-9}$ M FMLP, while the adhesion was reduced with $2x10^{-8}$ M FMLP.

5. Coating glass with serum significantly inhibited the adhesion of neutrophil leucocytes. The same response was found in the absence of divalent cations from the suspension medium.

The presence of the manganese ions in the suspension medium significantly increase the adhesion of neutrophil to glass coated with BSA. This suggeste that the manganese ions are an important divalent cation promoting the adhesion of neutrophil leucocyes.

Chapter 1

INTRODUCTION

During an acute inflammatory response, neutrophil leucocytes are the first cells to appear at the site of inflammation. They adhere to the surface of endothelial cells, migrate out of the blood vessel between the endothelial cells, and move towards the site of inflammation, where they phagocytose microorganisms or relese enzymes which digest damaged tissue, providing the first line of cellular defence against many bacteria.

It is therefore important to look at major functions of these cells in the inflammatory process. This study concerns adhesion and locomotion of neutrophil leucocytes in vitro using assays which are appropriate for modelling the early events of the inflammatory process.

In this introduction I wish to discuss briefly the activities of neutrophil leucocytes in inflammation, in particular their locomotion and adhesion. Pertussis toxin (PT) and filamentous haemagglutinin (FHA) will also be reviewed briefly. Both PT and FHA are considered as active virulence factors of Bordetella pertussis, the bacterium which causes whooping cough in infants and young children and which is associated with depressed phagocyte function. Since part of the study is concerned with adhesion which may mediated through the complement C3b receptor, the activation of the complement system will also be discussed.

1.1. NEUTROPHIL LEUCOCYTES IN GENERAL :-

1.1.1. Classification of white blood cells :-

Blood cells can be classified into two major categories, a myeloid series which includes erythrocytes, megakaryocytes, polymorphonuclear leucocytes, and mononuclear phagocytes, and the lymphoid line which gives rise to lymphocytes. Neutrophils, eosinophils and basophils are jointly classified as polymorphonucler leucocytes (or granulocytes). These cells make up 60% of circulating white blood cells. They contain many secretory granules and they take their individual names from the staining properties of the granules in their cytoplasm. They are end cells with no ability to multiply, little capacity for protein synthesis, and a short life span. The function and chemistry of these three types of granulocytes are different. Neutrophil leucocytes are numerically the largest set and are important in primary defence, their function being to destroy invading bacteria and remove damaged tissue (Dale & Foreman, 1984). Basophil leucocytes in certain immune reactions release histamine and other mediators which generate an inflammatory response (Foreman, 1984). Eosinophil leucocytes seem to have a primary role in killing parasites and a protective role in defence against invading organisms. They can participate in tissue damage in situations where they are inappropriately recruited. Also they are involved in hypersensitivity responses (Kay, 1984).

Monocytes (mononuclear phagocytes) are large mononuclear cells with a kidney-shaped nucleus. They accummulate at sites of inflammation in large numbers then differentiate into macrophages

and destroy microorganisms (Davis, 1984; Murphy 1976). Lymphocytes have a spherical nucleus and relatively little cytoplasm. The effector functions of lymphocytes include secretion of antibody (B cells); secretion of mediators responsible for delayed hypersensitivity reactions, and killing of specific target cells (cytotoxic T cells). Also they have roles in immune reactions (Murphy, 1976; Plaut, 1984).

1.1.2. Development of neutrophil leucocytes :-

In adults the only important site of neutrophil production from myeloid stem cells is in the bone marrow. Myeloid stem cells can give rise to several cell types including red cells, platelets, granulocytes and macrophages (Miller and Koffler, 1986). The earliest cell that can be identified as a neutrophil precusor is the myeloblast, which is approximately twice as large as the mature neutrophil and contains ribosomes and Golgi apparatus. Myeloblasts divide to produce promyelocytes, which also have extensive rough endoplasmic reticulum, mitochondria and prominent Golgi apparatus. Developing from this Golgi apparatus are the primary granules, which are referred to as azurophilic, because of their blue appearance when stained with Wright's stain. The next cell division produces the myelocyte at which stage the secondary granules develop. Secondary granules are also known as specific granules, because neutrophil leucocytes are the only cell containing this sort of granule. The normal period of development from myeloblast to myelocytes takes about 4 to 6 days (Davis and Gallin, 1981). During these stages granulocytes synthesize

proteins on rough endoplasmic reticulum which is then transferred to the Golgi apparatus, where it is concentrated and packaged into storage granules (Bainton, 1980). The maturation of myelocytes to mature neutrophils takes about 5 to 7 days. In this period the cells acquire their characteristic morphologic and functional features. Although various stages during this period can be distinguished there is no cell division. The earliest stage in this phase is the metamyelocyte, which has cytoplasm containing a full complement of granules. At this stage it becomes possible to distinguish neutrophilic from basophilic and eosinophilic metamyelocytes. The cell nucleus at this stage has a kidney-bean shape. The metamyelocyte has some phagocytic ability, but not as much as the band and mature neutrophil. The metamyelocyte matures to the band neutrophil, which has a sausage-shaped nucleus, and as the band cell matures the nucleus becomes lobulated (Murphy, 1976; Davis and Gallin, 1981).

1.1.3. Neutrophil structure :-

Under the light microscope the mature neutrophil has a diameter of 12-15 um, and contains a nucleus with 2-5 separate lobes linked by thin filaments of nuclear material. There is no nucleolus and so no synthesis of new ribosomes. The cytoplasm of the neutrophil contains very little endoplasmic reticulum, few mitochondria, few polyribosomes,and many glycogen granules; glycolysis, and not oxidative metabolism is the major ATP-generating mechanism (Dale, 1984).

The granules are the most obvious cytoplasmic components in neutrophil leucocytes. There are two types of granules, azurophil or primary granules and specific or secondary granules. The azurophil granules have a diameter of 0.5 um and make up 30% of the total number. They contain the usual acid hydrolases which act at the acid pH found in lysosomes; cathepsin G; lysozyme; hydrolytic enzymes; a haemoprotein with peroxidase activity (myeloperoxidase) and cationic proteins of low molecular weight that have bactericidal activity. The specific granules have a diameter of 0.2 μm, and contain lactoferrin; vitamin B12 binding activity; collagenase; lysozyme; and components capable of generating C5a by complement activation. In mature neutrophils, there is about one azurophilic granule to every two or three specific granules (Hoffstein, 1980; Stossel, 1974; Wright and Gallin, 1975; Bainton, 1975).

The contents of specific granules are secreted by neutrophils during nonspecific adherence to surfaces in vitro; a similar secretion of these granules may occur when neutrophils marginate and enter inflammatory sites in vivo (Wright and Gallin, 1977). Furthermore, phagocytosing neutrophils release granule products which generate C5a by activating the complement system (Ward and Hill, 1970; Wright and Gallin, 1975). Phagocytosing neutrophils also release a granule-associated protease capable of destroying the chemotactic activity of C5a (Wright and Gallin, 1975). It has been suggested (Wright and Gallin, 1977) that there are functional differences between specific granules and azurophil granules in terms of extracellular generation and inactivation of

chemotactic C5a fragment (C5a). These different effects of granule products have different pH optima for activity and distinct kinetics of release from neutrophils and together may contribute to the self-limited amplification and localization of the acute inflammatory response.

Another obvious component of neutrophil leucocytes is the plasma membrane, which is composed of phospholipids and proteins held together by noncovalent interactions. Microfilaments which are found beneath the plasma membrane are required for cell locomotion. Like many cells the mature neutrophil has a net negative surface charge, which might be important in the adhesion of neutrophil to surfaces. The neutrophil membrane has receptors for complement components C3b, C3bi, and C5a, for leukotrienes, for Fc piece of IgG antibody, for chemotactic peptides, for lectins, and for many other pharmacological agents such as adrenergic agonists, histamine and prostaglandins E1 and E2 (Stossel, 1974; Berlin et. al., 1975; Dale, 1984; Davis and Gallin, 1981).

1.1.4. Neutrophil function :-

The role of neutrophil leucocyte seems to be a general tissue maintenance or cellular defence system, which removes damaged or dying tissue, bacterial pathogens, small foreign bodies, or tissue which has been injured (Lackie, 1982). It is also a secretory cell, releasing the contents of its granules (enzymes) extracellularly, in response to various stimuli; it is capable of extracellular killing of large multicellular pathogens as well as

other tissue cells; it may have a role in the genesis of increased vascular permeability and oedema; and it is a significant source of platelet-activating factor (PAF) (Weissmann et.al., 1980; Dale, 1984). In order to carry out their defensive function, neutrophils must be capable of recognising the site of injury, increasing their adherence to the endothelial wall, moving towards the source of infection, engulfing (phagocytosis), killing and digesting the invading organisms, thus providing the first line of cellular defence system in the body.

The activation of neutrophil leucocytes in inflammation will be discussed briefly in the next section.

1.2. Inflammation :-

Inflammation is a complex process involving a variety of cell types in a coordinated series of actions and reactions. In 1979 Joris and Majno defined inflammation as a response of living tissue to local injury, leading to the local accumulation of blood cells and fluid. The types of injury which gives rise to inflammatory responses may be summarized into three groups :-

1) Physical : mechanical trauma, irradiation, cold or heat.
2) Chemical : acids, alkalis, phenols or other inflammatory irritants - turpentine, kaolin, carrageenan etc.
3) Infection and immunity : injury due to a living organism, such as viruses, bacteria, parasites or fungi, and immune responses to injury.

In general inflammation can be divided into two types, acute and chronic. These types of inflammation will be mentioned briefly in this section.

1.2.1. Acute inflammation :-

Acute inflammation is a highly complex process comprising different sequences of events which occur almost simultaneously, so that it is scarcely possible to give an acount of changes in strict chronological order (Passmore and Robson 1974). It also comprises the immediate and early response to an injurious agent, and the reaction, though relatively short, may last for hours or days (Robbins and Kumar, 1987).

The basic features of an inflammatory response may be summarized as follows :-

1) Rheological changes.

2) Vascular permeability changes.

3) Cellular changes.

1.2.1.1. Rheological changes :-

Immediately after an injury there may be a brief constriction of arterioles. It is followed by widespread dilation of arterioles and capillaries and by a rapid increase in blood flow as the precapillary sphincters open and the arterioles dilate. Concomitantly, the postcapillary venules also dilate and fill with the blood. The blood in the vessels is divided into two zones - a peripheral zone of almost cell-free plasma and an axial stream containing closely packed red and white cells. After mild injury, such as the application of histamine (Ebert and Graham, 1966), there is an increase in flow which lasts for 10 to 30 minutes and then gradually returns to normal. After more severe injury, rapid flow may last for several hours, to be followed by a gradual decrease in rate of flow in the still-dilated vessels (Hurley, 1972; Florey, 1970). In the case of very mild injury, the initial surge of blood flow is followed by slowing of flow, changes in intravascular pressure, and alterations in the orientation of the formed elements with respect to the vessel walls. Furthermore, the viscosity of the blood is increased, leading to both packing (sludging) of red cells and increased frictional resistance to flow (Robbins and Kumar 1987).

The time relationships of these vascular changes depend to some extent on the severity of the injury. Arteriolar dilation becomes evident within a few minutes of the injury. Slowing is apparent within 10 to 30 minutes.

1.2.1.2. Vascular permeability changes :-

From the earliest stage of the inflammation the walls of microvessels, which normally have a low permeability to macromolecules, become very permeable to blood proteins and white cells. This phenomenon may be explained as an adaptation of microvessels to supply blood proteins to the extravascular tissue space, and this has the secondary consequence of inducing tissue oedema.

These effects on the walls of microvessels are thought to be caused by release of inflammatory mediators such as prostaglandins (E1, E2, or I2), histamine, serotonin, bradykinin, C3a and C5a (anaphylatoxins), leukotriene C4, D4, E4, B4, oxygen metabolites and PAF-acether (Williams, 1981). Furthermore, in response to the inflammatory stimulus white blood cells, predominantly polymorphonuclear (PMN) neutrophils, accumulate in the early stages of inflammation.

The alteration in vascular permeability during the inflammatory reaction has been measured using different techniques, and these techniques can be summaried as follow :-

a) The Blueing technique : involves the intravascular injection of a dye such as Evans Blue which binds strongly to plasma albumin; leakage can then be visualised directly.

b) Radioisotope technique : plasma proteins can be labelled with, for example ^{125}I or ^{131}I and a quantitative study of extravasation made.

c) A more sophisticated technique has been developed which involves the injection of radioactive gas, $^{133}Xenon$, intradermally at a number of sites, together with some mediators of the inflammatory response, singly or combined at various doses, with or without specific blocking agents. The animal (most usually rabbit) is also given an intravenous injection of labelled plasma proteins, such as ^{125}I human serum albumin. The ^{133}Xe- counts in standard skin samples give a measure of relative blood flow, which is associated with vasodilation and increased microvascular hydrostatic pressure. With reference to the plasma concentration, the accumulation in the same skin samples of labelled protein is a measure of the amount of protein extravasated (Youlten 1984).

Polymorphonuclear (PMN) leucocytes play an important part in vascular permeability changes. In inflammatory reactions, Wedmore and Williams (1981) suggested that PMN leucocytes during their passage through the vessel wall, may secrete enzymes which break down substances present at junctions between adjacent endothelial cells, or that the enzymes may act on basement membrane underlying endothelial cells. Alternatively, PMN leucocytes may secrete a substance which affects endothelial cells, perhaps causing contraction. The interaction between PMN leucocytes and endothelial cells lining the blood vessel wall seems to increase vascular permeability to macromolecules (Williams, 1981). Wedmore

and Williams (1981) showed that C5a, FMLP, and leukotriene B4 in the presence of PMN leucocytes would increase vascular permeability and could be considered indirect-action mediators; bradykinin and histamine, however, increase vascular permeability by acting directly on vascular endothelial cells and are direct-action mediators.

1.2.1.3. Cellular changes :-

At the site of injury, white blood cells may well constitute an important aspect of the inflammatory reaction. The early stages of the response are characterised by neutrophil adhesion to the endothelium, emigration over and through the endothelium and movement through connective tissue probably directed by chemotactic cues. When neutrophils arrive, they will initiate a new set of activities including phagocytosis, and production of active oxygen species, and the release of granule contents. These activities will be briefly reviewed in this section

i) Adhesion of leucocytes to endothelium :-

Leucocytes circulate in the blood as non-adherent cells. To leave the bloodstream, they must adhere to the endothelial lining of post capillary venules. These adherent cells, in response to the inflammatory stimulus, move quickly between endothelial cells, which do not have complex junctional specialization in this part of microvasculature (Simionescu et.al. 1975), and then towards the site of injury. The adhesion formed must be suitable to support leucocyte locomotion over and between the endothelial cells. The

ability of leucocytes to move over the two-dimensional substratum of endothelial cells and to move in three dimensional matrices is essential for the subsequent emigration phase (diapedesis) and for tissue invasion, and migration requires adhesion changes (Lackie and Smith, 1980; Wilkinson and Lackie, 1979). It has been shown (Lackie, 1982) that the interactions between the neutrophil and the tissue matrix may affect the ability of the neutrophils to move. (See the following section).

ii) Emigration of the cells from blood vessels :-

After injury leucocytes stick to the vessel wall, and move rapidly over and between the endothelial cells through interendothelial cell junctions. By this active movement the leucocytes leave the blood vessel and enter the surrounding tissues (Florey, 1970).

It has been shown (Russo et. al., 1981) that the emigration of PMN leucocytes out of the blood vessels involves chemotactic factors and complex interactions with cellular and matrix components. The mechanism by which PMN leucocytes penetrate the natural tissue barriers such as basement membrane is still unknown, because the process is difficult to study in vitro. During inflammation neutrophils migrate more rapidly than monocytes and lymphocytes. A second phenomenon has also been noticed during the emigration of white blood cells and that is that some erythrocytes pass through the endothelium. This phenomenon might happen in the wake of emigrating polymorphs

and when the circulation is slow (Passmore and Robson, 1974; Florey, 1970).

iii) Locomotion :-

Leucocyte locomotion towards the site of inflammation is an important process during the acute inflammatory response. The direction of locomotion may be determined by the physical properties of the environment rather than being a response to stimulating factors and this was shown experimentally by Carter (1967) who also showed that adhesion, besides being necessary for locomotion, may also affect the rate and direction of locomotion. Furthermore, it has been reported that directional locomotion depends on chemotactic influence and may contribute to leucocyte accumulation in vivo. (Keller et.al., 1980; Keller, 1981).

The locomotion of neutrophil leucocytes can be divided into two categories :-

a) movement over two-dimensional surfaces, such as the endothelium in vivo or variously coated substrata in vitro.

b) movement through three dimensional matrices such as tissue in vivo or micropore filter and gels in vitro (Lackie, 1982). (see section 4).

iv) Chemokinesis and Chemotaxis :-

The rate of delivery of cells to an inflammatory site will be influenced both by the speed at which the cells are moving, and

the extent to which their movement is directed by chemotactic cues. Chemokinesis is defined as a reaction by which the speed or frequency of locomotion of cells or organisms is determined by substances in the environment (Keller et.al., 1977a), whereas chemotactic factors, when present as a gradient, actually direct the movement. Thus chemokinesis involves changes in absolute magnitude of movement parameters, whereas in a chemotactic response the probability of movement in one direction is different from that in any other. Arguably the crucial step in getting accumulation of cells at an inflammatory focus is the initial stimulation of locomotion, chemokinesis, but directed locomotion will be a more efficient way of getting cells rapidly to the site, and there is some evidence that chemotaxis does play a role in vivo. It is likely that in the early stages of an inflammatory response cells are merely stimulated to move randomly, and only near the site of damage does chemotaxis play a role in directing movement. A more extensive discussion of this is given in Lackie and Smith, 1980; Wilkinson and Lackie, 1979. Chemotaxis is defined as "the directed movement of a cell or organism in response to a chemical substance in the enviroment, usually a diffusible substance" (Lackie, 1986). Using time-lapse cinematography, chemotactic reactions in leucocytes have been described by a number of groups (McCutcheon, 1946; Robineaux, 1964; Ramsey, 1972; Zigmond, 1974; Allan and Wilkinson, 1978), and it has been shown that leucocytes migrate directionally towards sources of chemotactic factors. It has also been suggested that chemotaxis of leucocytes may play an important role in mechanisms

of repair such as the removal of damaged tissues. There is clearly a complex relationship between leucocyte adhesion, locomotion, chemotactic orientation and other leucocyte functions such as release of lysosomal enzymes, aggregation and the respiratory burst. (Keller, 1981). It is clear that some chemokinetic factors such as native human serum albumin (HSA) or fibrinogen have no chemotactic activity (Wilkinson et.al., 1977; Keller et.al., 1977b; Keller et.al., 1978; Wilkinson and Allan, 1978a), but all known chemotactic factors are positively chemokinetic. Because chemotactic factors may affect the adhesive properties of cells, and because increased adhesion may reduce the speed of movement, the accumulation of cells at an inflammatory focus is a complex problem in cell behaviour.

A technique for measuring chemotaxis was introduced by Boyden (1962). This technique uses a two-compartment chamber with a filter, usually 3 μm in pore-size, placed between them. The cells are allowed to migrate from the upper compartment through the filter to the attractant in the lower compartment. In this assay the attractant molecules diffuse up through the filter forming a gradient and the cells are stimulated to move directionally into the filter in response (Zigmond, 1978; Wilkinson and Lackie, 1979). In 1978 Nelson et. al., introduced a new method for the study of spontaneous migration and chemotaxis of human leucocytes under agarose.

During the past three decades, it has been shown that neutrophil leucocytes respond to many chemotactic factors, including complex lipids derived from arachidonic acid

metabolism, particulary the leukotrienes; formylated peptides such as tripeptide formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe); complement fragment C5a; and denatured proteins. Denaturation of haemoglobin by removal of the haem moiety to form globin produces a potent chemotactic factor (Lackie, 1986; Wilkinson, 1981). Furthermore, there is now good evidence that there are endogenous chemotactic factors for neutrophils and other cells in inflammatory reactions in vivo. The complement system is one of the sources of endogenous chemotactic factors in plasma. Similarly the coagulation system; the kinin system; and the fibrinolytic system may rapidly generate chemotctic factors. Moreover, there is evidence that the activation of the complement system is an important humoral reaction to injury (Wilkinson, 1974b). Recently it has been reported that neutrophil leucocytes and lymphocytes are stimulated by interleukin 8 a peptide which is produced by a variety of cells (Westwick et. al., 1989).

v) Phagocytosis :-

The process of phagocytosis is the first step in the destruction of foreign materials in the body. During inflammation phagocytes move towards the site of inflammation and recognize microorganisms which must adhere to the surface of the phagocytic cell, possibly through receptors. The plasma membranes of neutrophils and macrophages have receptors for the Fc portion of the immunoglobulin IgG and for a fragment of the third component of complement C3b, which help microorganisms coated with IgG or C3b to adhere to the surface of phagocyte

(Stossel,1974). After the microorganism has attached to the surface of the phagocyte pseudopods are extended around the microorganism until it is completely enclosed in a vacuole (phagosome). Within a minute cytoplasmic granules fuse with the phagosome and release their contents into the phagosome. However, if the particle is too large the vacuole may remain open and some enzymes may escape and attack host tissue (Davis et. al., 1970; Henson , 1971; Zurier et. al. ,1973). During phagocytosis, neutrophil leucocytes can generate superoxide anion (O2-), hydrogen peroxide (H2O2) and hydroxyl radical (OH.) from molecular oxygen. When these mediators are released from the vacuole they can kill bacteria and fungi and they can injure cells or membranes (Weissmann et.al., 1980). In the phagocytic process the neutrophil leucocytes produce inflammatory products such as endoperoxides, thromboxanes, hydroxy-, and hydroperoxy-acids and leukotrienes from arachidonic acid, some of which are metabolized to anti-inflammatory products such as stable prostaglandins which raise the intracellular level of cyclic AMP and so stop further release of neutrophilic enzymes. (Goldstein et. al.,1978; Weissmann et. al. ,1980; Samuelsson et. al.,1979; Zurier and Weissmann, 1972).

1.2.2. Chronic inflammation :-

Chronic inflammation results from injurious stimuli which last for weeks, months, or years. This inflammation may come about one of two ways, it may follow acute inflammation, or the response may be chronic almost from the beginning. The acute to chronic transition may occur as a result of three different mechanisms :-

(a) persistence of the initiating inflammatory stimulus. (b) the continued presence of the endogenous antigen. (c) endogenous factors which change the type of cells and their function at the site of inflammation. Chronic inflammation can be distinguished by the presence of fibroblasts, macrophages, lymphocytes and plasma cells. The macrophages are capable of phagocytosis to remove the excess protein which causes the oedema (Casley-Smith, 1980; Robbins and Kumar, 1986). The plasma cells and lymphocytes protect the body against most extracellular organisms by means of antibody formation (Passmore and Robson, 1974). Furthermore, there is an other type of chronic inflammation known as granulomatous. Granulomatous inflammation is defined as a special variety of chronic inflammation in which cells of the mononuclear phagocyte system are predominant (macrophages, epithelioid cells and multinucleated giant cells). The formation of granulomas in most cases involves the accumulation of these cells into suitable demarcated focal lesions called granulomas (Williams and Jones Williams, 1983).

1.3. ADHESION :-

1.3.1 Cell-cell adhesion :-

1.3.1.1 Cell adhesion mechanisms :-

There are at least two main types of theory advanced to explain the mechanism of cell adhesion: theories based on physico-chemical interactions (Curtis 1967,1973; Weiss and Harlos 1972; Bell 1978); (lyophobic colloid interactions) and receptor-ligand type theories which invoke specific receptors and ligands (e.g. antigen-antibody type interactions, CAMs).

1.3.1.2 Physico-chemical theories :-

The early treatment of interactions of small particles at close separation was published by Derjaguin and Landau (1941) and Verwey and Overbeek (1948), and is now often referred to as DLVO theory. Two forces are considered by the DLVO theory : the electrostatic-like charge repulsive force and the attractive Van der Waals-type force. Two kinds of adhesions between particles were predicted by DLVO theory, a strong and a weak adhesion. The strong interaction occurs with surfaces separated by a gap of 1-2 nm (the primary minimum). The weaker interaction is between surfaces separated by 6-30 nm (the secondary minimum). To bring the cells to the primary minimum a substantial repulsion must be overcome.

In 1960 Curtis suggested that the balance between electrostatic repulsion and attractive forces (London dispersion forces) determines the strength of cell-cell and cell-substratum

adhesion. A detailed consideration of the evidence for the applicability of DLVO theory to cell adhesion can be found in reviews by Bongrand (1988) and by Curtis (1988); Curtis et.al., (1986); Curtis and McMurray (1986).

The idea of cell-surface hydrophobicity has been invoked to explain the orientation of certain organisms at air/water, oil/water and solid/water interfaces. Marshall and Cruickshank (1973) showed a considerable increase in the adsorption of Hyphomicrobium vulgare ZV580 to hydrophobic glass and a decrease to glass treated with a non-ionic surfactant. Their results coupled with transmission electron micrographs showing the separation between the cell wall and the substrate surface and the adsorption of a negatively-charged AgI sol, led them to suggest that one end of the bacterium consisted of an extracellular hydrophobic polymer which preferentially adsorbed at an interface between a hydrophobic and aqueous medium. Corpe (1974) also described the the attachment of marine organisms via extracellular polymers. In an extensive study of the adhesion of Streptococcus mutans, Mukasa and Slade (1973) suggested that it was the adsorption of an extracellular glucan to the collector surface which caused the bacteria to attach. This suggested that the adsorption of extracellular polymers might cause the attachment of bacteria to surfaces via polymer-bridging. Some bacteria have specialised fibres extending from the cell surface which may also be able to bridge between the body of the bacterium and a collector surface. Cell-surface polymers appear to play an important part in bacterial attachment.

1.3.1.3 Receptor-ligand type theories :-

Specific interactions similar to those for antigen-antibody, enzyme-substrate, lectin-sugar, have been suggested as being responsible for the selectivity of cell adhesion. Basically, this theory suggested that the adhesion between animal tissue cells, and between cells and their extracellular matrix depends on the direct molecular binding of cell-surface components to specific ligands (Dolowy,1980).

1.3.1.3.1 Cell adhesion molecules (CAMs) :-

Cell adhesion molecules (CAMs) are large cell surface glycoproteins believed to be responsible for cell-cell binding during development and for stabilization of certain tissues in adult life. Primary CAMs such as liver cell adhesion molecule (L-CAM) and neural cell adhesion molecule (N-CAM) appear in early embryogenesis on derivatives of more than one germ layer. Secondary CAMs such as the neural-glial cell adhesion molecule (Ng-CAM) appear during later histogenesis to link cells of different types following cytodifferentiation. During development CAMs can undergo various local cell surface modulation either by chemical modification or by changes in their prevalence on particular cells (Edelman et. al., 1983; Crossin et. al., 1985). The neural cell adhesion molecule (N-CAM) and the liver cell adhesion molecule (L-CAM) are expressed in specific dynamically regulated patterns in many tissues during all stages of vertebrate development from pregastrulation embryo through the adult (Murray

et. al., 1986). These two primary CAMs have different molecular structure and are expressed in different cell groups during development in patterns that suggest that they act coordinately to effect intercellular interaction and border formation in cell collectives that are undergoing induction (Gallin et. al., 1987).

The L-CAM molecule was initially isolated from chicken liver epithelial tissue and mediates calcium-dependent adhesion in these tissues both in the embryo and in the adult. Previous studies suggested that L-CAM can may play a more general role in development than the mediation of interaction among liver cells. Also L-CAM is required for the formation of some junctional complexes (Gallin et. al. 1987).

The N-CAM molecule appears on the surface of muscle cells in parallel with the ability of the muscle cell surface to accept synapses, and has also been identified in chick retina and brain. This CAM appears to cause calcium-independent cell-cell adhesion in nervous tissue. Recent studies suggest that N-CAM mediated adhesion may participate in surface interactions of nerves with muscles (Sanes et. al., 1986; Gallin et. al., 1983; Covault and Sanes, 1986). Another recent suggestion is that molecules known as nectins are involved in cell adhesion. They are believed to be linking molecules bound to their appropriate receptor. The nectins contain various glycoproteins called Fibronectin, Laminin, Chondronectin, Vitronectin, Cytotactin, Thrombospondin and von Willebrand protein (Curtis, 1988).

1.3.1.3.2 Cadherins :-

Cellular interactions are believed to play roles in the regulation of cell growth, in development and in morphogenesis. Studies on cell adhesion have demonstrated that possibly some cell-type-specific adhesiveness resides in a class of cell-cell adhesion molecules termed cadherins, which were defined as the molecular components of the Ca^{2+} dependent cell adhesion system. Two types of cadherin have been described, E-cadherin and N-cadherin. The E-cadherin molecule was identified in mouse teratocarcinoma cells, and it was detected only in epithelial cells of various organs. This molecule seems to be identical to uvomorulin or cell-CAM 120/80, equivalent to chicken L-CAM, and did not cross-react with cadherins on other cell types. The N-cadherin molecule was found in the chick retina, and brain and was also present in muscle cells. Its distribution has been determined in the tissues of early embryos (Hatta and Takeichi, 1985; Yoshida et. at., 1984; Peyrieras et. at., 1983; Edelman et. at., 1983). Hatta and Takeichi (1986) suggested that the expression of N-cadherin molecules is associated with separation and sealing of cell layers in morphogenesis.

Studies on cell adhesion have described a specific contact receptor for intercellular adherens junctions. This receptor is known as adherens junction-specific cell adhesion molecule (A-CAM) and it is present at the junctional complex of cardiac muscle and of lens, as well as in cultured cells. There are at least two subfamilies of molecularly distinct adherens junctions. The first includes cell-cell junctions such as those between cultured cells

of various origins (myeloid cells, fibroblasts, etc.). The second group of adherens junctions consists of cell-substrate or cell-matrix adhesions such as focal contacts, specialized attachments to basement membranes etc. (Avnur and Geiger, 1981; Geiger et. al., 1982,1985). Furthermore it has been suggested that A-CAM is a surface glycoprotein of cell-cell adherens junctions in a large variety of mesenchymal, myeloid, and epithelial cells. Also it is a Ca^{2+} dependent adherens junction-specific membrane glycoprotein that is involved in intercellular adhesion in these sites (Volk and Geiger, 1986). There are similarities between some properties of A-CAM and N-cadherin molecule as shown using monoclonal antibody NCD-1 (Takieichi et.al., 1985). Moreover adherens junctions formed in the presence of divalent antibodies became essentially Ca^{2+} independent suggesting that cell-cell adhesion between them was primarily mediated by the antibodies. There is evidence, using monovalent Fab' fragments of anti-A-CAM antibody, that A-CAM participates in intercellular adhesion in adherens-type junctions and that it may be involved in microfilament bundle assembly (Volk and Geiger, 1986).

The third adhesion molecule to be isolated was Neuron-glial cell adhesion molecule (Ng-CAM) which first appears in development at the surface of early postmitotic neurons, and mediates the heterotypic binding of neurons to the support cells of nervous system (glia) by a heterophilic mechanism. This CAM is expressed only on neurons of the central nervous system and the peripheral nervous system during the later epoch of development concerned with neural histogenesis (Grumet et.al., 1984; Edelman,

1983; Thiery et.al.,1984). Futhermore it has been reported that in continuing development both N-CAM and Ng-CAM can both appear at the surface of individual neurons. Also there is evidence indicating that there are major differences in appearance times, distribution and modulation of the two neuronal CAMs (Gallin et. al., 1983; Edelman et.al., 1983; Thiery et.al., 1982).

1.3.1.4 Divalent cations :-

Many studies in vivo and in vitro have shown that divalent cations are required for adhesion. Garvin (1961, 1968) showed that the adhesiveness to glass bead columns by human polymorphonuclear neutrophils (PMN) in whole blood is dependent upon the presence of Mg^{2+}.The adhesion of rat PMN to glass bead columns coated with serum was also found to be affected by Mg^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+} and Cd^{2+} at millimolar concentrations, but Ca^{2+}, Sr^{2+} and Ba^{2+} were ineffective. Moreover there is evidence that cell adhesion has cation specificity, for example Rabinovitch and Destephano (1973) found that the spreading of macrophages and the adhesion of fibroblasts increased in presence of Mn^{2+} more than with any other divalent cation. In 1984 Trinkaus found that Ca^{2+} was the essential cation for cell adhesion in epithelial cells. Edwards et.al. (1987,1988) showed that the adhesion of BHK12 cells was enhanced by Mn^{2+}, Co^{2+}, Mg^{2+},Ca^{2+}, in that order. High concentration of Mn^{2+} and Mg^{2+} probably induces spreading of BHK12 by binding to the Ca^{2+}/Mg^{2+} site of Arg-Gly-Asp (RGD) receptors for adhesion proteins. Hornby (1973) showed that chick limb-bud cells were more adhesive in the presence of magnesium

ions than calcium and that strontium was more effective than barium. The role of divalent cations in the adhesion of polymorphonuclear leucocytes to the endothelial lining of blood vessel walls was studied by Hoover and others (1980) using a monolayer collection assay. They found that Mn^{2+} produced the highest adhesion followed by $Zn^{2+} > Ni^{2+} > Mg^{2+} > Ba^{2+} > Ca^{2+}$. In 1986 English and Gabig found that the adherence of neutrophil leucocytes stimulated by PMA (phorbol myristate acetate) was highly dependent on extracellular Mg^{2+} but not Ca^{2+}.

1.3.2. Cell-substratum adhesion :-

1.3.2.1. Cell surface receptors :-

It has become clear that cell adhesion, cell migration during embryogenesis, thrombosis and lymphocyte help and killing all involve related cell surface receptors. These interactions may be between two cells or between a cell and the extracellular matrix. A family of ten different but structurally related heterodimeric cell surface complexes (the integrins) has been defined. The integrins are transmembrane glycoproteins comprising noncovalent heterodimers that appear to link the extracellular environment with the cytoskeleton (Hynes, 1987). They interact with a variety of ligands such as extracellular matrix, complement and molecules on other cells. This family also includes cell surface receptors for extracellular matrix proteins, a major glycoprotein complex on platelet cell surfaces(IIb/IIIa), and groups of glycoprotein antigens on lymphoid and myeloid cells; all contain homologous subunits (Hynes, 1987). Using immunoselection with antibodies

that block cell adhesion and affinity chromatography with extracellular matrix ligands, the cell surface receptors for extracellular matrix glycoproteins such as fibronectin, vitronectin and laminin have been isolated (Ruoslahti and Pierschbacher, 1986). Many of these receptors belong to a group of substrate adhesion receptors that recognize Arg-Gly-Asp (RGD), a sequence present in their ligand structures which is thought to play a key role in cell adhesion.

A major glycoprotein complex found on platelet surfaces that plays a crucial role in aggregation and adhesion, contains two major surface glycoproteins called IIb and IIIa. These glycoproteins exist as a noncovalent 1:1 complex in the membrane, and serve as a receptor for fibrinogen, fibronectin, fibrin, von Willebrand factor and vitronectin. All these ligand proteins contain RGD sequences, which are involved in their binding to platelets (Pytela et.al., 1986; Ruggeri et.al., 1982; Bennett et.al., 1983; Jennings and Phillips, 1982). The IIb/IIIa glycoprotein complex is expressed on the surface of leucocytes where it may function as a receptor for fibronectin (Burns et.al., 1986). These glycoproteins have been reported to be a receptor mediating both platelet aggregation and leucocyte adhesion. It has been suggested that the IIb/IIIa complex may bind to other matrix protein such as thrombospondin and collagen which also contain RGD sequences (Plow et.al., 1985a; Shadle et.al., 1984).

Two interesting sets of surface antigens have been studied on lymphoid and myeloid cells. The first is a set of three

glycoproteins known as Mac-1, LFA-1 and p150,95. These glycoprotein receptors are cell surface α/β heterodimers that play a key role in leucocyte adhesion, and are now referred to as the leucocyte integrin subfamily. The LFA-1 molecule is expressed on all leucocytes and functions in cytotoxic T lymphocyte adhesion to target cells, participates in T helper cell responses, antibody dependent killing, and in lymphocyte and monocyte adhesion to endothelial cells and fibroblasts . Mac-1 and p150,95 are expressed on monocytes and neutrophils and on certain activated lymphocytes. They function as adhesion molecules in cell-cell and cell-substratum interactions, and as receptors for the complement fragment C3bi. They recognize the sequence Arg-Gly-Asp (RGD) in their ligands. Mac-1 appears to be identical to the complement receptor type 3 (CR3), which mediates adherence and phagocytosis of C3bi-coated particles by granulocytes and monocytes. (Springer et.al., 1984; Corbi et.al., 1988; Kishimoto et.al., 1987; Dimauche et.al., 1987).

In 1985 Gallin showed that the deficiency of the Mac-1 is associated with defective neutrophil adherence and spreading on a variety of surfaces and defective aggregation and chemotaxis to a variety of stimuli, as well as defective phagocytosis and respiratory burst responses to C3b and C3bi-coated particles.

A second set of antigens initially identified on T lymphocytes, is the very late antigen (VLA) protein family. These are a set of at least five related cell-surface heterodimers, including a fibronectin receptor structure, and other cell-substrate adhesion receptors. Using monoclonal antibodies to the common β subunit

and several of the α subunits, five VLA antigens have been identified on a variety of cell types other than lymphocytes (Hemler et.al., 1987). It has been suggested that the VLA proteins, LFA-1 , Mac-1, p150,95 family , the GP IIb/IIIa, vitronectin receptor family, and the Drosophila PS antigens have evolved as four subgroups in a highly conserved supergene family involved in fundamentally important functions, such as adhesion, migration, and embryogenesis.

All the surface glycoproteins (integrins) discussed above consist of two noncovalently linked subunits, the β subunits being around 95-130 Kd protein, α subunits being around 130-210 Kd protein, with the exception of the complex isolated from chicken cells (Hynes, 1987) which is trimeric. The common feature of the integrins is their role in cell-cell or cell-matrix interactions. Most of these receptors have been identified by their ability to bind to extracellular matrix glycoproteins (DeBruijn and Fey,1985).

1.3.3. Leucocyte adhesion :-

The organization of the immune system and its mobilization in response to foreign antigens are thought to be dependent on a network of transient cell-cell interactions. Two adhesion pathways using different receptor-ligand pairs are involved in these adhesion interaction, ICAM-1 being the ligand for LFA-1, and LFA-3 the ligand for CD2.

The intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoproteins originally defined by monoclonal antibody (mAb) that inhibits phorbol ester-stimulated leucocyte aggregation. The tissue distribution of ICAM-1 is consistent with its role as a ligand of LFA-1. ICAM-1 is widely expressed on cells of both hematopoietic and non-hematopoietic origin including lymphocytes, monocytes, fibroblasts, epithelial cells and endothelial cells. In vivo ICAM-1 expression is low in normal tissue but high in inflamed tissue or in lymph nodes draining sites of inflammation. In vitro the normally low basal expression of ICAM-1 appears in endothelial cells, dermal fibroblasts and keratinocytes which can be dramatically increased by treatment with the inflammatory mediators interferon- γ, IL-1, TNF, or lymphotoxin, and the increased expression of ICAM-1 directly correlates with increased LFA-1 dependent adhesion of lymphocytes to the induced cells (Dustin et.al., 1986; Pober et.al., 1986,1987; Cotran et.al., 1987).

In 1988 Makgoba and others demonstrated that anti-ICAM-1 mAb inhibited LFA-1-dependent interaction with some target cells in both studies of AIC (Antigen-independent conjugates) and CML (Cell-mediated lysis), indicating that ICAM-1 is a ligand for AIC formation, antigen-specific CTL (Cytotoxic T lymphocytes) recognition and cytolysis of particular target cells. The data also suggested the existence of alternative ligands for LFA-1. In 1987 Marlin and Springer showed that the interaction of LFA-1 with ICAM-1 required metabolic energy production, an intact cytoskeleton, the presence of Mg^{2+}, and was temperature dependent.

A second adhesion pathway has been shown to be dependent on the interactions between the cluster determinant-2 (CD2) and lymphocyte function associated antigen-3 (LFA-3) glycoproteins which permits T lymphocytes to interact with number of other cells. It has been shown that CD2 has homology with members of the immunoglobulin supergene family and it is T cell specific in its expression and utilization. LFA-3 is expressed on almost all cells and is generally not increased with activation. There is evidence which suggests that LFA-3 is the only ligand for CD2 (Selvaraj et.al., 1987; Dustin et.al., 1987).

Other immune adherence receptors such as Fc receptors and C3b (CR1) receptors are found on the surface of leucocytes. Fc receptors are found on numerous hematopoietic cells, monocytes, macrophages, all types of granulocytes, platelets, B and T lymphocytes and NK (natural killer) cells. They are involved in many important immunological functions including phagocytosis, clearing of immune complexes, antibody-dependent cytotoxicity, and the regulation of antibody production. It has also been suggested that the soluble forms of Fc receptors may be involved in the regulation of IgG production, but the structural relation of these receptors to other Fc receptors remains to be defined (Kinet, 1989). Different forms of Fc receptors have been identified by Unkeless et.al (1988). These forms are known as receptor I (FcRI) with high affinity for monomeric IgG, receptor II (FcRII) and receptor III (FcRIII) that have low affinity for monomeric IgG, but can effectively bind immune complexes by multiple receptor-ligand interactions. A comparison between the

different groups of homologous Fc receptors has been discussed by many workers (Ra et.al., 1989; Huppi et.al., 1988; Allen and Seed,1989; Hibbs et.al.,1988; Stuart et.al., 1987; Ravetch et.al., 1986; Scallon et.al., 1989). The study of soluble immune complex binding to guinea-pig peritoneal macrophages and neutrophils has revealed that Fc receptor expression by the two phagocyte types differs in two respects. Evidence from previous studies has showed that macrophges bind IgG1 and IgG2 via distinct receptors. Also macrophages and neutrophils differ greatly in the number of receptors per cell they possess for binding IgG2 (Coupland and Leslie, 1983).

Various fragments of the third component of complement, C3, bind to receptors present on the membranes of a number of cell types. C3b is bound by one set of receptors, designated CR3. In the presence of serum enzyme I the surface-bound C3b is rapidly cleaved to yield an altered form C3bi which is also recognized by receptors (CR3) on leucocytes. It has been shown that there are separate receptors for C3b and C3bi on human monocytes, and each type of receptor can independently mediate phagocytosis of C3b- or C3bi- coated particles (Wright et.al., 1983). Bound C3b or its cleavage products, C3bi also mediates attachment of the target material, such as antigen-antibody complex, micro-organisms, to effector cells such as mononuclear phagocytes, polymorphonuclear leucocytes and B lymphocytes (Fearon et.al., 1981; Siegel et.al., 1981) through CR2. The binding of C3b to lymphocytes may play a role in the development of B lymphocytes into memory cells and antibody-secreting cells, and may induce the secretion of a

chemotactic lymphokine (Sandilands and Whaley, 1983; Klaus and Humphrey, 1977).

1.3.4. Adhesion from flow :-

1.3.4.1. In vivo studies :-

In vivo observations demonstrate that polymorphonuclear leucocytes normally pass freely through the vessels and adhere only momentarily to the endothelial surface. During inflammation the local damage leads to changes in blood flow in the adjacent microvasculature partly through vasodilation and through the opening of additional capillary vessels. Within minutes leucocytes start to adhere along the vessel wall and this is shortly followed by migration through the endothelium to the site of inflammation. The rate at which leucocytes roll along the vessels walls is sensitive to blood flow (Atherton and Born 1972), and the possibility that leucocytes leave the axial stream and enter the marginal zone will be increased by decreased flow rate. The movement of neutrophils over the endothelium amd towards the focus of a lesion depends on the motor functions of neutrophils. Movement may by affected by the adhesive interactions of the cells with the tissue matrix. Chemokinetic and chemotactic factors may play a role in stimulating and directing the emigration of leucocytes (Wilkinson and Lackie, 1979; Lackie and Smith, 1980). By inducing shape change of transiently adherent cells chemokinetic factors may reduce the profile of the cell thereby reducing the distraction forces; spreading will increase the area over which adhesion can occur.

1.3.4.2. In vitro studies :-

The adhesion of red blood cells to foreign surfaces in the presence of flow has been studied by Mohandas et.al. (1974) using a parallel plate flow chamber method. The experimental technique is based on subjecting the adhered cells to a controlled fluid shear stress produced by a well-defined laminar fluid flow. This system is a fixed parallel-plate flow channel with a precise narrow gap width. It provides a uniform level of shear stress at a given flow rate and permits direct microscopic visualization of the process. The parallel plate flow chamber was modified by Doroszewski et.al. (1977) to investigate the adhesion of leukaemic cells. The adhesion of neutrophil leucocytes in a smiliar manner to that which occurs in vivo was investigated by Forrester and Lackie (1984) and Lackie and Forrester (1985) using the parallel plate flow chamber. A further modification to the parallel plate chamber design was made by Owens et.al. (1987).

Under the conditions of flow, Forrester and Lackie (1984) and Lackie and Forrester (1985) investigated the effect of divalent cations, various plasma proteins, chemotactic factors, and extracellular matrix components on the adhesion of human neutrophils. They found that neutrophil adhesion was reduced in the complete absence of divalent cations, and the addition of magnesium ions was enough to restore normal adhesiveness, but calcium ions would not do this. Coating of the chamber with human serum albumin (HSA) was found to be important, and a minimum concentration of 1mg/ml was required to reduce adhesion below that of uncoated glass. The results also indicated that neutrophil

adhesion was inhibited when the cells were suspended in alpha-2-macroglobulin, albumin, plasminogen, or anti-plasmin, but was less marked with fibrinogen. Also immunoglobulin G (IgG) appeared to have no effect on adhesion, but IgG complexed with antigen on the surface increased the adhesion. Moreover they showed that the adhesion of neutrophil leucocytes was increased by the chemotactic peptide fMet-Leu-Phe in the presence of autologous serum, but cells did not adhere well to surfaces coated with the chemotactic protein casein. Fibronectin appeared to reduce neutrophil adhesion. Lawrence et.al. (1987) investigated the effect of flow on the adhesion of polymorphonuclear leucocytes to vascular endothelium. Their results showed that slight changes in wall shear stresses can cause large changes in PMN adhesion to both stimulated and unstimulated endothelium, suggesting that the local control of blood flow (perhaps by vessel dilation) may be as significant as the presence of inflammatory mediators in determining where the arrest of leucocytes occurs. In 1987 Lackie et.al. showed that very rapid changes in adhesiveness happen when cells encounter an immune complex-coated surface.

This assay has been used extensively in my project because the assay allowed me to look at very rapid changes in cellular behaviour.

1.4. Locomotion :-

1.4.1. Mechanism of neutrophil locomotion :-

The locomotion of neutrophil leucocytes across the endothelium and through tissues has been studied by many investigators (Lackie and Smith, 1980; Keller, 1981; Lackie, 1982,1986; Stossel,1978; Zigmond, 1978). The movement of the cell has been described as amoeboid. This movement requires a reversible adhesion to the substratum which must resist the forces which move the cell body forward, but must be temporary so that the movement will continue. The cell uses the actomyosin motor system to generate contractile forces, and probably the process of actin gel-assembly for protrusion of the front (Wilkinson and Lackie, 1979). Locomotion requires energy (provided by glycolysis), a temperature between 25^{0}C and 40^{0}C and pH between 6.5 and 7.6. A moving neutrophil has a polarised morphology which is characterized by a pseudopod which is extended from the front or sides of a moving cell, a midregion containing the nucleus, and a knoblike tail. The pseudopods are thin layers of cytoplasm (about 0.2 μm thick) which exclude granules. The midsection of a moving cell contains most of the cytoplasmic granules and mitochondria. The granules are usually concentrated toward the front of the body but do not extend into the pseudopod. The nucleus remains toward the rear of the cell and the centriole is normally between the nuclear lobes and in front of the bulk of the nucleus. Direct observations have indicated that neutrophils are primarily adherent at the front, and it is not essential for locomotion that the tail adhere to the substratum. As cells turn, the tail can sometimes be seen to swing around to a new position,

indicating that it is not strongly stuck to the substratum.Tail fibres appear to prevent forward movement when the cell is moving on a rather adhesive substratum.

Keller (1983) and Haston & Shields (1984) described the locomotion of neutrophils as involving constriction rings in cell body which appear to move backward whether the cell is in the suspension, in a deformable matrix, or on a planar substratum. The mechanism of movement of human leucocytes over glass has been described as a contraction wave (Senda et.al., 1975). Brown (1982) discussed the locomotion of neutrophils in a three dimensional matrix of reconstituted collagen gel. The shape of the cells during the movement is more rounded when the turnover of attachments is fast (Lackie, 1986).

1.4.2. Locomotion of neutrophil leucocytes on 2-D substrata and in 3-D matrices :-

Neutrophil leucocytes require to form transient adhesions with the substratum in order to locomote over a planar substratum. Movement stops if there is excessive adhesion; cells may either be restrained by terminal attachments, or may become extensively flattened. Also in the absence of adhesion movement is impossible. Lackie and Wilkinson (1984) looked at the locomotion of neutrophil leucocytes on surfaces coated with an immune complex (BSA / anti BSA). They found that neutrophils adhere strongly to immune complexes and the movement was reduced. The movement of neutrophils over glass coated with fibronectin or with collagen was reduced and cells seem to be immobilized (Lackie

and Brown, 1982) probably because adhesion is low. On the other hand neutrophils are able to attach to, locomote in and invade three-dimensional matrices of collagen fibres. Scanning electron micrographs showed that neutrophils in collagen matrices seem to extend pseudopodia between collagen fibres. The adhesion of the PMN to the fibres of the matrix is not necessarily required for locomotion in three-dimensional matrices. In the presence of Mn^{2+} neutrophil leucocytes are able to adhere and locomote on planar collagen-coated glass. In three-dimentional matrices the movement was, however, reduced by the increased adhesion (Brown, 1982). Lackie et.al., (1987) found that the movement and the invasion of neutrophil leucocytes was inhibited in the presence of immune complexes in a collagen gel. Pentoxifylline was found to increase the locomotion of human neutrophil which had been pre-stimulated with FMLP at 10^{-9} M over protein-coated glass (Crocket et.al.,1988). This drug, it has been suggested, alters the deformability of blood cells, but why this should lead to an increase in the rate of movement is far from clear.

The next two sections (5 and 6) will discuss briefly the factors which were tested in this study.

1.5. Bordetella pertussis :-

Bordetella pertussis is a bacterium which causes whooping cough and which produces a variety of biologically active components which may individually or in conjunction with other components contribute to the susceptibility of the patient to secondary infection. Bordetella pertussis adheres specifically to

human ciliated respiratory-epithelial cells. It has been suggested that two bacterial components, Filamentous haemagglutinin and Pertussis toxin, act in concert as adhesins of Bordetella pertussis for human cilia (Tuomanen and Weiss,1985).

Filamentous haemagglutinin and Pertussis toxin will be reviewd briefly in this introduction because these factors seem to reduce host immunity and general leucocyte function.

1.5.1. Filamentous haemagglutinin :-

Bordetella pertussis produces two types of haemagglutinin, filamentous haemagglutinin (FHA) and leucocytosis-promoting factor haemagglutinin (LPF-HA). Filamentous haemagglutinin has a high haemagglutinating activity, and has no histamine-sensitizing activity in mice. Electron micrographs of FHA show that the filament is about 2x4 nm in size. FHA has been purified by various techniques (Morse and Morse, 1979; Irons and Maclennan, 1979). It has been found that antibodies against FHA protected mice from intracerebral infection by B. pertussis, suggesting that immunity to FHA is important in immunity to the disease. Anti-FHA was also found to enhance the clearance of B.pertussis from the lung and prevented the attachment of B.pertussis to mammalian cells in culture. The absence of FHA from the organism has been found to be associated with the loss of adherence to human ciliated respiratory epithelial cells in vitro. (Sato et.al., 1981; Arai and Munoz, 1979; Ashworth et.al., 1982; Tuomanen and Weiss, 1985).

1.5.2. Pertussis toxin :-

Pertussis toxin (PT) is one of the toxins produced by B.pertussis and appears to be a major virulence factor of the organism (Pittman, 1979). PT has been purified by many investigators (Cowell et.al., 1982). Many studies have been done to show the biological activities of PT. Ogawa et.al. (1983) showed that PT inhibits a number of immunopharmacological activities, including B cell-dependent and T cell mitogenicity. Shefcyk et.al. (1985) found that treatment of rabbit neutrophils with PT inhibits the increase in actin polymerization (actin associated with the cytoskeleton) produced by FMLP, leukotriene B_4 and the calcium ionophore A23187. On the other hand the PT does not affect the increase in cytoskeletal actin produced by phorbol 12-myristate 13-acetate. The sequence of reactions initiated by the activation of protein kinase C also was not affected by PT. The chemotaxis of human neutrophils in vitro, and the adherence of neutrophils to protein coated plastic and glass surfaces were inhibited by the presence of PT (Spangrude et.al. (1985). Lad et.al. (1985, 1986) showed that chemotaxis, granule enzyme secretion, superoxide generation, shape change, aggregation, and capping induced by chemotactic factors, were all inhibited by treating neutrophils with pertussis toxin.

1.6. Complement system :-

The complement system is a group of self-assembling plasma proteins which constitutes the principal humoral mediator of

antigen-antibody reactions. This system defends vertebrates against most bacterial infections. Activation of the complement system attracts phagocytic cells to sites of infection and enhances the ability of these cells to ingest and destroy microorganisms. The complement system is sensitive to heat (55°C for 30 minutes) and dialysis against water at 0°C for 18 to 36 hours. It has been shown that the complement system consist of 20 proteins and most of these components are proenzymes which are activated sequentially by proteolytic cleavage. Activated components bind tightly to membranes.

The complement system is activated by two major pathways. The classical pathway is activated by antibody-antigen complexes and the alternative pathway is directly activated by polysaccharides present in the cell envelopes of bacteria, yeast and protozoa as well as other means. The most important fragments produced by these pathways are C3a and C5a. It has been shown that C3a and C5a fragments are split off and released into the body fluids during complement activation. These fragments have several important actions. Both act directly on phagocytes (especially neutrophils) to stimulate the respiratory burst associated with production of oxygen metabolites. Also both are anaphylatoxins, and cause the release of histamine from mast cells and aid the increase in blood flow. C5a has a powerful effect in increasing vascular permeability if neutrophils are also present, and it is a chemotactic agent for neutrophils and monocytes, attracting them to the site of inflammation (Sandilands and Whaley, 1983; Whaley, 1983).

The aim of this project was to study the locomotion and the adhesion (under flow conditions) of neutrophil leucocytes *in vitro*. The effects of *Bordetella pertussis* components, the activation of complement system through the alternative pathway, chemotactic factor FMLP and manganese ions were investigated.

Chapter 2

Materials and Methods :-

2.1. Media :-

2.1.1. Balanced salt solution (Hanks Hepes = H2) (perlitre) :-

NaCl	8 g
KCl	0.4 g
$CaCl_2$	0.14 g
$MgCl_2.6H_2O$	0.2 g
D-Glucose	1 g
Hepes	2.388 g (10mM)
0.5% Phenol red	10 ml

The pH was adjusted to 7.5 with 1M NaOH.

2.1.2. Calcium and magnesium-free salts solution (Hepes saline = HS) (per litre) :-

NaCl	8 g
KCl	0.4 g
D-Glucose	1 g
Hepes	2.388 g
0.5% Phenol red	10 ml

The pH was adjusted to 7.4 with 1M NaOH.

2.2 Proteins :-

2.2.1. Foetal calf serum (FCS) :-

Foetal calf serum (FCS) was from various sources and was stored at -20°C. (All batches supported cell proliferation).

2.2.2. Bovine serum albumin (BAS) :-

Bovine serum albumin (BSA) (Fraction V powder: 96-99% pure) was obtained from Sigma (UK) Ltd and stock solutions in H2 were stored at -20°C.

2.3. Methods :-

2.3.1. Isolation of polymorphonuclear leucocytes :-

2.3.1.1. Human neutrophils :-

Human neutrophils were obtained from heparinized (200 units/ml) peripheral blood from healthy adult donors (20 ml) which was treated by dextran sedimentation (2 ml of Dextraven 110) followed by centrifugation on Ficoll-Hypaque (10 ml; Pharmacia, Uppsala) at 400g for 20 minutes. The pellet was washed three times in buffered balanced salt solution (BSS) and the contaminating red cells were removed by hypotonic lysis after the first wash. After the third wash the cells were resuspended in the appropriate medium for the experiment.

2.3.1.2. Rabbit peritoneal neutrophils :-

Rabbit peritoneal neutrophils were prepared as described by Lackie (1977). New Zealand White female rabbits were injected intraperitoneally with 500 ml of sterile 0.9% (W/V) NaCl containing 0.1% (W/V) oyster glycogen (Sigma Ltd.). The fluid was collected after 4 hours and the exudate contained > 95 % pure PMNs. The cells were stored in exudate fluid at 4°C and used within 3 days.

2.3.2. Preparation of neutrophils for adhesion and locomotion assays :-

Before using the peritoneal neutrophils the cells were washed in calcium and magnesium free salt solution (HS) then resuspended in buffered balanced salt solution (H2) at a final concentration of 2 - 1.5 x 10^6 cells/ml. The red cells were removed by adding 1 ml of distilled water to the cells for 30 seconds after the first wash.

2.3.3. Labelling of neutrophils :-

Neutrophil leucocytes were labelled by incubating 1ml of cells ($5x10^7$/ml in 0.5% BSA/H2) with 0.2 μl (200 μCi) of $Na_2\ ^{51}CrO_4$ (Amersham) for 30-45 minutes at 37°C. The radiolabelled neutrophils were washed three times with H2 or HS to remove BSA and free ^{51}chromate. Finally the cells were suspended in H2 at the desired concentration and used immediately.

2.3.4. Neutrophil movement assay :-

2.3.4.1. Automated tracking system :-

The automated system for cell tracking was described by Dow, Lackie and Crocket (1987). This system consists of a Newvicon-tube video camera (Panasonic) attached to a beam-splitting eye piece and a 12-inch monochrome monitor (Panasonic). The TV camera was also connected through a video digitizer unit (Watford Electronics) to a BBC Master microcomputer, which was provided with twin floppy disc drives, plotter, a printer and a monochrome monitor. An air curtain incubator (a modified domestic fan-heater)

was used to maintain the cells at 37°C. Successive video frames were stored at 30 second intervals for 30 minutes; each frame was obtained as a 640 x 256 pixel array and was stored within 2 seconds. In this system up to 50 cells were randomly selected and tracked for the 30 minute sequence. After the end of the experiment each track in turn is presented for further analysis (see chapter 3 for details) (fig 2.1; 2.2). Motile behaviour is measured in terms of two parameters, speed (μm. min-1) and persistence (seconds) using the method proposed by Dunn (1983) and used by Wilkinson et.al. (1984).

The statistical analysis (speed and persistence) for the film sequences was done using the non-parametric Mann-Whitney U-test .

In each experiment the cells were filmed between two glass coverslips which formed the upper and the lower surfaces of 16-18 mm diameter chamber located centrally on a stainless steel slide 700 μm thick. The chamber held approximately 200 μl. The chamber was allowed 10-15 minutes to warm up then the cells were tracked for two consecutive 30 minute periods.

2.3.5. Neutrophil adhesion under flow conditions :-

2.3.5.1. Flow chamber system :-

In 1984 Forrester and Lackie described a flow chamber adhesion assay for neutrophils, and this is the system which has been used in this study. The chamber consists of two glass microscope slides held together by a gasket of Nescofilm 150 μm thick (Nippon Shoji Kaishe Ltd, Osaka, Japan) in which an 4mm x 40mm channel had been cut. The upper microscope slide has two 4mm

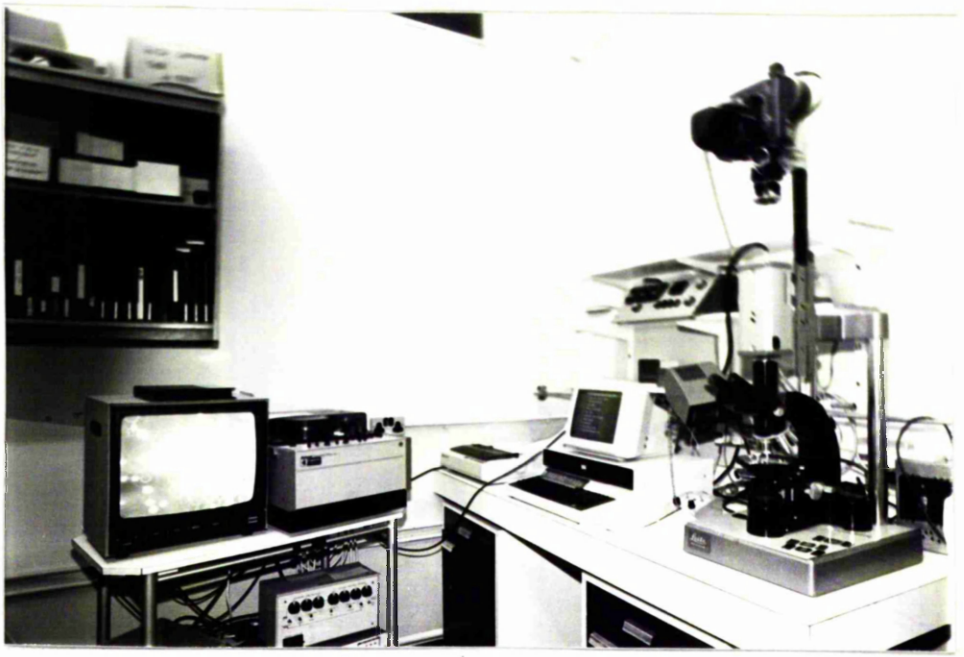


Fig 2.1 Photograph showing the whole setup of the tracking system equipment.

Fig 2.2 Photograph showing automated cell tracking. Photograph taken of monitor 14 minutes into a 30-min sequence. Cell tracks, with a x10 objective as here, cover an area 540x360 μm.

diameter holes coinciding with the ends of this channel which were used as inlet and outlet ports. The chamber was fixed in a metal and perspex clamping device with inlet and outlet ports that connected with the upper microscope slide holes. The cell suspensions were pumped through the chamber with a mechanical syringe drive (glass syringe or plastic syringe). The chamber was placed on the warm stage (an air-curtain) of an inverted Leitz microscope, and the cells were observed through a monochrome video camera, monitor system and recorded on a time-lapse video recorder (National : NV-8030) (Fig 2.3) (fig 2.4).

This flow chamber was employed in two different ways :-

(i) Kinetic method.

(ii) Distribution method.

(i) Kinetic method :-

In this assay the chamber was modified by pre-coating it with proteins or by adding proteins to the cell suspension. The flowing cell suspension was pumped through the chamber with a syringe drive at 0.2 μl/sec for 5 minutes. The number of adherent cells per unit area at different times was counted from the screen of the monochrome monitor. Adherent cells appear as white dots whereas non adherent cells appeared as blurs. Counts were made after 1, 2, 3, 4 and 5 minutes. The collection efficiency was calculated from the known cell delivery rate, cell concentration and the ratio of the area under observation to the total area of the chamber.

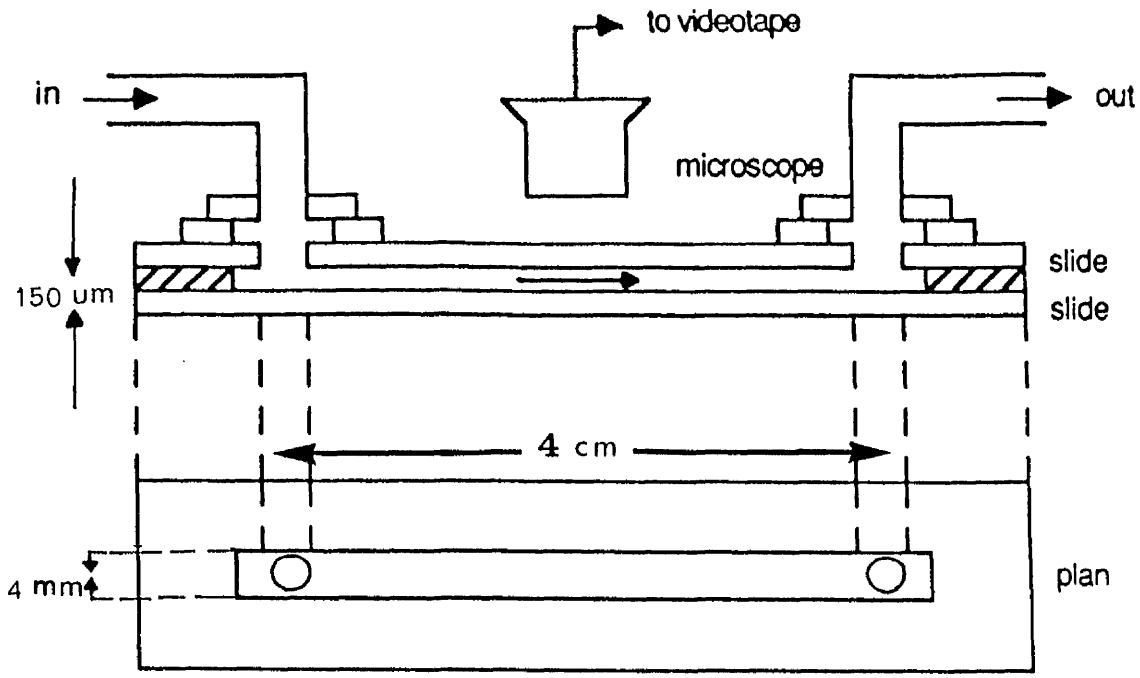


Fig 2.3 Schematic cross-section and plan view of the flow chamber showing the inlet and outlet ports which were drilled in a perspex block that is clamped over the glass chamber with silicone rubber gaskets between the perspex and the glass. (from Forrester and Lackie 1984).

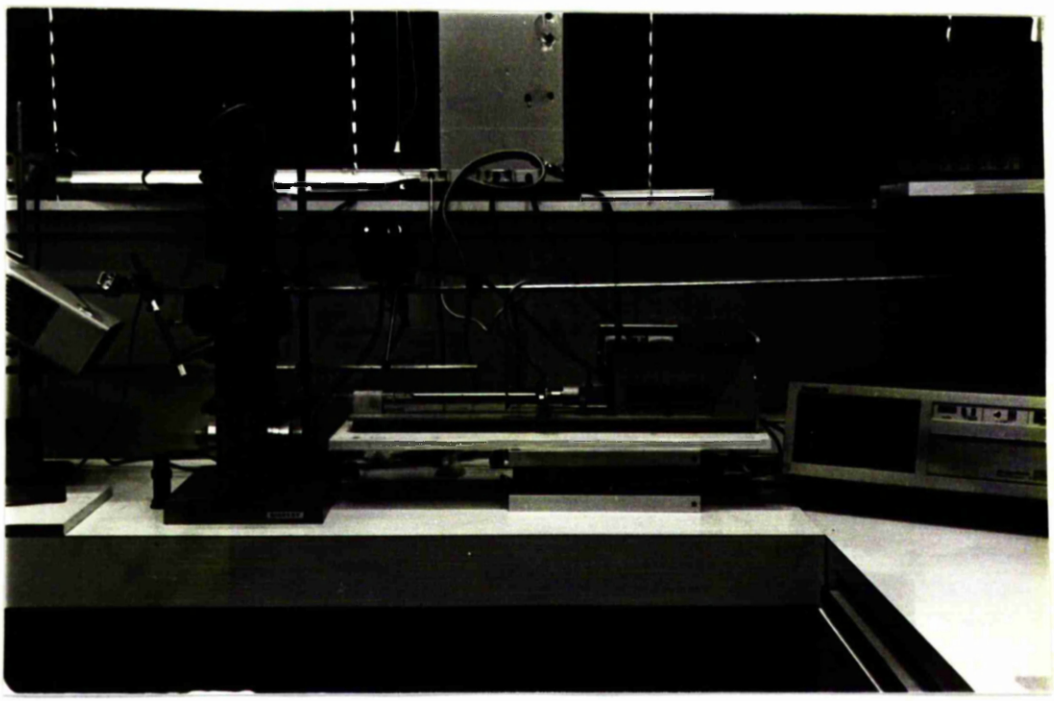


Fig 2.4 Photograph showing the whole setup of the flow chamber equipment.

(ii) Distribution method :-

Before the chamber was assembled, the lower surface of the chamber was coated one-third with air dried zymosan in agarose stripe (1.3 cm) (fig 2.5). The chamber was then rinsed with medium containing 0.5% BSA, to coat the uncovered glass of the chamber with protein. The cell suspension was perfused through the chamber for 5 minutes then the non-adherent cells were rinsed away with buffer (H2) for a minute. The number of adherent cells at different positions along the chamber was counted using a phase contrast microscope with an eye-piece grid.

In some experiments a chamber with two stripes of zymosan-agarose (0.4 cm) and three regions of uncoated glass (1.4 cm centrally, 0.5 cm upstream and downstream) was also used (fig 2.6).

2.3.6. Tube assay :-

A different adhesion-from-flow assay system was also used to investigate the time course of adhesion changes. Two syringes were used, one containing labelled neutrophil suspension (^{51}Cr) and the other containing the agent to be tested (chemotactic factor FMLP, serum, manganese, divalent cation-free medium). The two syringes were linked to a "Y" shaped manifold by two pieces of silicone rubber tubing (about 10 cm in length) the outflow from which then flowed through a sequence of 10 microcapillary tubes (each tube 3 cm in length and with a capacity 10 microlitres) which were linked together with silicone rubber tubes. Before the cells and the solution were pumped through; the microtubes were

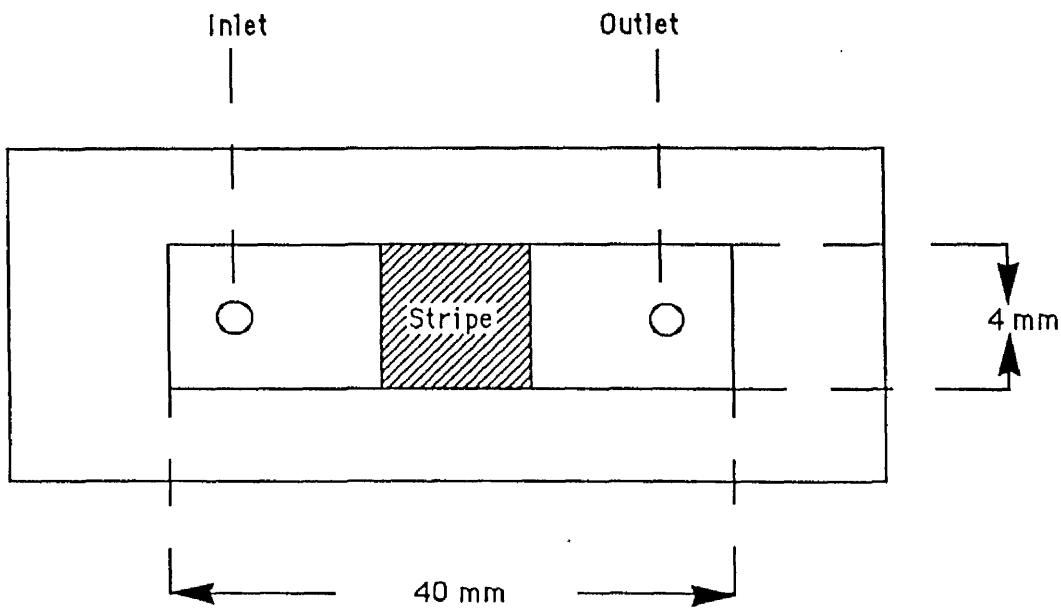


Fig 2.5 Diagrammatic plan view of the flow chamber showing the position of zymosan-agarose stripe which was dried on before the chamber was assembled.

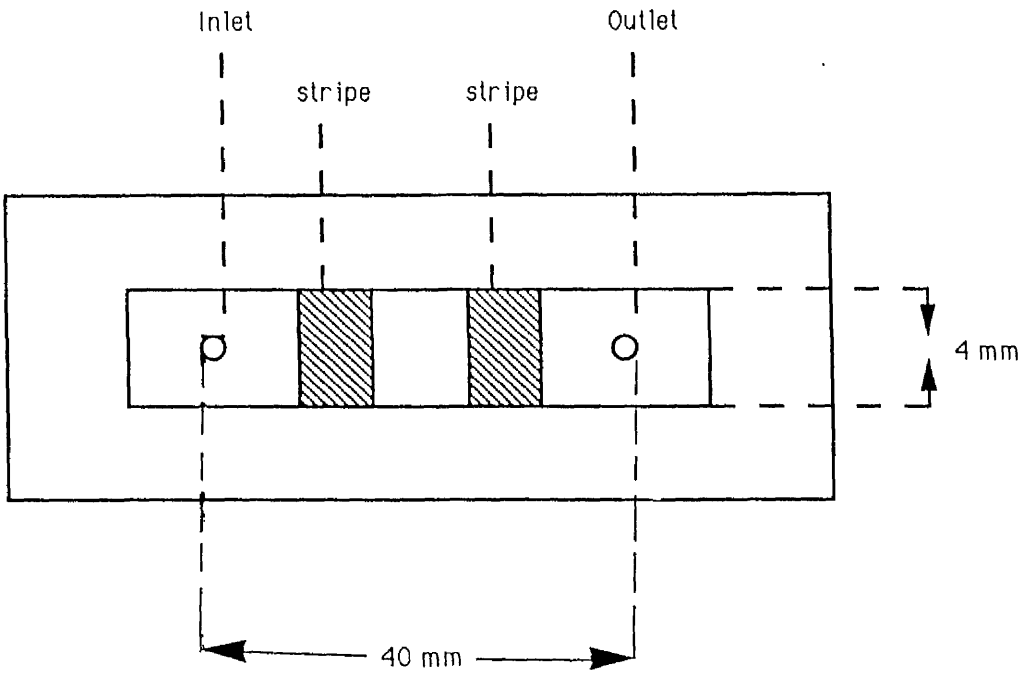


Fig 2.6 Diagrammatic plan view of the flow chamber showing the two zymosan-agarose stripes which were dried onto the lower surface of the chamber before assembly.

rinsed with H2 and then with 0.5% BSA. The cell suspension was pumped through for 20 minutes at 37°C with flow rate 1.25 μl/sec. The non adherent cells were rinsed out (at a high flow rate of 3.2 μl/sec.) with H2 for 2 minutes and the number of adherent cells in each tube was counted using a gamma counter (fig 2.7).

2.3.7. Complement receptor rosetting :-

2.3.7.1. Preparation of antibody- and complement-coated erythrocytes (EAC) :-

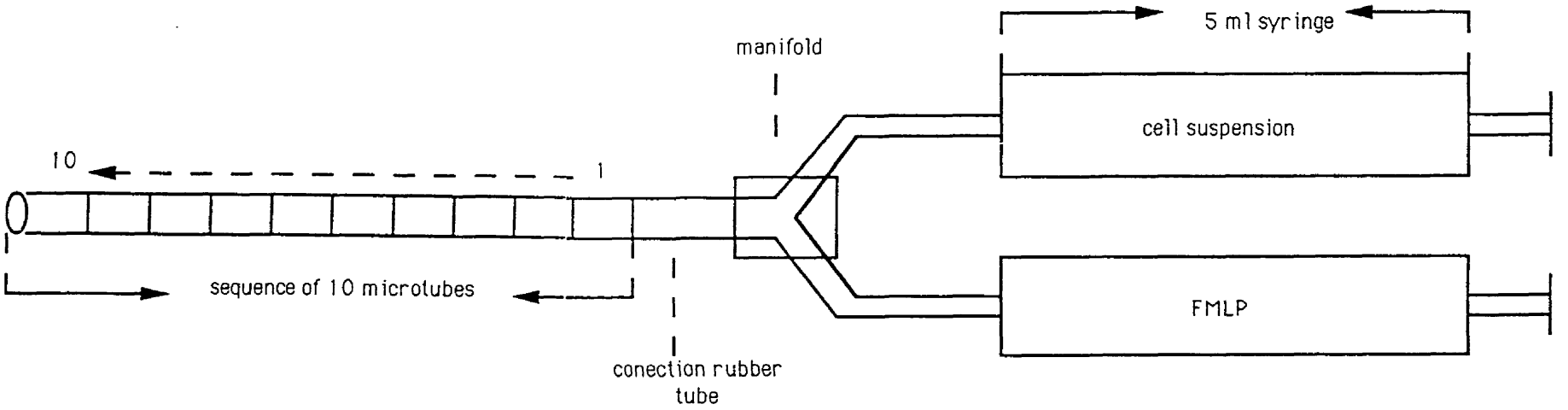
The antibody-complement coated erythrocytes (EAC) were prepared by incubating sheep erythrocytes (Scottish Antibody Production Unit; SAPU) with an equal volume of rabbit anti-sheep erythrocyte serum (haemolysin diluted 1:1000) at 37°C for 30 minutes. Following the incubation the antibody-coated erythrocytes (EA) were washed twice in buffer (H2) and resuspended in 5ml H2. An equal volume of fresh normal human serum diluted 1:80 was then added to the EA suspension and was incubated for 30 minutes at 37°C. The complement and antibody coated erythrocytes (EAC) were then washed twice in H2 and adjusted to a final concentration of 0.5% in H2.

The rosetting of EAC on neutrophils which had stuck to the zymosan stripe is described in chapter 4.

2.3.8. Preparation of filamentous haemagglutinin and pertussis toxin :-

Filamentous haemagglutinin and pertussis toxin were a gift from Dr.R.Parton, Microbiology Department, Glasgow University and

Fig 2.7 Diagrammatic plan view of the tube assay showing the Y-shaped manifold which connects the two syringes (containing the cell suspension and the chemotactic factor) and the sequence of 10 micro-tubes.


5 ml syringe
manifold
cell suspension
10
1
FMLP
sequence of 10 microtubes
conection rubber
tube

were stored at -20°C until use. They were diluted in Hanks Hepes (H2) prior to use.

2.3.9. Preparation of chemotactic factor FMLP :-

The chemotactic factor N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma; UK) was dissolved in DMSO to produce a stock of 10^{-2} M which was kept in small aliquots at - 20°C. FMLP was diluted in H2 before use.

2.3.10. Complement component activation through the alternative pathway :-

The complement system was activated by suspending the cells in 10% fresh rabbit serum before pumping them through a chamber containing a stripe of zymosan.

Serum was modified in various ways as follows :-

(1) Heat treated serum (HTS) : the serum was heated for 30 minutes at 57°C.

(2) Zymosan activated serum (ZAS) : 1mg/ml zymosan was added to the rabbit serum, incubated for 30 minutes at room temperature (20°-25°C) and then removed by centrifugtion for 5 minutes at 1500g.

(3) Heat-treated zymosan-activated serum (HT-ZAS) : fresh rabbit serum was activated (see 2 above) then the zymosan-activated serum was heated for 30 minutes at 57°C before being centrifuged for 5 minutes at 1500g to remove the zymosan.

(4) Serum dialysed against HS : dialysis tubing containing fresh rabbit serum was placed in HS and incubated overnight or for two days at 4°C with stirring. The medium was changed after 5 hours.
(5) Serum dialysed against H2 : the fresh rabbit serum was dialysed against H2.

2.3.11. Statistical analysis :-

Where the data were normally distributed standard t-tests have been used, but in many cases there was substantial overdispersal (with significant skew and kurtosis) and in these cases non-parametric methods (Mann-Whitney U-tests) have been used. On graphs the convention has been adopted of slowing significance on the following scheme :

*	$P < 0.05$
**	$P < 0.01$
***	$P < 0.001$

Chapter 3

3. Effect of Bordetella pertussis products on leucocyte behaviour :-

Introduction :-

Pertussis toxin (PT) and filamentous haemagglutinin (FHA) are considered to be important virulence factors of Bordetella pertussis, the bacterium which causes whooping cough in infants and young children. Different effects of these two proteins on the responses of a wide variety of cells, have been shown (Shefcyk et.al., 1985; Arai et.al., 1976; Ashworth et. al., 1982b). This chapter is concerned with the effects of pertussis toxin and filamentous haemagglutinin on the adhesion and the movement of neutrophil leucocytes in vitro. To test the influence of these factors on neutrophil adhesion and movement the flow chamber adhesion assay and an automated cell tracking system have been used.

3.1. Effect on neutrophil locomotion :-

3.1.1. Filamentous haemagglutinin :-

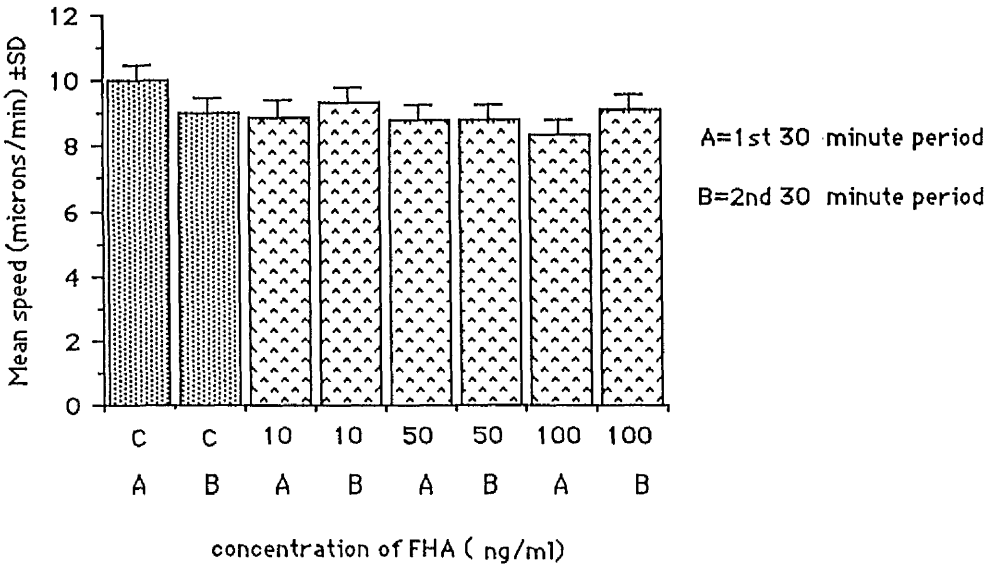
Using an automated cell tracking system the effect of filamentous haemagglutinin at different concentrations was tested. In these experiments, neutrophil leucocytes were suspended with 10% FCS in H2, and with high and low concentrations of FHA (1μg/ml, 0.5 μg/ml, 100ng/ml, 50ng/ml, 10ng/ml). The cells were tracked as described in methods (see tracking system). For each concentration of FHA the tracking was for two 30 minute periods.

The control which was used consisted of neutrophil leucocytes in 10% FCS. The movement parameters at different concentrations of FHA were compared with those of the control using Mann-Whitney U-test. There was no significant change in the speed with low concentrations of FHA (100ng/ml, 50ng/ml, 10ng/ml) as shown in figure 3.1, but the persistence of these cells was increased (figure 3.2). Figure 3.3 shows that at 1μg/ml the speed of neutrophil leucocytes was inhibited in the second 30 minutes (N1=89, N2=55, U=5.24, P< 0.1%) and at 0.5μg/ml there was a slight increase in the speed of the cells (N1=89, N2=79, U=3.22, P< 0.1%) in the second 30 minutes, but the persistence at these concentrations is no different from the control mean (figure 3.4).

The slight inhibition at high concentration of FHA may derive from an effect on adhesion of the cells (see section 3.2.1), but the effects were not dramatic.

3.1.2. Pertussis toxin :-

To examine the effect of pertussis toxin on neutrophil locomotion, the automated cell tracking system was also used. Neutrophil leucocytes were suspended in BSS with 20% FCS and different concentrations of PT (100ng/ml, 50ng/ml, 10ng/ml) were added to the suspension. The cells were then incubated for 30 minutes at 37^{o}C. The chemotactic factor FMLP at 2 x 10^{-9} M was added after incubation, to enhance the locomotion of the neutrophils. The cells then were tracked for two consecutive 30 minute periods. The incubation of the cells with PT has been done because the inhibitory effects of PT was noted after a pre-



N =	76	86	45	62	74	94	54	79
U =			1.41	1.54	1.26	1.9	0.68	1.23

n = 4

Control

FHA

- Comparison was made between the control in the first period and different concentrations of FHA in first period, and between the control in the second period and different concentrations of FHA in the second period.

N = number of the cells.

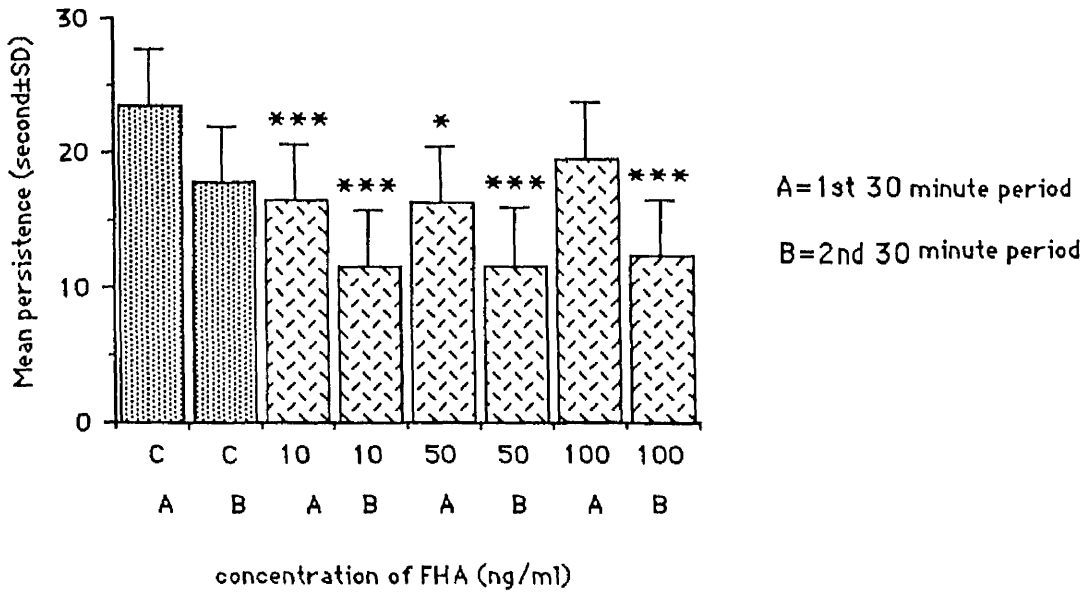
U = values of the Mann-Whitney comparsion.

n = number of replicates.

*** = Significant.

P< 0.001

Fig 3.1 The effect of low concentrations of FHA on the speed of neutrophil movement.



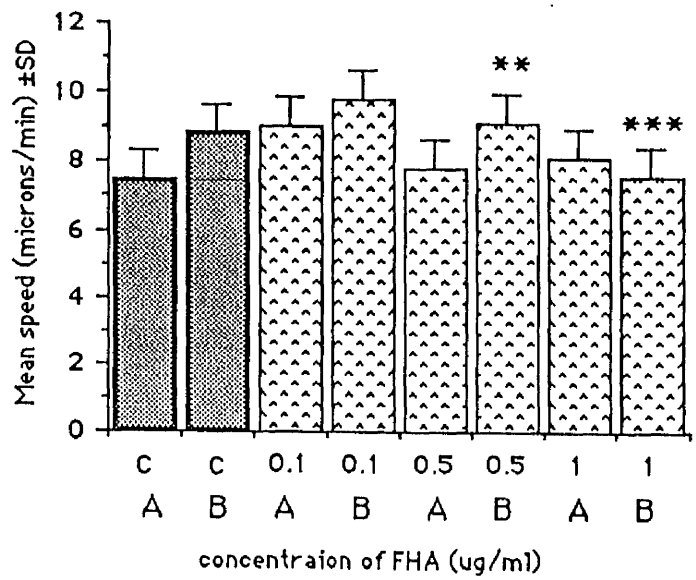
N =	76	86	45	62	74	94	54	79
U =			3.37	4.76	2.02	5.17	0.34	4.75

n = 4

Control

FHA

Fig 3.2 The persistence of neutrophil movement at low concentrations of FHA.



N =	64	89	47	58	81	79	43	52
U =			0.29	1.87	0.62	3.22	0.34	5.24

n = 4

Control

FHA

Fig 3.3 Effect of high concentrations of FHA on neutrophil speed.

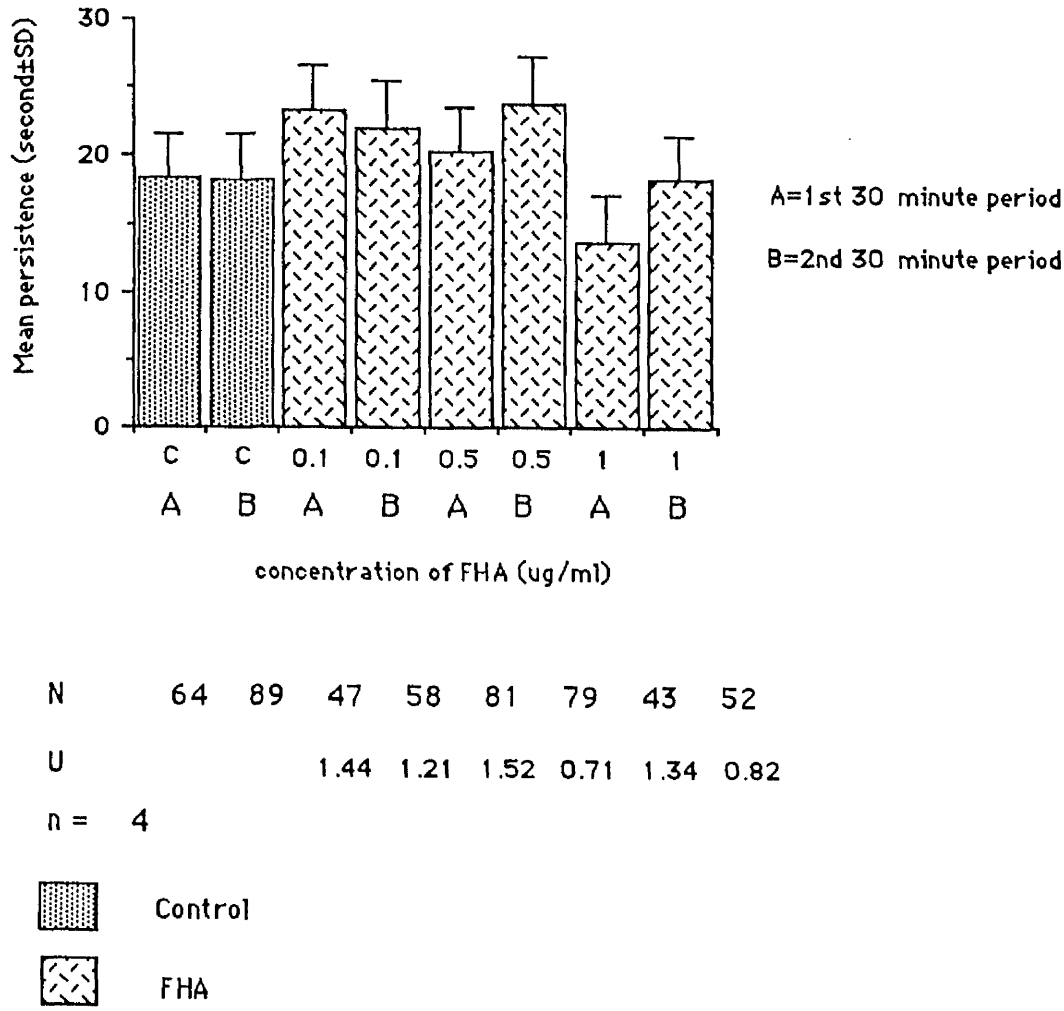


Fig 3.4 Effect of high concentrations of FHA on the persistence of neutrophil leucocytes.

incubation time of about 30-40 minutes (Craig, 1987). The speed and persistence of cells in different concentrations of PT were compared with speed and persistence of control cells, which were also stimulated with 2 x 10^{-9} M FMLP, using the Mann-Whitney U-test .

In figure 3.5 it seems that the treatment of neutrophil leucocytes with different concentrations of PT in the presence of chemotatic factor FMLP inhibits the speed of the cells; the most significant change was with 50ng/ml of PT in first and second periods of tracking (N1=182, N2=169, U=5.95 , N1=185, N2=151, U=3.40, P< 0.1%). Also there are significant differences in persistence between controls and cells treated with different concentrations of PT, and the greatest inhibition happened at 100ng/ml in two sequences of tracking (N1=182, N2=164, U=2.63 , N1=185, N2=107, U=5.27 , P< 0.1%) (figure 3.6).

The results obtained indicated that pre-incubation of neutrophil leucocytes with 50ng/ml PT at 37°C for 30 minutes inhibited the ability of the cells to move faster in response to the chemotactic factor FMLP in the first and second periods of tracking, while the inhibitory affect of FHA was noticed at 1μg/ml in the second 30 minutes. This difference in sensitivity (FHA 1μg/ml, PT 50 ng/ml) might suggest that PT is more likely to play an important part in inhibition of neutrophil movement than FHA. The results also indicated that FHA and PT required an incubation period of about 30 minutes with cells before any inhibitory effect is observed. Both PT and FHA might be suggested as important inhibitory factors for neutophil movement which might

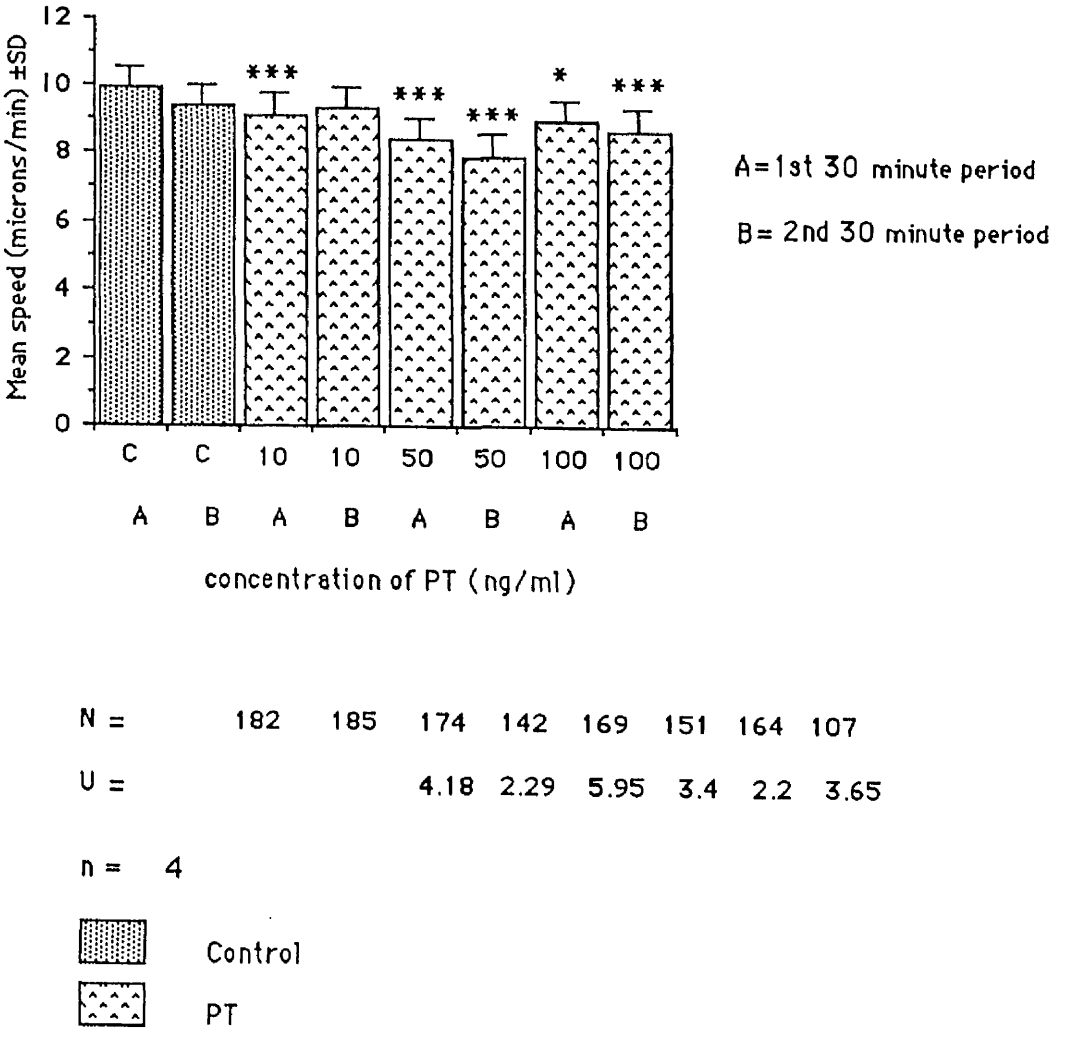


Fig 3.5 The speed of neutrophil movement at low concentrations of PT.

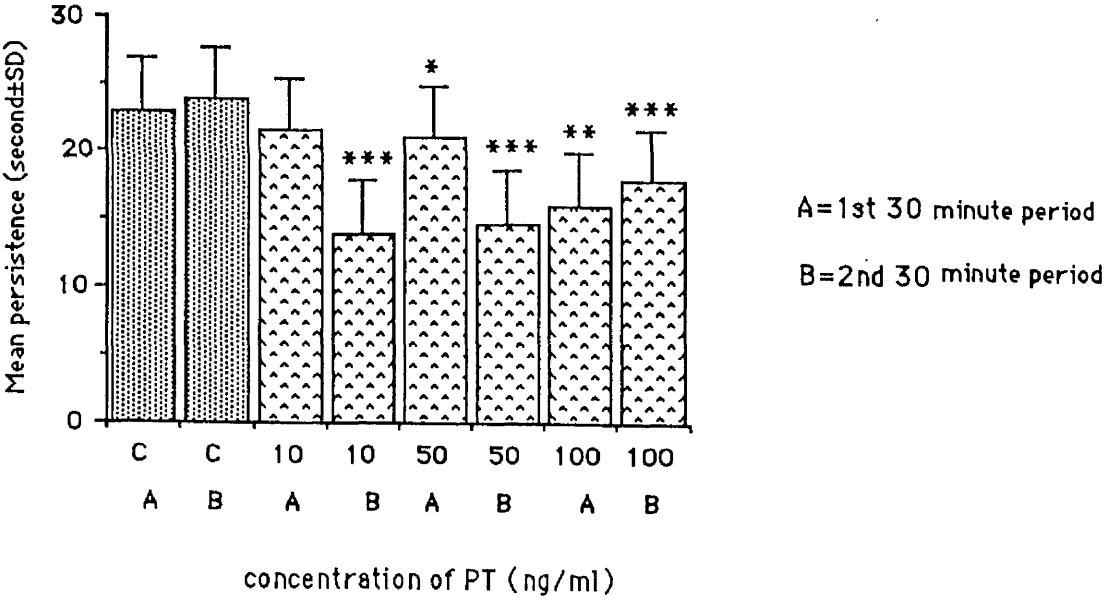


Fig 3.6 Effect of low concentrations of PT on the persistence of neutrophil locomotion.

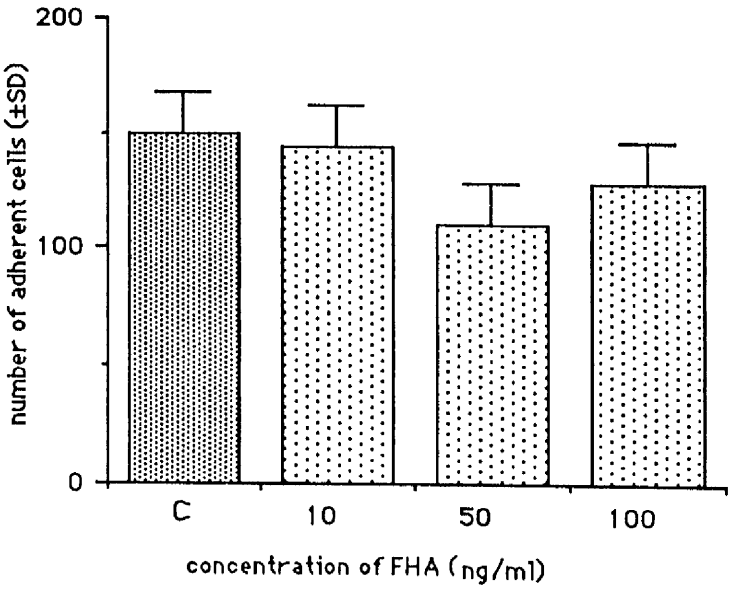
contribute to their action as virulence factors.

3.2. Effect on adhesion :-

3.2.1. Filamentous haemagglutinin :-

The adhesion of neutrophil leucocytes in response to FHA was tested using chambers coated with different concentrations of FHA (100ng/ml, 50ng/ml, 10ng/ml), the neutrophil leucocytes being suspended in 0.5% BSA in H2 at a cell concentration of 1.5 x 10^6 ml-1 (see the Kinetic method). As a control the chamber was coated with H2 and the cells were suspended with 0.5% BSA in H2. A comparison between the adhesion in the control and with different concentrations of FHA was done using Student t distribution-test.

The results indicated that there were no significant changes between the percentage of adherent cells in control chambers and those coated with various concentrations of FHA (100ng/ml, 50ng/ml) ($t=0.89, d.f.=5$, $t=1.41$, $d.f.=8$, $P< 0.1\%$) (figure 3.7). A comparison between the percentage of adhesion of FHA coated substratum and control was about 73% at 50ng/ml and about 94% at 100ng/ml. So the adhesion of neutrophil leucocytes was not affected by FHA. The collection efficiency, on clean glass and with FHA at different concentrations are shown in table 3.1 Figure 3.8 shows the adhesion of neutrophils to substratum coated with different concentrations of FHA and to clean glass after 1, 2, 3, 4 and 5 minutes of flow. The adhesion of neutrophils to substratum coated with 100 ng/ml FHA was shown in Figure 3.9. Flattening of the cells takes 2-3 minutes and once a cell has flattened it is unlikely to detach.



t.	0.25	1.41	0.89
d.f.	5	8	5

Control

FHA

Fig 3.7 Adhesion of rabbit peritoneal neutrophils to chambers pre-coated with different concentrations of FHA.

Flow time (min)	Collection efficiency (%)			
	control	10ng/ml	50ng/ml	100ng/ml
1	19	18	17	16
2	17	16	14	14
5	16	15	12	13

Table 3.1 Collection efficiency of neutrophil leucocytes on clean glass and different concentrations of FHA-coated glass.

n = number of replicates

n = 4

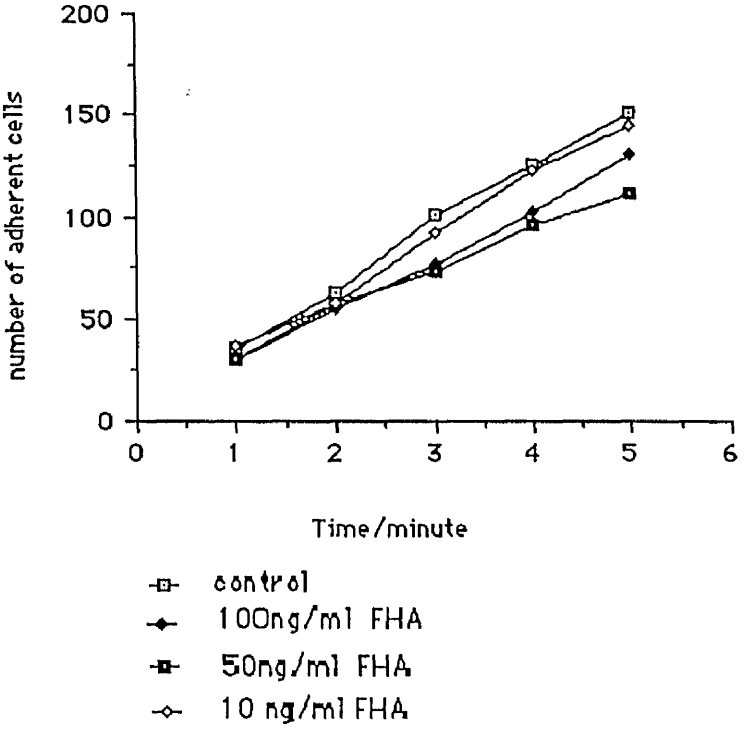
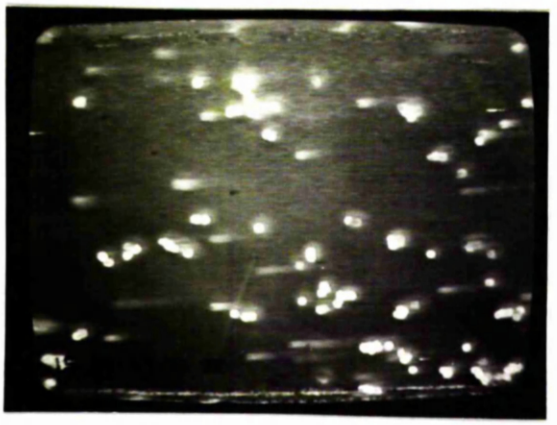


Fig 3.8 Adhesion of neutrophil leucocytes to the substratum coated with different concentrations of FHA and control after 1, 2, 3, 4 and 5 minutes.

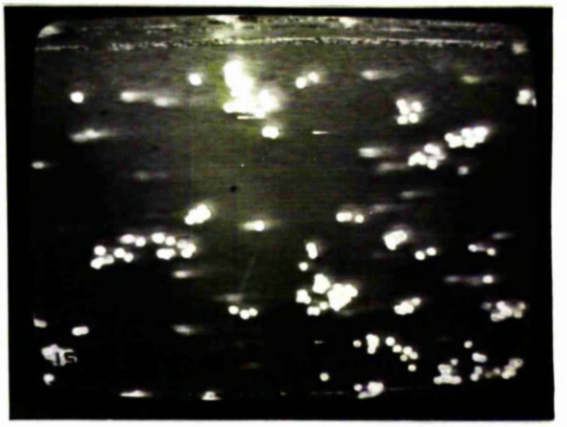
Fig 3.9 Four frames from a time-lapse videotape showing neutrophil adhesion in the flow chamber. The cells were suspended in 0.5% BSA and the chamber was coated with 100 ng/ml FHA. Photographs taken after 1 minute (A), 2 minutes (B), 3 minutes (C) and 5 minutes (D).

A

B



C



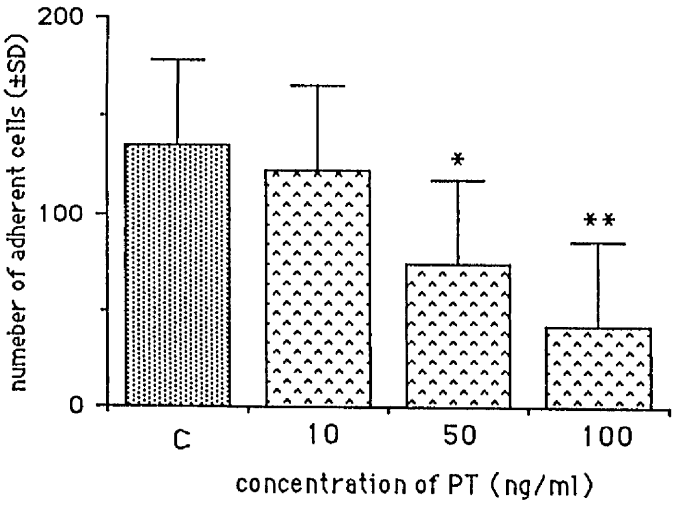
D

This result contrasts with that obtained by Craig (1987) who showed, in an aggregation assay, that FHA would increase the adhesion of rabbit neutrophils. The contrast between my results and his probably arises because of the different assay systems.

3.2.2. Pertussis toxin :-

The effect of pertussis toxin on neutrophil adhesion was tested by suspending neutrophil leucocytes with 0.5% (W/V) BSA in H2, and incubating them with different concentrations of PT (100ng/ml, 50ng/ml, 10ng/ml) for 30 minutes at 37°C before testing. Chemotactic factor FMLP at 2×10^{-7}M was then added to stimulate the cells for adhesion. The chamber was washed with H2, and the suspension pumped through for 5 minutes. The number of adherent cells was counted as described in Kinetic method (see method). Using Student t distribution-test the adhesion of neutrophils in presence of different concentrations of PT was compared with the neutrophil adhesion in controls which contained cells with 0.5% BSA and 2×10^{-7} M FMLP.

A marked inhibition of neutrophil adhesion was seen using 100ng/ml PT ($t=4.798$, d.f.=5, $P< 0.1\%$); there was also a slight inhibition with 50ng/ml ($t=3.375$, d.f.=5, $P< 0.1\%$) (figure 3.10). Table 3.2 shows the values of collection efficiency of cells at different concentrations of PT and control. The adhesion to clean glass of neutrophils which were pre-incubated with different concentrations of PT is shown in figure 3.11. Figure 3.12 shows the inhibition of neutrophil adhesion when the cells were pre-incubated with 100 ng /ml PT.



t.	0.57	3.375	4.798
d.f.	7	3	5

Control

PT

Fig 3.10 Effect of low concentrations of PT on neutrophil adhesion under flow conditions.

Flow time (min)	Collection efficiency (%)			
	control	10ng/ml	50ng/ml	100ng/ml
1	26.58	8.58	14.81	10.43
2	23.98	15.65	13.54	8.37
5	22.41	19.89	12.79	7.13

Table 3.2 Collection efficiency of neutrophil leucocytes with different concentrations of PT and clean glass.

n = 4

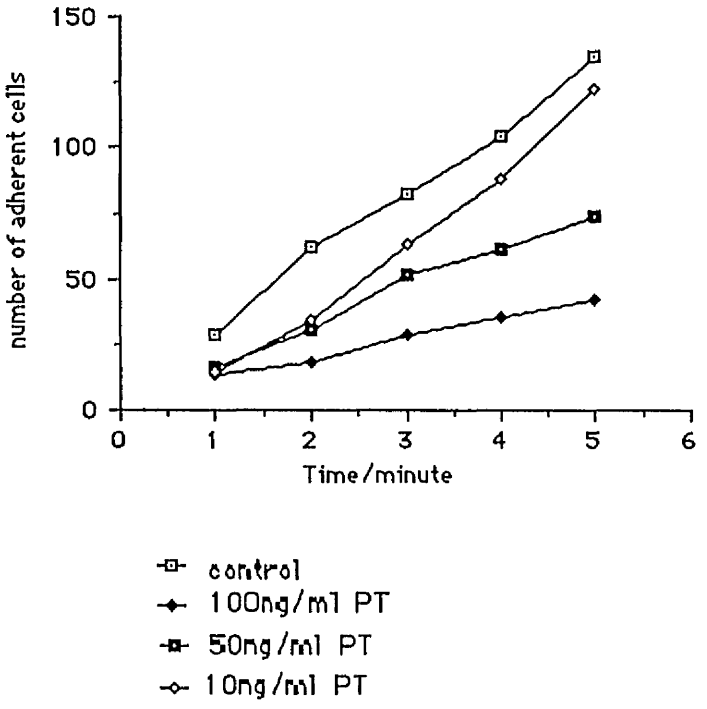
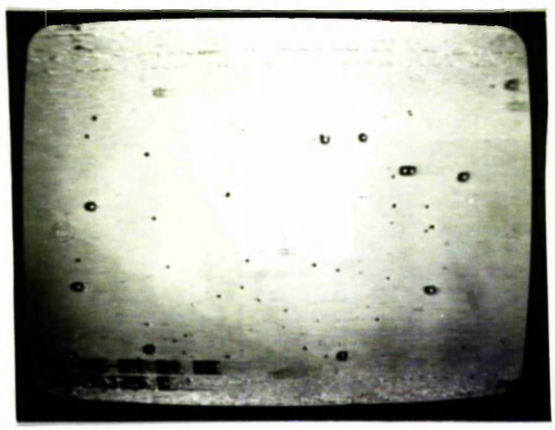
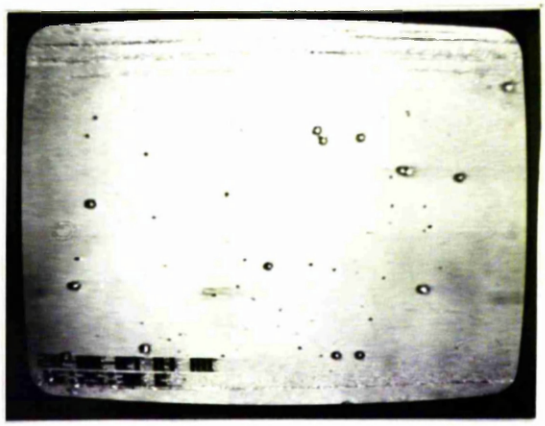


Fig 3.11 Effect of different concentrations of PT on neutrophil adhesion after 1, 2, 3, 4 and 5 minutes.

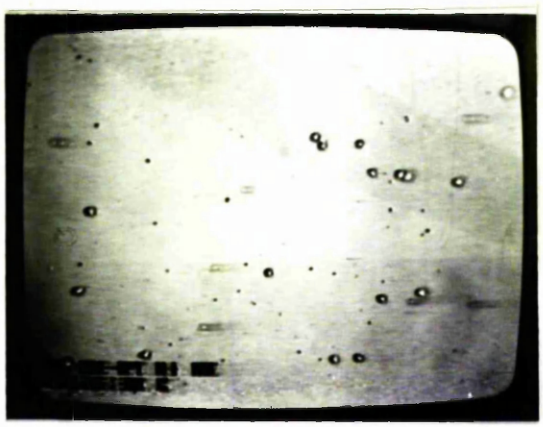
Fig 3.12 Four frames from a time-lapse videotape record of neutrophil adhesion in the flow chamber. The photographs showing the adhesion of neutrophils treated with 100 ng/ml PT and 2×10^{-7} M FMLP after 1 minute (A), 2 minutes (B), 3 minutes (C) and 5 minutes (D).



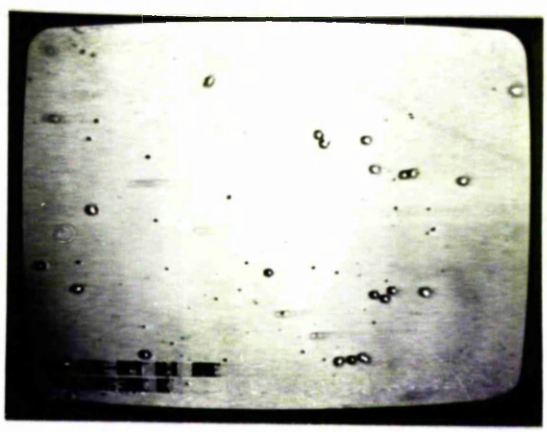
A



B



C



D

It is clear that incubation of neutrophils with pertussis toxin at 37°C for 30 minutes inhibited the enhancement of adhesion of rabbit peritoneal neutrophils brought about by the chemotactic factor FMLP. The degree of inhibition was increased with high concentration of PT.

The percentage of adhesion of neutrophils when the cells were pre-incubated with PT at 50ng/ml and 100ng/ml compared to controls was around 57% at 50ng/ml and 30% at 100ng/ml. This results might suggest that PT affected the adhesion of neutrophil leucocytes while FHA has no effect on neutrophil adhesion at these concentrations.

Previous studies showed that the adhesion of neutrophils to protein-coated glass coverslips was inhibited after a 2 hour treatment of neutrophils with 250ng/ml PT (Spangrude et.al. 1985).

Summary :-

1) The adhesion of neutrophil leucocytes is not affected by filamentous haemagglutinin, but the movement was very slightly inhibited.
2) Incubation of neutrophils with pertussis toxin inhibits the enhancement of adhesion and movement brought about by factor FMLP.

Chapter 4

Results II

4.1. Effect of complement components on neutrophil adhesion from flow :-

Introduction :-

The activation of the complement system in fresh serum is likely to play an important part in the inflammatory response by neutrophil leucocytes. The alternative pathway can be directly activated when polysaccharides such as yeast cell wall zymosan, or Gram negative bacterial endotoxin are added to the serum. The interaction of fresh serum with zymosan on neutrophil adhesion was studied using the flow chamber to measure the sudden change which might happen after the cells pass over different surfaces. For this experiment, neutrophils pass through a chamber in which one-third (1.3 cm^2) was coated with zymosan suspended in agarose and air dried before assembly (for more details see distribution method). The expectation was that complement would be activated when the cell suspension containing fresh serum passed over the zymosan stripe. The attachment of neutrophils to the zymosan-coated region was measured by counting the number of adherent cells at different positions along the chamber. Knowing the position of the stripe I could identify the change occurring when the properties of the chamber surface altered. The whole chamber was coated with BSA and the effects of adding serum treated in various ways was tested. In-addition to fresh normal serum I tested the following systems :-

- Heat treated serum (HTS). (inactivates complement system completely).
- zymosan activated serum (ZAS). (should have C5a and other complement components except C3b which binds to zymosan and is removed).
- Heat treated zymosan activated serum (HT-ZAS). (should leave C5a but no new complement activation possible).
- serum dialysed against HS (divalent cation free serum).
- serum dialysed against H2 (control for divalent cation free serum).

See tables 4.1 and 4.2.

4.1.1 The effect of normal and heat inactivated serum :-

The effect of activation of complement components in rabbit serum by the zymosan stripe is shown in figure 4.1. In these experiments neutrophil leucocytes were suspended with 10% fresh rabbit serum, the chamber was washed with 0.5% BSA, and the cells were pumped through the chamber for 5 minutes (see the distribution method).

Figure 4.1 shows that there is an increase in the number of cells adhering to the zymosan-agarose stripe particularly at the edge of the stripe, compared to the adhesion to the BSA coated regions. The number of adherent cells downstream was also higher than the comparable surface upstream of the complement activation stripe; upstream the cells are in normal serum, but over the stripe and downstream the cells are in zymosan activated serum. Thus activated serum enhanced the adhesion of neutrophils to

treatment of the serum (Abbreviation)	C3 activated	C3 system activatable	C5a present	C5a activatable
Normal serum (NS)	–	+	–	+
Zymosan activation (ZAS)	+	–	+	–
Heat treatment (HTS)	–	–	–	–
Heat treatment of ZAS (HT-ZAS)	–	–	+	–
Dialysed against HS (HS-S)	–	–	–	–
Dialysed against H2 (H2-S)	–	+	–	+

Table 4.1 Serum treatment : status of C3 and C5 systems.

Neutrophil Leucocytes suspension	Number of adherent cells %		
	Upstream	Stripe of Zymosan-agarose	Downstream
Fresh buffer	100% (n=4)	36% (n=4) **	102% (n=4)
10% Fresh serum	100% (n=5)	150% (n=5) ***	156% (n=5) ***
10% Heat treated serum	100% (n=5)	42% (n=5) ***	114% (n=5) ***
10% Zymosan activated serum	100% (n=6)	30% (n=6) ***	130% (n=6) **
10% Heat-treated zymosan-activated serum	100% (n=6)	23% (n=6) ***	110% (n=6)
10% divalent cation free-serum	100% (n=5)	90% (n=5)	112% (n=5)

The chamber was pretreated with 0.5% BSA containing buffer before the supension of rabbit neutrophils was pumped through.

*** P< 0.01 n= number of replicates.

Table 4.2 Number of adherent cells on the stripes of the chamber.

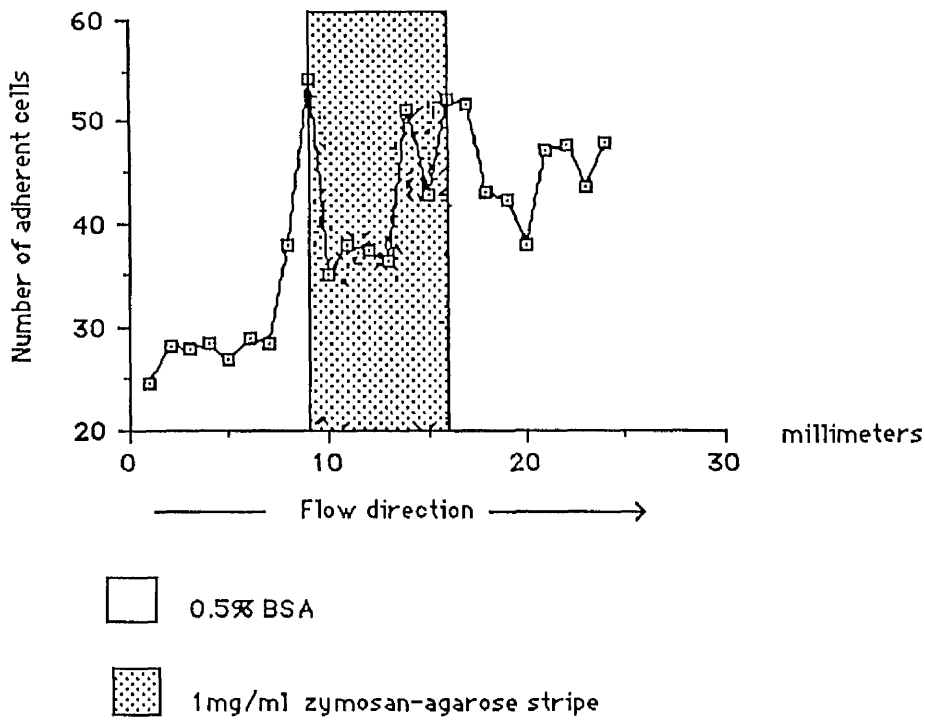


Fig 4.1 The effect of normal serum on neutrophil adhesion. The cells were suspended in 10% fresh rabbit serum then passed over substratum coated with zymosan-agarose or BSA.

zymosan and to BSA-coated glass.

Figure 4.2 shows that there are few adherent cells on the zymosan-agarose stripe when the cells were suspended in H2 and the chamber was washed with H2.

The effect of normal serum on neutrophil adhesion was also tested using chambers having two stripes of zymosan-agarose (0.4 cm apart), three regions of uncoated glass (1.4 cm midstream, 0.5 cm upstream and downstream). (see distribution method). The idea behind this treatment was to see how quickly neutrophils respond to the changes in substratum if there is more than one stripe. In these experiments the simple expectation is of the same response on both stripes.

The results obtained in figure 4.3, indicated that there were increases in the adhesion of neutrophils on the stripes of zymosan-agarose. A greater response was shown on the second stripe than the first stripe, which may come about because of the time taken for full activation of the complement system.

As a control the chamber was washed with H2 and the cells were suspended in H2. In the absence of serum the adhesion of neutrophils on the stripes of zymosan-agarose was inhibited compared to the adhesion on BSA-coated regions (fig 4.4).

The adhesion of neutrophil leucocytes was lower on zymosan-agarose stripes than on BSA stripes when the cells were suspended in 10% heat-inactivated serum (fig 4.5). Figure 4.6 shows the adhesion changes at the edge of the stripe.

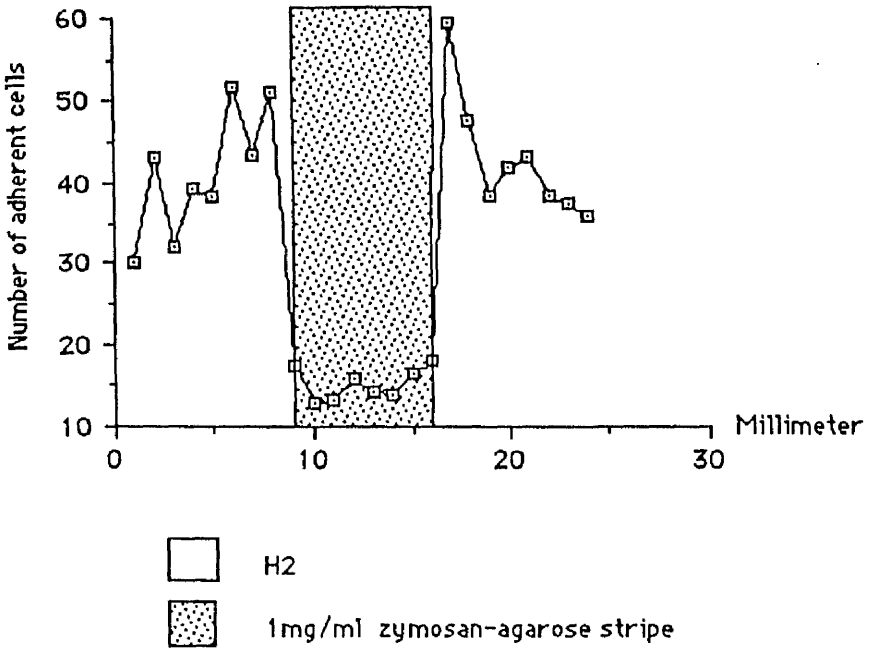


Fig 4.2 Adhesion of neutrophil leucocytes to a substratum coated with a stripe of zymosan-agarose. The cells were suspended in H2.

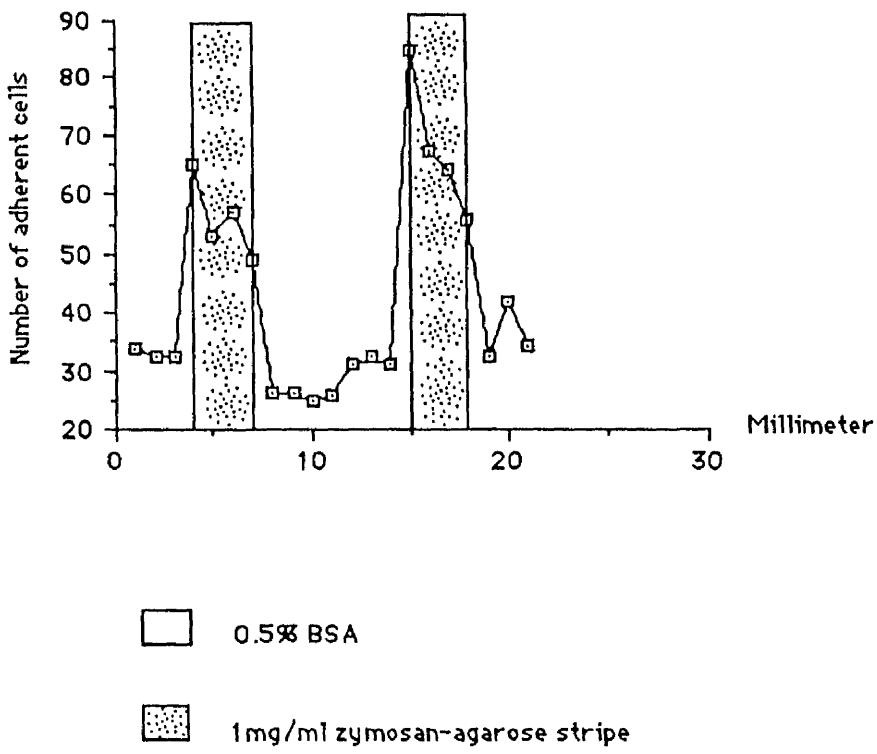


Fig 4.3 The effect of zymosan coated substratum on neutrophil adhesion. The cells were suspended in 10% fresh rabbit serum.

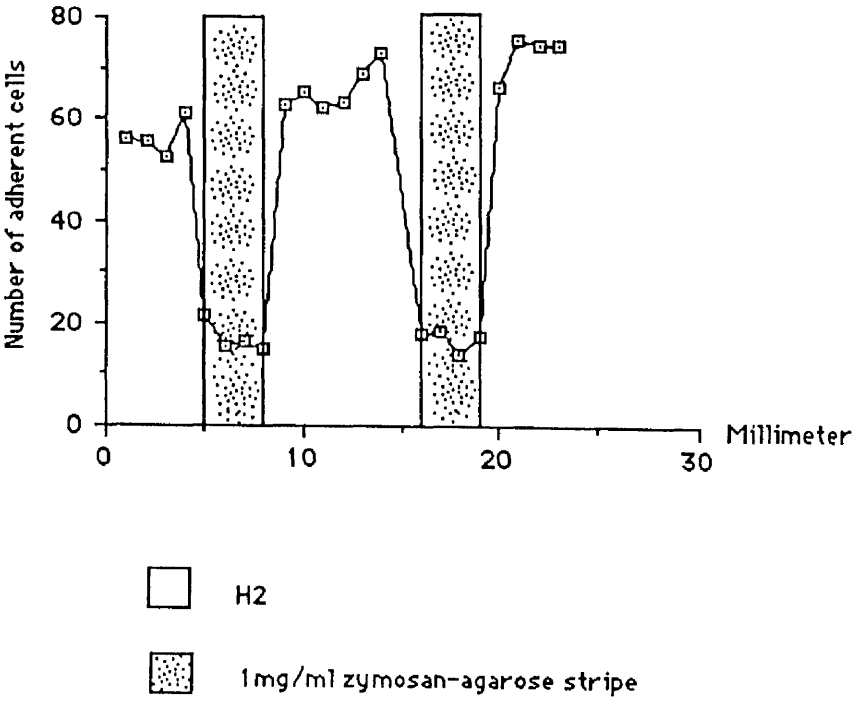


Fig 4.4 The effect of zymosan coated substratum on neutrophil adhesion. the cells were suspended. in H2.

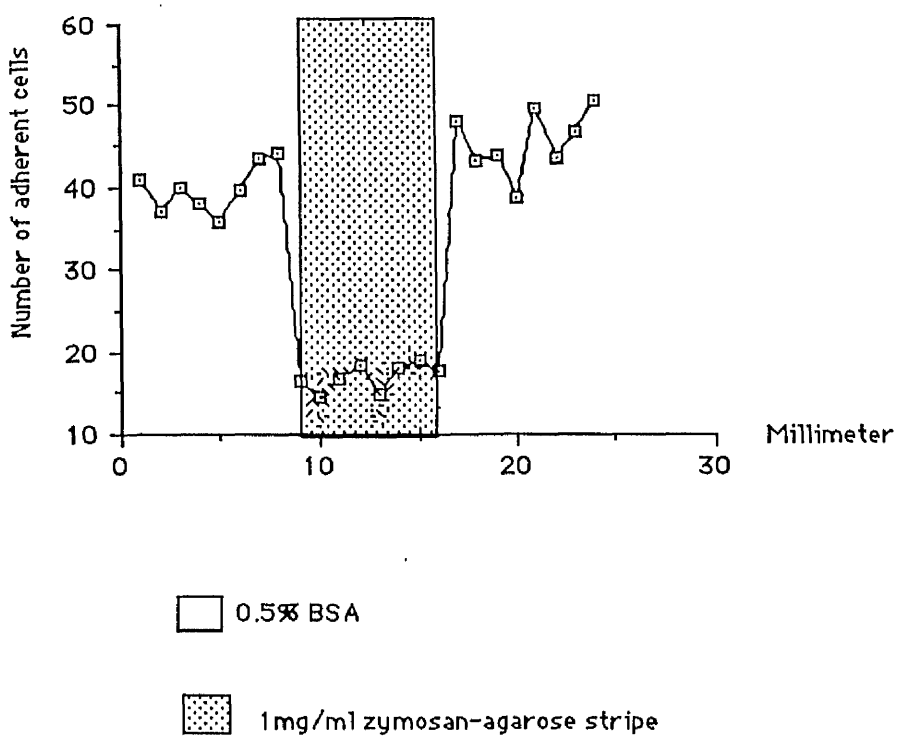


Fig 4.5 Effect of heat inactivated serum on neutrophil adhesion. The cells were suspended in 10% heat treated rabbit serum and the chamber was coated with a stripe of zymosan-agarose.

Fig 4.6 Photographs showing the accumulation of neutrophils on the zymosan-agarose stripe. The cells were suspended in 10 % fresh rabbit serum (A), and in 10% heat-treated serum (B).

A

B

Using a chamber which was coated with a stripe of zymosan in agarose, the effect of fresh serum was tested in a different way. In these experiments the chamber was washed with 10% fresh rabbit serum then rinsed with H2. Neutrophil leucocytes were suspended in H2 and pumped through the chamber for 5 minutes (for more details see distribution method).

The results are shown in figure 4.7. There were more adherent cells on the edge of the stripe, but fewer elsewhere. In this situation zymosan activated the serum but the only C3b was bound to the zymosan stripe, whereas in the system described previously there was free C3b in the suspending medium.

Figure 4.8 illustrates the same type of experiment done using a chamber with a stripe of zymosan only (no agarose having been used) in which there is also an increase in the adhesion of neutrophils to the zymosan stripe and to BSA coated regions downstream of the zymosan.

Greatest inhibition was seen on the stripe of zymosan-agarose when the chamber was washed with 10% heat inactivated serum and then with H2. The cells were suspended on H2 (figure 4.9). In this case the complement system had been inactivated by heating, and the decrease in number of adherent cells on the zymosan-agarose stripe is presumably a consequence of the adsorption of other proteins from the serum.

The next experiment was carried out to see whether C3b is the only complement component which enhances the adhesion of neutrophils to the zymosan stripe.

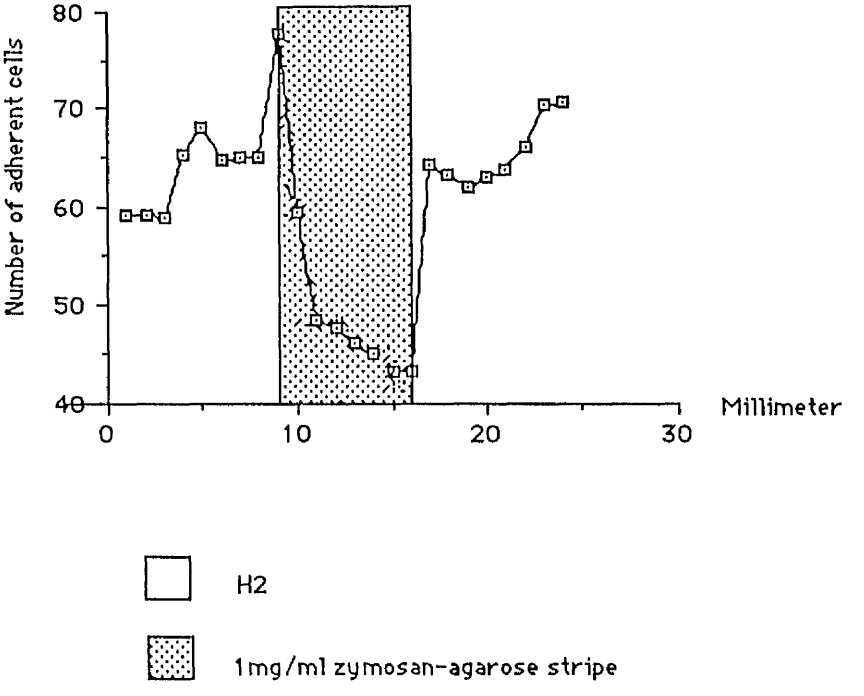


Fig 4.7 The adhesion of neutrophils to substratum coated with a stripe of zymosan-agarose.
The chamber was washed with 10% fresh rabbit serum, and the cells suspended in H2.

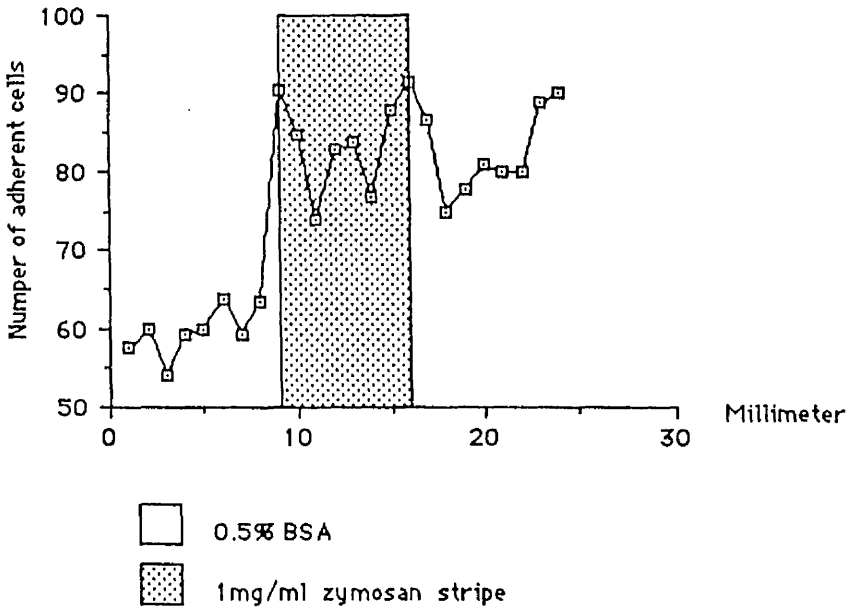


Fig 4.8 Neutrophil adhesion to substratum coated with a stripe of zymosan.

The cells were suspended in H2 and the chamber washed with 10% fresh rabbit serum

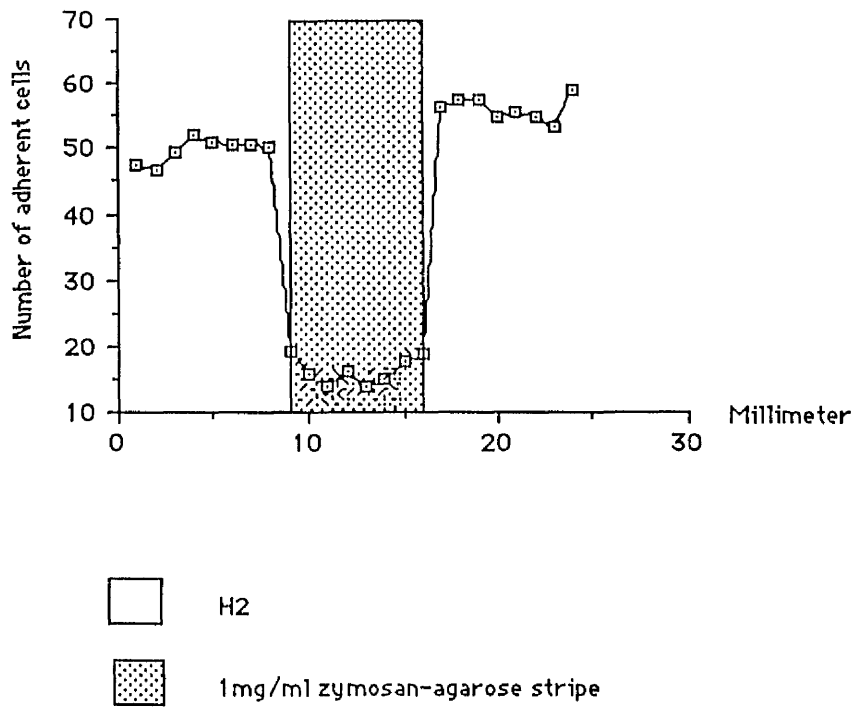


Fig 4.9 The effect of zymosan-coated substratum on neutrophil adhesion. The cells were suspended in H2, the chamber washed with 10% heat treated serum, and washed again with H2.

4.1.2 The effect of zymosan-activated serum (ZAS) and heated zymosan activated serum (HZAS) on neutrophil adhesion :-

In these experiments the complement system was activated, as described in methods (2.3.10), and the pre-activated serum was tested. The cells were suspended in 10% zymosan-activated serum and the chamber, which was coated with a stripe of zymosan-agarose, was pre-washed with 0.5% BSA. In this case the zymosan-activated serum contains all the complement components except C3b which will have bound to the zymosan and been removed.

When the complement system was pre-activated the adhesion of neutrophils on the stripe of zymosan-agarose was inhibited compared with their adhesion on BSA (fig 4.10).

The next experiment was done to test the effect of C5a on the adhesion of neutrophil leucocytes to the stripe of zymosan-agarose. The cells were suspended in 10% heated zymosan-activated serum (see methods 2.3.10), then pumped through a chamber which was coated with a stripe of zymosan-agarose and had been rinsed with 0.5% BSA. With this treatment, the serum should contain C5a, which is heat stable, but the complement system cannot be activated (fig 4.11).

The results of these experiments showed that there was less cell adhesion to the stripe of zymosan-agarose than on BSA-coated glass. Thus it seems that C5a does not affect neutrophil adhesion to stripe of zymosan-agarose, and that C3b mediates the adhesion of neutrophils to the zymosan-agarose.

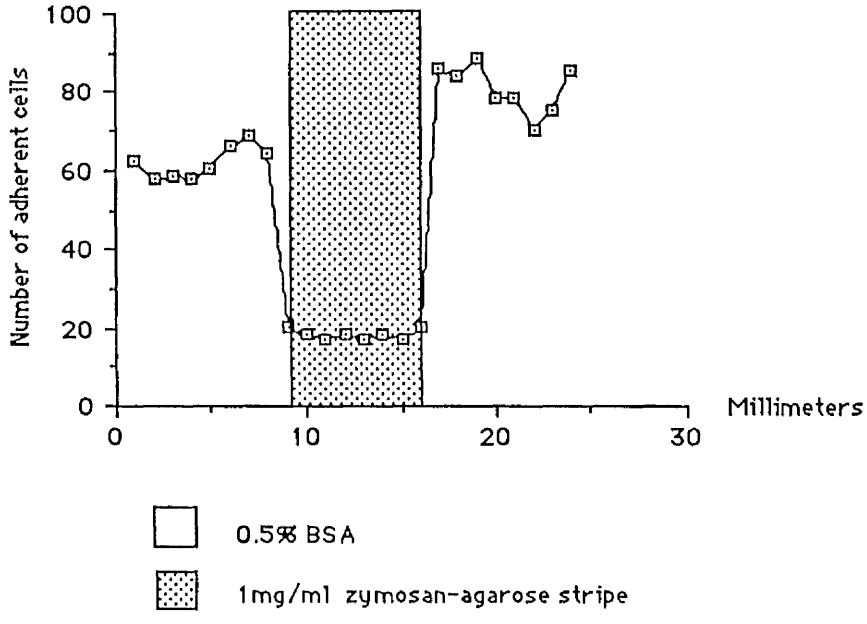


Fig 4.10 Effect of zymosan activated serum on the adhesion of neutrophil leucocytes to a zymosan-coated substratum.

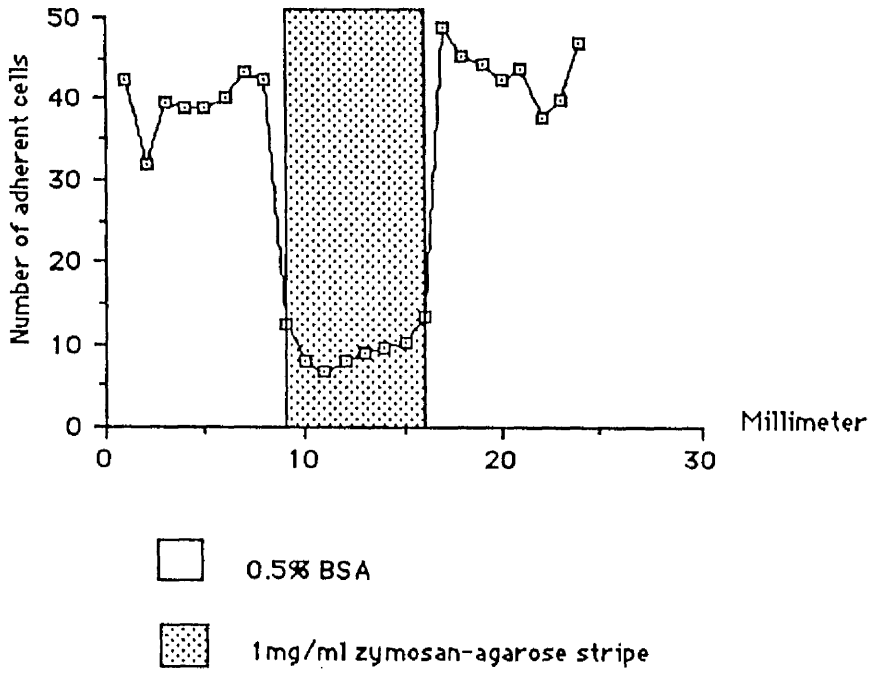


Fig 4.11 The effect of heat-treated zymosan activated serum on neutrophil adhesion.

4.1.3. Effect of divalent-cations (free serum) on the adhesion of neutrophils :-

Neutrophil adhesion is dependent on the presence of divalent cations (Hoover et.al. 1980, Garvin 1961). The effect of Mg^{2+} and Ca^{2+} in the activation of the complement system by zymosan was tested using the flow chamber assay (distribution method). In these experiments the rabbit serum was dialysed against HS to produce divalent cation-free serum (CMF-serum) (see methods 2.3.10). Neutrophil leucocytes were suspended in 10% CMF-serum, and the chamber with a stripe of zymosan-agarose was washed with 0.5% BSA. In the absence of Mg^{2+} and Ca^{2+} the adhesion of the cells was inhibited on a stripe of zymosan-agarose compared with BSA stripes (fig 4.12) presumably because the complement system could not be activated.

In the second assay, the serum was dialysed against H2 (H2-serum) (see methods 2.3.10), and the cells were suspended in HS with 10% H2-serum in HS. In this case the serum contains Ca^{2+} and Mg^{2+}, and (as shown in figure 4.13) there was an increase in the adhesion of neutrophils to the zymosan-agarose, more than the cell adhesion on BSA stripes.

So these results indicated that the enhancement of neutrophil adhesion brought about by activation of the complement system required divalent cations in the suspending medium. But which divalent cation is required? Is it Mg^{2+} to activate the alternative pathway or is it ions Mg^{2+} and Ca^{2+} as would be necessary to activate the classical pathway ? In our experiment we

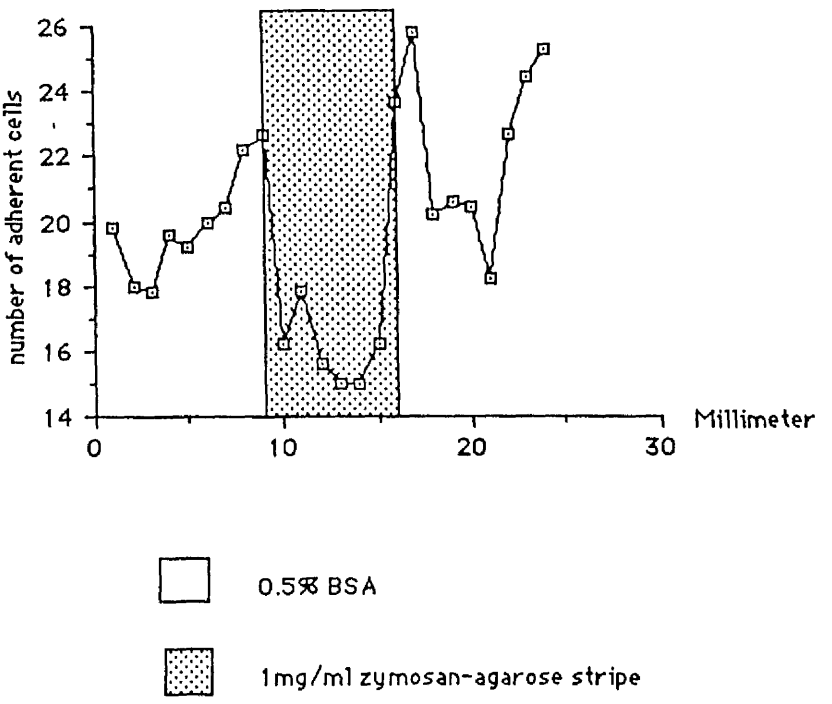


Fig 4.12 Effect of divalent cation-free serum on neutrophil adhesion.

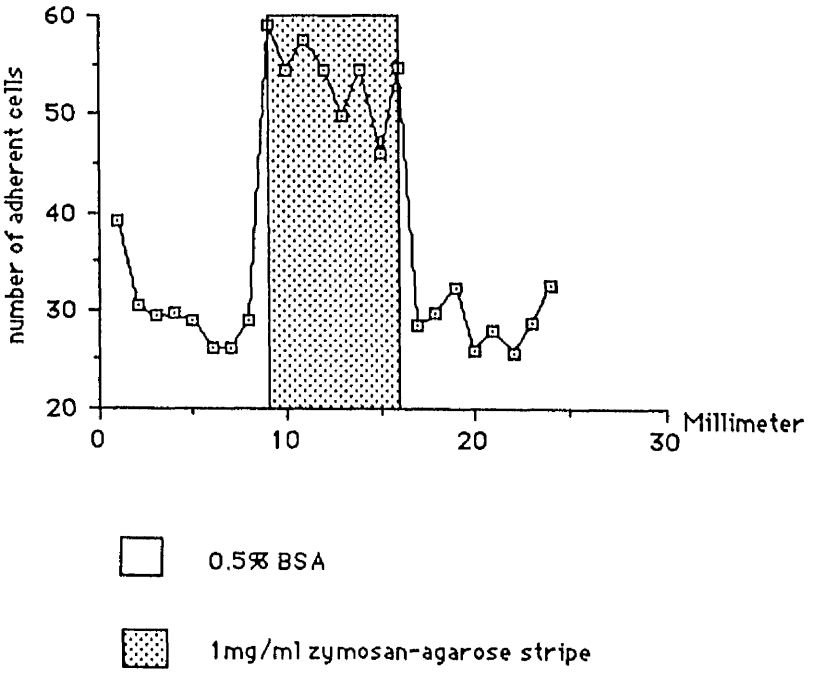


Fig 4.13 The effect of rabbit serum dialysed against H2 on neutrophil adhesion.

expected activation of the complement system through the alternative pathway, becouse there is no immune complex present.

The next experiments were to investigate which pathway of activation was involved.

4.1.4. Complement activation through the alternative pathway and the effect of MgCl :-

Activation of complement through the alternative pathway requires Mg^{2+} in the serum to produce more C3b and C5a. In present experiments the effect of $MgCl_2$ on the adhesion of neutrophil leucocytes on the stripe of zymosan-agarose was tested using fresh serum dialysed against HS (see methods 2.3.10). The cells were suspended in 10% serum dialysed against HS, and 1 mM MgCl.

As a control the cells were suspended in 10% serum dialysed against HS. As shown in figure 4.14, Mg^{2+} would restore adhesion to levels similar to those obtained with normal serum indicating that Mg^{2+} was the only important divalent cation, and the activation of the complement system by zymosan must therefore be through the alternative pathway.

4.1.5. Effect of agarose with normal serum on neutrophil adhesion:-

In previous experiments the stripe of zymosan on the chamber was prepared using zymosan suspended in agarose to fix the zymosan stripe to the chamber, but in this experiment the chamber was

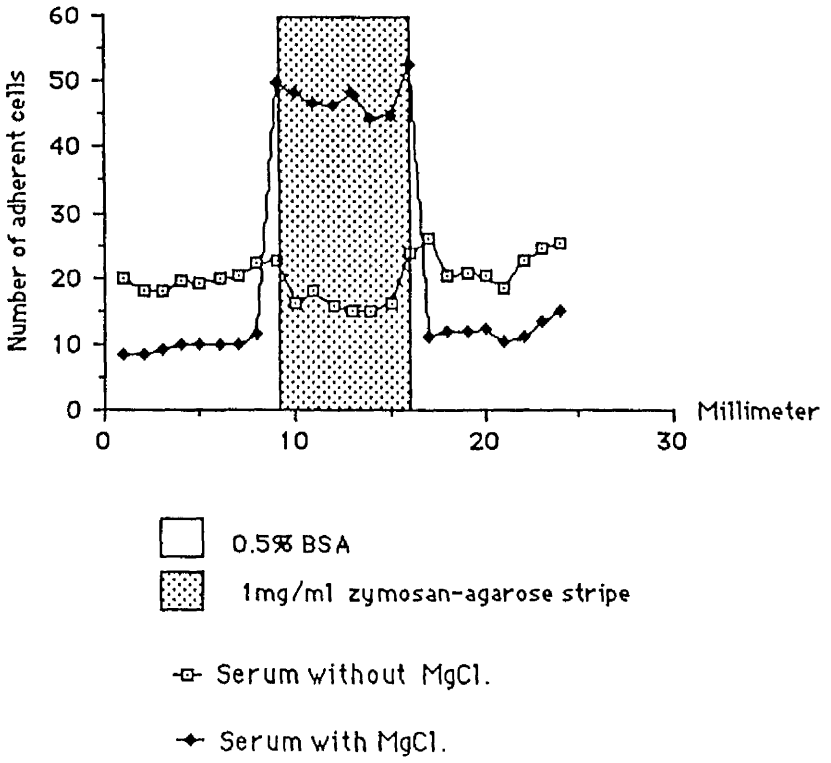


Fig 4.14 Effect of MgCl in the activation of the complement system through the alternative pathway on the adhesion of neutrophils.

coated with a stripe of agarose, and rinsed with 0.5% BSA. Neutrophils suspended in 10% fresh rabbit serum were then pumped through the chamber.

The result, shown in figure 4.15, there was a marked inhibition of adhesion of the cells on the stripe of agarose, more than the adhesion on BSA stripes. There is also a sudden increase on the cell adhesion immediately downstream of agarose presumably because there were more cells in the "marginal" zone (adjacent to the chamber wall) at this stage, and thus a much higher cell collection was possible; clearly the agarose does not affect the complement system.

4.1.6. The effect of the incubation of zymosan with serum on neutrophil adhesion :-

In these experiments, the chamber was coated with a stripe of zymosan and washed with 0.5% BSA. Fresh rabbit serum (10% in H2) was pumped through the chamber for 2 minutes, and the chamber was then incubated for 1 hour at 37°C, before being rinsed with H2 for 2 minutes. Cells, suspended in H2, were then passed through the chamber for 5 minutes. As a control a chamber was incubated with 0.5% BSA alone.

Figure 4.16 shows that there was a marked increase in adhesion on the chamber incubated with serum for 1 hour, compared to the control chamber incubated with BSA alone.

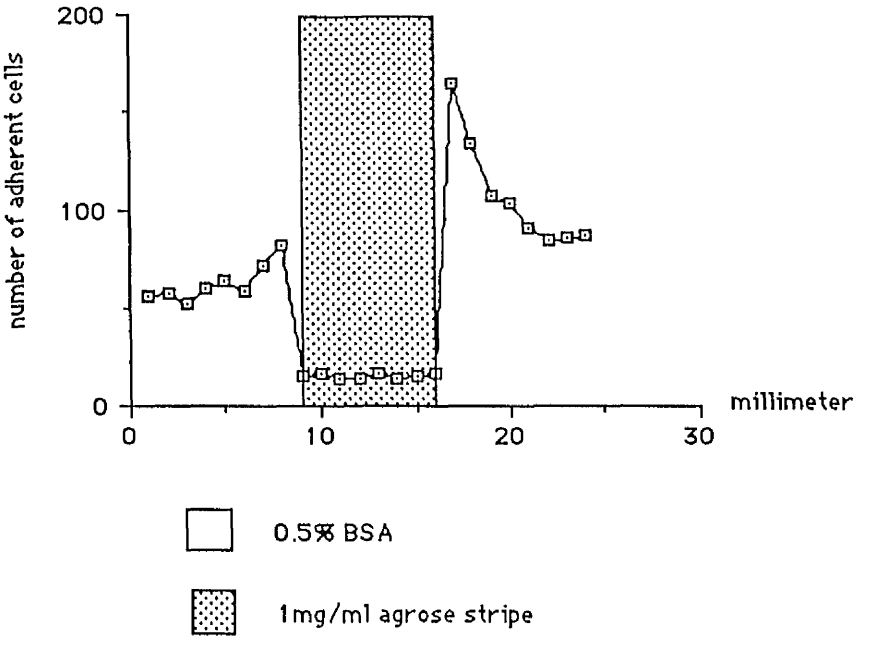


Fig 4.15 The effect of an agarose stripe on neutrophil adhesion. The cells were suspended in 10% fresh rabbit serum.

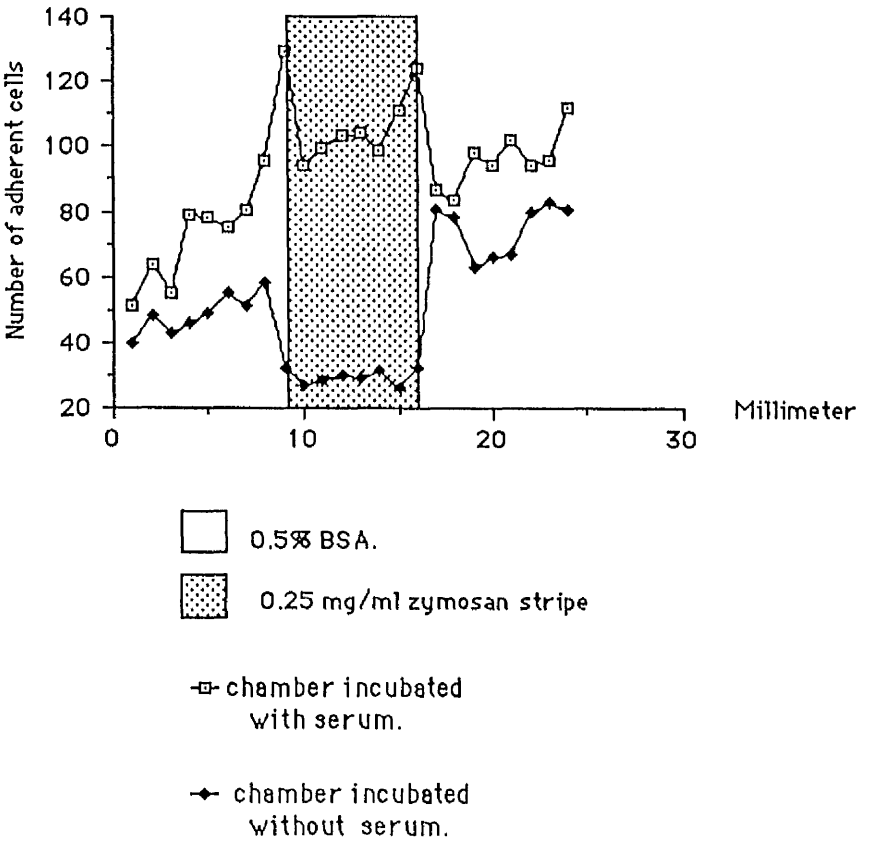


Fig 4.16 The effect of the incubation of the chamber with serum on neutrophil adhesion.

4.1.7. Complement receptor-mediated rosetting :-

The complement receptor rosetting assay was used to try to show the absence of C3b receptors on the dorsal surfaces of neutrophil leucocytes adhering to a zymosan stripe in the presence of serum. In this assay, neutrophils were suspended in H2 then $2x10^{-9}$M FMLP was added to the suspension to up-regulate complement receptors and increase the number of adherent cells. The chamber (with a zymosan stripe) was washed with 0.5% BSA and washed again with 10% fresh rabbit serum, then incubated for 60 minutes at 37°C. The cells were pumped through the chamber for 5 minutes and non-adherent cells were rinsed off with H2, then the chamber was incubated for 30 minutes at 37°C. Following this incubation period EAC (see methods 2.3.7) were added to the chamber which was then centrifuged for 5 minutes at 200g to bring about rosetting of erythrocytes on the dorsal surface of adherent neutrophil leucocytes. Centrifugation was done in a Shandon cytocentrifuge so that the centrifugal force was normal to the lower surface of the chamber. The chamber was then washed with H2, Trypan Blue/Glutaraldehyde added for 1-2 minutes, and finally rinsed with H2. As a control the chamber was washed with 0.5% BSA.

The results obtained (shown in figure 4.17) suggested that there are some indications of C3b/C3bi receptor redistribution on the leucocytes adherent to the stripe of zymosan but were not very clear cut because the assay did not work very well.

Figure 4.18 shows rosettes of erythrocye antibody-complement on neutrophils which have adhered to the zymosan-agarose stripe in

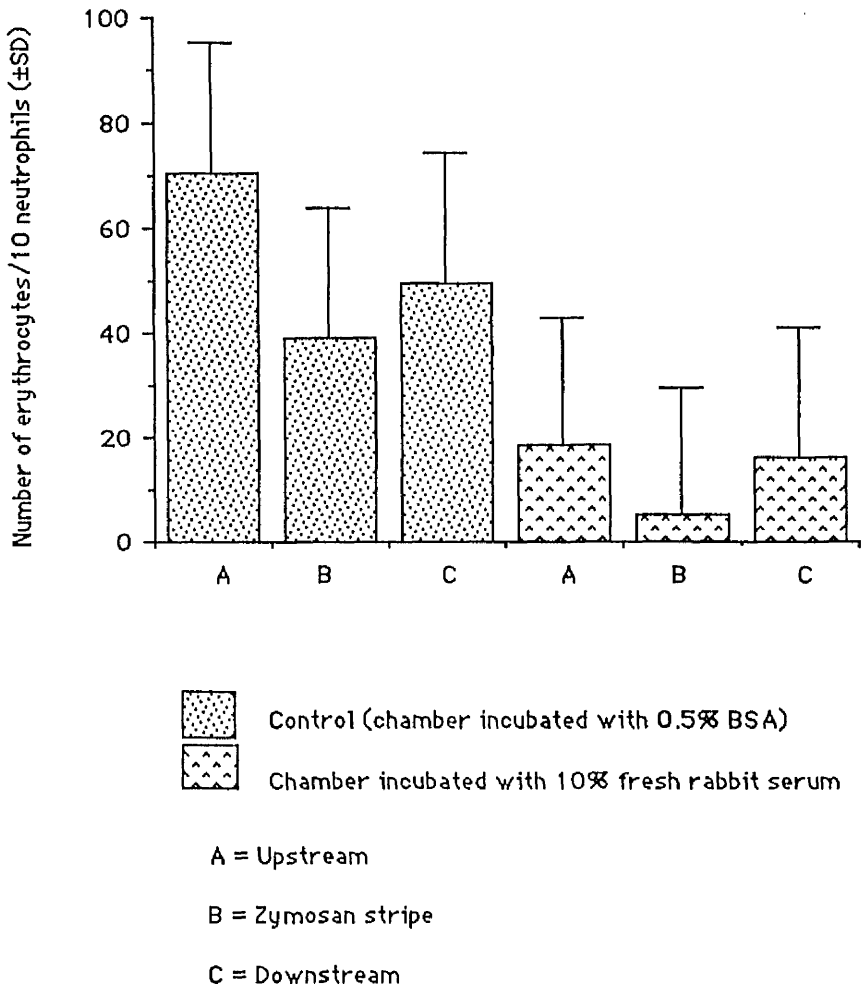
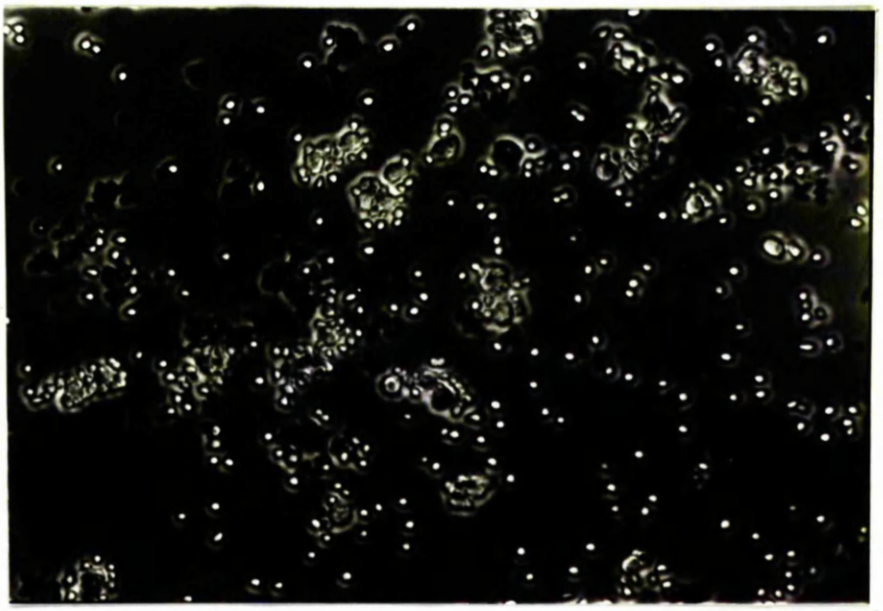


Fig 4.17 The erythrocyte antibody-complement rosettes on neutrophil leucocytes which have adhered to the zymosan stripe in the presence of serum.

Fig4.18 Photographs showing the rosette of erythrocyte antibody-complement on neutrophils which have adhered to (A) 0.5% BSA up stream, (B) zymosan-agarose stripe, (C) 0.5% BSA down stream.

A

B



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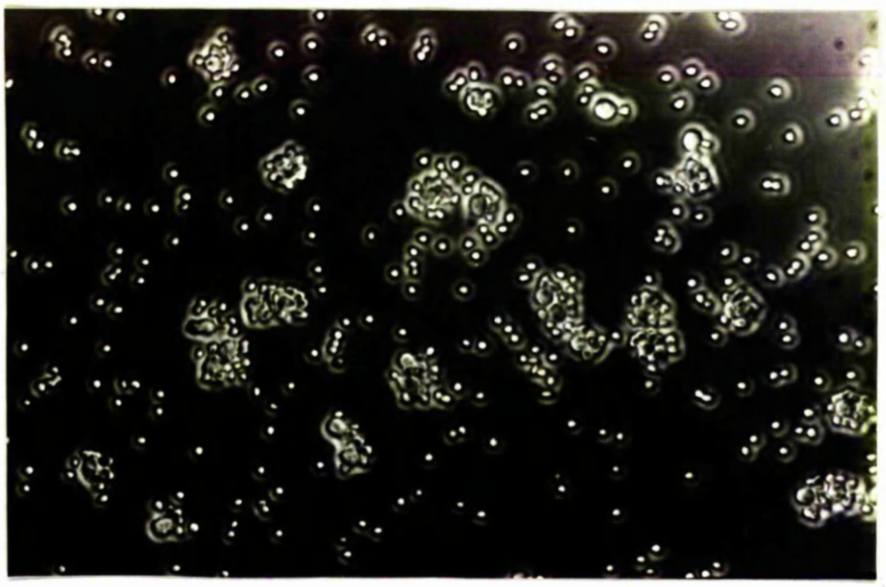

the presence of fresh serum.

4.2. Locomotion of neutrophil leucocytes in response to activation of the complement system :-

The movement of neutrophil leucocytes in response to the complement system was tested using an automated tracking sysytem. In these experiments the cells were suspended in H2 then 10% fresh rabbit serum and 0.25 mg/ml zymosan were added to the suspension. The control which was used consists of neutrophils suspended in H2, to which 0.25 mg/ml zymosan and 10% BSA were added. The speed and persistence of the cells treated with zymosan activated serum were compared with speed and persistence of the control cells.

The results in figure 4.19 show that there was an inhibition in the speed of the cells in both periods compared to the speed of control cells. The persistence of the cells is no different from the control (fig 4.20).

Summary :-

(1) The activation of the complement system through the alternative pathway greatly enhanced neutrophil adhesion from flow, and it seems that C3b was the important component in activated serum.

(2) The locomotion of neutrophil leucocytes was inhibited if the complement system was activated with zymosan.

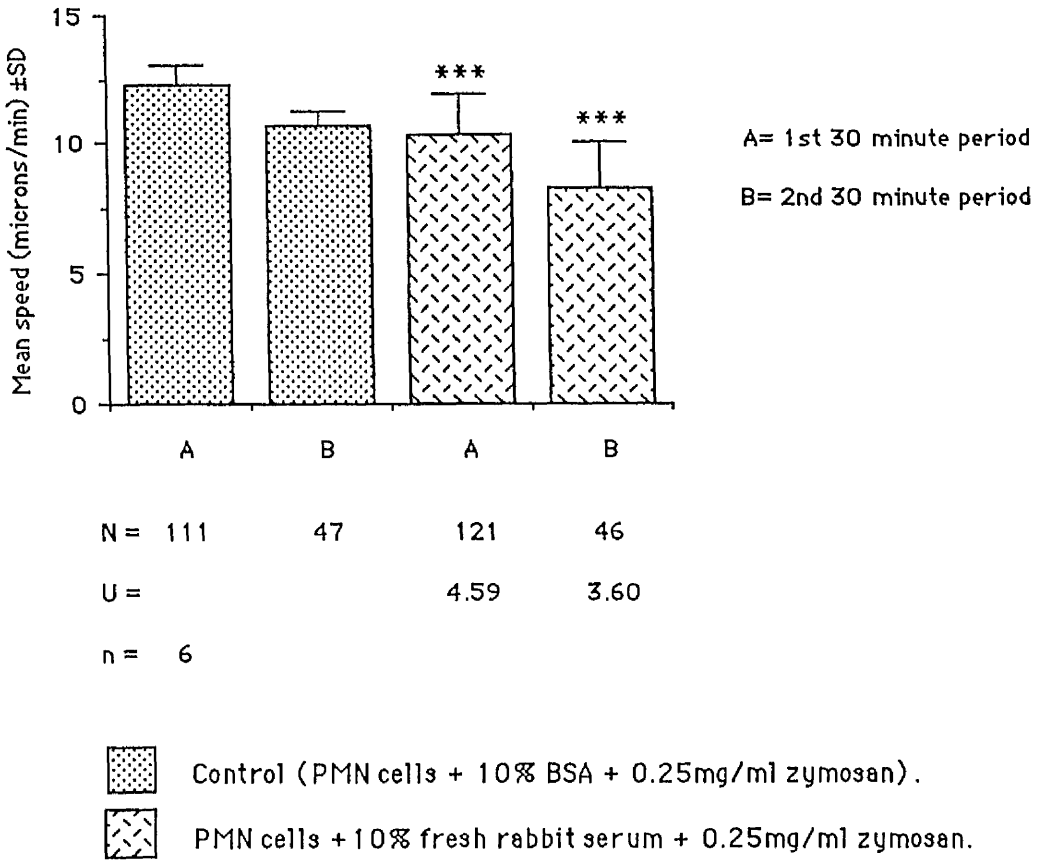

Fig 4.19 The speed of neutrophil leucocytes in response to the activation of the complement system through the alternative pathway.

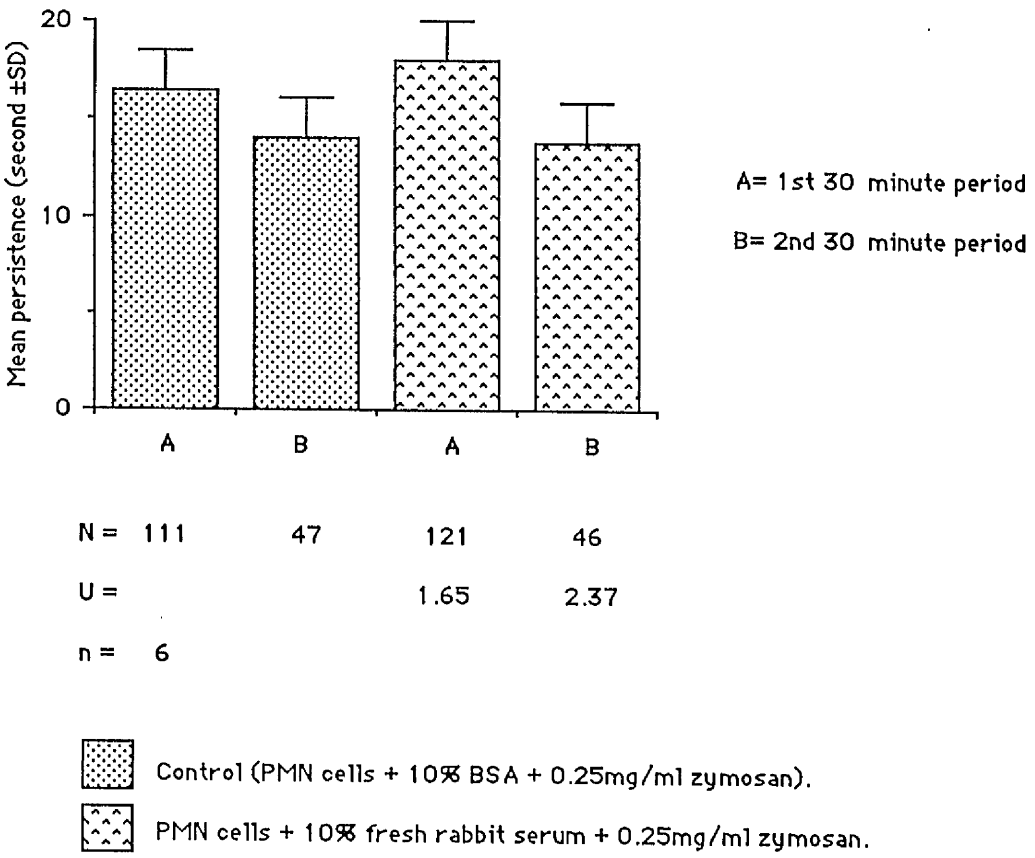

Fig 4.20 Neutrophil leucocyte persistence in response to the activation of the complement system through the alternative pathway.

Chapter 5

5.1. The speed of response of neutrophil leucocytes to a change in the suspending medium :-

Introduction :-

Neutrophil leucocytes are important cells, particularly in the early stages of defence system against infection. They must respond rapidly to a wide range of stimuli and though it is known that they are capable of recognizing a variety of chemotactic factors and phagocytosable particles, the speed of response has rarely been considered. Chemotactic factors influence the initial margination either by affecting the adhesiveness of the endothelial cells or of the neutrophils. The locomotion of neutrophils may also be directed by chemotactic factors.

In this chapter we shall discuss how quickly neutrophil leucocytes respond to a change in the suspending medium. This question relates to the problem which faces circulating leucocytes as they come in contact with local inflammatory factors. The effect of various agents (chemotactic factor FMLP, serum, manganese, and divalent cation free medium) on in vitro adhesion was studied using a flow assay (see methods 2.3.6) of a type which has not been used before. For convenience I will refer to this as the tube assay.

5.1.1. Effect of chemotactic factor FMLP :-

The effect of chemotactic factor FMLP on neutrophil adhesion was tested using the tube assay (see methods 2.3.6). In the present experiments labelled neutrophil suspension (^{51}Cr) and

solutions of FMLP at different concentrations ($2x10^{-7}$ M, $2x10^{-8}$ M, $2x10^{-9}$ M), were separately pumped through a Y-shaped manifold and then into a sequence of 10 micro-capillary tubes which had been coated with 0.5% BSA (see methods 2.3.6). In this case the contact between the neutrophils and FMLP happened when the solutions in both syringes were mixed in the manifold. It is therefore possible to estimate how long the cells take to respond to a change in suspension by knowing approximately how long the flowing neutrophils take from the contact point with FMLP until they reach the first micro-tube in which the adhesion changes.

As seen in figures 5.1, 5.2 the adhesion of neutrophils to a BSA-coated substratum slightly increased with FMLP at $2x10^{-7}$ M and $2x10^{-9}$ M while at $2x10^{-8}$ M the adhesion was inhibited.

The adhesion in all cases has been compared to the value of adhesion in control runs where labelled neutrophils are mixed with a solution of H2. The percentage adhesion at $2x10^{-7}$ M and $2x10^{-9}$ M were about 109% relative to control whereas at $2x10^{-8}$ M was 66% . Table 5.1 shows the adhesion of neutrophil leucocytes in response to chemotactic factor FMLP at different concentrations in each tube along the sequence. In these experiments we were expecting that the response of neutrophil adhesion to chemotactic factor FMLP might appear in the first few micro-tubes of the sequence. However the results obtained showed that all the micro-tubes give approximately the same response, possibly becouse the flow rate used in these experiments is inappropriate.

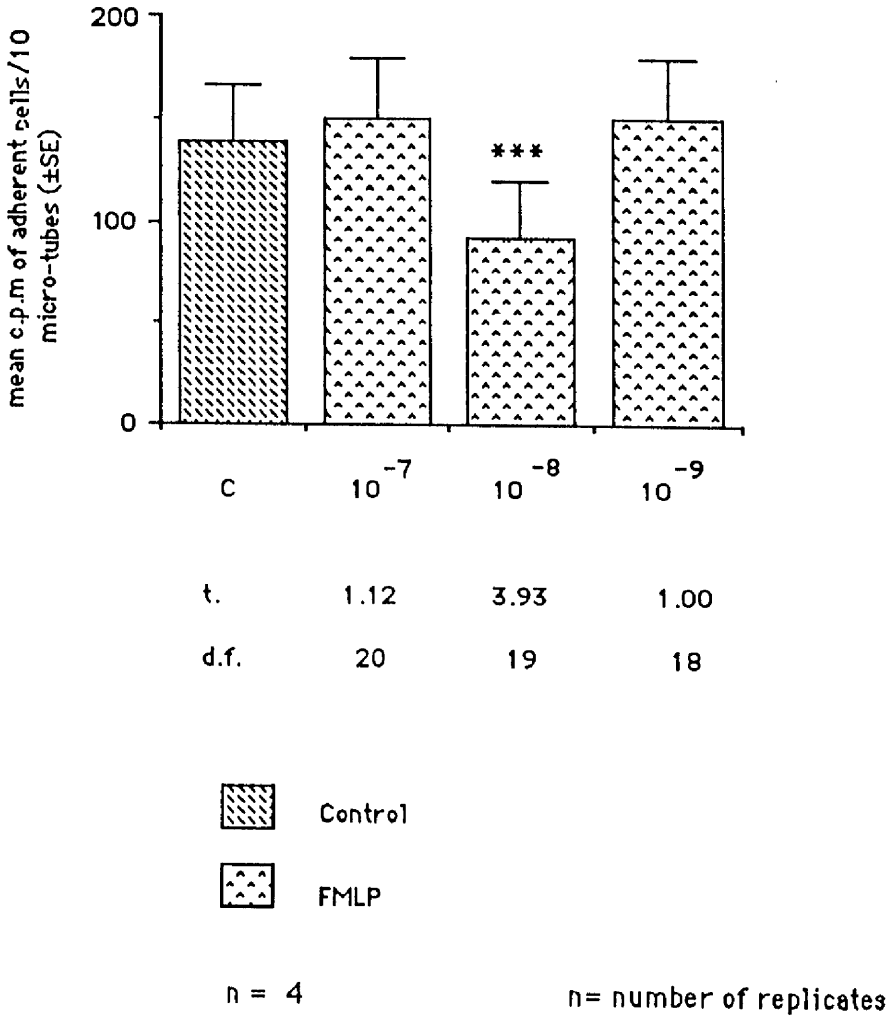

Fig 5.1 The response of neutrophil leucocytes to chemotactic factor FMLP.

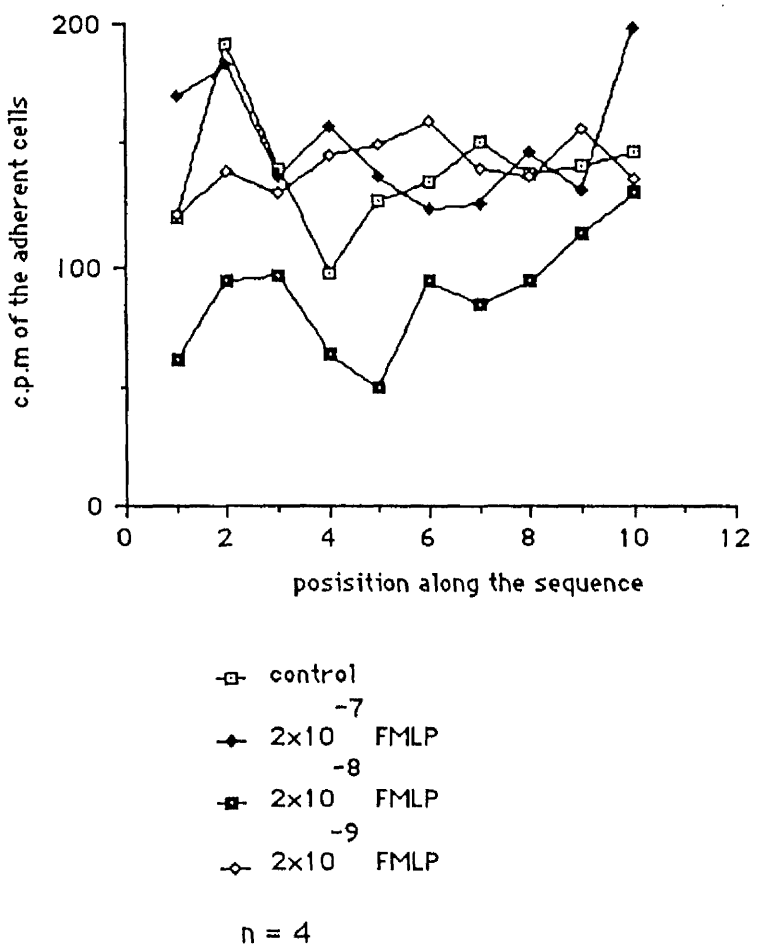

Fig 5.2 **Neutrophil adhesion in responses to chemotactic factor FMLP.**

treatment of neutrophil leucocytes	mean c.p.m.of adherent cells in each micro-tube along the sequence									
	1	2	3	4	5	6	7	8	9	10
Control	120.5	191.3	139.5	97.5	126.3	134	150.5	137	140	146
$2x10^{-7}$ FMLP	170.2	183.2	136.7	157.5	136.5	123.5	125.5	145.8	130.5	198
$2x10^{-8}$ FMLP	62	94	96	64	50	94	84.7	94	144	130
$2x10^{-9}$ FMLP	120.7	138	130	144.7	149.7	159	139.3	136	156	235.3

n = 4

Table 5.1 Effect of chemotactic factor FMLP on neutrophil adhesion.

Estimation of the time taken by neutrophils to respond to a change in the suspension medium was made by measuring the time taken after the cells were mixed with FMLP until they reach the first micro-tube in the sequence (table 5.2). The estimation of the time values will be discussed in the following chapter.

5.1.2. Effect of serum :-

As shown in figure 5.3, coating the micro-tubes with 10% fresh rabbit serum reduced the adhesion of neutrophils compared to the adhesion on control when the micro-tubes were coated with 0.5% BSA. The percentage of adhesion on serum relative to control was about 20%. This result indicated that serum coated glass is a very low adhesive substratum for neutrophil leucocytes.

5.1.3. Effect of divalent cations :-

The adhesion of rabbit peritoneal neutrophils to glass coated with protein (0.5% BSA) was inhibited in the absence of divalent cations from the cell suspension medium (fig 5.4).

Previous studies showed that the adhesion of human neutrophils to glass bead columns in whole blood is increased in the presence of Mg^{2+} (Garvin, 1961, 1968). Present experiments show that manganese ions increase the adhesion of rabbit neutrophils to glass coated with 0.5% BSA (fig 5.5). The percentage adhesion compared to control was about 247%.

This result indicated that manganese ions are even better than magnesium ions at promoting adhesion.

Number of experiment	Mixing time (seconds)
1	32
2	32
3	28
4	32
5	30
6	32
7	28
8	30

Table 5.2 The time elapsed between contact with chemotactic factor FMLP and reading the first tube in which adhesion was measured.

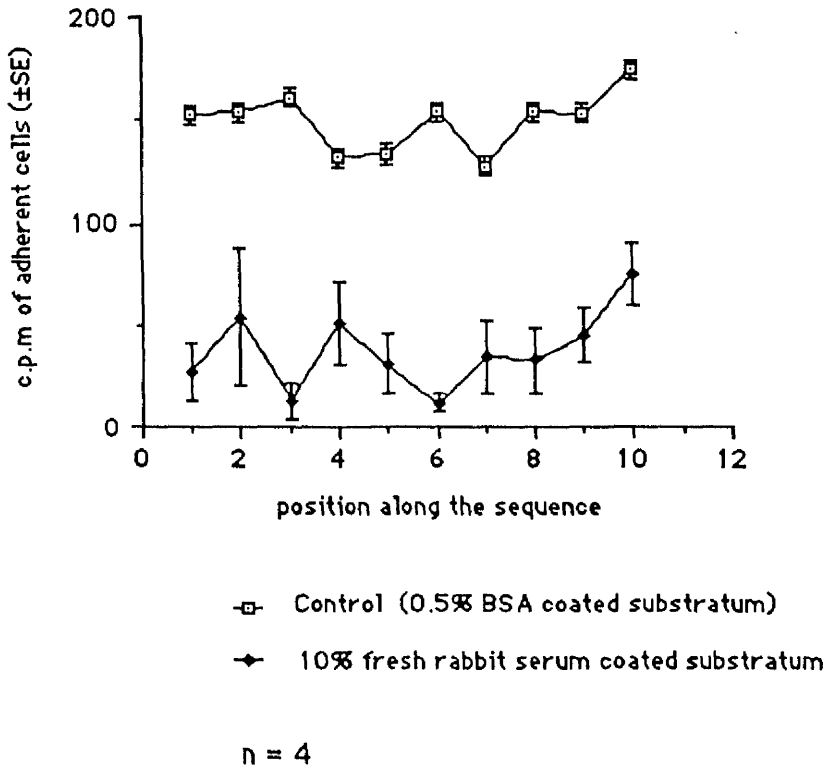

Fig 5.3 Adhesion of rabbit peritoneal neutrophils to substratum coated with 10% fresh rabbit serum.

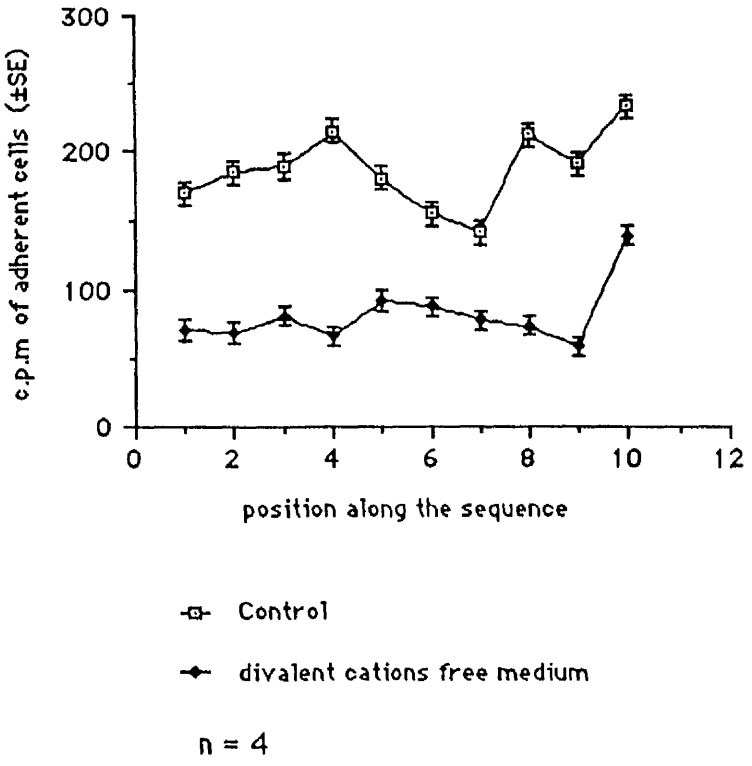

Fig 5.4 Effect of divalent cation- free medium on the the adhesion of neutrophil leucocytes.

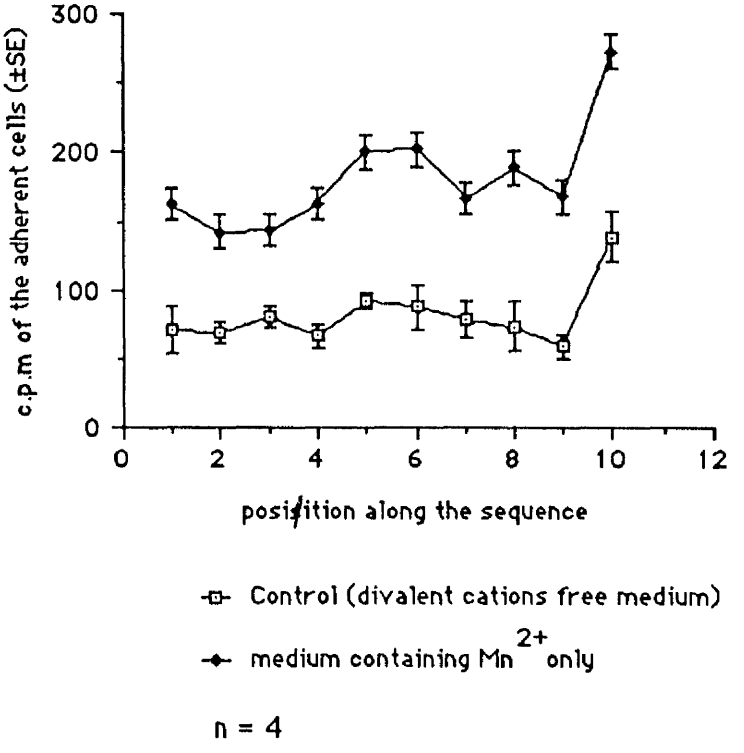

Fig 5.5 The effect of Mn^{2+} on the adhesion of neutrophil leucocytes to BSA coated glass.

Summary :-

1) Rabbit peritoneal neutrophils respond to the change in the suspension medium induced by chemotactic factor FMLP in less than 28 seconds.
2) The adhesion of neutrophil leucocytes was inhibited by coating the substratum with serum.
3) The absence of divalent cations from the suspension medium reduced the adhesion of neutrophils to glass coated with protein. Mn^{2+} is able to increase the adhesion.

Chapter 6

Discusssion

The adhesion and locomotion of leucocytes play an essential role in the emigration of these cells from the blood-stream into inflamed sites. The work described in this thesis is concerned with the adhesion and locomotion of neutrophil leucocytes in response to Bordetella pertussis components (filamentous haemagglutinin and pertussis toxin), following activation of the complement system through the alternative pathway, following the addition of chemotactic factor FMLP, as well as their behaviour on a serum coated substratum and in the presence of manganese ions. The flow chamber adhesion assay measured changes in neutrophil adhesion both in a realistic and reductionist manner (Lackie et.al 1987). An automated tracking assay has been used to measure the ability of the cells to move over a two dimensional substratum.

The main findings of this work are as follow :-

1. The enhancement of neutrophil adhesion and movement by chemotactic factor FMLP was inhibited by pre-incubation of neutrophils with PT.
2. Pre-coating a substratum with FHA had no effect on neutrophil adhesion, but movement was inhibited.
3. C3b is the important component produced by the activation of complement in terms of stimulating increases in the adhesion of neutrophil leucocytes. In the presence of activated complement the movement of neutrophils was reduced.

4. Rabbit peritoneal exudate neutrophils respond to a change in the suspension medium (the addition of chemotactic factor, FMLP) in less than 28 seconds.

6.1. Effect of products of Bordetella pertussis on neutrophil movement and adhesion :-

6.1.1. Effect of FHA and PT on neutrophil movement :-

The movement of the rabbit peritoneal neutrophils was reduced in the presence of FHA. The minimum dose of FHA which caused the significant inhibition was at 1 μg/ml.

The enhancement of neutrophil movement brought about by chemotactic factor FMLP was inhibited by pre-incubation of neutrophils with PT, but PT has no effect on the rate of unstimulated movement. This inhibitory effect was noticed at 50 ng/ml PT. The difference in sensitivity between FHA and PT (FHA 1 μg/ml, PT 50 ng/ml) suggested that PT is probably the more active virulence component in this respect.

Previous studies have shown that PT inhibits the metabolic responses of neutrophil leucocytes to FMLP in both human and rabbit neutrophils (Lad *et.al*, 1985; Becker *et.al*, 1985). Spangrude *et.al* (1985) reported that the motility of lymphocytes *in vitro* was inhibited by PT. This inhibition was observed after a 1 hour pre-treatment of the lymphocytes with a high dose of PT (4 μg/ml). Chemotaxis of human neutrophils was also inhibited in the presence of PT, a significant reduction being observed after a 2 hour pre-incubation of neutrophils with 50 ng/ml PT. Lad *et.al*,

(1985) reported that the chemotaxis of neutrophil leucocytes was inhibited after 1 hour incubation with 3 μg/ml PT. This difference in the reported results may be due to the extra hour of incubation time used in Spangrude's study. Craig (1987) showed that PT would inhibit the chemiluminescence response of rabbit peritoneal neutrophils to a chemotactic factor FMLP. The inhibitory effect of PT was seen after 30-40 minutes incubation period with cells. The metabolic response of rabbit peritoneal neutrophils to FMLP, measured using a chemiluminescence assay, was enhanced by the presence of FHA at 50 ng/ml (Craig, 1987). No enhancement effect by FHA was observed before 15 minutes incubation period with neutrophils. The present study shows that the inhibition of rabbit peritoneal neutrophil movement by PT and FHA was observed after an incubation period of about 30-40 minutes at 37°C. This suggests that both FHA and PT require an incubation period of about 30-40 minute with neutrophils before any inhibitory effect is observed. Difference between the incubation time and the effect of FHA on neutrophil response in my results and those of Craig may be due to the absence of FMLP in the present study and different assay systems.

Both FHA and PT might be suggested as important inhibitory factors for neutrophil movement which might contribute to their action as virulence factors.

6.1.2. Effect of FHA and PT on neutrophil adhesion :-

Under flow conditions the adhesion of rabbit peritoneal neutrophils was not significant affected by FHA at 100 ng/ml.

Craig (1987) showed that the aggregation of rabbit peritoneal neutrophil was enhanced by FHA. This enhancement of aggregation required about 100 ng/ml FHA. The discrepancy in the response may be because in Craig's work cell-cell aggregation was being measured (i.e. adhesion between identical particles) wheres in the flow experiments cell-substratum adhesion is being measured.

Pre-incubation of neutrophils with PT inhibited the enhanced adhesion of rabbit peritoneal neutrophils which was brought about by chemotactic factor FMLP. The inhibitory effect was observed after a pre-incubation time of about 30 minutes at 37°C. About 100 ng/ml PT was required to inhibit the adhesion of rabbit neutrophils. Spangrude et.al, (1985) reported that the FMLP-induced adhesion of human neutrophils was inhibited by PT. This inhibition of adherence was observed after pre-treatment of neutrophils with 250 ng/ml PT for 2 hour period while the minimum inhibition was noticed after 1 hour pre-treatment with PT. The variation between my results and Spangrude's is most likely to be due to the different assay system used in this study and the incubation period.

The results obtained in this study support the view that PT and FHA are important virulence factors of Bordetella pertussis. This work has shown that PT inhibits neutrophil adhesion and movement in response to the chemotactic factor FMLP, whereas activity which has already been induced is unaffected. FHA also appears to inhibit the movement of neutrophil leucocytes. The combination of these inhibitory effects may prevent the mounting of a normal bactericidal response by neutrophils and may account for the pre-

disposition to secondary infection which leads to the most serious side-effects of B.pertussis infection.

The present study has been done to investigate the effect of these factors (FHA and PT) on neutrophil movement and adhesion using a different assay system from those used before. Although PT will not alter the pre-existent activties of phagocytes it will hinder recriutment of additional cells, and will therefore depress host defences.

6.2. Neutrophil adhesion and movement following complement activation :-

The activation of the complement system in fresh rabbit serum by a zymosan stripe markedly enhanced the adhesion of rabbit peritoneal neutrophils under flow conditions. This enhancement of adhesion also occured if the zymosan stripe was pre-incubated with serum for 1 hour at 37°C before the neutrophils were passed over the stripe. The increase in neutrophil adhesion to the zymosan stripe in the presence of fresh rabbit serum was inhibited when the serum was heat-inactivated before use. Previous studies have shown that the adherence of human neutrophils to bovine aortic endothelial cells was enhanced by zymosan-activated human serum (Hoover et.al 1978). Cramer et.al (1986) showed that 10% human serum caused an increase in neutrophil adhesion to, and migration across, canine kidney epithelium cells. This increase also happened if the epithelium was pre-incubated with serum and was lost if the serum was pre-absorbed over kidney epithelium before use or was heat-inactivated. These results suggest that the

increase in neutrophil adhesion to a zymosan stripe or to epithelium involves heat labile components of serum.

The absence of C3b from the serum reduced the adhesion of rabbit peritoneal neutrophils to the zymosan stripe, whereas the presence of C5a and other complement components, except C3b, does not induce an increase of neutrophil adhesion. The most likely explanation for the effect of fresh serum on inducing greater adhesion is through the deposition of C3b or C3bi which is then bound by the leucocyte integrin Mac-1 (CR3). Previous studies with monoclonal antibodies against Fc and C3 receptors have indicated that the adhesion of neutrophil leucocytes to an epithelial surface was mediated predominantly by the receptors for C3b and C3bi (Cramer et.al 1986). Charo et.al (1985) found that the adherence of human neutrophils to monolayers of cultured human umbilical vein endothelial cells was reduced by C5a des Arg while the C5a enhanced the adhesion

The increase in neutrophil adhesion is inhibited in the absence of divalent cations from the serum. The presence of magnesium ions in the serum alone increases the adhesion of neutrophils to levels similar to those obtained with normal serum. This indicates that the magnesium ions are the only physiologically important divalent cation involved in the adhesion of neutrophils. Two roles for Mg^{2+} can be envisaged : one is the activation of the alternative complement system, the other through an effect on the C3bi receptor itself. The latter is the more probable in the light of the effect of Mn^{2+} which also markedly stimulate the response of fibroblasts to fibronectin (Edwerds et.al, 1988).

The locomotion of rabbit peritoneal neutrophils is inhibited in response to the activation of the complement system through the alternative pathway. Smith et.al (1979) found that the locomotion of neutrophil leucocytes on glass and in micropore filters was significantly reduced in the presence of zymosan activated serum. Crocket (1987) showed that coating the substratum with 10 μl serum (complement free) had no effect on neutrophil locomotion.

The results obtained in this study suggested that in vivo the plasma complement which has entered tissue during the phase of increased vascular permeability may activatad and release C3b and other complement components to attract leucocytes towards the inflammatory lesions. C3b might coat the particles so that neutrophils can bind to the particles by C3b receptors and ingest the particles. Also the release of C3b might serve as a signal to phagocytic cells (neutrophil, mononuclear phagocytes) to release the enzymes necessary for extracellular degradation of damaged tissue.

6.3. Estimation of neutrophil response-time :-

Using the tube flow assay the rapidity of response of neutrophil leucocytes to chemotactic factor FMLP at different concentrations was tested. By using this assay it is possible to estimate how long the neutrophil takes to respond to a change in the suspension medium. The importance of knowing the response time is related directly to the problem which is faced by leucocytes as they pass through the microvasculature adjacent to an inflammatory lesion.

In the present study significant changes in neutrophil adhesion were found within 28 seconds of exposure. Since there is a delay between mixing the peptide with the cells and measuring the adhesion (due to the manifold and rubber connection) the changes in neutrophil resoponse have already occurred during the delay time and the time resolution of the assay was inadequate as set up here. The significant changes in adhesiveness might have occurred in less than 28 seconds. O'Flaherty et.al (1977) found that effects of chemotactic factor could be detected within 20 seconds after exposure of the neutrophils.

The present data shows that FMLP at $2x10^{-8}$ M reduces the adhesion of rabbit peritoneal neutrophils to glass coated with BSA, while $2x10^{-7}$ M and $2x10^{-9}$ M of chemotactic peptide increased the adhesion, but this increase was not significant. Smith et.al (1979) found a significant enhancement on neutrophil adherence to BSA-coated glass when the cells were exposed to 0.1 nM FMLP. This concentration of FMLP was below that necessary to induce a detectable change in cell shape. Smith, Lackie and Wilkinson (1979) showed that the exposure of rabbit peritoneal neutrophils to chemotactic factors caused marked changes in the adhesion of neutrophils and they suggested that adhesive changes may affect neutrophil behaviour in vivo. Lackie and Smith (1980) showed that the adhesion of neutrophil in response to chemotactic factor FMLP was increased at $2x10^{-7}$ M, whereas $2x10^{-8}$ M and $2x10^{-9}$ M decreased neutrophil adhesion. The difference between the present results and Smith's can be explained partly on the basis of the differences in the assay technique and partly by chemotactic dose.

Many previous studies have shown the effect of chemotactic factors on the adhesion of neutrophils to endothelial cells. For example, Charo et.al (1985) showed that the adherence of neutrophils to endothelial cells was significantly reduced by low concentrations of chemotactic factors FMLP and C5a (0.1 nM). Higher concentrations (>50 nM FMLP and >1.0 nM C5a) of both chemotactic peptides enhanced the adhesion of neutrophils to endothelial cells and also caused decreased neutrophil migration. Hoover et.al (1978) found that the adherence of human neutrophils to aortic endothelial cells was enhanced by chemotactic factor FMLP.

Previously the effect of chemotactic factors on the aggregation and locomotion of neutrophil leucocytes have been studied (Smith et.al, 1979; Keller, 1981; Zigmond, 1978; Boyden, 1962; Wilkinson, 1974b, 1981; Wilkinson et.al, 1979). O'Flaherty et.al (1978) found that the FMLP at $5x10^{-8}$ M increased the aggregation of human neutrophils.

The complex dose- and time-dependence of the neutrophil response to chemotactic factors can be interpreted in terms of the responses required in an inflammatory response. If a chemotactic factor gradient is present between the site of inflammation and the blood vessel then it would be apropriate if, at the lower end of the gradient, there was enhanced movement and little secretory activity. At the upper end of the gradient near the lesion, reduced movement and enhanced release of lysosomal enzymes are to be expected. Near the vessel wall the concentrations of chemotactic factor will be low due to the

proximity of the circulating blood, and the rate of locomotion of neutrophils may increase. Once the neutrophil leaves the blood vessel and enters the tissue surrounding the site of injury the concentration of chemotactic factor increases. As the concentration of chemotactic factor increases neutrophils become trapped by their increased adhesion and concurrently there will be the secretion of granule contents and metabolic activation as is required at the site of inflammation (Lackie, 1977).

The adhesion of neutrophil leucocytes to glass coated with 10% serum was significantly inhibited in the present study. Similar observations were made by Smith et.al (1979) and by Vroman et.al (1977). These authors found that the pre-treatment of glass with albumin or serum reduced the attachment of neutrophils to treated surfaces.

The absence of divalent cations from the suspension medium reduced the adhesion of neutrophil leucocytes to glass coated with BSA (Forrester and Lackie, 1984). In the present study it has been shown that the adhesion of rabbit peritoneal neutrophils to glass coated with BSA increases in the presence of manganese ions. Previous studies have shown that manganese (and copper) ions increased the adhesion of human neutrophil to glass bead columns in whole blood more than magnesium ions (Garvin, 1968). Charo et.al (1985) found that the adherence of neutrophils to endothelial monolayers was dependent on the concentrations of extracellular magnesium.

Perhaps the most interesting extension of this study would be to develop and improve the tube assay system to study the rapidity of response of neutrophil leucocytes to a change in the suspension medium.

Chapter 7

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