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Neutrophil leucocyte adhesion under flow conditions.

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A thesis submitted for the degree of Master of Science, to the University of Glasgow.

Department of Cell Biology. November, 1987.

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Declaration

This thesis is the original work of the author.

Nedjma Chaabane.

Acknowledgments.

I would like to thank Professor A.S.G. Curtis for providing the facilities in his department.

My deepest thanks go to my supervisor Dr J.M. Lackie for his guidance, his discussions, encouragment, and patience throughout the work.

I would also like to thank my collegues H. Hameed and K.V. Crocket for introducing me to the computing facilities. My appreciation also to all members of the department and in particular to Dr J. Dow for helping me use the laser printer, and to Mr A. Hart for the photography.

My special thanks go to the Algerian government and the Ministry of Higher Education for the financial support, and to my family and friends for their moral support.

To my mother.

Abbreviation list:

BSA: Bovine Serum Albumin.

CR1: Receptor for C3b.

CR2: Receptor for C3bi and C3d.

CR3: Receptor for C3bi.

C5a: Complement fragment (Anaphylatoxin).

cAMP: Cyclic adenosine monphosphate.

Fn: Fibronectin.

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

HSA: Human Serum Albumin.

Hepes: N-2-Hydroxyethyl piperazine N'-2 ethane sulphonic acid.

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

H_2: Hanks Hepes (buffer balanced solution).

I CAM: Intercellular adhesion molecule.

LTB4: Leukotriene B4.

LFA-1: Lymphocyte function-associated antigen.

MAB: Monoclonal antibody.

PMN: Polymorphonuclear (neutrophil).

PG: Prostaglandin.

TNF: Tumor necrosis factor.

SUMMARY

Summary:

The adhesion of neutrophil leucocytes to overslips coated with plasma proteins has been studied under both static and flow conditions.

Neutrophils adhered poorly to both fibronectin- and cryoprecipitate-coated glass. The effect of the proteins tested on neutrophil adhesion was dose dependent, with a pronounced effect at 100 µg/ml of protein, which was then reduced at lower doses. Compared to clean glass (control), the effect of serum, HSA, fibronectin-depleted cryopreciptate, fibronectin-depleted plasma and Factor VIII was to reduce adhesion but by an insignificant amount, whereas, cryoprecipitate and in particular fibronectin dramatically inhibited adhesion. This suggests that plasma components in general are not effective in reducing leucocyte adhesion, although fibronectin-coated surfaces are very non-adhesive.

The speed with which neutrophils responded to chemotactic factors in the medium was tested in the flow chamber assay using a Y-shaped manifold for perfusing cells and chemotactic factors through a chamber pre-coated with BSA immediately after mixing.

Neutrophil response to FMLP was dose dependent. A high concentration of FMLP (10^{-7}M) induced a slight increase in the number of adherent cells to BSA coated chambers. Compared to 10^{-7}M the adhesion was less marked with 10^{-8}M, reached a minimum at between 10^{-9}, 10^{-10}M and

returned to control level at lower doses of the factor.

Zymosan-activated serum also enhanced neutrophil adhesion to BSA coated chambers, compared to normal serum, heat-inactivated serum and zymosan-treated heat-inactivated serum, which had little effect.

Stripes of conalbumin on the chambers locally trapped neutrophils which were suspended in 10% fresh serum containing a high titre of antibody to conalbumin. The response was relatively slow and not particularly dramatic unless antibody and an active complement-generating system was present. This seems to suggest that the local activation of complement through the classical pathway induces an extremly rapid response from cells.

The same response was found using stripes of zymosan-containing agarose and suspending the cells in normal (non-immune) rabbit serum, suggesting that complement activation through the alternative pathway is equally as effective in triggering the local trapping of cells.

CHAPTER 1

I) **Introduction:**

In order to consider the general topic of leucocyte adhesion it is necessary to look at two topics: the properties of leucocytes and the question of cell adhesion. In this introduction I will briefly look at the origin, development, and function of neutrophil leucocyte, review their activities in inflammation and in the second part move onto consideration of the problems of cell adhesion (i.e. hypothetical advances, methods for measuring etc.), before returning to the more specific topic of adhesiveness of neutrophils.

II) **General:**

1) **White blood cell classification:**

There are five types of nucleated cells in blood besides red blood cells (erythrocytes) and platelets; these are known as white blood cells (or leucocytes). Leucocytes can be divided into two major categories; mononuclear cells with round (lymphocytes) or kidney shaped nuclei (monocytes), and cells with a segmented or multilobed nuclei (polymorphonuclear leucocytes). Mononuclear leucocytes also differ from the polymorphonuclear leucocytes in that their cytoplasm does not contain obvious granules.

A more logical distinction is based on function (and ontogeny): myeloid cells (the mononuclear phagocytes or monocytes, and polymorphonuclear

granulocytes), and lymphoid cells (lymphocytes).

Polymorphonuclear leucocytes have segmented nuclei, and on the basis of their staining properties can be further subdivided into three classes, neutrophils, eosinophils, and basophils. The function of the basophils is not known in detail, but they participate in allergic reactions and are essential in some types of tissue damage such as serum sickness. Eosinophils seem to play a role in defence against parasitic infections and in the modulation of hypersensitivity reactions (Murphy, 1973). Neutrophils comprise the major class of white cells, accounting for 60% of the total white cell count in man. This figure varies somewhat from individual to individual and from species to species.

2) **The neutrophil leucocyte, origin, and function:**

2.1) **Origin and development:**

Neutrophils arise in the bone marrow from pluripotent stem cells of the myeloid series. A number of maturation stages are recognised on the basis of granule content and the process is well reveiwed by Cline & Gold, (1979); and Murphy, (1973). After approximately 14 days the mature neutrophil is released into circulation, a process which seems to require active movement and alteration in adhesion (perhaps) and it is the circulating neutrophil which is fully functional. It is the properties of these circulating

neutrophil which are of particular relevance to this thesis.

2.2) **Neutrophil function:**

The in vivo role of the neutrophil as a primary phagocytic cell is most conspicuous in acute inflammation, although it probably plays a role in general tissue maintenance (Wilkinson and Lackie, 1979). It must be emphasized that neutrophil function in host resistance to microbial invaders, and following damage, involves multiple steps and activities. Not only must the production and maturation of neutrophils in the marrow be regulated, but the cells must stick to vessel walls in an appropriate location, move over the endothelial cells and emigrate into tissues, must respond to gradients of chemotactic factors, adhere to and phagocytose bacteria, and damaged tissue components. Upon arrival at an inflammatory focus may require release of lysosomal enzymes in the environment as well as phagocytoses; effective antibacterial action depends upon a complex respiratory burst. Abnormality or interference anywhere along the line may result in the malfunction of the neutrophil.

2.3) **Neutrophil kinetics:**

The number of circulating neutrophils is dependent on the rate of production and maturation, regulated release from the marrow store, and their orderly

destruction. Upon appropriate stimulation, mature neutrophils leave the bone marrow and enter the circulation where they equilibrate with the marginated pool. Using radiolabelled cells ($DF^{32}P$) as a marker Athens et al. (1961) were able to calculate the total number of cells in the circulating and marginated pools. They estimated that the circulating granulocyte pool is approximately $0.3x10^9$/kg of blood with a marginated pool of $0.4x10^9$/kg of blood. The marrow supplies to this total granulocyte pool $1.5x10^9$ cells per kg per day and has in reserve $8.8x10^9$ cells per kg of blood stored.

3) **The inflammatory process:**

By inflammation is meant the defensive reactions of tissues to injury of any kind. Such reactions follow a broad general pattern but vary in detail according to the nature of the injurious agent and the site of the injury. The many types of injurious agents which give rise to an inflammatory response may be summarized as follows:

a) bacteria and their toxins, viruses, rickettsiae, fungi, protozoa and helminths;

b) trauma, which may be mechanical, thermal, electrical or chemical;

c) death (necrosis) of tissue arising as a result of loss of blood supply or from any other cause;

d) an immune reaction; e.g the interaction of an antigen and a specific antibody;

e) malignant neoplasms.

Inflammation may be divided broadly into two types; acute and chronic. In this Introduction chronic inflammation will not be reveiwed since my studies were related to neutrophil behaviour in acute inflammation.

3.1) **Acute inflammation:**

Acute inflammation is an immediate response of living tissue to injury: it is basically the same whatever the agent, and is triggered by a variety of chemicals (mediators) which can appear within the tissue in a matter of seconds and act primarily on the microcirculation. The main events which take place in an acute inflammatory reaction fall into two major categories:

a) Vascular events which comprise;

i) Changes in blood flow and calibre of microvessels.

ii) Changes in permeability.

b) Cellular events which comprise;

i) Leucocyte adhesion to endothelial cells lining the vessels.

ii) Leucocyte emigration towards the inflamed site.

a) **Vascular events:**

i) **Changes in blood flow and calibre:**

During and after injuries of various kinds there is often an initial vasoconstriction followed, after a few minutes, by arteriolar dilation. About 30 minutes after an acute injury the whole capillary bed is suffused with blood; capillaries which were closed open up and those already patent dilate. The flow continues to be rapid for a while; but as the reaction develops the blood in the dilated capillaries and venules begins to slow, and stasis may often occur.

ii) **Changes in permeability:**

During an inflammatory reaction the walls of the microvessels, which normally have a low permeability to macromolecules, become leaky to blood proteins. The increased permeability to proteins upsets the hydrodynamic equilibrium in the tissue resulting in fluid efflux from microvessels and, as a consequence tissue swelling (oedema). This phenomenon is thought to be caused by release of many chemicals (mediators) in the tissue. The chemical mediators of inflammatory oedema in man are unknown; there is little evidence that histamine, serotonin and bradykinin are important (Wedmore et al. 1981).

A recent technique for measuring oedema formation (Williams, 1981) involves injection of test materials (zymosan, FMLP, LTB4, PGE_2, histamine or bradykinin)

into rabbit dorsal skin and measuring plasma leakage using the local accumulation of intravenously injected ^{125}I-albumin mixed with Evans blue dye to indicate plasma leakage. Summarizing his work, Williams noted that plasma incubated with zymosan induced oedema, but only if prostaglandin was added (PGE_1). It has been deduced that this oedema response to zymosan was produced by two mediators acting synergistically: the vasodilator prostaglandin and a "permeability-increasing substance" probably C5a, the active component generated in plasma.

It has been suggested that neutrophils may be involved in the acute inflammatory response as mediators of increased vascular permeability: the hypothesis being that activated and adherent neutrophils discharge their contents of lysosomal enzymes and that these hydrolases increase vascular permeability. Furthermore, in experiments with carrageenan injected into the hindpaw of rats, by measuring the subsequent oedema, Di Rosa (1972) found that depletion of polymorphonuclear cells (PMN) leads to a slight reduction of the oedema normally associated with release of prostaglandin. Similarly, Williams (1981) reported that in normal rabbits leukotactic substances (FMLP, C5a, LTB4) were highly potent "permeability-increasing substances". However in animals depleted of circulating PMN, these chemotactic factors were ineffective, even when combined with PGE_2.

b) **Cellular events:**

i) **Neutrophil adhesion:**

Observations on the microcirculation in situ, such as on rabbit ear chambers, showed that immediately following any injury blood leucocytes (probably mainly neutrophils) adhere to the endothelial lining of post-capillary venules, the process referred to as margination. Neutrophil sticking has been reported to start after 10 minutes (Allison et al.,1955a), 30 minutes (Carscadden, 1927), 1 hour (Cliff, 1966), several hours (Florey and Grant, 1961): no doubt it depends upon the severity or nature of the injury. The adherent neutrophils move rapidly over and then between endothelial cells, which in this part of the microvasculature do not have complex junctional specialisations (Simionescu et al., 1975), and then towards the focus of the lesion. The margination phase must involve adhesion changes; the subsequent emigration phase (diapedesis) requires movement over the 2-D substratum of the endothelial cells and movement in a 3-D deformable matrix. Apart from neutrophils, other leucocytes, platelets, and red blood cells may also stick to the endothelial cell surface. It has been proposed that there is most probably some change in the endothelial cell surface allowing these cells to adhere (see section No.4).

ii) **Neutrophil emigration:**

The main morphological features of emigration of neutrophils are well established. Observation of living trasparent tissues showed that neutrophils emigrate by active amoeboid movement taking 2 to 9 minutes to pass through the venular wall, and thereafter moving through tissues at up to 20 μm per minute (Clark et al.1936). By relatively simple experiments, it has been shown that neutrophil emigration and increased permeability are completely separable phenomena. Thus Hurley (1972) noted the total absence of correlation between the escape of cells and of proteins following a single injection of saline, histamine, or serum. Permeability had returned to normal in each of these cases before emigration began.

The time course of neutrophil accumulation in an inflammatory exudate has been studied and the findings with relatively mild irritants (eg, serum and glycogen solutions), were that neutrophil rate of emigration reached a peak at approximately 4 hours and then declined rapidly (Hurley, Ryan and Friedman, 1966). Once the neutrophil arrives at the site of tissue injury a complex series of events occur that result in the destruction of invading bacteria.

4) **Neutrophil-endothelium interactions; in vivo studies.**

Although it is clear that during inflammation large number of neutrophils adhere to endothelium and

emigrate, the normal level of adhesion is less certain. Text-book descriptions of the process of margination often state that adhesion only occurs in injury, whereas microscopic observations of the microcirculation in vivo have suggested the opposite.

Early observations by Leeuwenhoek in the 1700s were made on the transparent tails of fish and tadpoles. Later on Waller and Cohnheim (1882) made accurate observations on the frog in the thin web of tissue between the toes and in the pinned-out tongue. More detailed descriptions come from observations on other transparent tissues such as the tadpole's tail (Clark et al.1936), mesentery (Janoff and Born, 1972), and in particular the regenerated vascular bed in rabbit ear chambers (Allison et al. 1955). In the tadpole tail cells were seen rolling over and over along the wall without any tendency to cling to the vessel wall. However, in the rabbit ear chamber (a stable plexus with active circulation), neutrophils were frequently seen rolling along the vessel walls or lying motionless at the edge of the moving stream.

Other evidence suggesting that neutrophil-endothelium adhesion is a normal occurrence comes from the study of neutrophil kinetics in vivo. It has been reported by Mauer, Athens et al. (1960) that when human neutrophils are labelled in vitro with ($DF^{32}P$), and then returned to the circulation of the donor, about

half of the labelled cells could not be found in the circulation at the end of the re-infusion. Thereafter the remaining labelled cells left the circulation in a random fashion with a mean half-life in circulation of 6.6 hours. It was then suggested that the immediate disappearence of half the infused cells was due to their rapid dilution in a larger pool than that calculated from the blood volume and the venous circulating granulocyte count. Athens et al., (1961a, 1961b) confirmed this suggestion and showed that there were two pools of granulocytes, designated as the circulating and the marginated pools, which were in equilibrium and were of about equal size. It was also calculated that in order to maintain a normal level of circulating neutrophils, approximatly 10^{11} cells must disappear from the blood -stream each day (Hirsch, 1973). Since neutrophils can only leave the blood steam by migrating through the endothelium adhesion must normally occur, and even if the figures for the half-life are underestimated, neutrophil-endothelium adhesion cannot be a rare event.

Both the observations on the microcirculation and those on neutrophil kinetics are consistent with the view that adhesion is a normal occurrence. The changes that occur during inflammation to produce an increase in adhesion are likely therefore to be of degree and not of an absolute "on-off" nature. This is important in the

consideration of possible mechanisms to account for the increase of adhesion.

4.1) **Localization of inflammatory response:**

Localised neutrophil adhesion in areas of inflammation suggests that a slight change in the local environment may be sufficient to induce the increased adhesion during inflammation. Several studies have been performed on the process and a number of hypotheses to account for local accumulation have been proposed:

1) Local changes in endothelium-cell adhesiveness.

2) Systemic changes in neutrophil adhesiveness.

1) **Local changes in endothelial-cell adhesiveness:**

This first hypothesis is one of the most attractive hypotheses to explain margination, since the endothelium is the only static component and is much more likely to be influenced by diffusible products of tissue damage or bacterial infection. Changes in the endothelium account for the local changes in capillary permeability and an observation frequently quoted in support of adhesive changes is that of Allison et al., (1955). These authors found in a rabbit ear chamber preparation that margination occured unilaterally on the wall of the vessel adjacent to a very local experimentally-induced injury. They also considered this to be in keeping with the concept that the vascular reaction was caused by

products of cellular damage which diffuse to the vessel from the site of injury. The published evidence for this was their plates, which showed neutrophils adhering to one side of the blood vessel at a sharp bend. The interpretation of these findings is complicated by the complex geometry of the vessel. Since margination requires only the adhesive interaction between leucocytes and endothelium exceeds the shearing stresses tending to distract the cells the local rheology must be taken into consideration.

Observations on the inflammatory response following injection of antigen-antibody complexes into tissue have shown that neutrophils stick on the side of the venules nearest to the injured area (Cliff, 1966), but this is not direct evidence for a change in the properties of endothelial- cell surface. Other observations which support the concept of endothelium change were made by Florey and Grant, (1961), and Grant, (1962), using U.V. light as the injuring stimulus. In these experiments neutrophil sticking occurred several hours after the injury. As these workers pointed out, the white blood cells that were adhering could not themselves have been injured by the U.V. stimulus; those white blood cells in the path of the light almost certainly were not the same ones that later returned to the site of the reaction and became attached to the vessel wall. This experiment suggested that the critical

alteration in the tissue is some change in the membrane of the endothelial cells which converted these cells from a non-adhesive to an adhesive state, but no clear morphological evidence was obtained that the endothelium became "sticky".

The rapidity of change in neutrophil-endothelium adhesion might be important.

2) **Systemic changes in leucocyte adhesiveness:**

The possibility that all leucocytes become adhesive and that margination occurs throughout the body seems an unlikely alternative model. Evidence for this model (i.e. that neutrophil adhesiveness can influence margination) comes from several observations. A dramatic illustration of the consequences of an alteration in neutrophil adhesiveness in vivo comes from studies on the neutropenia that can be induced by a variety of stimuli such as complement activation which can be initiated by injection of cobra venom factor (McCall, De Chatelet, Brown and Lachmann, 1974), exposing blood to foreign surface as in renal dialysis (Jensen et al., 1973), or in filtration leucophoresis (Schiffer et al., 1975 ; Hammerschmidt et al.,1978). Neutropenias are also induced by a variety of agents which affect neutrophil adhesiveness, notably endotoxin (Essex et al., 1953; Athens et al., 1961), and agents which tend to decrease blood flow (Bassen et al., 1952). The margination which brings about this neutropenia

leads to a massive pulmonary sequestration of neutrophils (Toren et al., 1970); probably because the capillary bed of the lung is the largest in the body, and is the first encountered by cells which have been altered.

An additional piece of evidence which points to change in neutrophils being important, rather change in the endothelium, comes from studies on neutrophil-endothelial interaction in vitro. By using monolayers of aortic endothelium grown in vitro and allowing neutrophils to settle on and adhere to these monolayers Lackie and Smith (1980) examined the influence of a variety of potential mediators and inhibitors on the strength of the interaction. In these experiments endothelial cells constituted a good substratum for neutrophil adhesion, but nearly all the changes in the interaction could be attributed to changes in the neutrophils, since pretreatment of the endothelium even with formaldehyde fixation, had little effect. Numerous other investigations on the interaction of neutrophil with cultured endothelial cells have been performed. Recent analyses have shown that IL-I induces a massive increase in the binding of neutrophils /monocytes to cultured human umbilical vein endothelium (Bevilacqua et al.1985; Fleming et al., 1985). In vivo studies support a proposed role for IL-I in controlling neutrophil-endothelial cell interactions during the acute inflammatory response (Cybulsky et al., 1985). These

alterations take several hours and involve protein synthesis. In contrast neutrophil-endothelial cell adhesion can be induced within seconds in vivo with mediators such as LTB4 (Bjork et al., 1985). It therefore seems that there may be several components to the adhesive interaction (Bevilacqua, 1985).

5) **Mechanisms of cell adhesion:**

Up until now there has been no clear answer to the question how cells adhere, but a number of hypotheses have been advanced to explain this phenomenon. These hypotheses can be divided into two major categories: those which depend upon the surface properties, the physico-chemical interactions, and those which depend upon more specific interactions of the receptor-ligand type.

i) **Physico-chemical theory:**

Curtis (1960) proposed that cell-cell adhesion was determined by the interactions between the electrostatic forces of repulsion and attractive forces attributed to London dispersion forces. This is based on analogy with the theoretical work on colloid flocculation done by Derjaguin, Landau, Verwey and Overbeek, and which has since been known as the DLVO theory. The DLVO theory predicts two types of adhesion between particles: a strong and usually irreversible adhesion between surfaces separated by 1-2 nm, and a weaker interaction between surfaces separated by a gap of 6-30 nm. The strong interaction is in the so-called primary minimum, the weaker interaction in the secondary minimum, a substantial repulsion having to be overcome to bring cells to the primary minimum.

Another physico-chemical interaction which may be

of importance is exemplified by the interaction of enterobacteria and other model particles with animal cells and other surfaces. This interaction could be based on specific structures on the surface of the microbe and complementary structures on the host cell similar to the fit between antigen and antibody. An alternative explanation, which has long been popular, particularly in connection with phagocytosis by polymorphonuclear cells, is that the surface properties of the "outermost" interface of bacteria and other particles, as well as of phagocyte cells determine the outcome of the particle/phagocyte interaction. The main physico-chemical properties that may be involved are hydrophobicity and charge (Fenn, 1923; Mudd et al., 1934; Van Oss, 1975; 1978).

Although it is inescapable that long-range attractive forces of electrodynamic origin must contribute to adhesion between cell surfaces, it may well be that their contribution to intercellular adhesion is small.

ii) **Receptor-ligand theory:**

Many workers favour this second theory, proposing that adhesion between cells of animal tissues, and between cells and their extra-cellular matrix involves binding by molecules present at the cell-surface (probably intrinsic to the plasma membrane) to specific

ligand. If such an hypothesis is accepted then it should be possible to identify the molecules involved.

Identification of the molecules involved in cell-cell adhesion:

There have been several approaches used in the identification of molecules that may be involved directly or indirectly in cell-cell adhesion. These are as follows:

a) The identification of the components of intercellular junctions.

b) Detection of specific molecules by production of antibodies to cell adhesion molecules that would specifically block adhesion.

a) **Identification of the components of intercellular junctions:**

Morphological studies of specialized cell junctions are of importance when considering the mechanisms of cell adhesion but of little relevance to neutrophils which form only trasient adhesions.

b) **Identification of cell adhesion molecules (CAM's):**

It has been proposed that cell-cell recognition occurs by means of local cell surface modulation of a small number of proteins rather than by expression of large numbers of different cell surface markers. Several

different CAM's have been found in a number of vertebrate species, in different tissues such as liver and striated muscle and even in a single complex structure such as the brain, where different molecules specific for neurons and glia have been identified.

Based on Gerisch's (1980) technique using univalent antibody fragments as tools for the analysis of cell interactions in the slime mould, Dictyostelium discoideum; Edelman et al., (1984) have identified and characterised a series of CAM's from chick embryos. These moleculesare, N-CAM, which is present on all neurons in the central and peripheral nervous system, L-CAM, originally isolated intact from embryonic liver cells, and Ng-CAM for neuron-glia isolated from neurons.

Once the CAM's were purified (by means of classical chromatographic fractionation and affinity chromatography, using the antibodies to CAM's), Edelman and his colleagues could cleave them with enzymes and could construct linear maps of N-CAM and L-CAM. All of these well characterized CAM's are large cell surface glycoproteins; the evidence suggests that they are also intrinsic membrane proteins (Hoffman et al., 1982; Gennarini et al., 1984 a,b).

Although much is now known about specific adhesion molecules (CAMs) in solid tissues it is unlikely that the same molecules are used by cells which are normally

in suspension, and switch to being adherent without protein synthesis. The precedent is, however, important and it now seems that analogous molecules are involved in leucocyte adhesion - possibly molecules with some ancestral homology i.e. belonging to a super-family of adhesion proteins.

It has been recently found (Dustin et al., 1986), that an adhesion molecule ICAM-1 (intercellular adhesion molecule-1) was expressed both on non-hematopoietic cells (vascular endothelial cells, thymic epithelial cells, certain other epithelial cells, and fibroblasts) and on hematopoietic cells (tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal center den ritic cells in tonsils, lymph nodes, and Peyer's patches). As has been reported, ICAM-1 is a cell surface glycoprotein originally defined by a monoclonal (MAb) that inhibits phorbol ester-stimulated leucocyte aggregation.

Moreover, ICAM-1 induction on endothelial cells by inflammatory mediators may facilitate margination and extravasation of T and possibly B lymphocytes at sites of inflammation or localized immune response. ICAM-1 has also been reported to have some properties in common with the papain and trypsin sensitive molecule proposed to be LFA-1 ligand (Davington et al., 1981; and Growkowski et al., 1985). Reporting their work, Dustin et al., (1986), suggested that ICAM-1 is important in

leucocyte adhesion and is regulated in a manner consistent with its being an important molecule in inflammatory and immune responses.

C) **Molecules involved in leucocyte adhesion:**

The development of monoclonal antibodies (MAb) specific for murine leucocyte antigens (Springer et al., 1979) gave a good opportunity to understand the molecular basis of leucocyte adherence. Some of these MAb which will block adhesion were shown to bind to a common antigen on neutrophils and monocytes called Mo1 (also known as Mac-1), while other MAb bound to a different antigen called "lymphocyte function-associated antigen"(LFA-1). LFA-1 was found on lymphocytes (T cells and natural killer cells), B cells, monocytes, and neutrophils. Cell-surface molecules that share a common beta subunit of Mr= 95,000 and which all seem to function in adhesion reactions have now been characterized in both mice and humans.

The LFA-1 molecule participates in T-lymphocyte-mediated killing and other leucocyte cell-cell adhesion reactions (Arnaout et al., 1982). The Mac-1 molecule appears identical to the receptor for C3bi (CR3) on monocytes and granulocytes and also functions in adhesion of these cells to protein-coated surfaces. Furthermore, MAb specific for p150,95, a third member of the Mac-1 and LFA-1 leucocyte adhesion protein family,

have been identified and used to study the biochemistry and cellular expression of p150,95 (Springer et al., 1986).

These molecules constitute a family of structurally and functionally related, high molecular weight, human leucocyte surface glycoproteins (Sanchez et al., 1983). Each of these molecules contains an alpha and a beta subunit, noncovalently associated in an αβ structures. They share an identical β subunit and are distinguished by their α subunits, which have different isoelectric points, molecular weights, and cell distributions and are immunologically non-crossreactive.

Studies related to the deficiency of these molecules in relation to leucocyte (neutrophil) dysfunction have been recently performed (Springer et al., 1983; Anderson et al.,1985). A group of patients with recurring bacterial infections and a defect in mobilization of neutrophils and monocytes into inflammatory sites have been found to be genetically deficient in the expression of the LFA-1 and Mac-1 molecules (Springer et al., 1985). Neither the αM, αL, or β subunits were expressed on the surface of leucocytes from these patients. Moreover, these authors noted that the patient's granulocytes showed multiple defects in adhesion-related functions, including adherence and spreading on protein-coated surfaces, cell-cell aggregation, and chemotaxis. All these and other observations about this glycoprotein-family

suggest that it is of central importance in the regulation of leucocyte adherence reactions.

Among other cell-surface receptors expressed on neutrophils (as mentioned in a previous section), are those for the complement opsonins C3b and C3bi, which are designated as CR1 for the C3b receptor and as CR3 for the C3bi receptor (Fearon et al., 1983). CR1 is a polymorphic glycoprotein of 160-260 kilodaltons, found on monocytes, B lymphocytes, some T lymphocytes, eosinophils, mast cells, erythrocytes, and glomerular podocytes. CR3 on the other hand, is LFA-1 (see previous section) a heterodimer consisting of polypeptide of α=160.000 and β=90.000 daltons, resides on neutrophils, monocytes, some macrophages, and large granular lymphocytes. It has been reported (Fearon et al., 1983b) that unstimulated neutrophils express only 5000 CR1 molecule per cell, whereas activation with chemoattractants such as C5a increases expression to as high as 50-75,000 receptors per cell. Similar increases in expression have been demonstrated in vivo during the complement activation caused by haemodialysis (Lee et al., 1984), and subsequent studies have shown that CR3 on neutrophils exhibits similar augmentation of expression on exposure to chemoattractants in vitro and in vivo (Arnaout et al., 1984). All these findings suggest that these receptors play a critical role in facilitating phagocytosis of bacteria and other

microorganisms that are coated with C3b and C3bi after activation of the complement system.

Other recent studies suggest that membrane glycoproteins may also play a critical role in the adhesive interactions of neutrophils. Schwartz et al., (1985) reported that the neutrophil membrane antigen CDw18 recognized by MAb 60.3 (murine monoclonal antibody) is required for neutrophil adhesion to artificial and cellular substrates. These authors reported that CDw18- neutrophils from patients suffering recurrent infections without pus formation, failed to migrate to sites of infection in vivo, did not spread on plastic, and did not show augmented adherence to cultured endothelium when stimulated. In addition neutrophils from these membrane glycoprotein-deficient patients, secrete lactoferrin normally in response to soluble stimuli. It thus appears that the membrane glycoproteins of the CDw18 complex rather than secreted lactoferrin are primarily responsible for the increase in neutrophil adhesiveness following activation.

Burns and his collegues (1986) found that the IIb-IIIa glycoprotein complex, which functions as the receptor for fibrinogen on platelets and is central to platelet aggregation, is related to the LFA-1 super-family of adhesion glycoproteins and is expressed on the surface of leucocytes where it may function as a receptor for fibronectin. Pommier et al., (1984) reported that neutrophils displayed only low levels of

the IIb-IIIa antigen complex (using MAb 25E11 which specifically identifies the IIb-IIIa glycoprotein complex), and subsequently these cells have been reported to express only low numbers of receptors for fibronectin. Furthermore, it has recently been reported that the related cell surface receptors; LFA-1 (on leucocytes) and IIb-IIIa (on platelets) share features with RGD-recognising surface protein of other cells such as fibroblasts in that they all contain homologous B subunits (Richard. O.Hynes, 1987).

6) **Neutrophil adhesion: in vitro studies:**

Since in vivo studies of the microcirculation have somehow failed to show which of these cells (neutrophils or endothelial cells) change their properties and become more adhesive in an inflammatory response, many recent investigations have been concentrated on the in vitro work in order to try to understand the phenomenon of neutrophil-endothelial interaction. These in vitro studies fall into three main categories:

- Neutrophil adhesion to artificial substrata.
- Neutrophil-neutrophil adhesion (Neutrophil aggregation).
- Neutrophil adhesion to cultured endothelial monolayers.

1) **Neutrophil adhesion to artificial substrata:**

A wide variety of methods have been used to measure neutrophil-substratum adhesion. All these experiments rely on the ability of artificial surfaces such as glass to bind protein films rapidly and irreversibly, and it is to the adsorbed protein that cells adhere. The retention of cells on glass bead columns (Garvin, 1968; Kvarstein, 1969 a,b; Lorente et al., 1978) and to nylon fibre columns (MacGregor et al., 1974; Schiffer et al., 1977) has been used. Others have used glass capillary tubes distracting the cells by centrifugation (Bryant et

al., 1972). In addition, glass coverslips attached to the walls of modified Payling-Wright rotator flasks (Banks and Mitchell, 1973a,b,c) or static coverslips with distraction either by centrifugation (Gallin et al., 1978) or by passing through an air/fluid interface (Lackie and De Bono, 1972). Some other studies have used whole anti-coagulated blood or leucocyte rich-plasma and these, although more realistic models of the in vivo situation, are more difficult to interpret since, for example, platelets may adhere first and modify the substratum.

Regarding adhesion of neutrophil leucocytes to substrata, extensive range of agents has been tested and a number of factors affecting this adhesion have also been studied, and these will be briefly discussed in the following paragraphs:

1) **Temperature:**

The adhesion of cells to glass differs in many respects from adhesion to biologically more realistic substrata. In the absence of serum adhesion to clean glass is neither temperature nor divalent cation dependent and does not require metabolic activity by the cells (Grinnell, 1978). However, in the presence of proteins a minimum temperature of 20° C is required for neutrophil adhesion. Garvin (1961) reported that the optimal temperature range for neutrophil adhesion was

30-40° C, and Lichtman (1979) found it to be abolished at 4° C.

2) **Divalent cations:**

Neutrophil adhesion is also dependent on the presence of divalent cations. Garvin (1961) found that adhesion to glass using whole blood was dependent on the presence of Mg^{2+}; Kvarstein (1969b) also found that Mg^{2+} stimulated adhesion, while Ca^{2+} had no effect. Chelation of divalent cations by EDTA was found to decrease markedly the adherence of neutrophils in heparinized whole blood to glass beads (Penny et al., 1966). Other studies using a range of divalent cations have shown that not only Mg^{2+} is capable of promoting adhesion, but so will Ba^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+}. For example, Rabinovich et al., (1973) using other cell types have found that fibroblasts and macrophages adhere better in the presence of Mn^{2+} than other divalent cations.

3) **Plasma proteins:**

Since plasma is the normal environment of neutrophils, the influence of plasma proteins is of particular relevance. It has been reported that although albumin-coating glass reduced neutrophil adhesion only slightly, it made the surface suitable for locomotion (Lackie and Smith, 1980). Kvarstein (1969b) showed that fibrinogen reduced neutrophil adhesion and plasma

fibronectin (cold-insoluble globulin), has been found to be a very poor adhesive substratum for neutrophils (Brown and Lackie, 1980).

4) **Others:**

A variety of other substances have been examined for effects on adhesion to surfaces. Adhesion of neutrophils was also found to be inhibited by agents which decrease their secretory activity (and vice versa). The local anaesthetics lidocaine and tetracaine (Schiffer et al., 1977), alcohol and chlorpromazine (Mac Gregor et al., 1977), histamine (Bryant et al., 1974) and prostacyclin (PGI_2) may also produce significant inhibition of neutrophil adhesion. PGE_2 was found to inhibit neutrophil adhesion to glass in vitro (Boxer et al., 1980), but not to endothelium in vivo (Higgs et al., 1980). Colchicine was also reported to inhibit neutrophil adhesion to nylon fibres, but not to glass (Boxer et al., 1979). Conversely, neutrophil adhesion has been found to be enhanced by chemotactic factors, such as C5a (Fehr et al., 1977) and FMLP (Smith et al., 1979), and by vasoactive amines like serotonin and metabolites of arachidonate. The chemotactic factors which are regarded as the main factors in enhancing neutrophil adhesion are discussed in the section on PMN adhesion to endothelial monolayers (section 3), because this model of adhesion has recently been the most commonly used assay for investigating neutrophil adhesion in

vitro.

2) **Neutrophil-neutrophil adhesion (neutrophil aggregation):**

Polymorphonuclear neutrophil leucocytes (PMN) have received much attention in terms of general study of cell-cell adhesion, because they can be obtained in large number and much is known about their general biology. An estimate of the strength of cell-cell adhesion is provided by observing the rate and extent of aggregation of cells in a shaken suspension and aggregation can be measured by following the decline in total particle number with time. Using this technique, the aggregation of rabbit peritoneal neutrophils was investigated by Lackie, (1974, 1977) who reported that aggregation was essentially complete by 30 minutes, and could be inhibited by microtubule-blocking agents such as colchicine and vinblastine, and by increasing the intracellular concentration of cAMP. It has also been reported that the aggregation of other cell types such as BHK-21 clone 13 cells could be inhibited by these microtubule-blocking agents.

Looking at the effect of chemotactic factors and using an assay for measuring neutrophil aggregation and cell size with a Coulter counter equipped with a mean cell volume computer and volume analyzer, O'Flaherty et al., (1977) found that chemotactic agents (C5 and C3 fragments) induced a rapid, reversible aggregation and a

slow, sustained swelling of individual leucocytes. These authors claimed that the former effect may underlie the induction of neutropenia and the latter can be an expression of pseudopod formation. Further studies have been performed (O'Flaherty et al., 1978a) examining the ability of C5a and FMLP to induce aggregation and swelling in various preparations of human leucocytes. They found that human neutrophils responded with more prominent swelling but with less prominent aggregation compared with rabbit peritoneal neutrophils. Unlike rabbit peritoneal neutrophils, human neutrophils would adhere spontanously to plastic surfaces and chemotactic factors cited above enhanced this adherence. They also found that lymphocytes were unresponsive to these factors, whereas Ficoll-Hypaque sedimented mononuclear cells (containing a mixture of lymphocytes and monocytes) were responsive indicating that monocytes behave in a manner similar to the human neutrophil.

In addition O'Flaherty (1978b) showed that in the presence of Ca^{2+} and Mg^{2+}, C5a, FMLP, and ionophore A23187 aggregated human neutrophils. Aggregation induced by the two chemotactic factors (C5a and FMLP) was transient and reversed within 2 to 4 minutes after exposure, compared to the aggregation induced by A23187 which was sustained and continued to increase over 15 minutes. Moreover, the authors mentioned that cells preincubated for 4 minutes with a chemotactic factor, exposed to divalent cations and then rechallenged with

the same chemotactic factor showed a minimal aggregation response, ie, cells could be "desensitized" to the original stimulus. A final suggestion was that chemotactic factors induce responsive cells to develop a hyperadherent cytoplasmic membrane. Aggregation and increased adhesiveness to plastic surfaces may reflect this induction.

3) **Neutrophil adhesion to cultured endothelium:**

Interaction of circulating neutrophils with the vascular endothelial cells lining the blood vessels is one of the initial responses characterizing the acute inflammatory response. Although neutrophil adhesion and emigration have been studied (Wilkinson, 1982; Grant, 1973), the mechanisms by which neutrophils recognize and adhere to endothelial cells and subsequently migrate through interendothelial cell junctions are still not understood.

Because the adherence of the circulating neutrophils to the vascular endothelium is a critical step in the establishment of a leucocyte-rich inflammatory infiltration, besides the difficulty of in vivo result interpretations, many investigators have analyzed the adherence of neutrophils to vascular endothelial cells (VEC) monolayers grown in vitro (Beesley et al., 1978; Hoover et al., 1980). Monolayers of VEC from human (e.g., Umbilical cord vein) and animal

(e.g., aorta, pulmonary arteries,etc.) sources can be grown in culture (Jaffe et al., 1973; Gimbrone et al., 1976).

Neutrophils have been demonstrated to adhere preferentially to cultured endothelial cells compared to other cultured cell types (e.g., epithelial cells, fibroblasts, B16 melanoma cells and smooth muscle cells (Lackie et al., 1977; Mac Gregor et al., 1978; Hoover et al., 1978; Beesley et al., 1978). This preference is observed even when the isolated neutrophil and cultured endothelium are of different species.

Knowledge of factors responsible for provoking adherence of PMN to endothelial surfaces is incomplete. Recent work, however, has identified a number of variables (e.g., temperature, divalent cations, and chemotactic factors) that might influence adherence of neutrophils to endothelial monolayers.

Using a centrifugation assay to measure the adherence of PMN to cultured cells, Charo et al., (1985) found that this adherence was temperature dependent since they demonstrated that almost all the PMN incubated with endothelial monolayers at 4° C attached very loosely, (were removed by a relative centrifugal force of 10g). In contrast PMN incubated with monolayers at 37° C became tightly adherent. They claimed that the molecular "bond" that was formed between PMN and endothelial cells at 37° C (but not at 4°C) is unknown.

Adherence of neutrophils to both umbilical vein and

bovine aortic endothelial cells has been demonstrated to be dependent on the presence of extracellular Mg^{2+}, but not Ca^{2+} (Charo et al., 1985). Although Beesley et al., (1978) found Ca^{2+} to be more effective than Mg^{2+} in increasing adherence of porcine PMN to porcine aortic endothelial cells, Hoover et al. found that Mg^{2+} was more effective than Ca^{2+} in enhancing attachment of human PMN to bovine aortic endothelial cells. Adhesion of neutrophils to endothelial monolayers, besides being temperature and divalent cation dependent, has also been shown to be proportional to the initial neutrophil concentration (Beesley et al., 1978), to be enhanced by the presence of erythrocytes and reduced by the presence of platelets (Pearson et al., 1979), or by pretreatment with agents that interfere with the metabolism of arachidonic acid via a non-cyclooxygenase-dependent mechanisms (Buchanan et al., 1983).

The study of chemotactic factors and their effects on neutrophil adhesion to endothelial monolayers has recently received much attention. Several investigators have reported that factors which are chemotactic for neutrophils, such as C5a, FMLP, Zymosan-activated serum, and leukotriene B4 (LTB4) been shown to increase the attachment of PMN to EM. (Smith and Lackie, 1979; Hoover et al., 1980; Gimbrone et al., 1984; Tonnesen et al., 1984). Hoover and collegues, for example, reported that adherence of PMN to bovine aortic endothelial cells

was enhanced by trypsinized human C5, Zymosan-activated human serum, and by formylated peptides (fMet—Ala and FMLP). Using isolated C5-derived peptides, Tonnesen et al., (1984) reported that C5a and C5a des-Arg, as well as FMLP, would augment adherence of human PMN to human umbilical vein endothelial cells. Others, however, have suggested that C5a des-Arg may not influence PMN adhesiveness at all (Tedesco et al., 1981; Fehr and Huber,1984). Because of these conflicting results, Charo et al., (1985, 1986) used a sensitive centrifugation assay to examine the effects of chemotactic factors on both the extent and the strength of PMN adhesion to monolayers of human umbilical vein endothelial cells. These investigators have confirmed that the chemotactic factors FMLP, C5a, and C5a des-Arg not only increased the number of PMN that adhered to endothelial cells, but also increased the strength of adherence. They also found that these factors enhanced PMN adhesiveness in a concentration-dependent manner, (ie, low concentration of C5a and FMLP significantly reduced PMN adherence to endothelial cells and conversely). All these findings suggest that the increased neutrophil adherence to endothelial cells induced by C5a, C5a des-Arg and FMLP is due in large part to an effect of the agents on neutrophils because these stimuli can increase the adherence of neutrophils to an inert surface, such as protein-coated plastic. On the other hand, LTB4 has been found to increase the adherence of neutrophils to

endothelial cells by a direct action of the stimulus on the endothelial cells (Hoover et al., 1984).

Although most of the previous studies on the effects of agents which influence neutrophil-endothelium interaction have been focussed on the PMN, and specific receptors for chemotactic factors such as FMLP and some others are known to exist, the way in which binding of the chemotactic agent modulates the increase in adhesion is not yet known.

It has also been suggested by Harlan (1985) that specific (secondary) granules contents may play an important role in neutrophil adhesion to endothelium:

1) degranulation stimuli decrease the negative surface charge and increase the adhesiveness of neutrophils (Lackie, 1977; Gallin, 1980);

2) exocytosis of specific granules occurs during adherence in vitro and during exudation in vivo (Wright and Gallin, 1979);

3) specific granules contain proteins that promote neutrophil adherence to surfaces (Blockenstedt et al., 1980).

Boxer and collegues (1981, 1982) have proposed the basic lactoferrin as the critical specific granule constituent which promotes adhesion. In support of this hypothesis they noted that purified lactoferrin promoted neutrophil adherence to cultured endothelial cells but

recent evidence seems to rule this out.

More recent studies were performed on the stimulation of the adherence of neutrophils to umbilical vein endothelial cells (UVE) by human recombinant tumor necrosis factor (TNF) (Gamble et al., 1985). This cytokine (TNF) was found to enhance the adherence of PMN to human (UVE) and this enhancement is achieved by effects both on PMN and UVE. The mechanism of the action of TNF on neutrophils is unknown but, the rapidity of the effect suggests the induction of surface expression of adhesion-promoting molecules. One such molecule is the receptor for complement component C3bi (CR3), since monoclonal antibodies against certain epitopes of this molecule block adhesion-dependent reactions.

7) **Adhesion from flow:**

a) **In vivo,**

Observations of the microcirculation in transparent tissues have shown that the flow of blood within vessels is normally laminar or streamline. In this type of flow the velocity increases and the lateral pressure decreases progressively from the vascular wall to the centre of the stream.

Immediately after injuries, or in an inflammatory response, flow through the dilated vessels and through their draining venules becomes extremely rapid. In arteriols and venules (but not in capillaries whose diameter is about that of an erythrocyte the flowing blood is divided into two zones; a peripheral zone of almost cell-free plasma and an axial stream containing blood corpuscles (cells). As flow slows, and long before stasis is apparent, leucocytes begin to appear in the marginal plasma stream of the venules, and to stick from time to time to the venular wall.

b) **Use of the parallel-plate flow chamber:**

The parallel-plate system was first used by Mohandas et al., (1973; 1974) to investigate the adhesion of red blood cells to foreign surfaces in the presence of flow. This system provides a uniform level of shear stress at a given flow condition and permits direct microscopic visualization of the detachment

process. In other words, this technique involves first letting cells settle and adhere to the surface under investigation. The adhered cells are then exposed to a well-defined fluid shear force and the number of cells which detach from the surface as a function of time and applied force is monitored.

The design of the parallel-plate flow chamber was modified by Doroszewski and his co-workers (Doroszewski et al., 1977; Zachara and Doroszewski, 1978), and used in investigating the adhesion of leukaemic cells. The flow chamber assay was also applied by Forrester and Lackie, (1984) to measure neutrophil adhesion in a manner which resembles much more closely the interaction which must occur in vivo. The fluid shear stress in the chamber (around $4x10^{-11}$ N) approximates to that of the post capillary venule where margination normally occurs (Lackie and Forrester, 1985). In addition, the flow chamber assay is regarded as a particularly appropriate technique for looking at the adhesive behaviour of blood cells (leucocytes), since this is effectively what these cells must do in order to leave the circulation in vivo. One advantage of the flow chamber method is that it is insensitive to the extent of speading, since the adhesion must be established before the cells can exhibit any spreading.

Very few studies on neutrophil adhesion from flow have been performed. Forrester and Lackie, (1984) looked

at human neutrophil (PMN) adhesion to various plasma components, connective tissue component and chemotactic factor-coated chambers. Their results showed that human serum albumin (HSA) reduced PMN adhesion at a certain concentration (1mg/ml) compared to uncoated chambers. Adhesion to alpha-2-macroglobulin coated surfaces was markedly reduced (Forrester et al., 1983) and glass coated with transferrin was also shown to be a non-adhesive substratum for PMN. The effect of chemotactic factors was also tested and the findings demonstrated that PMN did not adhere to surfaces coated with the chemotactic factor casein (1mg/ml) but preincubation of PMN in the same concentration of casein increased their adhesion to human albumin-coated glass. Neutrophils were also found to be more adhesive when treated with fMLP. On the other hand, coating the surface with collagen (either type I from tendon or type IV from basement membrane), or with fibronectin, reduced adhesion implying that PMN would not tend to stick to the sub-endothelial basement membrane (Lackie and Forrester, 1985).

Since adhesion to endothelial cells lining the blood vessels, is the first stage of a neutrophil's response to an inflammation, and since little previous work has been done on this process under conditions resembling the flow of blood, the present work was mainly based on the study of (rabbit peritoneal neutrophil) adhesion from flow and the effect of various

factors on this adhesion and on the rate at which cells changed their properties.

CHAPTER 2

Materials and methods:

1) Isolation of cells:

a) Rabbit neutrophils:

Peritoneal exudates were elicited in female New Zealand white rabbit by injection of approximately 500 ml of sterile 0.9% w/v NaCl containing 0.1% w/v oyster glycogen. Peritoneal fluid containing > 95% pure neutrophils was collected after 4 hours, and stored in 20 ml aliquots for up to 3 days at 4°C (Lackie, 1974). Normally about 300 ml of exudate ($1-3x10^6$ cells/ml) fluid was obtained after this procedure.

b) Human neutrophils:

20 ml peripheral blood was taken from healthy adult donors, anticoagulated with heparin, and a leucocyte-rich supernatent taken off after dextran sedimentation of red blood cells (2 ml Dextran 110/20 ml blood). The leucocyte-rich fraction was separated on Ficoll-Hypaque (10 ml, Pharmacia, Uppsala) 400g/30 min. The cells were washed 3 times in Hepes-buffered balanced salts solution (HS), and contaminating red cells were removed after the first wash by hypotonic lysis. Finally pure washed neutrophils were resuspended in buffered salts solution (H_2) and BSA at a desired concentration.

II) **Preparation of peritoneal neutrophils:**

When needed for use, neutrophils in peritonal exudate were taken from stock, shaken and pelleted at 400 g. These were then washed 2-3 times with HS and the contaminating erythrocytes were lysed by adding 0.5 ml distilled water for about 10 seconds after the first wash, and then resuspended in H_2+BSA at a desired concentration.

III) **Neutrophil adhesion assays:**

1) **Adhesion to glass coverslips:**

A number of 13 mm diameter coverslips were placed in "Linbro" plastic multi-welled tray, these were coated with proteins for 30 mins, and then rinsed with H_2. A suspension of neutrophils (0.5 ml) was added to the coverslips to give a final volume of 1 ml and the tray was then incubated at 37°C for 30 mins. The coverslips were then removed and non-adherent cells were rinsed by either dipping the coverslips through an air/H_2 interface, 5, 10, or 20 times, or by using a "stirred rinse". The latter method consisted of 300 ml flat beaker containing H_2 stirred at a moderate speed by means of a magnetic stirrer. The coverslip held by forceps was then dipped vertically into the rotating medium for 1 minute in order to wash off the non-adherent cells. The number of adherent cells was then

determined by one of two methods:

i) The coverslips were fixed for 30 mins in buffered formalin, rinsed with 70% Ethanol, then with distilled water, stained in Giemsa (1:10 dist.H2O) for 20 mins, rinsed and dehydrated in isopropanol, air dried and finally mounted in DPX. Using the x25 objective and 10x10 eyepiece grid, the cells at 10 random squares were counted.

ii) The radioactivity bound to coverslips was counted in a Wilj 2001 Gamma counter, having used ^{51}Cr-labelled neutrophils.

2) **Labelling of neutrophils:**

Isolated neutrophils were washed as mentioned before. The cell pellet was resuspended in 1 ml of 0.5% w/v BSA and 100-200 μCi of sodium chromate solution (Radiochemical Centre, Amersham) were added. The cells were incubated for 30-45 mins at 37°C, shaken every 10 mins of incubation, washed three times in HS or H_2 to remove BSA and free chromate, and finally cells were resuspended in the appropriate medium (usually H_2 containing BSA) at the desired concentration.

3) **Preparation of coverslips:**

Glass coverslips (13 mm diameter, Chance Propper Ltd) for use in adhesion assays were boiled in detergent (Decon) for few minutes, soaked overnight, rinsed for several hours in tap water, washed in distilled water

and in absolute Ethanol, and then dried before use, (the same procedure was applied for the preparation of the flow chamber slides).

2) **Neutrophil adhesion from flow:**

a) **The flow chamber:**

As described by Lackie and Forrester (1984), the flow chamber is made up of two glass microscope slides held together by a Nescofilm gasket (Nippon Shoji Kaisha Ltd, Osaka, Japan), 150 ʌm thick within which a channel (40x4 mm) had been cut using a steel template. The ends of this channel coincide with two 4-5 mm diameter (drilled holes) in the upper slide which serve as inlet and outlet ports. The chamber was then placed in a metal and Perspex (lucite) clamping device with inlet and outlet ports that also coincide with the 4 mm holes in the upper microscope slide (Fig. 2-1).

The lower surface of the chamber, to which the cells attach, can be modified by pre-coating it with proteins, either by pre-incubating the chamber or by treating the lower slide before assembly as shown in (Fig. 2-2). Alternatively the cell suspension, which is pumped through the chamber with a syringe drive (2 ml glass syringe "Sigma") at around 0.2 ʌl/sec can contain the proteins to be tested . The flowing cells were viewed through an inverted microscope and the number of adherent cells were observed using a monochrome video

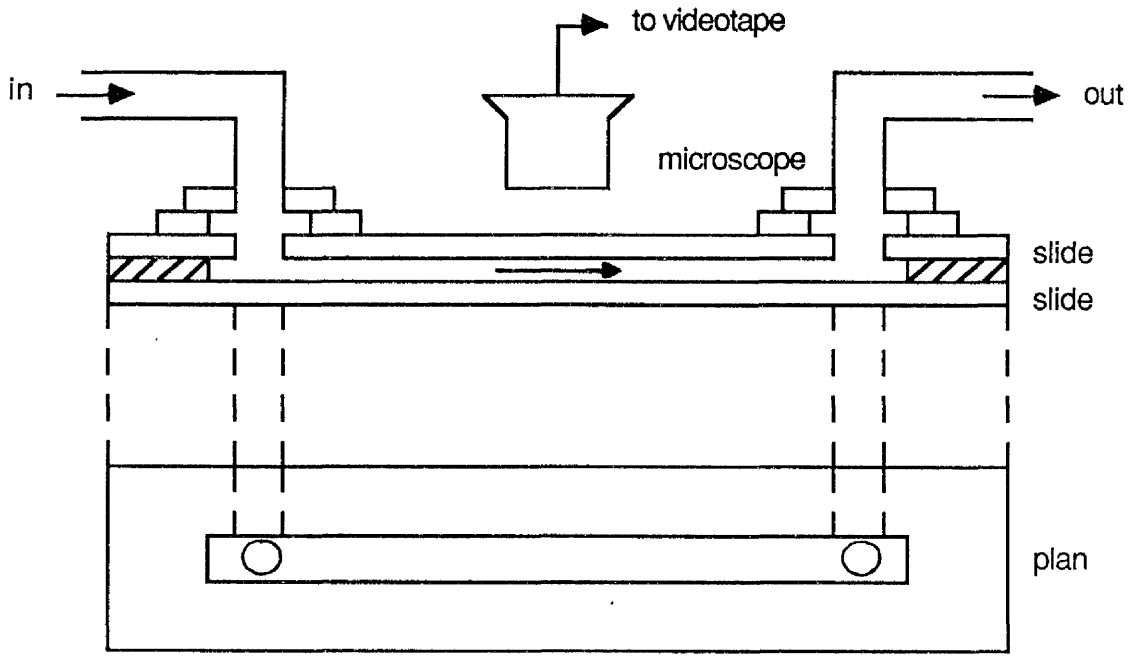


Fig. 2-1: Cross section and plan view of the flow chamber. The inlet and outlet ports are drilled in a perspex block which is clamped over the glass. (From Forrester and Lackie, 1984).

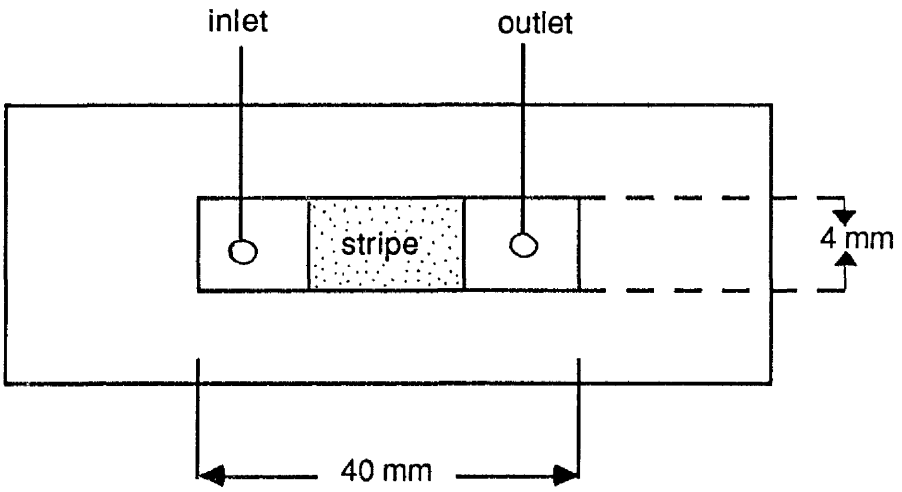


Fig. 2-2: Diagrammatic plan view of the flow chamber showing the position of the conalbumin or zymosan in agarose stripe which was dried on before the chamber was assembled.

camera and monitor and recorded for analysis on a time-lapse film video recorder (National; VTR NV-8030) (Fig. 2-3). Two methods of scoring were used:

i) **Direct count on one area with time:**

Using the time-lapse function for recording and then viewing at normal speed, cells attached to the chamber could easily be counted as white dots, whilst unattached cells just appeared as blurs. The counts were plotted as number of adherent cells/unit area/unit time or converted to collection efficiency when cell delivery rate is known.

ii) **Count of number of adherent cells at different positions along the chamber:**

After having air-dried proteins onto one third of the lower surface, the chamber was assembled, rinsed with BSA containing medium, and then a cell suspension was perfused through. Five minutes later, the cell suspension was replaced with buffer, the chamber rinsed for a minute at higher flow rate and then the adherent cells were counted at different positions along the chamber, using a phase contrast microscope with an eye-piece grid.

In some other experiments using the flow chamber, a slight modification has been performed. Two syringes, one containing the cell suspension and one the chemotactic factor at double concentration were used.

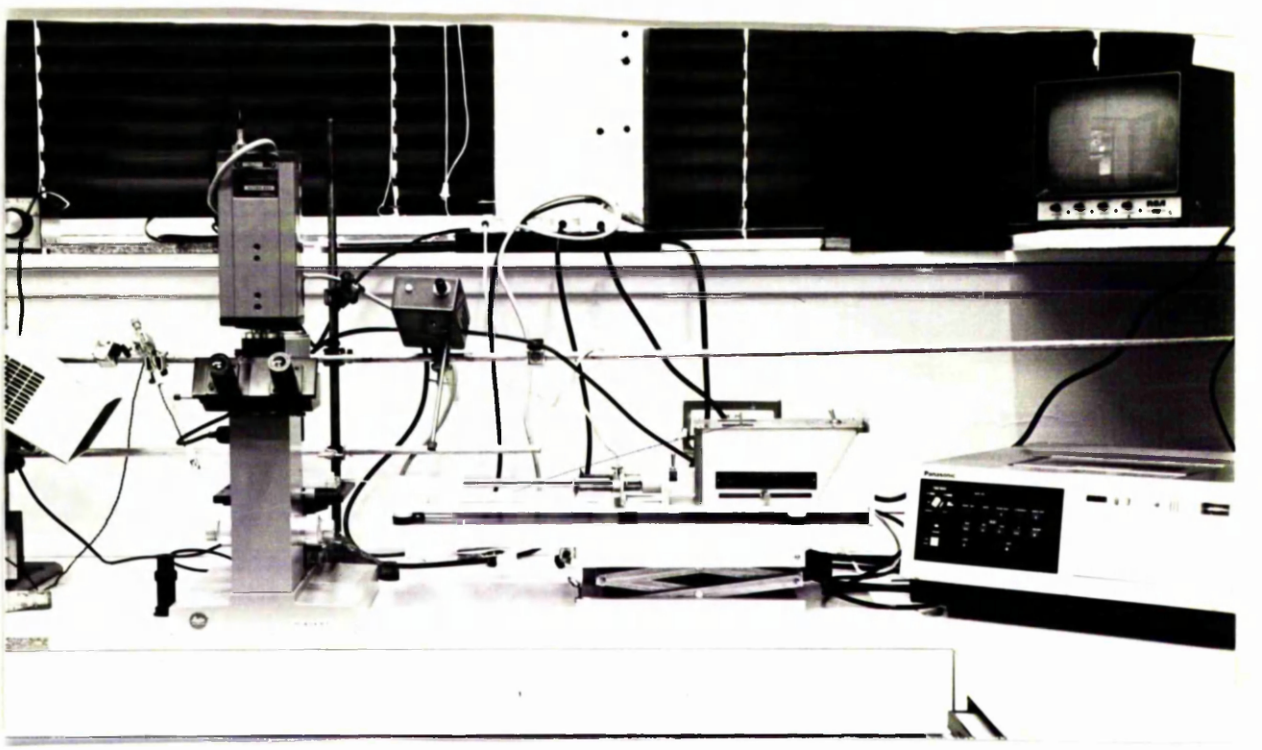


Fig. 2-3: Photograph showing the whole set up of the flow chamber equipment.

These two were linked to a "Y" shaped manifold which was directly connected to the chamber (Fig. 2-4).

IV) **Proteins:**

A range of plasma proteins were used to coat the substrata on which neutrophils adhered. These proteins were as follows:

1) Cryoprecipitate.
2) Fibronectin.
3) Cryoprecipitate depleted of fibronectin.
4) Plasma.
5) Plasma depleted of fibronectin.
6) Serum.
7) Human albumin.
8) Factor VIII.

1) **Cryoprecipitate:**

A precipitate which forms when freezing plasma to -20°C and then thawing it to 4°C, was generously supplied as was factor VIII, by Dr D. Maharadj (Department of Haematology, Royal Infirmary, Glasgow). This residue is a complex mixture of substances, the exact mixture not having been identified yet. It contains substantial amounts of:

- Factor VIII.
- Fibronectin (about 4 to 5 times plasma level, i.e. about 2 mg/ml).
- Immunoglobulins (mainly IgM and IgA).

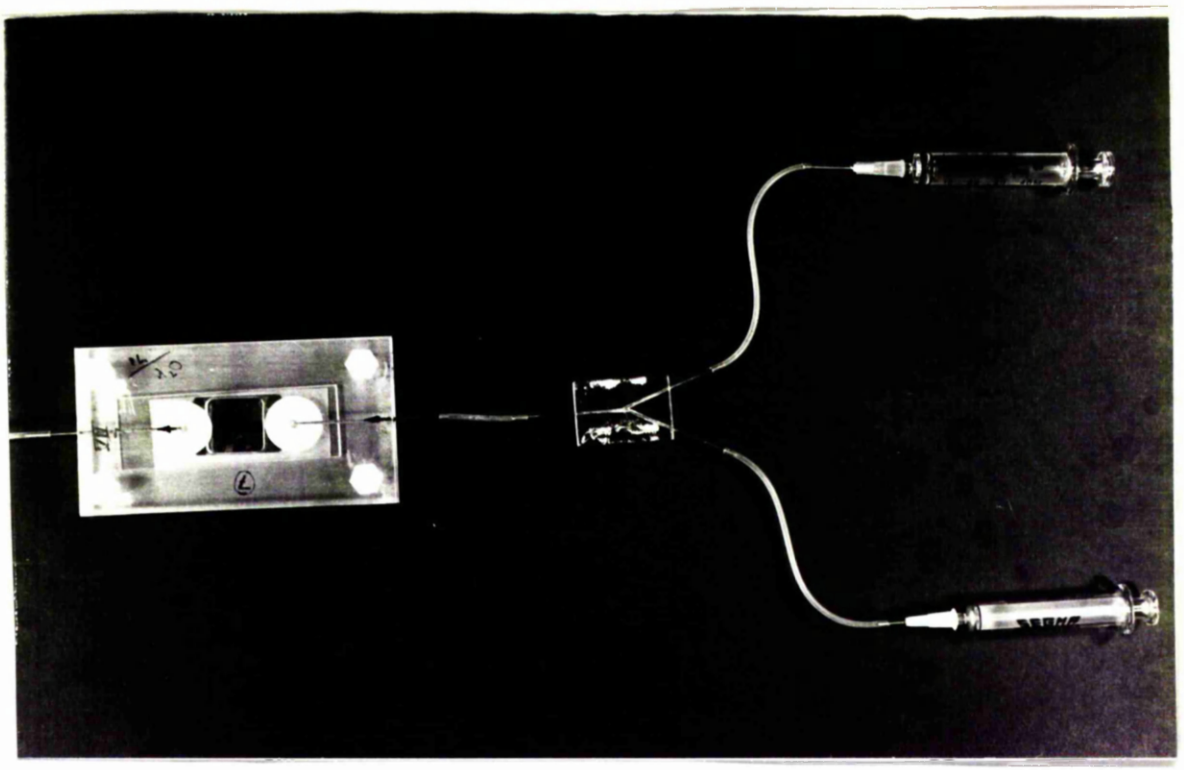


Fig. 2-4: Photograph showing the Y-shaped manifold which serves as connector between the two syringes (containing the cell suspension and the chemotactic peptides) and the chamber.

2) **Fibronectin:**

This protein (Gift from G. Campbell., Cell Biology Department, Glasgow University) was isolated from bovine serum by affinity chromatography on a gelatin-sepharose column according to the method of (ENGVALL & RUOSLAHTI, 1977). The eluate was adjusted to give a concentration of 1 mg/ml fibronectin in 8M urea; this was then diluted to the desired concentrations with H_2. Bashes of fibronectin were tested for serum impurities by SDS-polyacrylamide gel electrophoresis.

3) **Conalbumin (Ovotransferrin):**

(Iron complex; from chicken egg white), was obtained from "Sigma" and used at a concentration of 1mg/ml. High titre rabbit anti-ovotransferrin was kindly supplied by Dr. X. Alvarez-Hernandez, Department of Bacteriology and Immunology, Glasgow University. The protocol for obtaining this antiserum was as follows: Ovotransferrin saturated with iron (1 mg of protein binding with 1.5 ʌg iron) was emulsified with the same volume of complete Freund's adjuvant (Difco, Detroit, Mich.), and injected in 2 ml volumes intramuscularly. Injections were done at 15 d intervals, and the titre was tested after the fourth injection. The equivalence point, with complement, is 1 ʌl antiserum to 4 ʌg ovotransferrin. When activated, the equivalent point is 1 ʌl antiserum to 8 ʌg ovotransferrin.

V) **Chemotactic factors:**

1) **FMLP:**

The synthetic chemotactic factor N-formyl-Methionyl-Leucyl-Phenylalanine (FMLP), was supplied from "Sigma" and was dissolved in DMSO at 10^{-2} M, and stored in small aliquots. When needed for use, these batches were diluted in H_2.

2) **Complement component activation:**

The activation of complement component was initiated by both classical and alternative pathways. The former was induced in situ by formation of immune complexes, when treating the lower surface with conalbumin, then perfusing through the cell suspension with 10% serum containing a high titre of antibody to conalbumin. Complement was also activated by the alternative pathway by incubating zymosan (yeast cell wall) with fresh serum for 30 min at 37°C, then removing zymosan by centrifugation. Heat-treated serum was similarly treated with zymosan to produce "HtZa "; Heat-treated Zymosan-activated Serum

VI) **Media Recipes:**

1) **Calcium and Magnesium free salts solution (Hepes saline, HS):**

NaCl 8 g/l

KCl 0.4 g/l

Glucose 1 g/l

Hepes 2.388 g/l

Phenol red (1%) 2 ml/l pH to 7.4 with 1N NaOH.

2) **Balanced salts solution (Hanks Hepes=H_2):**

NaCl 8 g/l

KCl 0.4 g/l

$CaCl_2$ 0.14 g/l

$MgCl_2.6\ H_2O$ 0.2 g/l

Glucose 1 g/l

Hepes 2.388 g/l

Phenol red (1%) if required 2 ml/l, pH to 7.4 with 1N NaOH.

3) **Buffered formalin contained (per litre):**

Formaldehyde (40%) 100 ml

$Na_2HPO_4.2\ H2O$ 6.5 g

NaH_2PO_4 4 g

$MgCO_3$ to excess.

CHAPTER 3

Results I.

This chapter will mainly deal with the effect of plasma proteins on the adhesion of neutrophil leucocytes both in a conventional static assay and in the flow chamber. The first results obtained with the flow chamber in the present work, which has concentrated on the adhesion of neutrophils to glass substrata, were cross-checked and compared with a more conventional static assay.

Since plasma proteins are normal components of the neutrophil environment, a wide range of these components was used to coat the surfaces on which neutrophil adhesion took place. Fibronectin and cryoprecipitate were looked at in greatest detail.

It has been reported that the intravenous administration of cryoprecipitate is useful in treating patients suffering from septic shock (Saba et al.,1978), a condition in which there is massive infection with gram negative bacteria, as may occur with a gut wound, or in a patient with severe burns. Endotoxins released from bacteria "activate" neutrophil leucocytes causing them to adhere non-specifically to the endothelium. Since the largest capillary bed is in the lungs, many neutrophils marginate there, causing an inflammatory response which leads to accumulation of fluid in the lungs; many patients die from this acute respiratory distress syndrome. Treatment with cryoprecipitate may

help by reducing neutrophil adhesion, thus relieving the symptoms, (i.e. probably by acting on neutrophils themselves and preventing them from adhering in the capillary bed of the lungs.

Cryoprecipitate is also important in treating patients suffering from haemophilia A because it contains a higher concentration of Factor VIII than does plasma. In view of the importance of this protein in blood clotting, it might also have an important effect on neutrophil adhesion in vitro, so that cryoprecipitate was first tested using the static assay as described below.

I) **Neutrophil adhesion to coverslips coated with plasma proteins:**

As described in Materials and Methods, the adhesion of chromate-labelled neutrophils to protein pre-coated coverslips was tested, the number of adherent cells being estimated by counting the radioactivity of the coverslips in the Gamma counter; the results were expressed as percentage of control values. The stock solution of the proteins (1mg/ml) used in these experiments was diluted to the following concentrations for coating the coverslips or the chambers; 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1 µg/ml.

a) **Effect of cryoprecipitate:**

As seen in Fig.3-1 the adhesion of neutrophils to cryoprecipitate-coated coverslips was greatly reduced when a concentration of 100 µg/ml was used, but at concentrations below 100 µg/ml, the adhesion was seen to increase progressively until it reached the control level. In all cases the adhesion has been compared to the value of adhesion to clean glass which has been taken as 100%.

b) **Effect of Fibronectin and Fibronectin-depleted cryoprecipitate:**

Fibronectin (isolated from bovine serum by gelatin affinity chromatography) also reduced neutrophil adhesion to glass coverslips. There was also a significant reduction ($p<0.001$) at the concentration of 100 µg/ml and 50 µg/ml (Fig.3-2). As with cryoprecipitate, the reduction in adhesion was lost at lower concentrations.

Coating the substratum with fibronectin-depleted cryoprecipitate had no marked effect on adhesion (Fig.3-3). The percentage adhesion relative to control was about 75% at 100 µg/ml protein concentration, whereas with cryoprecipitate containing fibronectin (Fig.3-1) the adhesion at this concentration was only around 40%. This difference in figures probably

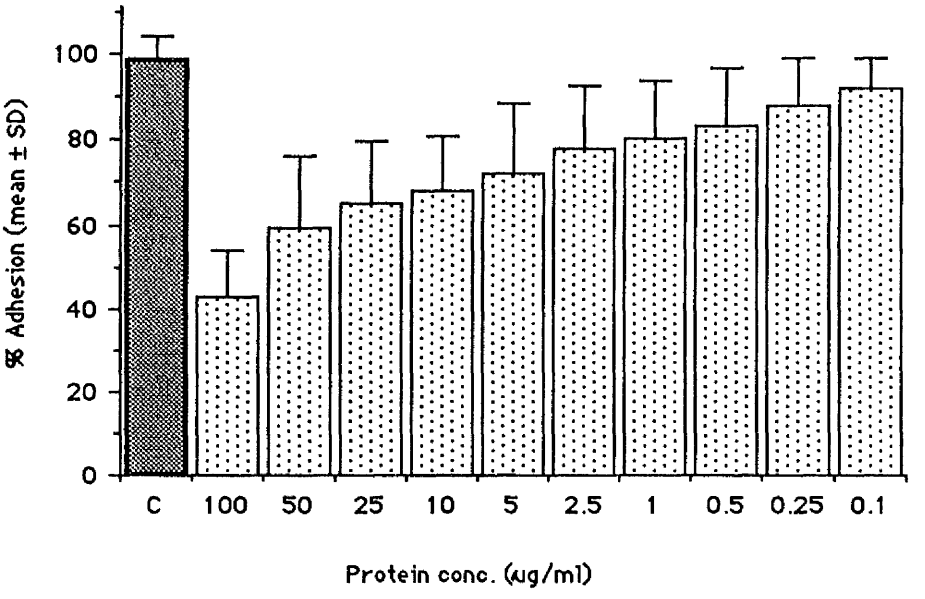


Fig. 3-1 : Adhesion of rabbit peritoneal neutrophils to coverslips pre-coated with different concentrations of cryoprecipitate. Bars represent standard deviations of the mean and adhesion is relative to clean glass as 100%.

C: Clean Glass.

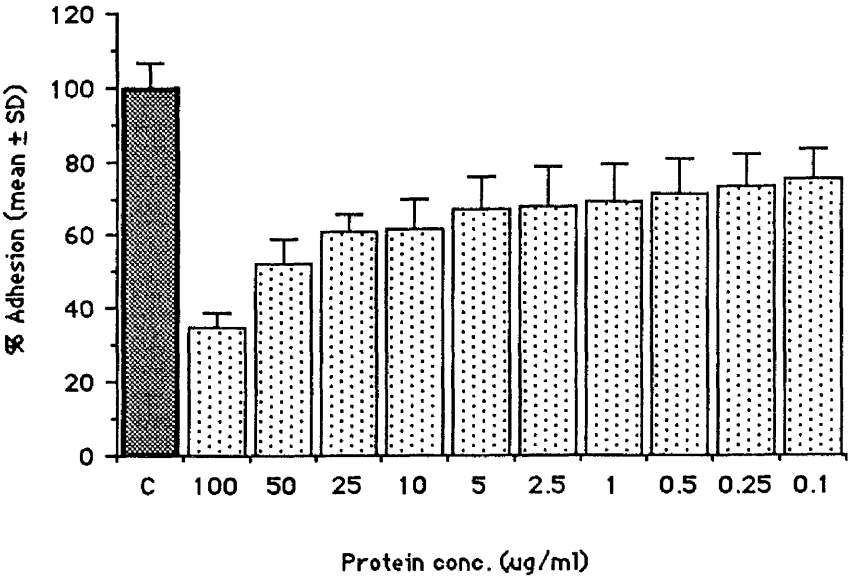


Fig. 3-2: Neutrophil adhesion to coverslips coated with various concentrations of Fibronectin.

C: Clean Glass.

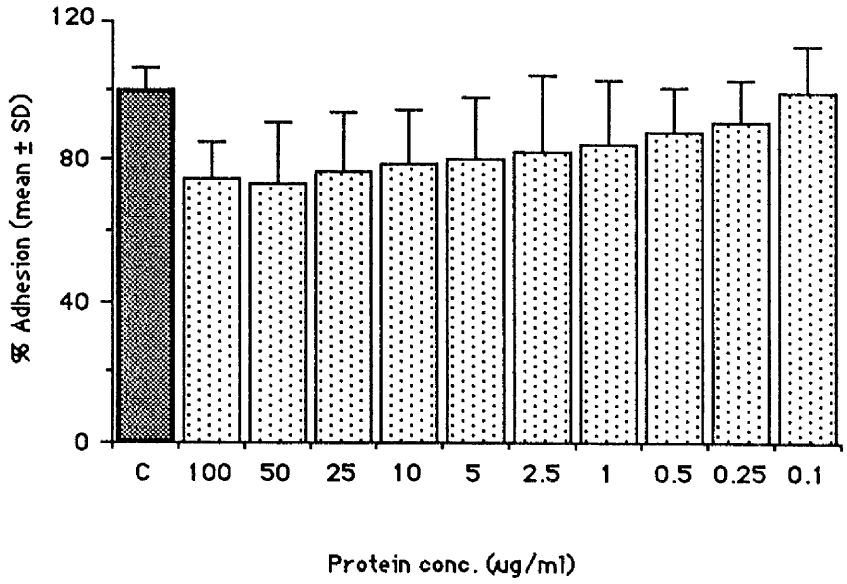


Fig. 3-3: Neutrophil adhesion to coverslips coated with various concentrations of Fibronectin-depleted cryoprecipitate.

C: Clean Glass.

implies that neutrophils do not tend to stick to cryoprecipitate because this contains a great amount of fibronectin, which has been shown to be a very low adhesive substratum for neutrophil leucocytes.

c) **Effect of serum, human albumin, and Factor VIII:**

As seen in Figs.3-4 and 3-5, serum and human serum albumin (HSA) decreased neutrophil adhesion (74%, 70%) but the effect was less dramatic than that produced by fibronectin and cryoprecipitate both of which significantly reduced adhesion.

Factor VIII (the clotting factor) was even less effective at reducing adhesion than serum and HSA; adhesion to Factor VIII went from 78% at the highest concentrations up to 90% at the lowest concentrations (Fig.3-6).

In all experiments, the effect of the proteins tested on neutrophil adhesion was dose dependent, with a pronounced effect at 100 ug/ml of protein, which was then reduced at lower doses. Thereafter, this dose (100 ug/ml) was adopted as a convenient standard, and differences between all proteins were tested at this concentration. The comparison and degrees of significance are shown in Fig.3-7. Compared to clean glass all proteins seemed to decrease the adhesion of neutrophils, the effect of serum, HSA, cryoprecipitate-depleted fibronectin and Factor VIII were more or less

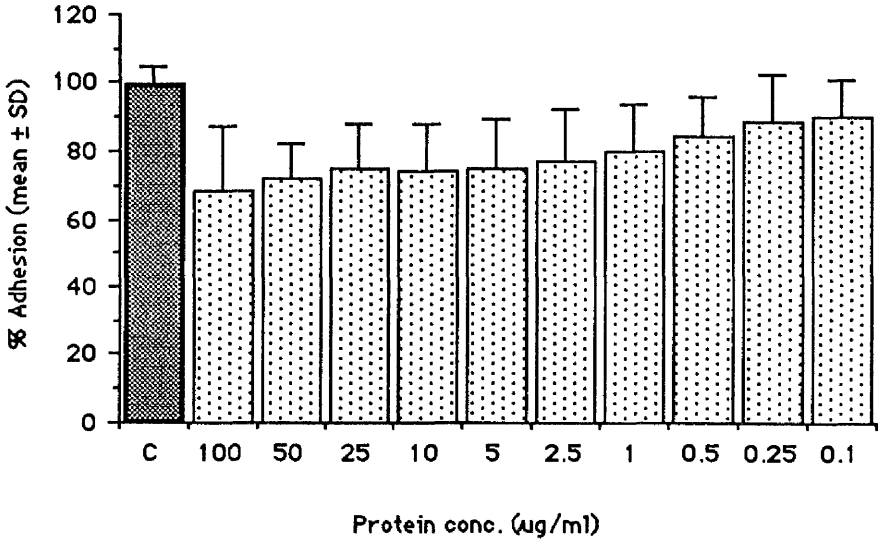


Fig. 3-4: Neutrophil adhesion to coverslips costed with variuos concentrations of serum.

C: Clean Glass.

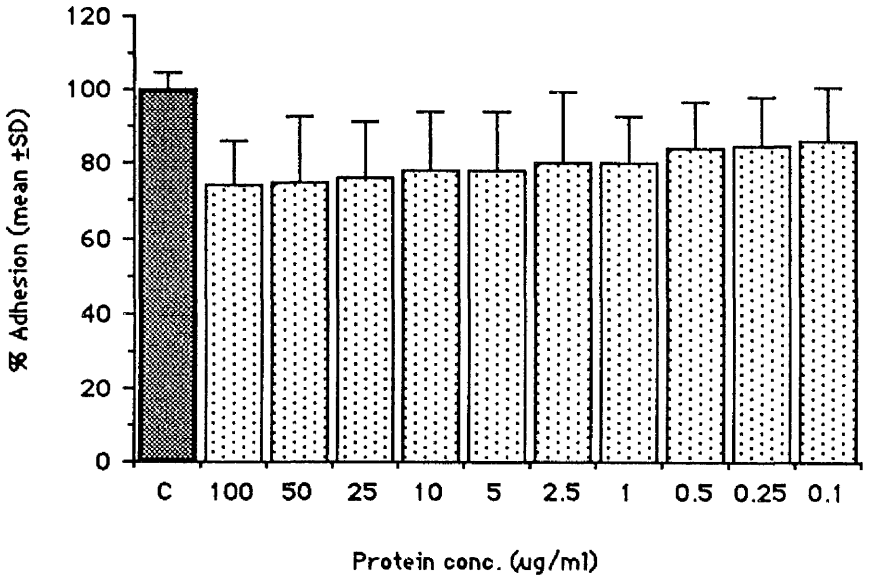


Fig. 3-5: Neutrophil adhesion to coverslips coated with different concentrations of HSA.

C: Clean Glass.

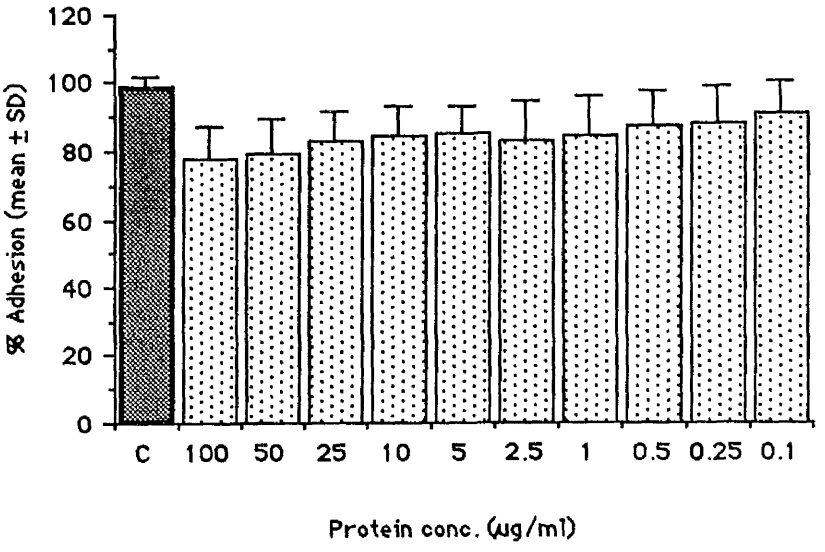


Fig. 3-6: Neutrophil adhesion to coverslips coated with diffrent concentrations of Factor VIII.

C: Clean Glass.

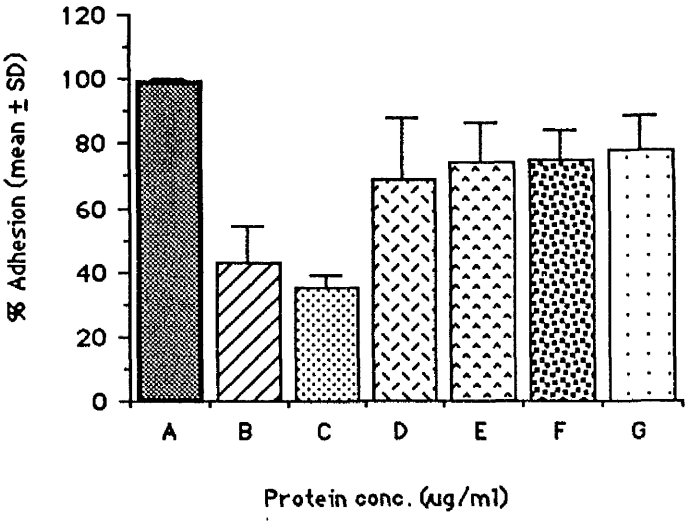


Fig. 3-7: Neutrophil adhesion to coverslips pre-coated with 100 ug/ml of different proteins using a static assay.

- clean glass
- cryoprecipitate
- Fibronectin
- serum
- Albumin
- fibronectin-depleted cryoprecipitate
- Factor VIII

the same and not significant, however, cryoprecipitate and especially fibronectin dramatically inhibited the adhesion of neutrophils to coated coverslips.

Although adhesion to coverslips (static assay) has the advantage of being much faster, and easier to use to test several fractions simultaneously, this assay does not measure one single cellular function, since a distraction assay of this type will be affected by the extent of cell spreading, and is not a simple adhesion-dependent function . Unlike the flow chamber, this assay does not mimic the in vivo behaviour of blood cells, and because these cells circulate in a rather complex environment, it would undoubtedly be naive to suppose that any single, simple, assay will suffice to measure their behaviour. The flow chamber represents a more realistic and effective assay for measuring neutrophil adhesion in a manner which resembles much more closely the interaction which occurs in vivo.

II) **Neutrophil adhesion from flow; effect of plasma proteins:**

Having found a marked inhibition with both fibronectin and cryoprecipitate in the static assay the adhesion of rabbit peritoneal neutrophils to plasma protein-coated glass under condition of flow was studied using a special constructed flow chamber, as described in Materials and Methods. Results obtained from this

technique were expressed as number of adherent cells per unit time and the collection efficiency could be calculated knowing the cell concentration, the flow rate, and the ratio of the area under observation to the total area of the chamber.

Similar results to those on coverslips were obtained. Glass coated with a 100 µg/ml solution of the plasma proteins used in previous section, was less adhesive for neutrophils. A very marked reduction was obtained by pre-coating the chamber with fibronectin or cryoprecipitate. Moreover, depleting cryoprecipitate of fibronectin gave results that were not significantly different from control (Fig.3-8).

Coating the chamber with plasma also reduced adhesion. If, however, the chamber was pre-coated with plasma depleted of fibronectin, the surface trapped cells very effectively (Fig.3-9). The collection efficiency, the number of cells trapped as a proportion of those delivered to the area being observed, rose from about 9% with cryoprecipitate to 30% with fibronectin-depleted cryoprecipitate (Table 3-1), and from 11% with plasma to 30% with fibronectin-depleted plasma (Table 3-2).

Interestingly, Factor VIII did not markedly affect adhesion compared to serum which also decreased adhesion (Fig.3-10). Collection efficiency of neutrophils to serum- and Factor VIII-coated chambers are shown in

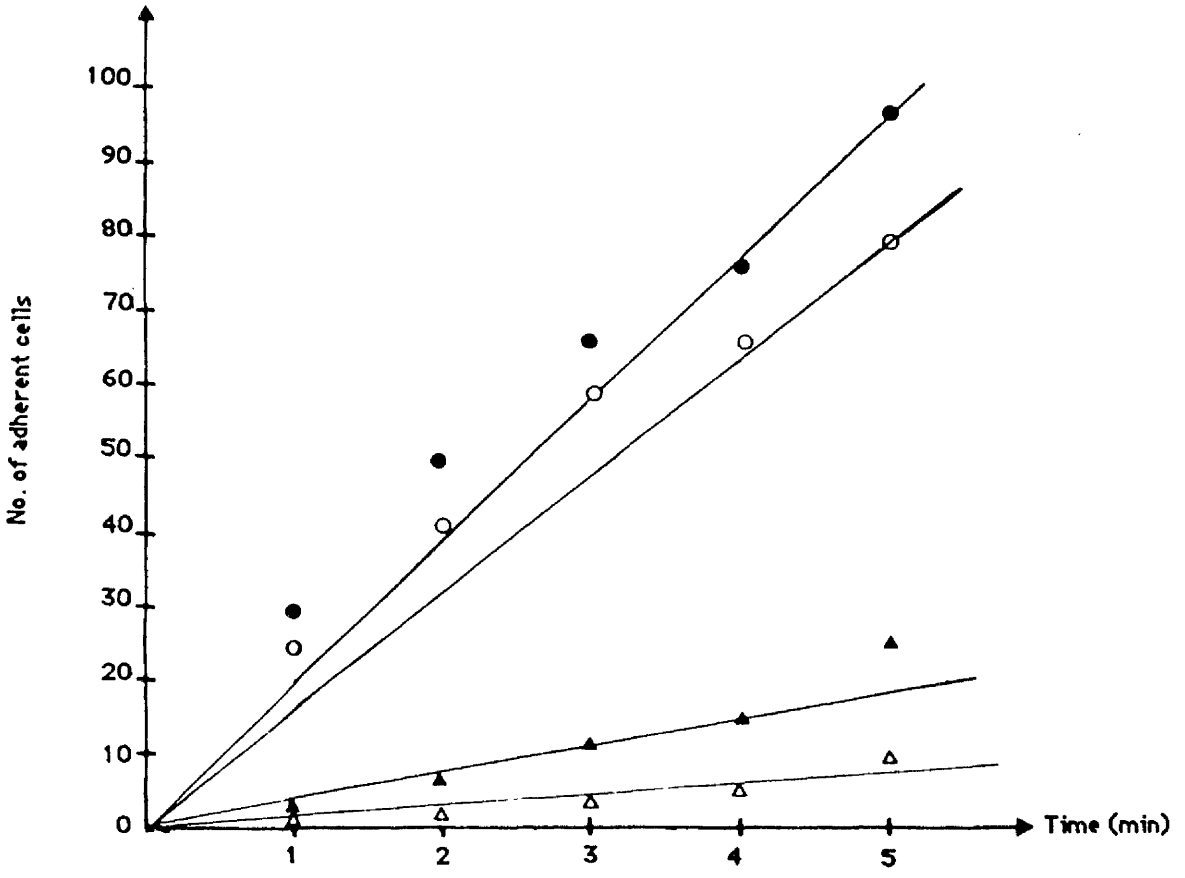


Fig. 3-8: Neutrophil adhesion to chambers coated with 100 µg/ml cryoprecipitate (▲—▲), Fibronectin (△—△), Fibronectin-depleted cryoprecipitate (O-O). (Clean glass ●-●).

Flow time	Collection efficiency (%)			
(min)	Control	FN	Cryo	Cryo-FN
1	41	5	2	40
2	38	7	3	34
5	36	8	3	30

Table 3-1: Collection efficiency of rabbit neutrophils on clean glass, Fibronectin (FN), cryoprecipitate (Cryo), and Fibronectin-depleted cryoprecipitate (cryo-FN).

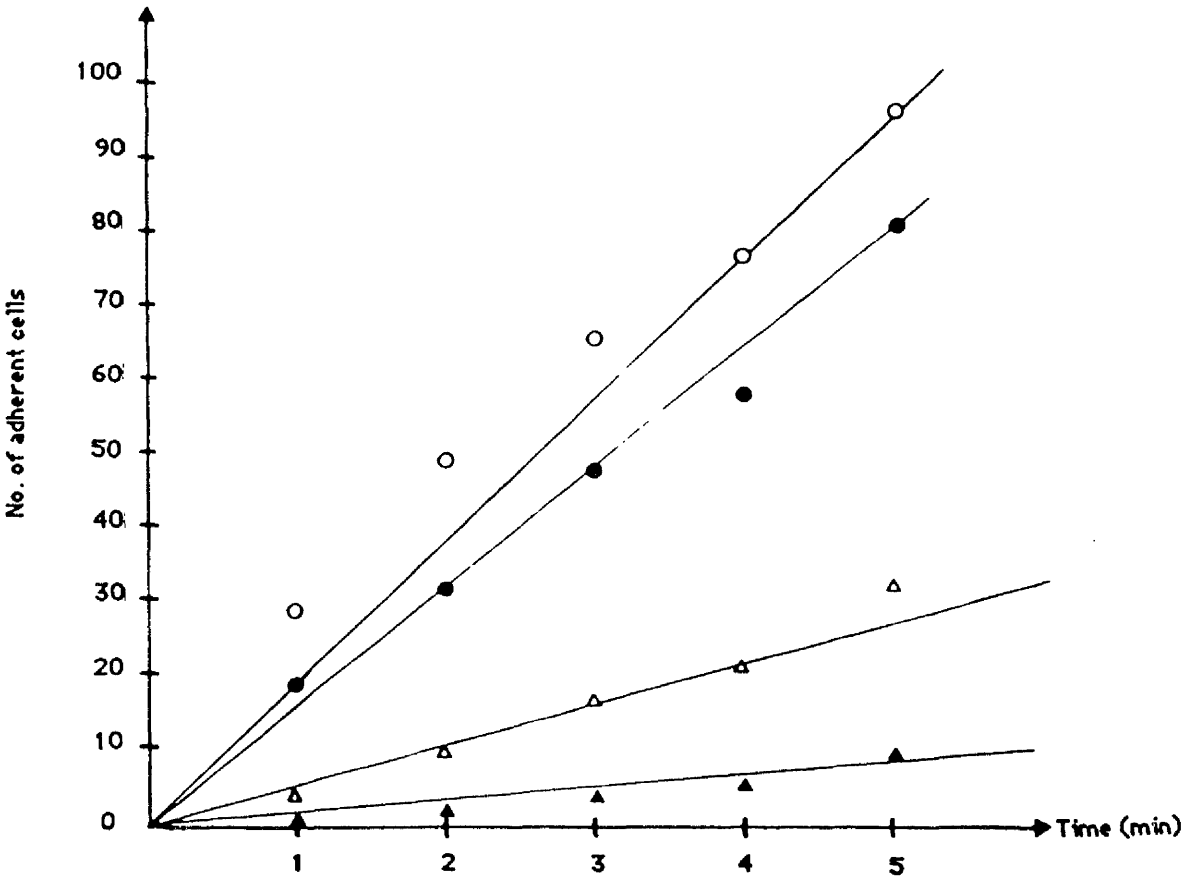


Fig. 3-9: Neutrophil adhesion to chambers coated with 100 µg/ml Plasma (△—△), Fibronectin (▲—▲), Fibronectin-depleted Plasma (●-●). (Clean glass O-O).

Flow time	Collection efficiency (%)			
(min)	Control	FN	Plasma	Plasma-FN
1	41	2	10	30
2	38	3	11	30
5	36	3	11	30

Table 3-2: Collection efficiency of rabbit neutrophils on clean glass, fibronectin (FN), plasma, and fibronectin-depleted plasma (plasma-FN) coated glass at various times after starting.

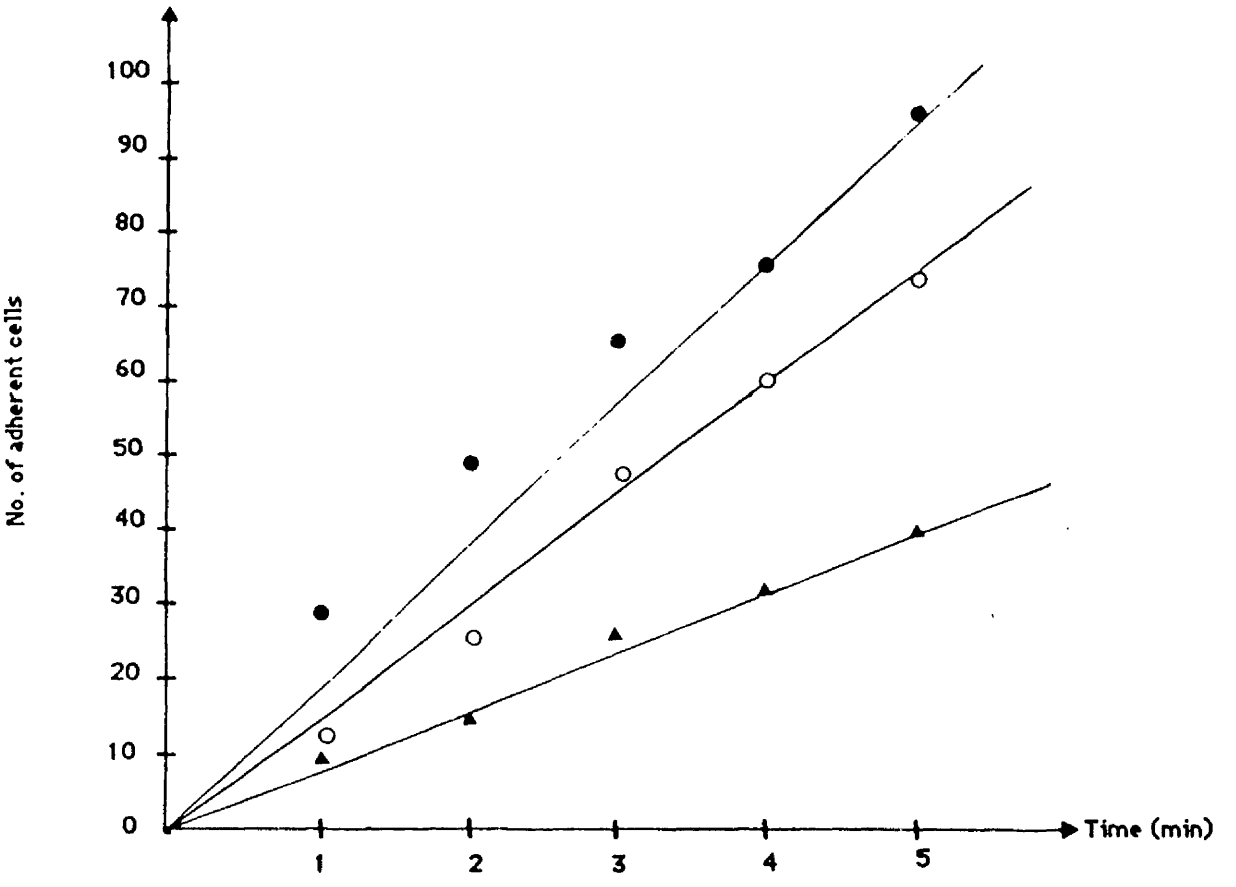


Fig. 3-10: Neutrophil adhesion to chambers coated with 100 µg/ml Factor VIII (O-O), serum (▲—▲). (Clean glass ●-●).

Flow time	Collection efficiency (%)		
(min)	Control	Serum	Factor VIII
1	41	15	25
2	38	15	26
5	36	14	27

Table 3-3: Collection efficiency of rabbit neutrophils on clean glass, serum, and Factor VIII-coated glass at various times after starting.

Table 3-3.

A summary of the result giving a comparison of the effect of the different proteins on neutrophil adhesion under condition of flow (expressed as collection efficiency) is shown in Fig. 3-11.

Summary:

1) Fibronectin and cryoprecipitate, but not the rest of the plasma proteins used, significantly inhibited neutrophil adhesion in both the static assay and in the flow chamber.

2) The effect on neutrophil adhesion was dose dependent for all proteins used.

3) As in the vascular system plasma has properties necessary for maintaining a non-adhesive environment for cells, this component is considered as low adhesive medium (in vitro), and its components when adsorbed onto a glass substratum reduced neutrophil leucocyte adhesion. Although their effects varied quantitatively during the experiments, the relative effect of the different proteins was always consistent.

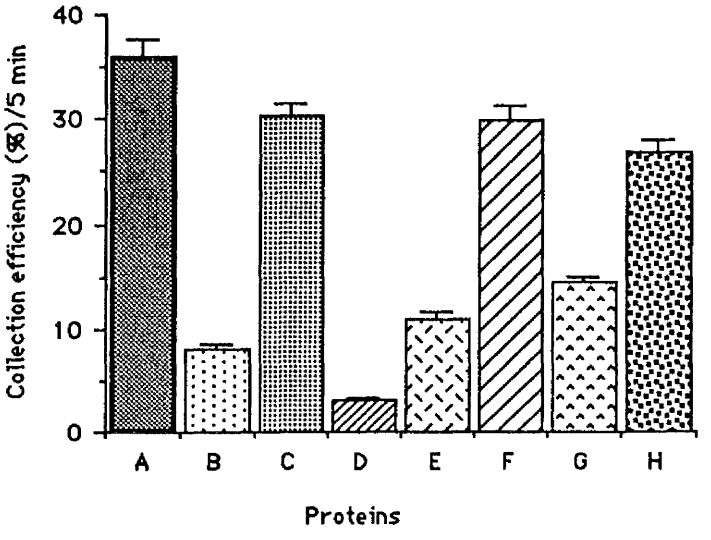


Fig. 3-11 : Effect and comparison of 100 µg/ml of different proteins on neutrophil adhesion from flow.

- Clean Glass
- cryoprecipitate
- Fibronectin-depleted cryoprecipitate
- Fibronectin
- plasma
- Fibronectin-depleted plasma
- serum
- Factor VIII

CHAPTER 4

Results II.

I) Effect of chemotactic factors on neutrophil adhesion from flow:

Neutrophil leucocytes respond to many different stimuli, and to function normally in defense against infection or as scavengers of damaged tissue they must be capable of recognizing a wide variety both of phagocytosable particles, and chemotactic factors. Chemotactic factors for neutrophils fall into 4 categories:

* N-terminal blocked peptides, of which the best known is FMLP.
* Products of arachidonic acid metabolism, particularly the leukotrienes.
* A complement-derived peptide C5a generated by proteolytic cleavage of C5 in both the classical and alternate pathways of complement activation.
* Denatured proteins.

Chemotactic factors diffusing from the inflammatory focus might act primarily on the neutrophils to stimulate adherence to the endothelium, which would require that the adhesiveness is capable of rapid modulation. Accordingly, the effect of the chemotactic factor FMLP and zymosan-activated serum on neutrophil adhesion was studied using the flow chamber to measure the changes which might occur immediately after the

cells and the chemotactic factor come in contact.

a) **Effect of FMLP:**

Using the flow chamber the effect of the chemotactic factor FMLP at different doses was first tested. These experiments were done in a slightly different way from those reported in the previous chapter: A neutrophil suspension and a solution of FMLP were perfused through a "Y" shaped manifold by means of two pieces of rubber tubes (about 10 cm) in length, and the manifold was then directly connected to the flow chamber (for more detail see materials and methods).

This sort of modification in the system has not been used before, and the idea behind this experiment was to try to measure the time course of neutrophil response to the chemotactic factor. Contact between the cells and the chemotactic factor occurs in the manifold when the solutions in both syringes were mixed and consequently, knowing approximately how long it takes for a flowing neutrophil to reach the area of the flow chamber in which adhesion is scored, it is possible to estimate how long it takes for the cell to respond to a change in the suspension.

The time estimate shown in Table 4-1 was made by measuring the time between switching on the syringe drive and the time at which the first cells (already mixed with the chemotactic factor) appeared in the area of the chamber where the adhesion was scored. The

No. of experiments	Mixing time (sec.)
1	26 (6)*
2	20 (6)
3	21 (6)
4	23 (6)
5	20 (6)
6	20 (6)
7	21 (6)

Table 4-1: Time course at which cells were mixed with the chemotactic factor. Results represent the mean time taken from 7 experiments ; * : number of replicates.

figures represent the mean time measured from 7 separate experiments.

The results in Fig.4-1 show that a high dose (10^{-7}M) of FMLP clearly induced an increase in the number of adherent cells to BSA coated chambers. Adhesion was reduced to about half at 10^{-8}M compared either to control (cells treated only with H_2), or to higher dose of FMLP. Adhesion reached a minimum at 10^{-9} and 10^{-10}M, and returned to control levels at lower concentrations.

In general chemotactic factors appear to decrease adhesion of neutrophils in vitro unless added above the optimum dose for chemotaxis, at concentrations which would induce secretion and the metabolic burst. The effect of various doses of FMLP on neutrophil adhesion from flow show a rather peculiar dose response curve (Fig 4-1) where it can be seen that a slight increase in adhesion occurred only at 10^{-7}M of FMLP. 10^{-7} M is the lowest dose which will give chemiluminescence response (Lackie & Lawrence, 1987).

Addition of chemotactic stimulus (FMLP) also caused shape change as illustrated in (Fig.4-2), cells tended to be more flattened rather than rounded.

b) **Effect of zymosan-activated serum:**

Under the same conditions and using the previous procedure for looking at the effect of FMLP on neutrophil adhesion from flow, normal serum (NS), zymosan-activated serum (ZAS), heat-inactivated serum (HIS) and heat

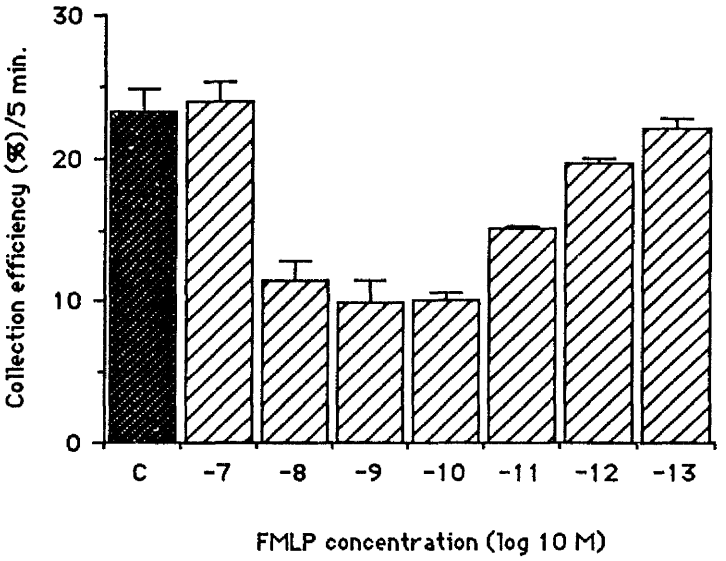


Fig. 4-1 : Effect of different FMLP doses on neutrophil adhesion to BSA coated chambers.

C : Control.

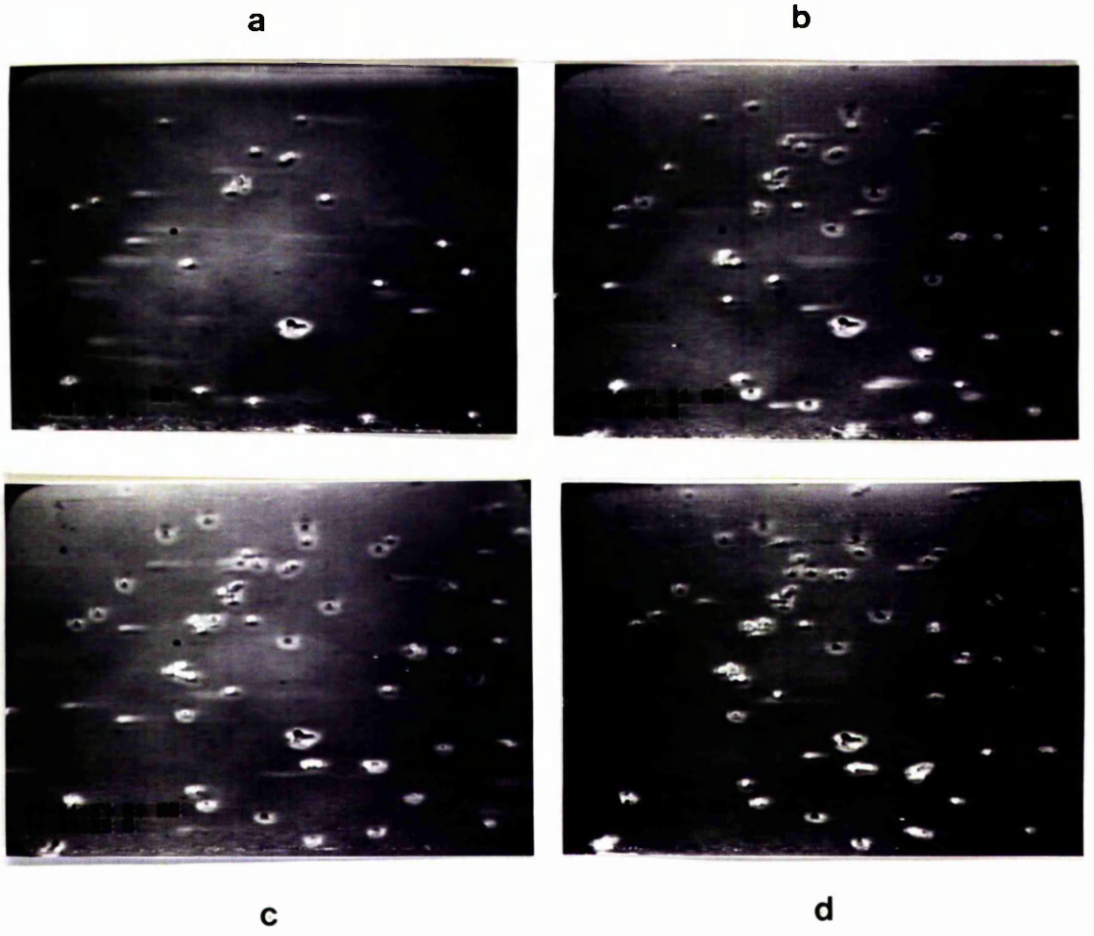


Fig. 4-2: Four frames from a time-lapse videotape record of neutrophil adhesion in the flow chamber. Photographs show the increase of adherent cells treated with FMLP 10^{-7}M after 1 minute (A), 2 minutes (B), 3 minutes (C), and 5 minutes (D). Flattening of cells started after the first minute.

inactivated serum treated with zymosan (ZIS) were tested for their effect on neutrophil adhesion to BSA-coated chambers were compared.

As seen in Fig.4-3 ZAS (10%), which contains C5a as chemotactic factor enhanced adhesion to greater extent than comparable concentration of normal serum or heat-inactivated serum. Chape change also occurred when cells were treated with zymosan-activated serum (Fig. 4-4). Zymosan treatment of heat-inactivated serum also had little effect on the adhesion of neutrophils to BSA coated chambers. But since the main purpose of this experiment was measuring the time course for neutrophils to respond to a change which occurred in the suspension, the stimulus is not so important as the effect.

Following treatment with FMLP and 10% ZAS, significant changes in neutrophil adhesiveness occurred within 20 sec as illustrated earlier in this section; this seems a very long time for a neutrophil which is travelling downstream at the sort of speed, known to occur in vivo. Comparable speeds of cell travel are found in the flow chamber, where the wall shear stress is comparable to that found in the post capillary venules. Taking the lowest possible estimate of cell speed (6 ʌm/sec) based on the movement of cells near the chamber wall (Forrester and Lackie, 1984), the neutrophil would have travelled about 120 um in 20 sec. Thus if the altered behaviour takes 20 sec the response will be some way

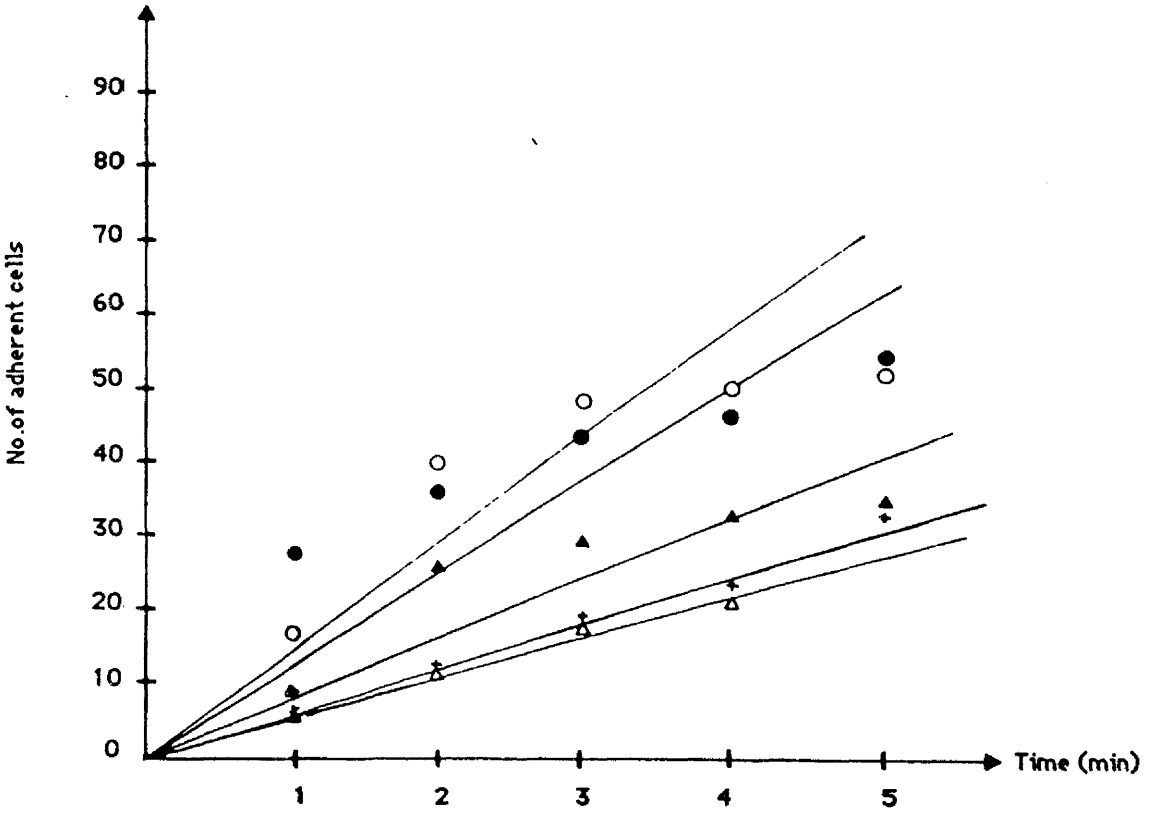


Fig. 4-3: Neutrophil adhesion to chambers coated with 100 µg/ml zymosan-activated serum (O–O), zymosan-inactivated serum (▲–▲), normal serum (+–+), heat-inactivated serum (△–△). (Clean glass ●–●).

Flow time	Collection efficiency (%)				
(min)	Control	NS	ZAS	HIS	ZIS
1	26	4	19	4	8
2	19	4	15	6	6
5	15	4	12	4	5

Table 4-2: Effect of zymosan-activated serum on neutrophil adhesion to BSA coated chambers compared to normal serum (NS) heat-inactivated serum (HIS), and zymosan-inactivated serum (ZIS).

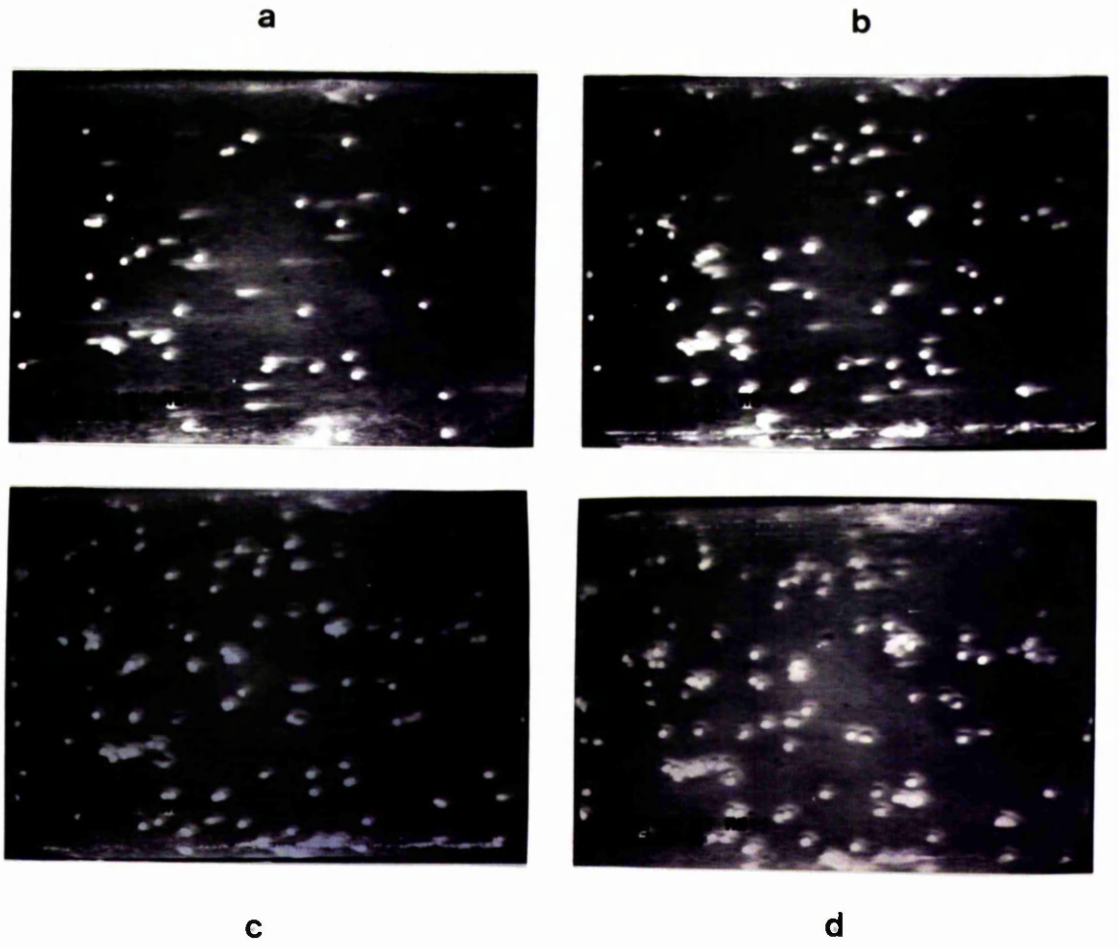


Fig. 4-4: Frames from a time-lapse videotape of neutrophil adhesion in the flow chamber. Photographs show the increase in adherent cells (treated with zymosan-activated serum) after 1 minute (A), 2 minutes (B), 3 minutes (C), and 5 minutes (D). Flattening of cells started after the first minute.

away from the site; it would take a neutrophil about 10 min to travel this distance (in a straight line). In my experiments, neutrophil response to the chemotactic factors occurred almost within the first third of the chamber where the adhesion was measured. This implies, that the changes could have occurred earlier in the manifold, because there was a delay between the mixing of the peptide with the cells, and the measurement of adhesion. Although this problem could have been reduced somewhat by redesigning the chamber and the manifold, a more direct approach to the question was used and will be discussed in the next section.

II) **Neutrophil response to immune complexes:**

Complement activation is an efficient way of attracting many leucocytes to the focus of inflammation. The major chemotactic peptide generated during activation of complement is C5a which is probably extremely important as a chemotactic factor, because it can be generated both by classical and alternative pathways. The former pathway will be triggered when Ab reacts with Ag in presence of fresh serum; neutrophil response to immune-complex formation was studied using the flow chamber assay. As mentioned earlier, this also provided a more direct approach to estimating how rapidly the cells respond to the changes which occured on the surface. Stripes of proteins were air-dried in the centre of the lower surface of the flow chamber

(before this latter was assembled), so that neutrophils flowing through the chamber passed over three main surfaces (1.3 cm apart); firstly a non-coated position; secondly the protein-coated position; and the other uncoated position (see Fig 2-2 in Materials and Methods). The attraction of neutrophils to the protein stripe was measured by scoring the number of adherent cells at different positions along the chamber, and knowing where the stripe is situated, I could determine how far downstream of the boundary protein the change occurred.

Using conalbumin as the protein stripe, the neutrophil response was very slow (Fig.4-5), but when cells were suspended in 10% fresh serum containing a high titre of antibody to conalbumin, the response was very rapid and occured within the first few microns of the stripe edge (Fig 4-6). Suspending cells in heat-treated serum did not however, did not seem to have much effect, and the neutrophil response was slower and less marked (Fig.4-7). Figure 4-8, shows the changes which occurred at the edge of the stripe.

Looking at these results, one can say that the complement activation through the classical pathway induces a very rapid response for neutrophil leucocytes.

III) **Neutrophil response to complement activation through the alternative pathway:**

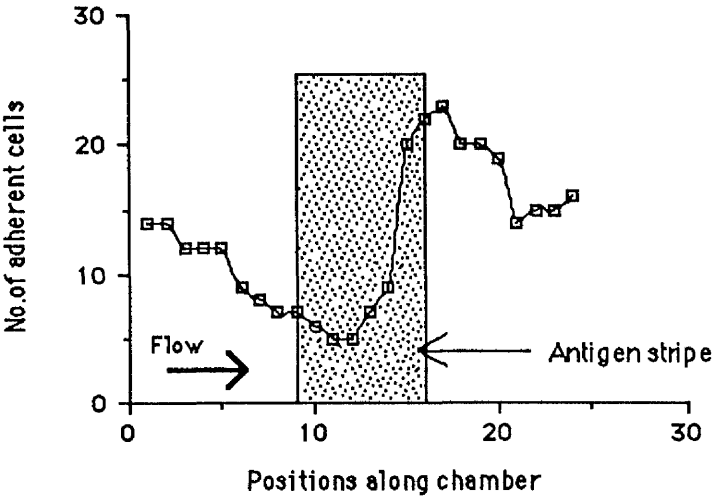


Fig. 4-5: Number of adherent neutrophils at different positions along the chamber. The chamber was pretreated with BSA-containing buffer before the suspension of the rabbit neutrophils was pumped through. The position of the conalbumin stripe is shown.

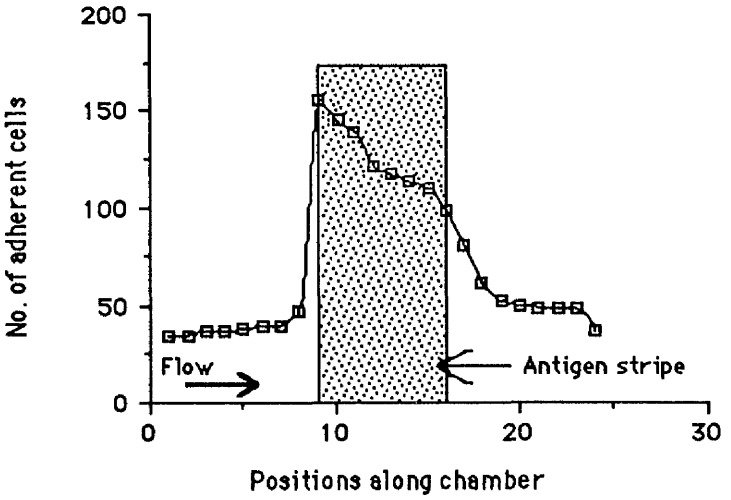


Fig. 4-6: As for control (Fig. 4-5), but the neutrophils were suspended in buffer containing 10% rabbit serum with a high anti-conalbumin titre.

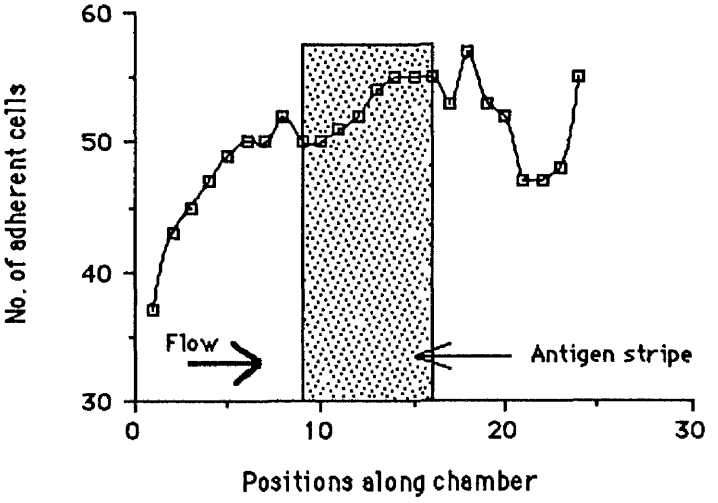
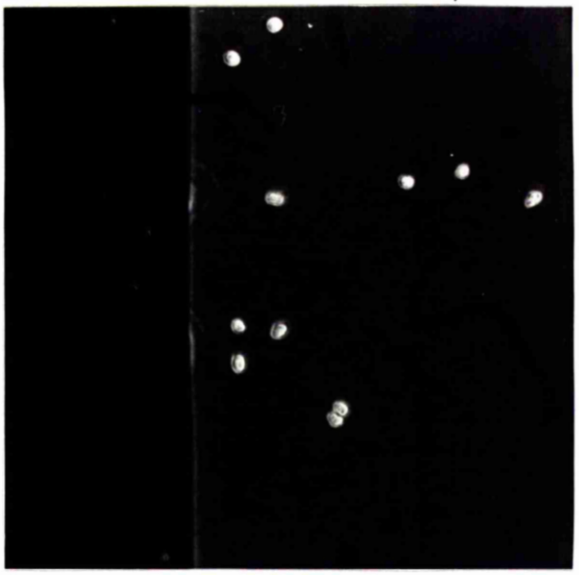


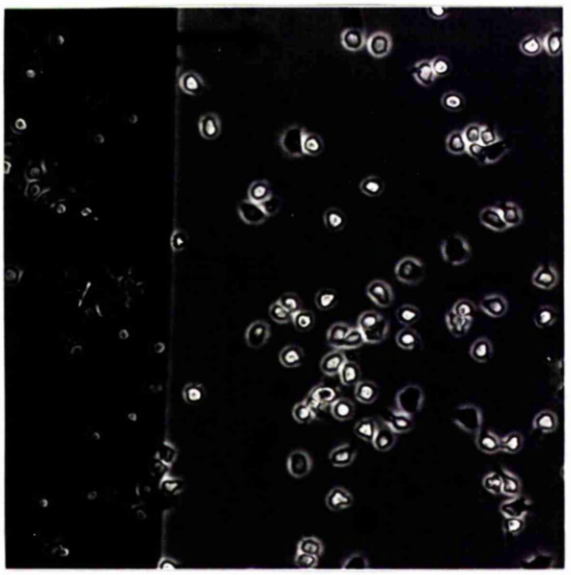
Fig. 4-7: As for control (Fig. 4-5), but neutrophils were suspended in buffer containing heat-treated serum (56°C, 30 min).

Fig. 4-8: Photographs showing the accumulation of neutrophils on the antigen stripe (conalbumin). Cells were suspended in H_2 only (A), in 10% fresh serum containing high titre of anticonalbu - min (B), and in heat-treated serum (C). Arrows show the flow direction.

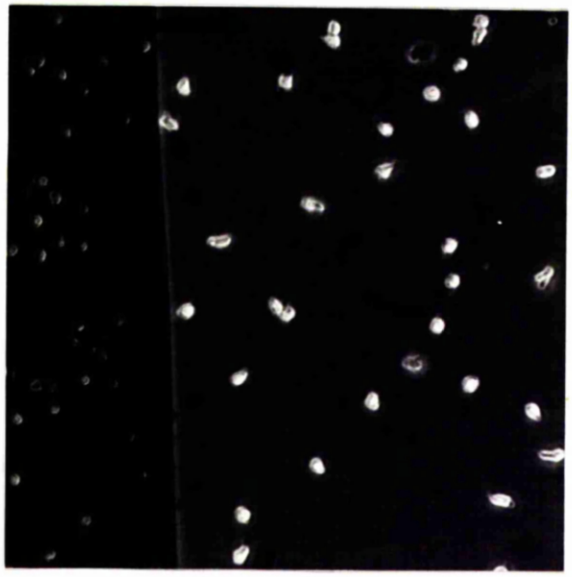
A



B



C



Complement activation through the alternative pathway usually occurs when polysaccharides such as yeast cell wall zymosan, or Gram negative bacterial endotoxin are added to serum. In the present experiments, stripes of zymosan-containing agarose were air-dried on the lower surface of the chamber and cells were suspended in (non-immune) fresh serum, so that the activation of the complement components could be induced when the cell suspension containing the fresh serum flowed over the zymosan stripe. Similar results to neutrophil response to immune complexes were found. The zymosan stripe did not attract many cells when they were only suspended in H_2 (Fig.4-9), but adding 10% fresh serum to the cells suspension rapidly activated neutrophil response (Fig.4-10). No significant response was seen when cells were suspended in heat-inactivated serum (Fig.4-11). Almost similar results were obtained when the zymosan-containing agarose stripes were pre-washed with fresh serum (Fig 4-12). Suprisingly, there was almost no difference in trapping cells when the zymosan stripe was pre-washed with heat-inactivated serum (Fig 4-13) although this latter has been inactivated in a similar way as the heat-treated serum used in cell suspension.

Summary:

1) The chemotactic factor FMLP had a concentration-dependent effect on the adherence of rabbit peritoneal neutrophils to BSA coated chambers. The complement-

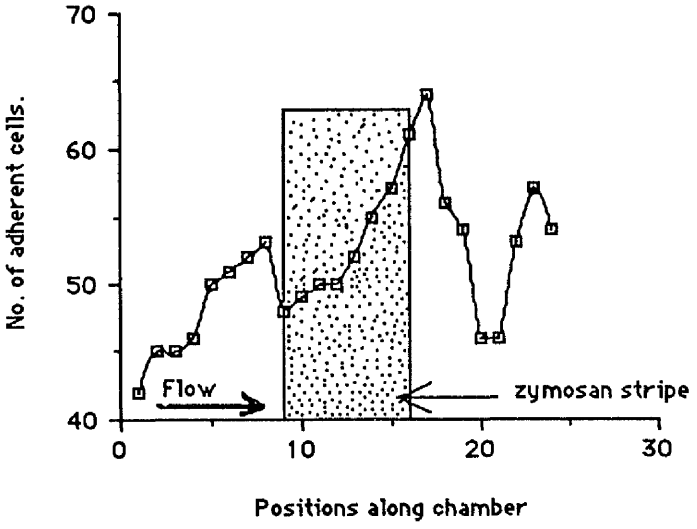


Fig. 4-9: Number of adherent cells at different positions along the chamber. The chamber was pre-washed with BSA-containing buffer before the suspension of rabbit neutrophils was pumped through. The position of the zymosan stripe is shown.

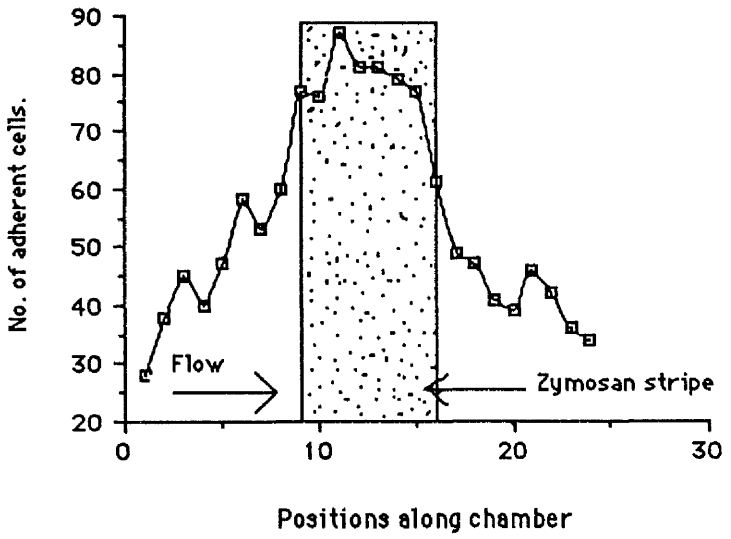


Fig. 4-10 : As control (Fig.4-9), but the neutrophils were suspended in buffer containing 10% non-immune rabbit fresh serum.

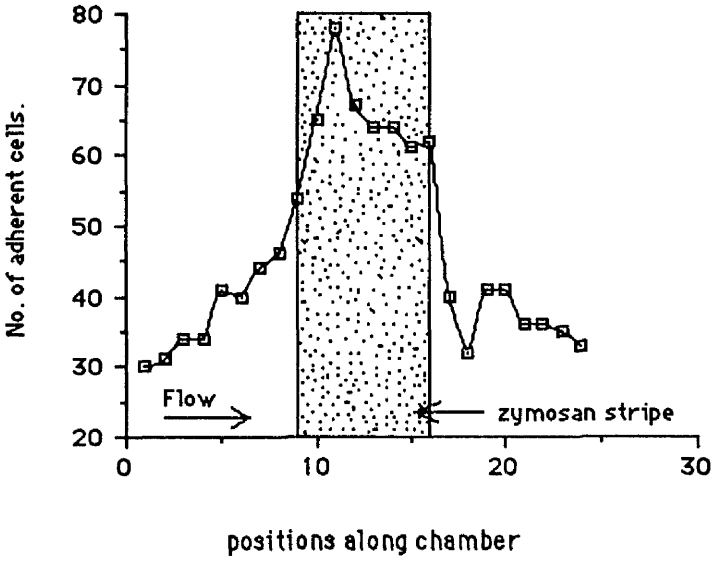


Fig. 4-11: As control (Fig. 4-9), but the neutrophils were suspended in buffer containing heat-treated serum.

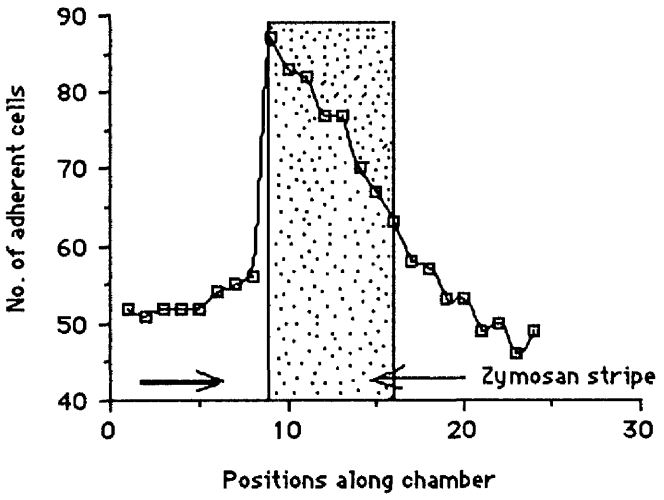


Fig. 4-12: Number of adherent neutrophils at different positions along the chamber. The chamber was pretreated with 10% fresh serum before cells in H_2 were pumped through.

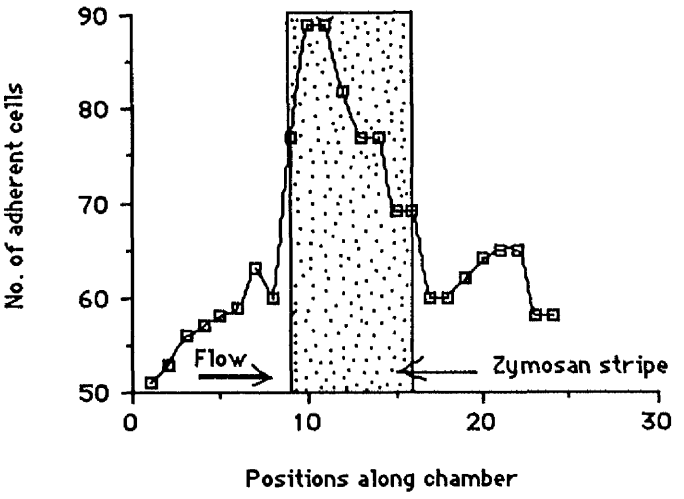


Fig. 4-13: As for (4-12), but the chamber was pretreated with 10% heat-inactivated serum.

derived chemotactic peptide C5a, probably generated by zymosan-activated serum also increased adherence of neutrophils to BSA coated chambers, compared to normal serum and to heat-inactivated serum.

2) Rabbit peritoneal neutrophils responded rapidly to a change in the surface of the flow chamber. This occurred when immune complexes were formed provided the serum was untreated or when the complement system was activated through the alternative pathway.

CHAPTER 5

Discussion:

As a prelude to their emigration into tissues, leucocytes must form an adhesion to the lining of the vessel. The adhesiveness of neutrophil leucocytes has therefore received considerable attention although most adhesion assays are poor models of the physiological situation.

The present study describes a reliable and reproducible assay for cell adhesion, which has been applied to the adhesive interactions of neutrophil leucocytes. Neutrophil adhesion to surfaces coated with various plasma components, the materials with which these cells are normally in contact have been tested, as have neutrophil adhesion changes in response to chemotactic peptides (FMLP), zymosan-activated serum, immune complexes, and to complement activation. The assay involves the adhesion of the neutrophil from flow, and as such models the adhesive interaction involved in margination in vivo.

The main findings of this work can be summarized as follows:

1. Several (but not all) plasma proteins tested, slightly decreased the adhesiveness of rabbit peritoneal neutrophils. Fibronectin and cryoprecipitate brought about a dramatic inhibition of neutrophil adhesion.

2. Neutrophil adhesion to BSA coated chambers was stimulated by the chemotactic factor FMLP, and by 10%

zymosan-activated serum. The response was rapid and dose dependent; neutrophil adhesiveness was increased at higher doses of FMLP, went to a minimum at optimum doses for chemotaxis and then returned to control level at lower concentrations.

3. Rabbit peritoneal neutrophils rapidly responded to a local change induced by contact with a new substratum (conalbumin, or zymosan-containing agarose) in the presence of freshly activated complement.

These findings will be discussed in turn below:

1. **Effect of plasma proteins on neutrophil adhesion:**

Although the various plasma proteins tested varied in the extent to which they reduced adhesion, the medium (plasma) in which neutrophil leucocytes normally circulate can be considered as a "low adhesion buffer". The inhibition of neutrophil adhesion to fibronectin-coated surfaces is the most marked effect, and perhaps the most surprising, and is discussed in detail below.

It has been argued by Grinnell (1978) and Grinnell & Feld (1979) that fibronectin is essential for the adhesion and spreading of fibroblasts. They found that cells that were constitutionally unable to synthesize fibronectin were unable to adhere well and spread on polystyrene surfaces unless medium was supplemented with fibronectin or serum, or unless fibronectin was adsorbed to the substrate. Unlike fibroblasts, the adhesion of neutrophils to fibronectin coated surfaces was found to

be very weak.

It has been recently found (Burns et al., 1986) that the IIb-IIIa glycoprotein complex (which functions as the receptor for fibrinogen on platelets and is central to platelet aggregation) is also expressed on the surface of leucocytes, and could, in principle at least, function as a receptor for fibronectin. The findings reported in this work are not in accord with the idea that Fn/receptor interactions are important in neutrophil adhesion. Since neutrophil adhesion to fibronectin, using both the static assay and the flow chamber, showed a very low binding of neutrophils to fibronectin-coated surfaces. In fact, we do not know that neutrophils actually bind to fibronectin on a surface, though the presence of a receptor may make this possible. Moreover, this low adhesion on a fibronectin coated surface, could be because the binding of fibronectin does not trigger spreading and the Receptor-ligand interaction contributes little to the adhesive interaction. The low adhesion to fibronectin found in this study confirms observations of Forrester & Lackie, (1984); and of Brown & Lackie, (1982). They are also consistent with those of Pommier et al., (1984) who reported that neutrophils had very low levels of IIb-IIIa antigen complex, as judged by the binding of a monoclonal antibody (25E11) which specifically identifies the IIb-IIIa glycoproteins complex.

The observation that neutrophils do not adhere to adsorbed fibronectin, confirmed here by the flow chamber assay, is also consistent with the general pathological view; that in vivo complete endothelial cell removal leads to platelet sticking (platelets will stick and spread on fibronectin coated surfaces in vitro) rather than neutrophil adhesion.

Because cryoprecipitate contains a high fibronectin concentration (2mg/ml), this may also explain the marked decrease in neutrophil adhesion which was found when cryoprecipitate-coated substrata were used for adhesion. This is further supported by the observation that removing Fibronectin from cryoprecipitate makes in a poor inhibition.

As a general conclusion one can say that plasma and serum contain at least one (or possibly other) components which will produce non-adhesive surfaces for neutrophils in vitro. It therefore seems likely that, in the vascular system, they contribute to maintaining a non-adhesive environment for cells.

2. **Effect of chemotactic factors on neutrophil adhesion from flow:**

Although circulating freely, neutrophil leucocytes can leave the blood stream and infiltrate any site of tissue damage or microbial invasion. Whether formed by the host or released by bacteria, these factors cause neutrophil leucocytes to marginate along nearby

endothelial surfaces, move extravascularly and accumulate near sites where a chemotactin has been generated.

The effects of chemotactic factors on neutrophil behaviour have been widely studied. The initial response for neutrophils to chemotactic factors appears to be a stimulation of locomotion (Zigmond & Sullivan, 1979) and a transient increase in adhesion which can be detected in high-shear aggregation assays (O'Flaherty et al., 1978). The effect of chemotactic factors on neutrophil adhesion was was studied by Smith, R.P.C., et al., (1979), who reported that in the early stages of exposure the effects of low concentration of chemotactic factor (FMLP) was to decrease the adhesiveness of neutrophils as can be shown using low-shear aggregometry or adhesion to substratum.

Using a rather different adhesion assay, the flow chamber where the shear forces are approximately similar to those found in the post capillary venules (around $4x10^{-11}$ N), the effects of the potent chemotactic factor (FMLP) and zymosan-activated serum on neutrophil adhesion to BSA-coated chambers were tested. By using the flow chamber it is possible to look at the kinetics of attachment of leucocytes to the lower surface of the chamber, but a rather different question can be addressed using the flow method; how long does it take for a cell to respond to a change which may occur either in the suspending medium or in the surface of the

chamber? This timing of response is very important because it relates very directly to the problems faced by leucocytes when they pass an area of inflammation.

In these experiments, I found significant changes in adhesiveness within 20 secs of exposure, but this time resolution (due to the design of the "Y" shaped manifold), seems inadequate because the changes could have occurred during the delay between the mixing of the peptide with the cells and then measuring the adhesion.

The dose response curve of the potent chemotactic factor FMLP (confirmed here by the flow chamber) is consistent with those results obtained by Lackie & Smith, (1980). High concentrations of FMLP (above the chemotactically "optimum" level), rapidly induced an adhesion increase, whereas the effect of low concentrations (10^{-9}, 10^{-10} M) of the chemotactic factor at the same time of exposure was to decrease, in a dose-dependent fashion, the adhesiveness of neutrophils.

This dose response curve (increased adhesion at high concentrations and decreased adhesion at lower concentration of the chemotactic factor) may be related to the response of neutrophil leucocytes to chemotactic gradients and various potent chemotactic factors associated with the inflammatory response. Leucocytes must not only detect and respond to a gradient of chemotactic factor but must also accumulate at the source. Near the vessel wall chemotactic factor

concentration is low due to the proximity of the circulating blood; once the neutrophil has marginated reducing adhesion may actually facilitate its movement through the extracellular matrix. As the focus of damage where the chemotactic factors are mostly concentrated is approached, the adhesiveness increases and the cells may be trapped. Neutrophils moving up-gradient towards the focus of the lesion become trapped by their increased adhesion which is also associated with the secretion of lyzosomal enzymes (Lackie, 1977) which are required at the inflammation focus.

Many recent investigations have been concerned with the effect of chemotactic factors on neutrophil leucocyte aggregation as well as their adhesion to artificial and cellular substrata. For instance, Charo et al., (1986) found that high concentrations of FMLP and C5a enhanced human neutrophil adhesiveness to endothelial monolayers in a concentration-dependent manner. Similar effects, have been observed in studies of neutrophil leucocyte adherence to artificial surfaces (e.g. plastic, glass). Smith et al., (1979) for example, found that low chemotactic concentration of FMLP (0.1 to 1.0 nM) decreased adherence of rabbit peritoneal neutrophils to cultured porcine aortic endothelial cells, whereas higher concentrations of FMLP (>10 nM) which decreased directed migration of rabbit PMN augmented adherence.

Although, numerous recent studies on the effect of

FMLP and other chemotactic factor on leucocyte adhesion have been investigated, comparison of results remains difficult because of the diversity of the assays used. It has, for instance, been reported that FMLP can decrease adhesion, be ineffective or produce an increase, depending on time of exposure, concentration and probably differences in the test procedure (Smith et al., 1979; Fehr & Dahinden, 1979; Keller, Wissler and Damerau, 1981). The complexity of the dose-response relationship and the rapid time course of some effects undoubtedly contribute to the confusion in this area.

Moreover, it is not clear whether the adhesion changes observed in this study (using the flow chamber and the manifold) are relevant to the events occurring during margination of neutrophils. But, in general the adhesion changes are of some interest because of the varying requirments for adhesion as the neutrophil adheres to the wall of the blood vessel, moves through a three-dimensional matrix and then becomes trapped in the lesion.

On the whole, the precise mechanism by which high concentrations of FMLP or complement (C5) derived chemotactic peptides augment the adhesiveness of neutrophil leucocytes is unknown. Although altered surface charge (Gallin, J.I., 1980) may be an important factor, the role of granule constituents (Bockenstedt et al., 1980; and Oseas et al., 1981), oxygenation products

of arachidonic acid (Spagnuolo et al., 1984), and plasma membrane proteins (Todd et al., 1984; Arnaout et al., 1984) also have to be considered. Concerning the latter, evidence has been presented recently that chemotactic factors may augment leucocyte adherence by provoking translocation of "adhesive glycoproteins" from an intercellular pool (perhaps in specific granules) to the cell surface.

3. **Neutrophil response to complement activation:**

Some other features of the inflammatory lesion such as the deposition (formation) of immune complexes may also contribute to adhesive trapping of leucocytes.

Rabbit peritoneal neutrophil response to immune complexes, confirmed here by the flow chamber assay, showed that cells suspended in 10% rabbit serum containing a high titre of antibody to conalbumin, were very rapidly trapped by a conalbumin stripe on the lower surface of the chamber (see chapter IV). In this experiment, I have tried to model the situation which may occur when immune complexes are deposited on the wall of microvessels adjacent to a lesion. If localization of neutrophils near an inflamed site depends upon altering the cell-vessel wall adhesion then deposition of immune complexes seem to be effective: wherever immune complexes are developed or deposited then the adhesion of neutrophil leucocytes will be increased.

Although the adhesion assay used here was slightly modified, the results obtained confirm those found by Lackie and Forrester (1985) also using the a flow system. These showed that non-immune IgG either present at physiological levels in the suspending medium or preadsorbed to the chamber did not affect adhesion, but, when the chambers were coated with BSA and then with a purified IgG directed against BSA the surface trapped cells very effectively.

One possible mechanism for neutrophil accumulation on immune complexes is that these cells become attached to substratum-bound immunoglobulin by their Fc receptors (Wilkinson & Lackie, 1985). It is also worth pointing out that as complement is present in the extracellular fluid, immune complexes formed within the tissues will activate the classical pathway and localized complement activation would result in the formation of chemotactic factor (probably C5a). This mediator could promote neutrophil margination, chemotaxis and subsequent local accumulation of these cells.

Using dried stripes of zymosan-containing agarose as substratum within the flow chamber as described before, rabbit peritoneal neutrophils suspended in fresh serum rapidly got trapped as they were flowing over the stripe. Pre-washing chambers (initally pre-coated with zymosan-containing agarose) with fresh serum also rapidly trapped the cells almost in the manner when cells were suspended in fresh serum-containing medium.

The changes in cellular behaviour, which turned out to be very rapid as shown in this kind of experiment, seem to suggest that not only the local activation of complement through the classical pathway elicits an extremely marked increase in margination, but so does the alternative pathway, which would have been activated by the zymosan stripe. On the whole, it seems that the adhesive interaction must be extremely rapid, otherwise the cells would have swept away downstream; the change induced by contact with a new substratum in the presence of freshly activated complement is sufficiently fast to permit a local response rather a down stream response.

The obvious extension of this project which can be followed using the flow chamber is to use an endothelial cell monolayer as the lower surface of the chamber. In this respect the use of chamber designed by Owens, Gingell & Rutter (1987) may prove more convenient system for looking at neutrophil adhesion to endothelial monolayer under flow conditions.

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