In Vitro Studies of the Adhesive Interactions of Neutrophil Granulocytes

A thesis submitted for the degree of

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March 1980

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Acknowledgements

I wish to thank my supervisor, Dr. J. M. Lackie for his patience and encouragement throughout this work, Professor A. S. G. Curtis for having me in his department and everybody else in the Cell Biology Department for help and advice. During this work I was in receipt of a Medical Research Council Studentship.

Contents

Summary

The adhesion of rabbit neutrophil granulocytes to cultured pig aortic endothelial cells, to serum coated glass and to each other has been studied under a variety of conditions using adhesion-to-substratum and aggregation assays.

Under standard conditions, in the presence of balanced salt solution (BSS) and serum alone a number of variables were found to alter the level of adhesion to serum coated glass and endothelium. Changes in time of incubation, cell number, serum concentration and assay volume all altered the observed level of adhesion. Adhesion to serum coated glass was higher than to endothelial cells.

The inflammatory mediators histamine and 5-hydroxytryptamine (5-HT) had slight inhibitory effects while bradykinin had no significant effect on adhesion.

Anti-inflammatory drugs were examined. Aspirin, hydrocortisone and colchicine all reduced adhesion. Prednisolone, indamethacin and amino-n-caproic acid had no significant effect on adhesion.

A range of miscellaneous agents were tested for effects on adhesion. These were aminophylline, formalin, heparin and several sugars and had slight, if any, inhibitory effects on adhesion.

A range of chemotactic factors were shown to have diverse effects on adhesion. At chemotactically optimal concentrations, α_s-casein, β-casein, alkali-denatured human serum albumin (HSA) and several synthetic n-formyl-di-and tri-peptides reduced neutrophil adhesion

after short ($\ll$ 30 min.) periods of incubation.

Detailed study of one such peptide, n-formyl methionyl-leucyl-phenylalanine revealed that at higher than chemotactic optimum concentrations, neutrophil adhesion was less reduced and that after longer times of incubation, was increased.

An inverse relationship was observed between neutrophil adhesiveness and locomotion. This was shown at various concentrations and times.

In parallel with increased neutrophil adhesion, an increase in lysosomal enzyme secretion was demonstrated. This, and the adhesive increase were inhibited by the presence of hydrocortisone sodium succinate.

No increases in endothelial adhesiveness induced by chemotactic factors were ever observed.

It was concluded that the adhesive changes observed were not related to the process of margination in vivo but that they may be related to the pattern of neutrophil movement in vivo.

CHAPTER 1

General

The white cells (leucocytes) of mammalian blood migrate out of the blood vessels in a variety of locations and in various circumstances. To do this they must recognize the appropriate site, adhere, and move over and through the endothelium into the tissues. The problems raised by these processes have been the subject of investigation for many years and much is now understood about the events that occur e.g. during inflammation or in lymphocyte recirculation. The control of the initial adhesion of white cells to endothelium, however, is still obscure and it is on this subject, with particular reference to neutrophil granulocyctes and inflammation, that this introduction will concentrate.

White blood cell terminology and distribution

Granulocytes comprise the largest group of leucocytes and are characterized by the presence of large cytoplasmic granules and by a nucleus that contains several bodies. There are three subclasses of granulocyte, based on whether their granules stain with acidic dyes (eosinophil granulocytes), basic dyes (basophil granulocytes) or neutral dyes (neutrophil granulocytes). This latter group is commonly referred to by a variety of synonyms including neutrophil, polymorph, polymorphonuclear leucocyte, PMN. The other broad class of leucocytes are the lymphoid or mononuclear leucocytes. Most of these are lymphocytes which are further broadly subdivided into classes of T and B lymphocytes. The remaining lymphoid leucocytes are monocytes (mononuclear phagocytes) which differentiate into macrophages, generally found in tissues and rarely in blood.

This classification system is the strictly correct one but another common usage of some of the terms exists. Leucocyte is commonly used

to refer to granulocytes so that white cells are classified as leucocytes, lymphocytes and monocytes.

The proportions of the various classes of white cells for man and the rabbit are given below.

	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Man	59	2.7	0.5	34	4
Rabbit	46	2	5	39	8

(Source: Biology Data Book (1964) ed. D.S. Dittmerr (Federation of American Societies for Experimental Biology)).

The distributions and fates of the various classes of white cell are quite different, reflecting the very different roles they play. After release from the bone marrow, granulocytes spend only a few hours in the blood after which they migrate into the tissues where several days are usually spent before they either die or are excreted across mucous membrane (Cline, 1975). Once a mature granulocyte enters the tissues it does not normally return to the circulation. The cells are incapable of division. Monocytes also migrate from the blood into tissues where they proliferate and differentiate into macrophages. Lymphocytes display a range of life cycles and distribution patterns, some involving recirculation between blood and lymph for years. Cline (1975) reviews the whole field of leucocyte production, distribution and life cycle.

Neutrophil granulocyte function

The pivotal role of the neutrophil granulocyte in the body's defence against infection or injury is now well known. In the tissues their major function is the engulfment and digestion of invading micro-organisms and damaged tissue. This function is achieved in several (arbitarily defined) stages.

1. Adhesion to the endothelial cells lining the small blood vessels.
2. Initiation of cell movement.
3. Movement over and through the endothelium.
4. Movement towards the damaged or infected site.
5. Phagocytosis of the offending material.

This process (which probably occurs under normal circumstances) is only one observable component of an extremely complex series of responses of living tissue to injury which constitute the acute inflammatory response. Of the stages of this process, the first is one of the least understood. It is not known what initiates adhesion or how that adhesion occurs. Much work has been done on the subjects of cell adhesion and of inflammation and many excellent reviews exist on both. Curtis (1973), Marchase, Vosbeck and Roth (1976), Edwards (1977) and Grinnell (1978) have reviewed aspects of cell adhesion, while inflammation is well covered by the books and reviews of Hurley (1972), Zweifach, Grant and McCluskey (1973), Ryan and Majno (1977) and Vane and Ferreira (1978). Accordingly only those aspects of adhesion and inflammation necessary for the appreciation of the significance of the studies that have been performed will be discussed.

Early vascular and cellular events in inflammation

Most of the events in inflammation involving the vascular system take place in that part of the vascular system known as the microcirculation. The blood vessels of this are the small arteries (30-250 μm internal diameter), arterioles (30-20μm), capillaries (down to 2μm), post capillary venules (20-50μm) and the small veins (50-250 μm). Vessels > 300μm internal diameter are visible to the naked eye and are not considered part of the microcirculation. The rate of blood flow in the microcirculation is slow, ranging from 0.2 - 4.0mm

sec^{-1}, compared with rates of up to 800 mm sec^{-1} in the aorta and large arteries and $\sim$ 150 mm sec^{-1} in the veins. Flow is generally laminar and due to the geometry of the blood vessels and the rheologic properties of blood, divided into an axial stream containing the cells and a peripheral or marginal cell free zone adjacent to the endothelium.

Three main events characterize the early stages of an acute inflammatory response.

1. Changes in vascular flow and calibre.
2. Changes in vascular permeability.
3. Adhesion and emigration of leucocytes.

Changes in flow and calibre

Immediately after injury there may be a transient constriction of arterioles which does not always occur and which is not regarded as being of importance. Following this there is a widespread dilation of arterioles and venules in the area of injury and the opening up of many previously closed vessels. Since volume flow is proportional to the fourth power of the radius, blood flow increases markedly and may increase as much as ten fold (Aschheim and Zweifach, 1961). Initially flow through the dilated vessels is rapid and this stage is of variable duration. After mild stimuli such as the local application of histamine, rapid flow lasts 10-15 minutes but after more severe injury may last for several hours (Hurley, 1978). Following this high flow phase, a low flow phase occurs and often stasis develops. The cause of slowing of flow is not clear but leakage of fluid from the vessels and the concomitant viscosity increase may largely account for the observations.

Permeability changes

From the earliest stages of inflammation, the vessel walls of the microcirculation become much more permeable to plasma proteins. Use of the techniques of vascular labelling (e.g. with carbon particles) and electron microscopy have elucidated the main features of the response. Increased permeability can be induced by many substances such as histamine, 5-hydroxytryptamine (5-HT), bradykinin. These agents cause transient opening of gaps between endothelial cells and leakage from venules and small veins but have no effect on the capillaries (Majno, Palade and Schoefl, 1961). Loss of plasma will result in an increase the blood viscosity in the inflamed area.

Leucocyte adhesion and emigration

Soon after injury, as the rate of blood flow decreases, leucocytes begin to appear in greater numbers in the marginal bloodstream and adhere to the luminal surface of the endothelium. Following adhesion, the cells move over the endothelial surface and into the surrounding tissues. The time course of adhesion following injury is very variable and widely differing times have been reported following differing injuries in different tissues. Sticking has been reported to start after 10 minutes (Allison, Smith and Wood, 1955a), 30 minutes (Carscadden, 1927), one hour (Cliff, 1966) and several hours (Florey and Grant, 1961). Adhesion and emigration occur predominantly in the post-capillary venules (Ryan and Majno, 1977). Migration is between the endothelial cells. Early suggestions that lymphocytes migrate through endothelial cells (Marchesi and Gowans, 1964) have been refuted by the observations of Schoefl (1972). Emigration is by an active cell movement and Clark, Clark and Rex (1936) reported that polymorphs passed through the venular

wall in 2 - 9 minutes. Movements through tissues at speeds of up to 20μm min^{-1} have been observed (Clark *et al.*, 1936). Leucocyte emigration and increased permeability are independent phenomena. The evidence for this is largely the difference in time course and magnitude of the two responses. Grant and Wood (1928) showed that the increase in permeability following histamine injection was not accompanied by significant leucocyte emigration. Hurley (1972), summarizing his work, noted the lack of correlation between permeability and emigration following single injections of saline, histamine or serum. Permeability had returned to normal in each of these cases before emigration started. Hurley, Ryan and Friedman (1966) studied the time course of leucocyte accumulation in inflammatory exudates. For mild irritants such as serum or glycogen solutions, neutrophil accumulation reached a peak at 4 hours and declined rapidly thereafter. As the response progressed, monocyte accumulation became more prominent.

Once in the tissues, it is almost certain that leucocytes are guided to a site of damage or infection by a chemotactic response. This view is supported by an abundance of *in vitro* evidence (Wilkinson, 1974) and although *in vivo* evidence is scarce and more difficult to interpret, *in vivo* chemotaxis has been reported (Buckley, 1963).

Neutrophil-endothelial adhesion: in vivo observations of the microcirculation

While it is clear that during inflammation large numbers of leucocytes adhere to endothelium and migrate, the normal level of adhesion is less certain. It seems important to understand what constitutes 'normal' in order that the exact nature of the pathological changes can be understood and so that explanations can be advanced based on this

understanding. Text-book descriptions of the process of margination often state that adhesion does not normally occur. Such statements may be misleading.

At the light microscopic level, the work of the Clarks (Clark and Clark, 1935; Clark *et al.*, 1936) is one of the most extensive studies performed. Leucocytes and endothelium were observed in the tails of amphibians and in rabbit ear chambers. It was argued that increased leucocyte adhesion following injury was due to a change in endothelial 'consistency' and the diagram of a series of changes has frequently been reproduced. It is worth noting however that the state referred to as 'normal - no sticking' (Clark and Clark, 1935) is described in text, for the tadpole (p.394).

"Under usual conditions the cells all slip or roll over and over along the wall without any tendency to cling to the vessel walls" and for the rabbit, (p.416):

"In observing a stable vascular plexus with active circulation, leukocytes are frequently seen rolling along the vessel walls or lying motionless at the edge of the moving stream. This phenomenon is particularly noticeable in the wider venous capillaries and venules and.....may be mistaken for leukocyte sticking."

It was concluded (p.434) that the slight reactions observed very frequently were not pathological, due to their high rate of occurrence.

These observations seem to indicate that although firm adhesion to endothelium may not occur normally, there is a definite attraction between leucocytes and endothelium which causes their rolling behaviour.

Another very extensive piece of work in this field is that of Vejlens (1938) who studied the distribution of leucocytes in the vascular

system. He regularly observed leucocytes rolling or gliding along vessel walls under normal circumstances, especially in the "para-capillary" vessels and venules. No leucocytes were observed in the arterioles. He also observed rolling leucocytes where blood velocity was reduced and the number of leucocytes seen was related to the blood velocity. Commenting that Cohnheim in 1882 had ascribed the appearance of marginal white corpuscles in large numbers to reduced blood velocity, Vejlens further stated: "...it is a characteristic feature in all circumstances where the circulation velocity is reduced and the white blood cells become marginal that polymorphonuclear leukocytes dominate."

Rolling of white blood cells along, or adhesion to, endothelial walls in normal circumstances has since been reported by a number of workers (Allison, Smith and Wood, 1955a; Zweifach, 1973; Atherton and Born, 1972; Schmid-Schoenbein, Yuan-Cheng Fung and Zweifach, 1975). A general criticism of this work, however, is that the trauma involved in the manipulations necessary to observe the microvasculature has generated a mild inflammatory response and resultant margination. Although it is virtually impossible to refute this criticism, the wide-spread observation of the phenomenon and its persistence for several hours argue against it. Moreover, there is another quite different body of experimental work, on neutrophil kinetics *in vivo*, which supports the view that normally, large numbers of leucocytes are marginated.

Neutrophil kinetic studies

Mauer, Athens, Ashenbrucker *et al* (1960) reported that when human granulocytes are labelled *in vitro* with radioactive diisopropyl fluoro-phosphate ($DF^{32}P$) and then returned to the circulation of the donor, about half the labelled cells could not be found in the circulation at

the completion of the infusion. Thereafter the remaining labelled cells left the circulation in a random fashion with mean half-time disappearance of 6.6 hours. Since cell damage and elution of label could not be demonstrated, it was suggested that the immediate disappearance of half the infused cells was due to their rapid dilution in a larger pool than that calculated from the blood volume and venous granulocyte count. Athens *et al* (1961a, 1961b) confirmed this suggestion and showed that there were two pools of granulocytes, the circulating and the marginated, which were in equilibrium and which were of about equal size. It was calculated that in order to maintain a normal level of circulating neutrophils, approximately 10^{11} must disappear from a man's bloodstream each day (see Hirsch, 1973). Since neutrophils can only leave the bloodstream by migrating through endothelium, this figure indicates that a high level of neutrophil-endothelial adhesion must normally occur. All these data are based on the use of DFP as a label. DFP is a highly toxic esterase inhibitor (Merck Index, 1978) and the results of these experiments may need to be treated with caution.

On balance however, these results in conjunction with the *in vivo* microscopic observations suggest that neutrophil-endothelial adhesion is a normal occurence. The changes that occur during inflammation to produce an increase in adhesion are likely therefore to be only of degree and not of an absolute "on-off" nature. This is important in the consideration of possible mechanisms to account for the increased adhesion.

Models of cell adhesion

Two broad classes of hypothesis have been advanced to account for the process of cell adhesion. Briefly, these attribute adhesion to:

1) long range physical forces determined by the average bulk properties of the cell surface (Curtis, 1960).

or 2) direct molecular binding between surface components either directly between cells (e.g. Roseman, 1970) or via some extracellular cross linking agent (e.g. Moscona, 1968).

Curtis (1960) proposed that cell-cell adhesions were determined by the interactions between electrostatic forces of repulsion and attractive forces attributed to London dispersion forces. Such forces exist between cell particles in aqueous suspension and this theory has become known as the DLVO theory after the authors (Derjaguin, Landau., Verwey and Overbeek) who in the 1940s explained the flocculation of lyophobic colloid particles in terms of these interactions. The DLVO theory predicts that two sorts of adhesion may form between particles, a strong and usually irreversible adhesion with surfaces in molecular contact, and a relatively weak reversible interaction occurring with surfaces separated by a gap of 10-20nm. This latter is caused by the occurrence of a secondary minimum in the energy of repulsion due to the different rates of decrease of attraction and repulsion with distance.

If this model is relevant to the process of neutrophil-endothelial adhesion, then such factors as the net surface charge of the interacting cells may be important in determining the degree of adhesion.

Complex carbohydrates of the cell surface have been particularly favoured as possible adhesive surface components by Roseman and colleagues (Oppenheimer, Edidin, Orr and Roseman, 1969; Roseman, 1970; Roth, 1973). These molecules have attractions for such a role, especially because of their position and their potential information

content. These workers' hypothesis, that surface-located glycosyl transferases and their substrates constituted complementary species in cell-cell adhesion, has not however, been supported by conclusive evidence and is now rather out of favour.

Recognition of carbohydrates by lectin-like carbohydrate binding proteins is another hypothetical mechanism of cell adhesion that has been proposed. The involvement of carbohydrates in adhesion has been suggested by Edwards, Dysart and Hughes (1976). The role of complex carbohydrates in adhesion has been reviewed by Hughes (1975). Changes in the membrane proteins or carbohydrates then are possibly involved in controlling the level of neutrophil-endothelial adhesion.

Interest has focussed recently on the role of large external proteins termed fibronectins in cell adhesion. Yamada and Olden (1978) have recently reviewed this field. It is of interest that endothelial cells produce fibronectin *in vitro* (Jaffe and Mosher, 1978), that it appears to be localized on the underside of the cells (Birdwell, Gospodarowicz and Nicolson, 1978) and that more is formed as the cells are cultured after confluence (Zetter, Martin, Birdwell and Gospodarowicz, 1978). It is possible that fibronectin affects neutrophil adhesion.

Most models of cell adhesion have concentrated on explaining how the actual sticking takes place between cell surfaces. However, cell shape may also affect adhesion. Spreading and flattening may also be involved. Thus the internal elements of the cell controlling these variables (the cytoskeleton) might modulate the degree of adhesion either by controlling the movement of membrane components or by affecting the rigidity of a cell. Rees, Lloyd and Thom (1977) have embodied similar ideas in their considerations of 'grip' and 'stick' in adhesion.

Several studies have been performed on the mechanism of neutrophil-endothelial adhesion. Electron microscopic studies have failed to detect any alteration in the endothelial surface (Marchesi and Florey, 1960; Florey and Grant, 1961; Williamson and Grisham, 1961). These observations led to the suggestion by Spector and Willoughby (1963) that changes occurring at the molecular level may involve electrochemical forces operative at the cell surface causing a reduction in the energy of repulsion. It has also been suggested that both divalent cations and cationic proteins may act as ligands between negatively charged cell surfaces causing adhesion (Bangham, 1964; Janoff and Zweifach, 1964; Thompson, Papadimitriou and Walters, 1967). For calcium ions to act as bridging agents a gap between the binding chemical groups e.g. carboxyl of 5Å is necessary. Electron microscopic observations revealed a gap of 150 - 600Å thickness however. Jones (1970) used colloidal iron to stain the acid mucosubstances which are known to be present on the endothelial cell surface (Buonassisi, 1973; Buonassisi and Root, 1975) and found such substances to fill the gap between adhering neutrophils and endothelium, a "cell coat" of 100 - 200Å thickness being demonstrated on both types of cell. Juxtaposition of carboxyl groups in these "cell coats" would allow calcium cross-linking. Curtis (1973) has pointed out the shortcomings of the early evidence for "cell coats" but it is now generally accepted that all cells have a surface layer of carbohydrate, and the term "cell coat" is now realised to be a slight misnomer.

Possible mechanisms leading to increased neutrophil-endothelial adhesion

Theoretically the level of adhesion of neutrophils to endothelium will depend on the number of neutrophils in the blood vessel, the force

of distraction at the vessel wall and the strength of the attraction between the neutrophils and the endothelium. Since neutrophils have a tendency to adhere to endothelium under normal circumstances, a slight change in the local environment may be sufficient to induce the increased adhesion during inflammation. Four classes of hypothesis to explain increased adhesion, not necessarily mutually exclusive, can be formulated to account for the increased adhesion. These are:

1. Increase in neutrophil number.
2. Reduction in the force of distraction.
3. Increase in neutrophil adhesiveness.
4. Increase in endothelial adhesiveness.

These hypotheses will be discussed in turn.

Increase in neutrophil number

This simple hypothesis is that as blood volume flow increases through an inflamed area, the increased number of neutrophils flowing in the vessels results in a proportional increase in the number adhering to endothelium. Such a mechanism has been proposed to account for increased lymphocyte traffic in lymph nodes (Hay and Hobbs, 1977). Conversely, Freitas and de Sousa (1976) and Ford, Sedgley, Sparshott and Smith (1976) have ascribed apparent decreased lymph node entry ability of lymphocytes to the paucity of cells in the bloodstream. On its own, this seems an inadequate explanation however, since most observers agree that neutrophil adhesion begins to increase after the initial period of high blood flow has receded. Mayrowitz, Wiedeman and Tuma (1977) made continuous measurements of vessel diameter and blood velocity in the microcirculation of the bat wing. In a total of ten experiments, the volume of blood flowing after occlusion of vessels

was found to increase in three experiments, to decrease in three and to remain unchanged in four experiments. In spite of this variation, the number of rolling leucocytes at the vessel wall was increased in all ten experiments. This independence of blood volume and number of adherent leucocytes argues against the possibility that increased adhesion is simply the result of the presence of increased cell numbers in the blood vessels.

Reduction in the force of distraction

The shear force or shear stress at the vessel walls is given by the equation (Atherton and Born, 1973):

$$\gamma = \frac{4\bar{V}\eta}{r_0}$$

where $\bar{V}$ is the mean blood velocity, η is the blood viscosity and r_0 is the vessel radius. Changes in any of these variables, which occur during inflammation, will affect the shear force and hence in theory the number of adherent leucocytes. It is possible that the apparent specificity of neutrophils' adhesion to endothelium in the post-capillary venules during inflammation (Ryan and Majno, 1977) may be accounted for by the rheology of the circulation. Due to the increase in radius of the vessels beyond the capillaries and the concomitant slowing of flow the wall shear stress drops markedly in this area. That rheological factors may be important is also suggested by the fact that it is during the slow flow phase of the blood that adhesion predominantly occurs during inflammation.

Plasma loss in many injuries occurs predominantly from the post-capillary venules. This loss of plasma will produce an increase in the viscosity of the blood., Wells (1973) concluded that following injury an almost exponential increase in viscosity would occur,

especially in the post-capillary venules. This will apparently increase the shear stress at the vessel wall. However, the increase in viscosity will lead to a reduction in the flow rate which will reduce the shear stress.

Red blood cells may affect the level of adhesion of leucocytes. It has been suggested, (Vejlens, 1938), that clumping of red blood cells will increase the proportion of leucocytes in the marginal flow and may thus assist adhesion. Using high speed cine photomicrography and large scale modelling of bloodvessels, Schmid-Schoenbein *et al* (1975) found the shear force to be strongly influenced by the haematocrit. This varied from 20% - 40% in vessels of ∿40μm diameter in the rabbit omentum. A white blood cell sticking to endothelium was calculated to be subject to a shear force in the range 4×10^{-5} - 235×10^{-5} dynes. The shear stress on an individual cell was calculated to very between 50 - 1060 dynes cm^{-2}. This is rather higher than the value of 5 dynes cm^{-2} calculated from *in vivo* experiments by Atherton and Born (1973a). The exact value further depended on the velocity of the blood, the size and motion of the white blood cell and the size of the blood vessel.

Several workers have reported a dependence of the number of rolling leucocytes on the rate of flow in the blood vessel (Vejlens, 1938; Atherton and Born, 1973a). Beesley, Pearson, Carleton *et al* (1978) reported that the degree of *in vitro* neutrophil-endothelial adhesion was inversely proportional to the flow rate of the suspending medium. Clark and Clark (1935) observed that after heat injury, the very rapid flow present normally in the arteries and veins interfered temporarily with leucocytes sticking but that there was no relation between sticking of leucocytes and slowing of the stream in venules with more moderate

circulation. Born, Planker and Richardson (1979) suggested that blood flow rate changes were more likely than changes in the properties of granulocytes or endothelium to account for differences in the number of rolling granulocytes in different segments of the microcirculation. This seems to contradict the earlier observation (Atherton and Born, 1972) that leucocyte adhesion increased in the absence of any flow rate changes. That flow rate changes may not account for increased adhesion is further suggested by the observation of Graham, Robert, Ratnoff and Moses (1965) that arteriolar dilation and rapid venous flow occurred simultaneously with leucocyte sticking and emigration in response to injection of activated Hageman factor. The detailed studies of Mayrowitz *et al* (1977) also indicate that in some circumstances flow rate reduction is insufficient stimulus to induce adhesion *in vivo*. No evidence was found of leucocyte adhesion in arterioles that had previously been occluded to produce stasis for periods of 1 - 25 minutes. In further experiments examining vessels in which marginating leucocytes were present, stasis led to a five-fold increase in the number of marginated cells and it was concluded that although flow stasis was not sufficient stimulus to induce leucocyte adherence, that it did augment pre-existing vessel wall interactions. As this work was restricted to the arterioles where marginal leucocytes are rarely observed normally, it seems possible that stasis alone might induce adhesion in venules where marginating leucocytes have frequently been observed under normal circumstances (see *in vivo* observations of the micro-circulation).

Changes in vessel wall adhesiveness

On theoretical grounds a change in the vessel wall is one of the most attractive hypotheses to explain margination. It appears to be

the simplest way of localizing the response and the endothelium is one of the first components involved in the margination process to be reached by any stimulatory signal from damaged tissue.

Many workers place the change responsible for increased leucocyte adhesion during inflammation in the vessel wall (Clark and Clark, 1935; Grant, 1973; Hurley, 1978). The evidence for this is often circumstantial and no rigorous analysis has been performed. Allison *et al* (1955a) reported that by localizing injury to one side of a blood vessel, unilateral leucocyte adhesion could be demonstrated. They 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 to support this however, was one plate showing leucocytes adhering to one side of a blood vessel at a sharp bend. Rheologic factors could clearly influence the intravascular distribution of cells. Further, other interpretations of the observation are possible. The work of Cliff (1966) in which the inflammatory reaction following injection of antigen-antibody complexes was observed, also showed leucocytes sticking on the side of the venules nearest to the site of injection. However, even "frequent" observation of such an event does not on its own constitute hard evidence. Florey and Grant (1961) using UV light as the injuring stimulus showed that leucocyte sticking began several hours after the injury. They concluded that since the neutrophils which stuck almost certainly could not have been those in the path of the light at the time of injury that a change in the endothelial cells must have occurred. No direct evidence for a change in the cell surface was given however and rheological factors were not considered.

The results of Grant (1962) from similar experiments led to the suggestion that the critical alteration in the tissue (leading to neutrophil adhesion) caused by the UV stimulus was some change in the membrane of the endothelial cell which coverted it from a non-adhesive to an adhesive state. Again, however, no evidence for an actual adhesive change was given.

Direct injury to endothelial cells certainly causes white cell sticking *in vivo*. Using a laser to inflict microburns on the walls of vessels perfused with dye-saline solutions, it was shown that leucocytes which could not have been injured adhered only to the injured site at first when blood flow was restored (Grant and Becker, 1965, 1966; Grant, 1973). It was therefore suggested that changes in the endothelial cell were responsible for the adhesive reaction in inflammatory states. However it is doubtful whether any valid comparison can be drawn between the condition of the surface of an endothelial cell directly damaged by laser induced heating and one affected by a putative adhesion stimulating agent associated with tissue injury.

Changes in leucocyte adhesiveness

A change in the adhesiveness of circulating leucocytes leading to increased adhesion is one of the least attractive hypotheses to explain margination. To induce such a change a signal presumably has to enter the bloodstream where it and the putative hyperadherent neutrophils will be carried away from the site of inflammation. This problem of localization may be overcome if a signal affected already marginated leucocytes or if it seeped into the bloodstream during conditions of slow flow or stasis.

Conflicting *in vivo* observations have been reported. Grant (1973)

observed that leucocytes which broke off laser induced thrombi re-adhered to the vessel wall at undamaged endothelial sites, suggesting that they had become more adhesive. Cliff (1966) and Zweifach (1973) observed leucocyte clumping and multi-layering on the walls of inflamed vessels suggesting that leucocyte adhesiveness had increased. Clark and Clark (1935) on the other hand reported that leucocytes did not re-adhere at undamaged sites. Allison *et al* (1955a) reported similar observations.

That neutrophil adhesiveness can influence margination is indicated by another series of observations. Profound transient neutropenias can be induced *in vivo* by a variety of stimuli such as cobra venom factor (McCall, De Chatelet, Brown and Lachmann, 1974), renal dialysis (Jensen, Brubaker, Nolph *et al.*, 1973; Macgregor, 1977), or filtration leucophoresis (Schiffer, Aisner and Wiernik, 1975; Hammerschmidt, Craddock, McCullough *et al.*, 1978). These reductions in the number of circulating neutrophils have been ascribed to increased margination, especially in the lungs (although this may not be of significance since about 50% of the body's capillaries are in the lungs) (Craddock, Fehr, Brigham *et al.*, 1977) and increased *in vitro* neutrophil adhesiveness has been found after such stimuli (Fehr and Jacob, 1977; O'Flaherty, Craddock and Jacob, 1978; Macgregor, 1977). Lentnek, Schreiber and Macgregor (1976) found that the adhesiveness of granulocytes from patients with acute inflammatory diseases (such as osteomyelitis, pneumonia, vasculitis and thrombophlebitis) was twice that from normal patients. Increased neutrophil adhesiveness was shown to be due to the presence in the plasma of an adherence augmenting factor. Essentially identical results were obtained using rabbits. It was concluded that the degree of adhesiveness of the

neutrophils affected the extent of margination. Goodman, Way and Irwin (1979) observed reductions in circulating neutrophil numbers following endotoxin injection into rabbits. Concurrent observations of blood vessels using ear chambers revealed leucocytes sticking to endothelium in both vessels and arterioles within minutes of injection.

It does not seem possible yet on the basis of the published evidence to decide firmly in favour of any one mechanism to account for increased adhesion during inflammation. Adhesive change has often been assumed to completely account for margination. This assumption does not seem entirely justified but nevertheless evidence exists which clearly shows that adhesive changes do occur and it seems likely that these have a role in margination.

Experimental manipulation of adhesion in vivo

Consideration of the *in vivo* adhesive behaviour of neutrophils and endothelium either in normal circumstances or in response to injury has indicated that a wide range of potential factors may be involved in the increased level of leucocyte adhesion during inflammation. Another approach to the clarification of the factors inducing increased adhesion has been to study the effect of a variety of substances likely to be involved in inducing or preventing adhesion.

Table I shows the effect of agents which have been examined for effects on *in vivo* adhesion.

TABLE I Agents studied *in vivo* for effects on adhesion

Agent	Effect*	Reference
Gelatin	+	Fahraeus, 1929
Casein	+	Atherton and Born, 1972
E.coli extract	+	Atherton and Born, 1972
Neutrophil granule fraction	+	Janoff and Zweifach, 1964
Activated Hageman Factor	+	Graham *et al.*, 1965
EDTA	-	Thompson *et al.*, 1967
Cortisone	-	Allison, Smith and Wood, 1955b
Hydrocortisone	-	Grant, Palmer and Sanders, 1962
Lidocaine	-	Giddon and Lindhe, 1972
Prostacyclin	-	Higgs, Moncada and Vane, 1978
Heparin	0	Grant, Palmer and Sanders, 1962
Warfarin	0	Allison and Lancaster, 1960, 1961
Fibrin	0	Allison and Lancaster, 1960, 1961
Fibrinogen	0	Allison and Lancaster, 1960, 1961
EACA	0	Allison, Lancaster and Crosthwaite, 1963
Papain	0	Allison, Lancaster and Baxter, 1962
Bromelin	0	Allison, Lancaster and Baxter, 1962
Hyaluronidase	0	Allison, Lancaster and Baxter, 1962
Vitamin A	0	Allison, Lancaster and Baxter, 1962
Histamine	0	Atherton and Born, 1972
HSA	0	Atherton and Born, 1972
PGE_1	0	Atherton and Born, 1973b
PGE_2	0	Atherton and Born, 1973b
$PGF_{2\alpha}$	0	Atherton and Born, 1973b

* - = decrease; + = increase; 0 = no effect

A further vast range of substances has been tested for effects on leucocyte emigration or accumulation, often with contradictory results (reviewed by Grant, 1973; di Rosa, 1978).

The range of possible mechanisms and substances causing increased adhesion is large and complex. With few exceptions (Atherton and Born, 1972, 1973a, b; Giddon and Lindhe, 1972) the results reported have been qualitative and while they more clearly define the factors which are or are not effective, knowledge of the phenomenon has not yet reached a level where analysis and understanding replace description. Many investigators have turned to the examination of the isolated components of the interaction in the hope that controlled experimental manipulation *in vitro* yielding quantitative results would shed light on the process of adhesion.

In vitro experimental studies: a) leucocyte-endothelial interactions

Recent advances in the culture of endothelial cells from a variety of sources (Jaffe, Nachman, Becker and Minick, 1973; Gimbrone, Cotran and Folkman, 1974; Booyse, Sedlak and Rafelson, 1975) have allowed the *in vitro* study of leucocyte-endothelial interactions.

Using monolayers of pig aortic endothelial cells on glass coverslips de Bono (1976) studied the adhesion of pig lymphocytes. Quantitation was by microscopic counting of the number of cells per unit area. In an attempt to elucidate the mechanism of adhesion the lymphocytes, the endothelium and the medium were modified in turn. Adhesion was found to be prevented by treatment of the lymphocytes, but not endothelium, with formaldehyde, microtubule disrupting agents, by treatment of the endothelium with papain, neuraminidase or gluteraldehyde or by the presence in the medium of azide or EDTA. The adherence of lymphocytes

to cultured pig aortic endothelium was greater than to any other cells examined, suggesting a degree of adhesive specificity. Culture of endothelium under flowing medium led to a reduction in adhesion. Calcium and magnesium divalent cations were required for optimal adhesion.

A number of workers have investigated neutrophil-endothelial adhesion. Lackie and de Bono (1977) showed that rabbit peritoneal neutrophils adhered well to pig aortic endothelial cell cultures. The species differences in these experiments did not seem to be a problem since the adhesion of the neutrophils to fibroblasts from several species was similar and low and the adhesion to human umbilical vein endothelium did not differ from that shown to pig aorticendothelium. Hoover, Briggs and Karnovsky (1978), Macgregor, Macarak and Kefalides (1978) and Beesley *et al.*, (1978) reported that neutrophils adhered preferentially to endothelial cells compared with a wide range of cells from other tissues and species and adhesion to endothelium from different species was shown to be similar (Hoover *et al.*, 1978). The divalent cations Mg++ and Ca++ were both required for optimal adhesion (Hoover *et al.*, 1978; Beesley *et al.*, 1978). Adhesion could be modified by enzyme treatment of endothelium. Neuraminidase treatment (Hoover *et al.*, 1978) increased and trypsin treatment (Macgregor *et al* 1978) decreased adhesion. The effect of trypsin treatment was reversible after 24 hours incubation of endothelial cells. No requirement for plasma components was demonstrated for adhesion (Beesley *et al* 1978; Macgregor *et al.*, 1978) although plasma from patients with acute inflammation and plasma from patients treated with prednisone increased and decreased adhesion respectively (Macgregor *et al.*, 1978). These investigators also concluded that neutrophil adherence was a random phenomenon and that the adhering

neutrophils were not a subpopulation. Hoover *et al* (1978) found that treatment of neutrophils or endothelium with chemotactic factors rendered them more adhesive, suggesting an explanation for the *in vivo* observations of Atherton and Born (1972). Culture of endothelial cells under flow conditions before use in assays reduced adhesion (Beesley *et al*, 1978) a result similar to that of de Bono (1976) for lymphocyte adhesion to endothelium.

Conflicting results have been reported on the effects of cyclic nucleotides on neutrophil-endothelial adhesion. Macgregor *et al* (1978) found that cyclic GMP and cyclic AMP respectively increased and decreased adhesion while Pearson, Carleton, Beesley *et al* (1979) found cAMP to have no effect and dibutyryl cAMP and adenosine to increase adhesion.

Prostaglandins which are found in increased quantities in inflamed sites have been shown to increase adhesion to endothelium by Pearson *et al* (1979) who suggested that these molecules may have a role in the modulation of granulocyte adhesion to endothelial cells. These workers also discovered that the presence of erythrocytes and platelets in the adhesion assays respectively enhanced and diminished neutrophil adhesion. The effect of the erythrocytes was accountable by a large increase in the number of migrated cells (Beesley *et al.*, 1979).

Sacks, Moldow, Craddock *et al* (1978) reported that when exposed to activated complement, granulocytes induce endothelial damage. This injury is mediated primarily by oxygen radicals produced by the granulocytes. Studies with cytochalasin B suggested that close approximation of granulocytes and endothelium was necessary for maximal cell injury.

b) Neutrophil - surface interactions

The adhesion of neutrophils to a variety of inert artifical surfaces has been widely studied. These experiments are all open to the general criticism that the adhesion to such a surface may not resemble adhesion to another cell. For adhesion to adsorbed protein films, however, cell-surface adhesion seems to very closely resemble cell-cell adhesion (Grinnell, 1978). It is important to remember that for neutrophil adhesion, it is two different cell types that are interacting and therefore that results based on the use of only one cellular component may be incomplete or misleading.

A variety of systems for assaying adhesion have been developed which fall into two broad classes. These are firstly, columns of glass beads or glass wool through which blood or leucocyte suspensions are passed and secondly, plane substrata onto which blood or leucocyte suspensions are allowed to settle after which non-adherent cells are removed by rinsing or inversion. For both these classes of method cell adhesion and spreading will effect the apparent level of adhesion, since they depend on the removal of non-adherent cells. The characteristics of neutrophil adhesion to surfaces will be discussed under a series of subject headings.

(i) Energy requirements

The energy requirements for adhesion have been investigated using metabolic inhibitors by several workers. Garvin (1961) reported that cyanide and dinitrophenol had no effect on human neutrophil adhesion (in whole blood) to siliconized glass bead columns, while iodoacetamide eliminated adhesion. An intact glycolytic pathway was concluded to be essential for adhesion. Allison, Lancaster and Crosthwaite (1963)

found however that iodoacetamide at the concentration used by Garvin (1961) killed most of the cells in their experiments. Using mono-iodoacetate, antimycin, 2-deoxyglucose and oligomycin, Kvarstein (1969b) concluded that adhesion was an energy requiring process deriving energy from both the glycolytic and oxidative pathways. Banks and Mitchell (1973b) also concluded that energy from the glycolitic pathway is involved in adhesion. It has been suggested by Curtis (1973) however that alkylation of cell surface sulfhydryl groups by iodoacetamide could account for the reduced adhesion observed in the presence of this agent. However, since even under aerobic conditions neutrophils derive most (>90%) of their energy from glycolysis (Hirsch, 1973) it seems likely that adhesion will depend on this source of energy.

(ii) Temperature

Fenn (1923a, b) studied the adhesion of leucocytes to a variety of solid surfaces including glass, mica, coal and paraffin. In the absence of serum adhesion was not temperature dependent but in its presence an optimal temperature of 20°C was found. Garvin (1961) reported adhesion to glass to be greatest in the range 30°C-40°C and Kvarstein (1969a) found that adhesion increased with increasing temperature. Lichtman and Weed (1972) found adhesion to be abolished at 4°C, as did Bryant and Sutcliffe (1972) who also found that adhesion was reduced at temperatures greater than 37°C. These results all probably reflect the requirement of metabolic energy for adhesion although it is possible that changes in membrane fluidity may also be involved.

(iii) Divalent cations

Divalent cations have been implicated in a wide range of cellular activities and their effects on neutrophil adhesion have been extensively

studied. Garvin (1961) reported that the adhesiveness to glass, using whole blood, was dependent on the presence of magnesium ions. Further observations (Garvin, 1968) using rat peritoneal neutrophils and serum coated beads showed that a variety of divalent cations were effective in promoting adhesion but that calcium, strontium and barium ions were ineffective. A possible auxilliary role for calcium ions when added with magnesium ions was suggested. Bryant, Desprez, Van Way and Rogers (1966) reported that adhesion to glass was dependent on the presence of magnesium ions and independent of calcium ions. Kvarstein (1969a) and Macgregor, Spagnuolo and Lentnek (1974) reported that neutrophil adhesion was reduced in the presence of EDTA and Kvarstein (1969c) found magnesium ions to markedly stimulate adhesion to glass while calcium ions had no effect. Banks and Mitchell (1973b), Beesley *et al* (1978), Bryant and Sutcliffe (1972), Penny, Galton, Scott and Eisen (1966) all reported that adhesion (in the presence of blood or serum) requires magnesium or calcium ions.

Although the requirement of extracellular divalent cations for adhesion seems clear, the role these ions play in adhesion is less certain.

(iv) Serum and plasma (factors)

In the absence of serum, Fenn (1923a) found that adhesion was not temperature dependent. In its presence, temperature dependence was demonstrated. Serum inhibited adhesion and spreading of leucocytes on glass, the inhibition being maximal at 8% serum. Penny *et al* (1966) and Banks and Mitchell (1973b)reported that adhesion to glass was reduced in the absence of serum and suspension of the cells in saline though. Bryant *et al* (1966) showed that adhesion was partially dependent on heat

labile plasma factors. In a thorough study, Kvarstein (1969c) concluded that plasma contains factors are important for adhesion, that fibrinogen stimulated adhesiveness and suggested that serum may contain a heat-labile factor which inhibits adhesion. Garvin (1968) and Smith, Hollers, Patrick and Hassett (1979) reported neutrophil adhesion to be much higher to glass in the absence of serum. Garvin (1968) reported that the removal of complement had no significant effect on adhesiveness of neutrophils to serum coated glass. Using whole blood and measuring adhesion to nylon wool columns, Lentnek, Schreiber and Macgregor (1976) showed that plasma from patients suffering from acute inflammatory disease contained a factor that augmented the adhesiveness of normal granulocytes. Exudate fluid from experimental inflammation (sterile peritonitis) in rabbits was also shown to contain such a factor. Macgregor (1977) showed that the human factor was heat stable and suggested that a heat-labile co-factor, possibly complement, was also required for augmented adherence. Fehr and Jacob (1977) also suggested that increased granulocyte adhesion to glass wool columns depended on complement. McGillen and Phair (1979) found that adherence of neutrophils to nylon fibre was plasma independent and distinct from the process of 'augmented adherence' which was dependent on plasma activated by zymosan. Heating and anti-C3 blocked the augmentation of adherence seen with zymosan activated plasma.

(v) Other proteins

Smith *et al* (1979) reported that bovine serum albumin (BSA) reduced adhesion to glass. Keller, Barandun, Kistler and Ploem (1979) found that human serum albumin (HSA) and fibrinogen both reduced adhesion to glass. Endotoxin has been shown by Macgregor (1977) and Schneir, Gall,

Carpe and Boggs (1977) to increase adhesion.

(vi) Inflammatory mediators

A variety of substances associated with inflammation have been examined for effects on adhesion to surfaces. Bradykinin and activated Hageman factor were found by Penny *et al* (1966) respectively to reduce and have no effect on the level of adhesion. Banks and Mitchell (1973c) found that 5-HT increased white cell adhesion to glass flasks. Histamine was reported by Bryant and Sutcliffe (1974) to reduce adhesion of neutrophils to glass as did PGE_1, which was also found by Lomnitzer Rabson and Koornhof (1976b) to abrogate the enhancement of adhesion induced by cytochalasin B. PGE_2 was found by Pearson *et al* (1979) to increase adhesion of neutrophils to serum coated glass coverslips.

(vii) Chemotactic factors

Recently the effects on adhesion of chemotactic factors have been examined. Gallin, Wright and Schiffmann (1978) found that incubation in 10^{-5}M formyl - methionyl, leucyl, phenylanine (FMLP) increased the adhesion of human neutrophils to plastic petri dishes after a 30 minute incubation. Studying much earlier times, Smith *et al* (1979) observed that several chemotactic factors caused a significant increase in neutrophil attachment to protein coated glass. Fehr and Dahinden (1978) found that chemotactic factors had various effects on neutrophil adhesion to petri dishes. O'Flaherty, Kreutzer and Ward (1978) found that chemotactic factors increased adhesion to plastic vials. Smith, Lackie and Wilkinson (1979) found diverse effects of chemotactic factors on adhesion. These studies will be described more fully in the discussion (Chapter 8).

(viii) Anti-inflammatory agents

Penny *et al* (1966) reported that addition of hydrocortisone to adhesion assays had variable effects but that oral administration of prednisolone reduced neutrophil adhesion consistently. Macgregor *et al* (1974) reported that administration to patients of salicylates and glucocorticoids caused a reduction in granulocyte adhesion but that direct addition to *in vitro* assays had no effect. Similar results were reported for a range of anti-inflammatory compounds (Macgregor, 1976). Schnier *et al* (1977) obtained similar results using hydrocortisone. Banks and Mitchell (1973c) found that addition of aspirin to assays reduced adhesion. Pearson *et al* (1979) found that pre-incubation of granulocytes with 10^{-3}M aspirin led to a significant increase in adhesion to serum coated glass.

(ix) Cytoskeletal components

Microtubule and microfilament disrupting agents have been investigated with varying results. Cytochalasin B was shown by Lomnitzer *et al* (1976b) to increase and by Boxer, Allen, Watanabe *et al* (1978) and Gallin *et al* (1978) to decrease adhesion. Colchicine was reported to decrease adhesion by Boxer *et al*., (1978) and Macgregor (1976) but to have no effect by Lomnitzer *et al*., (1976b). Schneir *et al*., (1977) reported that vinblastine reduced adhesion while Lomnitzer *et al* (1976b) found no effect.

(x) Nucleotides

Contradictory results have also been reported on the effects of nucleotides on the level of adhesion of neutrophils. Bryant and Sutcliffe (1974) reported that a variety of agents which increase intracellular cAMP levels reduced adhesion to glass. Similar results were reported by Lomnitzer *et al* (1976a) and it was further shown (Lomnitzer *et al*.,

1976b) that DBcAMP and theophylline could abrogate increased adhesion induced by cytochalasin B. cAMP itself had no effect on adhesion. On the other hand Boxer *et al* (1978) found that a threefold increase in intracellular cAMP had no effect on adhesion. Banks and Mitchell (1973b) found ADP to increase adhesion and adenosine (1973c) to inhibit adhesion while Pearson *et al* (1979) found adenosine and DBcAMP to increase adhesion. Macgregor (1977) found that epinephrine caused a transient decrease in granulocyte adhesion to glass wool, however Banks and Mitchell (1973c) found adrenaline, noradrenaline and isoprenaline to enhance adhesion.

(xi) Miscellaneous studies

Ectosulphydryl groups have been implicated in cell adhesion. Giordano and Lichtman (1973) however concluded that such groups are either not present or do not participate in the adhesion of neutrophils.

Lichtman and Weed (1972) compared immature and mature granulocytes. Immature cells, characterized by a high neuraminidase susceptible negative surface charge density and high rigidity exhibited low adhesiveness. Neuraminidase treatment of immature cells did not increase adhesiveness.

c) Neutrophil-neutrophil adhesion

The study of neutrophil-neutrophil adhesion, usually brought about by agitation of cell suspensions, may not appear to be especially relevant to the study of margination but is a sensitive method and does yield information about the adhesiveness of these cells. Many similarities with the requirements for adhesion to substrata have become apparent.

Brittingham (1958) observed that normal leucocytes separated from

whole blood (but not leucocytes from defibrinated blood) were rapidly agglutinated when suspended in normal serum. This was prevented by EDTA. In a biophysical study, Wilkins, Ottewill and Bangham (1962a, 1962b) studied the flocculation of sheep peritoneal leucocytes. From experiments using a variety of cations it was concluded that secondary minimum effects (see DLVO theory - models of adhesion) did not play an important part in the flocculation of these cells.

The aggregation of rabbit peritoneal neutrophils in the presence of a rough strain of pneumococcus has been studied using a semi-quantitative visual scoring method (Allison *et al* 1963; Allison and Lancaster, 1964, 1967). In the absence of bacteria, only slight, loose clumping occurred. Upon the addition of bacteria, irreversible clumping and phagocytosis occurred. This aggregation was temperature, pH and divalent cation dependent and independent of the presence of complement or of any requirement for metabolic energy as evidenced by the lack of effect of iodoacetamide, DNP, sodium cyanide or sodium fluoride. Epsilon amino-n-caproic acid (EACA) at high concentrations (0.3 - 0.5M) blocked clumping. The proteolytic enzymes varidase and hyaluronidase had no effect, but trypsin had variable, concentration dependent effects. ADP, AMP, ATP and other nucleotides failed to modify clumping.

Lackie (1977) found that AMP, ADP and ATP had only minor inhibitory effects on the aggregation of rabbit peritoneal neutrophils and (Lackie, 1974), that adenosine, DBcAMP or cAMP inhibited aggregation while guanosine nucleotides had no effect. Increased aggregation following phagocytosis has been observed on several occasions (Bryant *et al.*, 1966; Talstad, 1972; Lackie, 1977). Various bacteria and bacterial

endotoxins have also been shown to enhance aggregation (Thorne, Oliver and Lackie, 1977). Berlin and Ukena (1972) found that concanavalin A increased agglutination of neutrophils and that this effect could be reduced by low concentrations of colchicine and vinblastine. Divalent cation dependence of neutrophil - neutrophil adhesion was confirmed by Gray, Tsan and Wagner (1977). Vasoactive amines, non-steroidal anti-inflammatory drugs, autonomic agonists and cholinergic agents had little effect on and cytocholasin B markedly enhanced aggregation (Lackie, 1977).

The effects of chemotactic factors on aggregation have been extensively studied recently (see for example: O'Flaherty, Kreutzer and Ward, 1977a; Craddock, Hammerschmidt, White *et al.*, 1977). The central observation is that after exposure to chemotactic factors while being stirred at high speeds, granulocytes transiently aggregate. The maximal effect is observed after 1 minute's exposure and by 8 minutes aggregation is reversed. It is tempting to speculate that a chemotactic factor induced transient increase in neutrophil adhesiveness may be the mechanism responsible for margination *in vivo*, (see discussion, Chapter 8).

Conclusion and aims of project

Since the first report by Dutrochet in 1824, many descriptions of the process of leucocyte adhesion to endothelium and emigration have appeared. Development of new experimental systems and techniques has yielded an almost complete morphological description of these processes. Despite this large volume of descriptive material, however, the circumstances in which leucocyte adhesion occurs, the means of control of the level of adhesion and the mechanism of adhesion itself still require considerable clarification.

One clear result is that following injury to tissues, as part of the inflammatory response, leucocyte adhesion is markedly increased and is one of the earliest observable cellular events of an inflammatory response. An understanding of the processes leading to this increased adhesion and of the mechanism of adhesion has been the goal, so far unrealized, of a large number of investigators for many years.

This lack of success undoubtedly stems from the complexity of the changes that occur in response to injury but also stems from the difficulty of finding suitable experimental models with which to examine the phenomenon.

The aim of this project is to model neutrophil - endothelial adhesive interactions *in vitro* and by experimental manipulation of conditions to determine if and how adhesive changes occur which might affect the events observed *in vivo* during inflammation.

CHAPTER 2

Media

1. Growth medium for endothelial and other cells was Glasgow-modified Eagles medium containing 10% foetal calf serum (Gibco). The gas phase was 5% CO_2, 95% air. Cells were also cultured on occasions in Waymouth's medium (Gibco) containing 20% foetal calf serum.

2. Modified Hanks Balanced Salt Solution (BSS) contained (per litre):

NaCl	8g
KCl	0.4g
$MgCl_2.6H_2O$	0.2g
$CaCl_2$	0.14g
Glucose	1g
HEPES	2.388g (10mM)
Phenol Red	10ml

The pH was adjusted to 7.4 with 1N NaOH.

3. Phosphate buffered saline (PBS) contained:

NaCl	0.14M
KCl	217mM
Na_2HPO_4	8.1mM
KH_2PO_4	1.5mM
$CaCl_2$	0.7mM
$MgCl_2$	0.5mM

4. Versene in PBS contained (per litre):

NaCl	8g
KCl	0.2g
$Na_2HPO_4 2H_2O$	1.15g
KH_2PO_4	0.2g
EDTA	0.2g
Phenol red	10ml

5. Trypsin solution was 0.25% w/v(Difco 1:250) in Tris-saline (pH 7.4).

6. Tris-saline contained (per litre):

NaCl	8g
KCl	0.4g
$Na_2HPO_4 2H_2O$	0.1g
Glucose	1g
Tris (hydroxymethyl) amino methane	3g
Penicillin	10^5 units
Streptomycin	0.1g

7. Geys solution contained (per litre):

NaCl	8g
KCl	0.375g
$Na_2HPO_4 2H_2O$	0.15g
KH_2PO_4	0.025g
$NaHCO_3$	0.25g
Glucose	2g
$CaCl_2$	0.275g
$MgCl_2$ 6 H_2O	0.201g

The pH was adhusted to 7.2 with IN NaOH.

8. Buffered formalin contained (per litre):

Formaldehyde (40%)	100ml
$Na_2HPO_4 2H_2O$	6.5g
NaH_2PO_4	4g
Mg CO_3	to excess

9. Acid citrate dextrose contained (per litre):

Trisodium citrate (dihydrate)	22g
Citric acid	8g
Glucose	24.5g

Chemicals

All chemotactic factors, α_s-casein, β-casein (gifts from Dr. D.G. Dalgleish, Hannah Dairy Research Institute, Ayr), alkali denatured human serum albumin (HSA), N-formyl-methionyl-phenylalanine (f-met-phe), N-formyl-tri-tyrosine (f-tri-tyr) and N-formyl-methionyl-leucyl-phenylalanine (FMLP) (gifts from Dr. P.C. Wilkinson, Department of Bacteriology & Immunology, University of Glasgow) were stored at -20°C in BSS. FMLP (Miles, Yeda) was similarly stored. Since freeze-thawing apparently led to a loss of activity, stocks of chemotactic factors were stored in small volumes and the concentrations required for experiments made up from these, using glass universal bottles and one glass pipette for each serial dilution.

All other chemicals were commercially obtained.

Cells

1. Rabbit peritoneal neutrophils were obtained as described by Lackie (1977). New Zealand White rabbits were injected intraperitoneally with 400-500ml of sterile 0.9% NaCl solution containing 0.1% oyster glycogen (Sigma) and the fluid drained off after approximately 4 hours. At this time the exudate contains mostly (>95%) neutrophil granulocytes. The exudate was collected into plastic beakers to prevent cell loss through adhesion to the vessel, filtered through surgical gauze to remove debris and fibrin clots and either used immediately or stored at 4°C in plastic universal bottles for up to 3 days, during which time viability remains high.

2. Rabbit peripheral blood neutrophils were obtained using a method similar to that described by Henson (1971). New Zealand White rabbits were bled from the ear into 1/7 volume acid citrate dextrose in plastic

universal bottles, which were then centrifuged at room temperature for 20 minutes at 550g. The platelet rich plasma and buffy coat were removed with a siliconized bent tipped pasteur pipette, the bottle filled up to its original volume with 2.5% gelatin (Difco) in 0.9% saline, mixed and the red cells left to sediment at 1g for 30 minutes at 37°C. The supernatant was removed and centrifuged at room temperature (to prevent setting of the gelatin) for 10 minutes at 400g. The resultant pellet was resuspended in 1ml distilled water for one minute to lyse contaminating red blood cells, BSS added to fill the bottle and the cells centrifuged at 400g for 5 minutes. This procedure yielded cell suspensions of 50-85% neutrophils (mean 69%), of which >95% were viable. Monocytes and lymphocytes were the major contaminating cell types. Yields of $>10^7$ white cells per 10 mls blood were regularly obtained.

3. Neutrophil washing. Before use in experiments, neutrophils from either source were washed in calcium and magnesium free (CMF) - BSS, CMF-BSS containing 1mM EDTA and BSS before final resuspension in BSS. Centrifugations were for 5 minutes at 400g. If necessary, contaminating red blood cells in peritoneal exudate suspensions were lysed as described for blood cell suspensions. Before use cell suspensions were filtered through 10μm plastic mesh ('Nitex') to remove clumps.

4. Neutrophil viability was determined by nigrosine exclusion. Approximately 5.10^6 cells were suspended in 1ml BSS containing 0.2% nigrosine for 5 minutes at room temperature and the number of dead (staining) cells then counted.

5. Neutrophil purity was determined by counting cytocentrifuge

preparations of cells. Approximately 5.10^5 in 0.5ml BSS were centrifuged onto clear glass slides at 1200 rpm for 2 minutes using a Shandon Cytospin. The resultant smears were fixed in 90% methanol for 5 minutes, rinsed in H_2O, stained with Giemsa (1/10 in H_2O) for 15 minutes, rinsed with H_2O to remove excess stain, air dried and mounted in DPX.

6. Endothelial cells were obtained from the aortas of freshly slaughtered pigs using a method based on that described by Booyse, Sedlak and Rafelson (1975). Cannulated aortas were cleaned of external fat and tissue, clamped at the small blood vessels with crocodile clips, washed with BSS to remove blood, drained, ligated, refilled with a sterile solution of 0.05% collagenase (Sigma, type II) in BSS and incubated for 20 minutes at 37°C. After decanting the enzyme solution, the aortas were gently rinsed with BSS to remove residual collagenase, massaged lightly along their length to loosen the sheets of endothelial cells, filled with growth medium and shaken several times. The resultant cell suspension was dispensed into plastic $25cm^2$ culture bottles in 5ml aliquots. Growth medium was changed after 24 hours and thereafter every 3-4 days. Confluent monolayers of polygonal cells were formed within 3-6 days depending on the initial plating density and viability. Cells were subcultured using trypsin/EDTA as described by Edwards and Campbell (1971). After discarding the growth medium, cultures were rinsed twice with Tris-saline and once with trypsin/versene. Several minutes after the trypsin/versene rinse, the cells rounded up and were freed from the culture bottle by gentle tapping. Fresh medium was added and the appropriate volume dispensed into fresh culture vessels.

Adhesion assays

1. Adhesion to substratum assay. In principle this assay is identical to that described by Walther, Ohman and Roseman (1973). Assays of neutrophil adhesion to coverslips with either confluent cell monolayers or serum coats as substratum were performed in "Linbro" plastic multi-welled trays (Flow). A suspension of neutrophils (10^6 cells/well) was added to BSS or test solution containing wells to give a final volume of 1ml and the tray incubated at 37°C. The coverslips were then removed and non-adherent cells rinsed off by dipping edge-wise 5 times in BSS at 37°C. The number of adherent cells was then determined by either of two methods:

   a) the coverslips were fixed overnight in buffered formalin, rinsed in H_2O, stained in Giemsa (1/10 in H_2O) for 30 minutes, rinsed with 90% isopropyl alcohol, rinsed with absolute isopropyl alcohol and dehydrated for 10 minutes in absolute isopropyl alcohol, air dried and mounted in DPX. Using a X25 or X40 objective and a 10 x 10 eyepiece grid, the cells in a total of at least 10 fields per coverslip were counted at random along 2 diameters at right angles to each other and the mean number per unit area calculated.

   b) The radioactivity bound to the coverslips was counted in a Wilj 2001 gamma counter, having used ^{51}Cr labelled cells.

2. Labelling of neutrophils. Neutrophils were suspended in 1ml BSS ($\sim 5 \times 10^7$ cells) to which was added $\sim$100μCi $Na_2\ ^{51}Cr\ O_4$ (100-300 mCi/mg Cr) in 0.1ml isotonic saline (The Radiochemical Centre, Amersham). After incubation at 37°C for 30 minutes the cells were washed 3 times in BSS, filtered through 10μm plastic mesh ('Nitex') to remove clumps

and used immediately.

3. Aggregation assays.

a) Shaking bath assay. Aggregation was measured using a method based on that of Lackie (1974). Cell suspensions ($\sim 10^6$ cells) in BSS were placed in plastic vials containing either BSS or test solutions to give a final volume of 1ml. The vials were then shaken at 37°C at 120 cycles per minute using a reciprocating shaker bath (stroke 4cm). Total particle number was estimated at various times by removal of samples (0.5ml usually) of cell suspension and counting electronically on a Coulter Counter model A with 100μm orifice tube, threshold setting 050 and aperture current 4.

b) Couette viscometer assay. Aggregation was measured using the method introduced by Curtis (1969). Approximately 10^6 cells in BSS were added to the chamber of a Couette viscometer in a volume of 0.7 - 1.0 ml and allowed to aggregate using a preset shear rate of 10 sec^{-1}. Samples were withdrawm with a pasteur pipette at 7 minute intervals from 0 - 28 minutes and the total number of particles counted on unlined haemocytometers using a Quantimet Image Analysing Computer.

Preparation of coverslips

Glass coverslips (13mm diameter, Chance Propper Ltd) for use in adhesion assays or for cell culture were boiled in detergent ('Decon') for a few minutes, soaked overnight, rinsed for several hours in tap-water, washed several times in distilled water and then in absolute ethanol. They were then dried and/or sterilized by dry heat at 160°C before use.

Staining of endothelial cell junctions

Endothelial cells were outlined by silver staining using the method described by de Bono (1976). Cell monolayers were washed with 5% glucose, 10mM HEPES in distilled water, incubated for 30 seconds with 0.05% silver nitrate in 5% glucose, washed with glucose-Hepes, incubated for 1 minute with 1% NH_4 Br/4% $CoBr_2$ in distilled H_2O and fixed in buffered formalin for 30 minutes.

Enzyme release

To measure enzyme release during aggregation, 1ml samples of cell suspension ($\sim 10^7$ cells) were aggregated using the shaker bath for 1 hour. A sample of 0.1ml was removed for counting and the remaining 0.9ml centrifuged in a Jencons Microcentrifuge for 30 seconds. The supernatant was removed for enzyme assays and the cell pellet resuspended in 0.5% Triton X-100 in BSS before enzyme assays were performed.

a) Lactate dehydrogenase was assayed by following the rate of breakdown of NADH at room temperature. To 3ml cuvettes were added in the following order:

2.7ml phosphate buffer (0.067M, pH 7.4)

0.1ml 3mM NADH

0.1ml enzyme solution

0.1ml 0.01M sodium pyruvate.

The change in absorbance at 340nm was then followed using a Varian Techtron recording spectrophotometer.

b) Lysozyme was assayed by following the loss of turbidity of a suspension of Micrococcus lysodykticus.

To 1.9ml of a suspension of M. lysodykticus ($O.D_{450}$ 0.3) in 0.1M phosphate buffer pH 6.5 was added 0.1ml of the enzyme solution and the

decrease in optical density followed at 450nm using a Varian Techtron recording spectrophotometer.

Neutrophil locomotion

Neutrophil locomotion was assayed using a modified Boyden chamber micropore filter assay described by Wilkinson (1974) pp.168-172. Briefly, sawn off tuberculin syringe barrels to which 3μm pore size micropore filters (Sartorius) had been glued, were filled with neutrophil suspensions in Geys solution ($\sim 10^6$ cells). These were then lowered into beakers containing the test solution and incubated at 37°C during which the neutrophils migrate through the filter. After the appropriate time the filters were removed, fixed and stained and mounted on coverslips in DPX. The distance migrated was assessed using the leading front method of Zigmond and Hirsch (1973). Briefly, using the fine focus micrometer scale on a microscope, the distance was measured from the top of the filter to the furthest focal plane containing 2 neutrophils. Five measurements were made on each filter and for each test 2 or 4 replicate filters were used.

Depending on the experiment, chemotactic factor was present in either or both of the two compartments of the assay.

CHAPTER 3

Endothelial cell culture

Endothelial cells were isolated from the aortae of freshly slaughtered pigs as described in materials and methods. This procedure yielded a suspension containing single cells, clumps of cells and sheets of up to several hundred cells, (Plate 1). These generally settled and adhered to the culture flasks within 1 - 2 hours (Plate 2) with spreading starting within the following six hours. Cells were usually all spread within 24 hours, except for large clumps where cells in the centre were compressed and spread more slowly only as peripheral cells moved out from the clump (Plates 3, 4).

Cell viability, as judged by the number of floating cells after 24 hours was very variable. Levels of approximately 90% were often obtained, but viability of only a few percent of the isolated cells was not uncommon. This did not appear to be a problem, however, as the viable cells grew and divided to yield confluent monolayers indistinguishable morphologically from those of high original viability.

Also present in the cell suspensions were a variety of blood cells. These did not appear to adhere to the culture bottles and their numbers were low compared to endothelial cells, the great majority of them having been removed by rinsing. Any remaining blood cells were removed when the culture medium was changed after 24 hours.

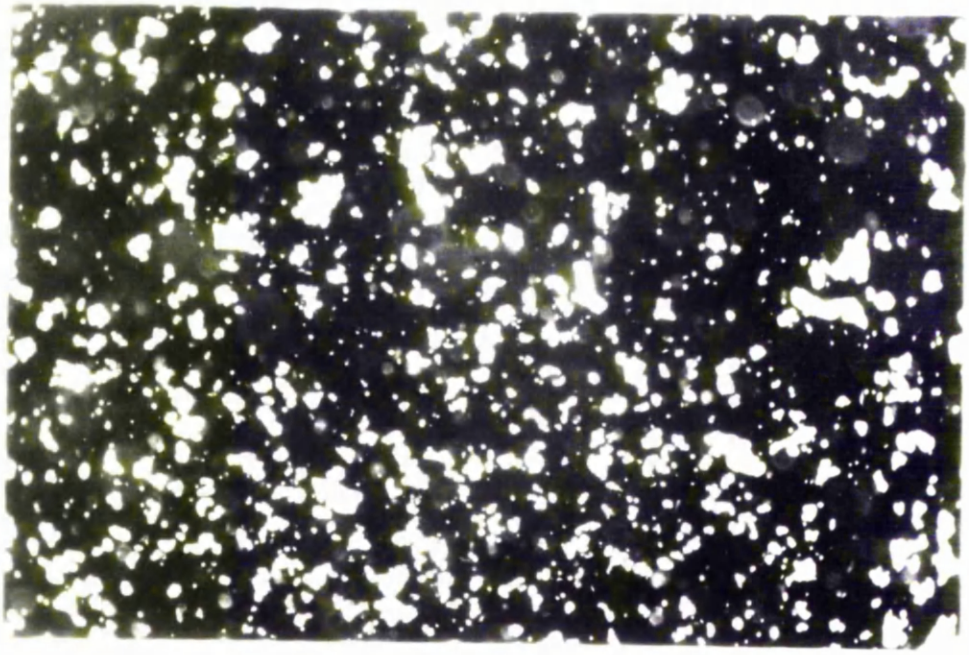
Depending on the density of initial plating and viability, monolayer cultures of polygonal cells were obtained within 2 - 6 days. At this stage the cells were large (average approximately 30μ x 50μ)

PLATE 1

Endothelial culture immediately after isolation. Note the numerous large 'rafts' of cells. (Dark field, x 50)

PLATE 2

'Raft' of endothelium, 1 hour after isolation. (Phase contrast, x 240)



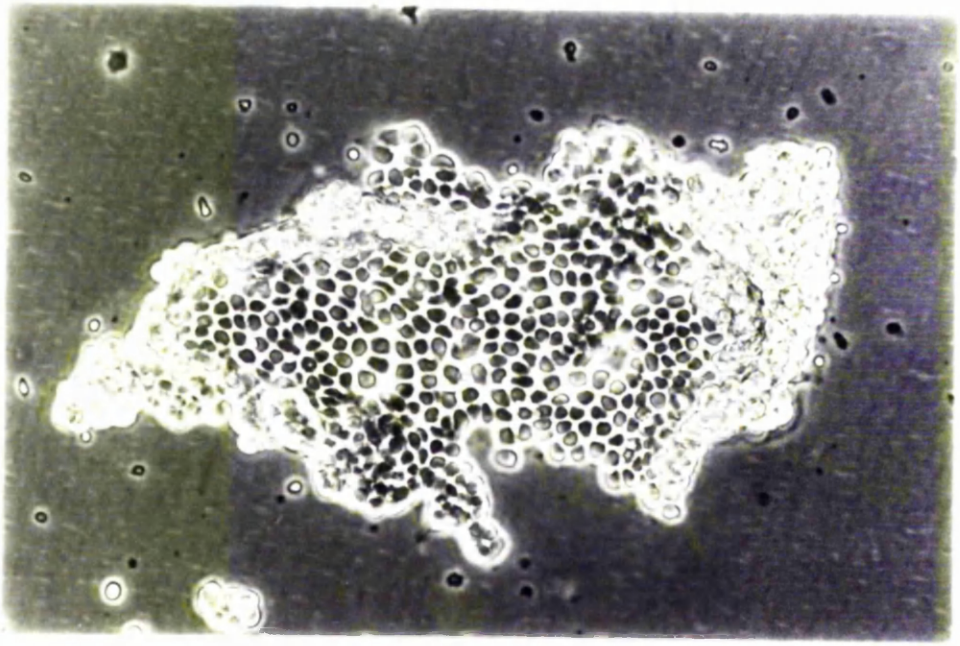
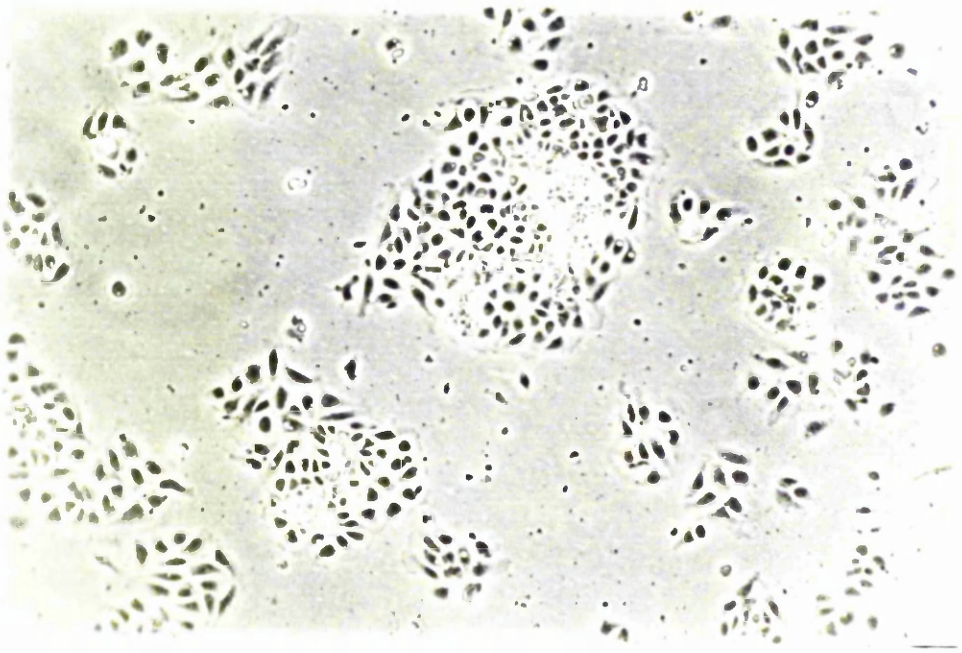


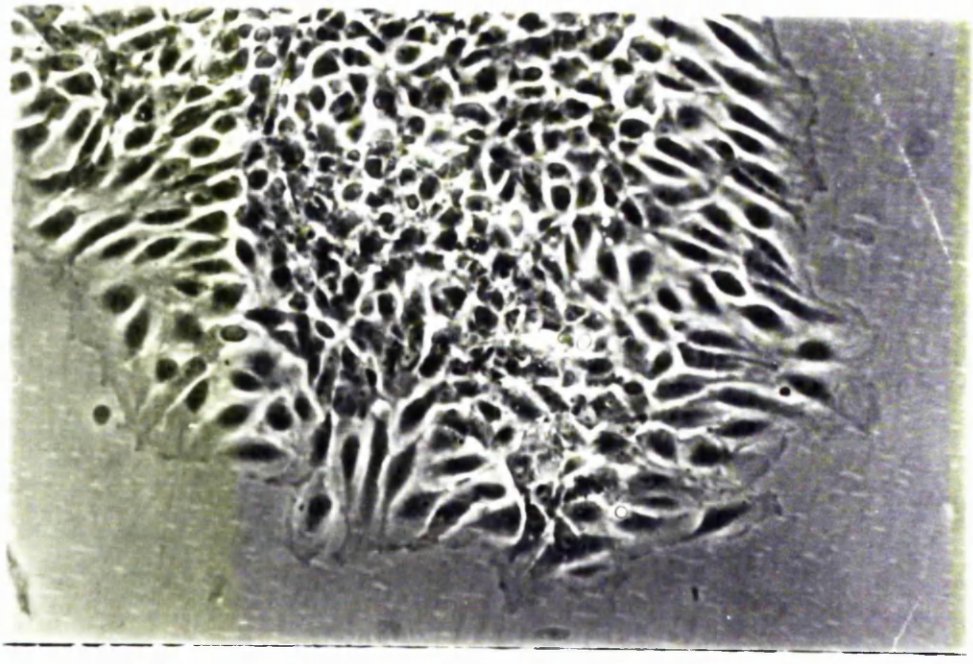
PLATE 3

Endothelial culture, 24 hours after isolation. (Phase contrast, x 120)

PLATE 4

Endothelial 'raft', 24 hours after isolation. (Phase contrast, x 240)





and continued dividing, becoming smaller (approximately 20μ x 30μ) and more closely packed (plates 5, 6), essentially as described by Booyse *et al* (1975), for bovine aortic endothelial cells.

Plate 7 shows a culture in which contaminating non-endothelial cells are overgrowing an area of the endothelial monolayer. These are probably smooth muscle or fibroblast cells and no attempt was made to further identify these, but they do show that observation of contaminant cell types is straightforward. The purity of cultures, as judged by cellular morphology when confluent, was variable. It is unlikely that any of the cultures obtained were absolutely pure endothelium since after passaging several times and especially when left post confluency, a small number of contaminating cells became apparent in most cultures. However, since primary, first and second passage cultures with no visible contaminating cells were routinely obtainable, no further attempts to improve purity were made. The appearance of the cells during growth in primary culture altered quite dramatically, especially at the edges of islands of cells. The cells were often elongated,bipolar and their appearance resembled fibroblast morphology rather than endothelial. However, when confluent, monolayers of polygonal cells were invariably formed.

No rigorous identification of the cells as endothelial was performed although specific markers for these cells are known. It was felt that as a well documented method was being followed which was known to yield endothelial cultures as shown by the presence of Weibel-Palade bodies, pinocytotic vesicles and immunologically by the presence of factor VIII antigen (Booyse *et al* 1975) and as the cultures were identical morphologically to those obtained by other workers, that

PLATE 5

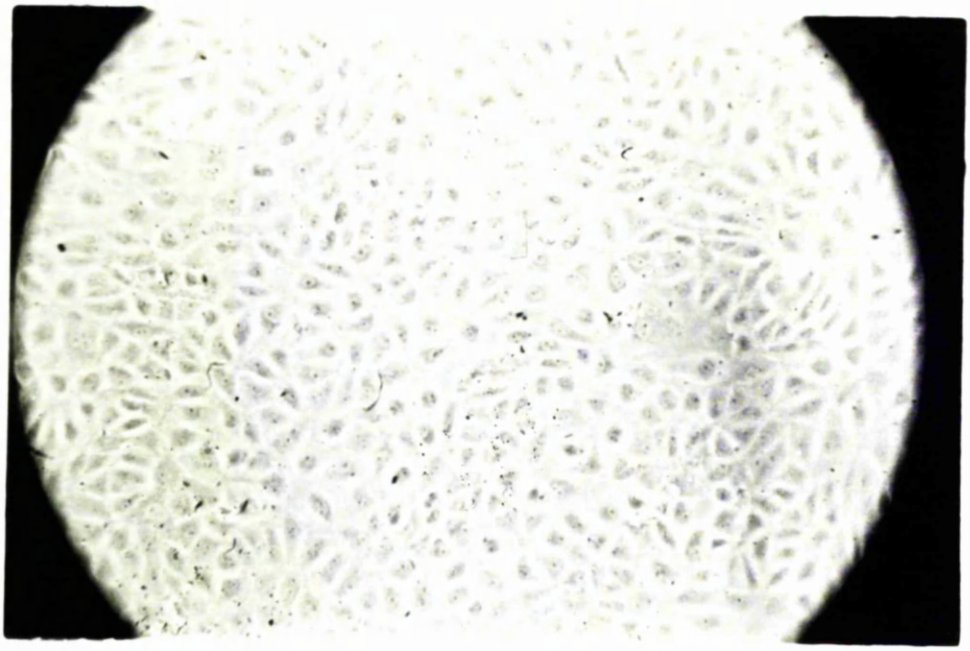
Confluent endothelium

(Phase contrast, x 160)

PLATE 6

Confluent endothelium

(Phase contrast, x 80)



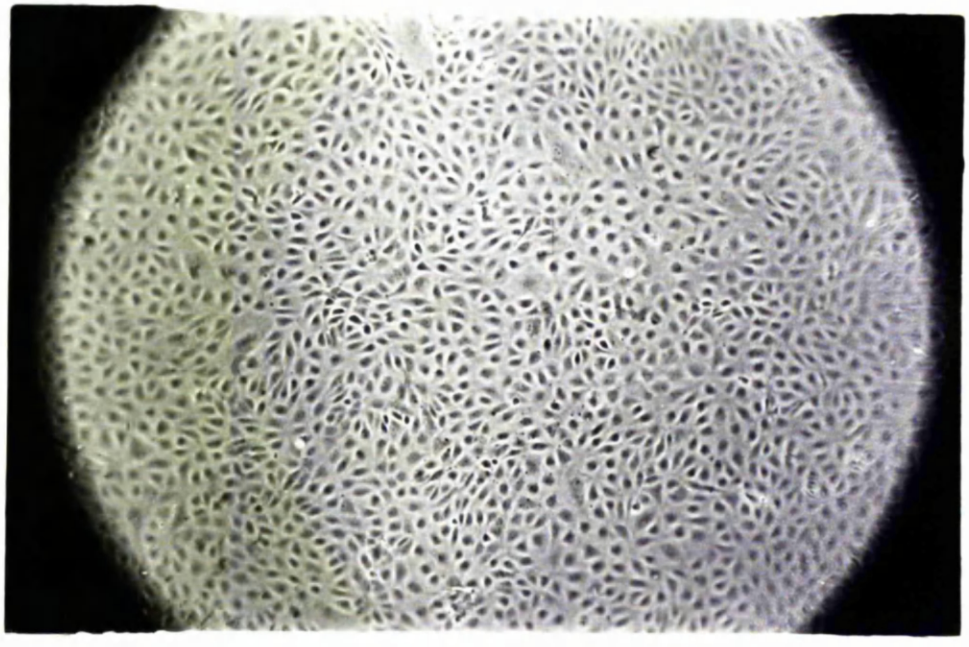
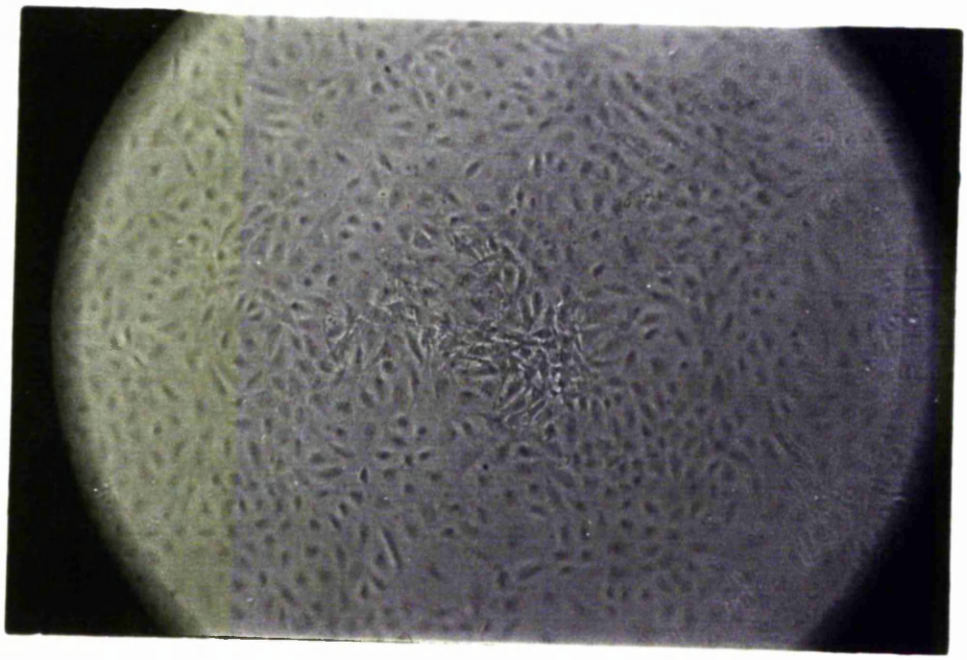


PLATE 7

Confluent endothelium, showing contaminating cells.

(Phase contrast, x 80)



further characterization was not necessary. Further, stained sections of aortae before and after collagenase digestion (plates 8, 9) showed that endothelium had been removed and that no other morphological changes were apparent.

Several different growth media have been reported for culture of endothelial cells. Lackie and de Bono (1977) used Waymouths medium supplemented with 20% foetal calf serum (FCS). Booyse *et al* (1975) used RPMI-1640, 35% FCS. Medium 199, 20% FCS was used by Wagner and Matthews (1975). Dulbecco's modified Eagles medium, 10% calf serum and 100 ng ml^{-1} of fibroblast growth factor has been used. (Gospodarowitz, Moran and Braun, 1977.) Since preliminary results using endothelial cells cultured in Waymouths medium (Lackie and de Bono, 1977) had been reported this medium was initially used. However, comparison of adhesiveness (see next chapter) using Glasgow modified Eagles medium, 10% FCS, revealed no difference in behaviour and this medium was therefore routinely used.

After subculture using trypsin/EDTA viability remained high. No attempt was made to obtain perfect single cell suspensions. The doubling time for cultures was 30-40 hours.

For use in adhesion assays it is important that the cell monolayers used are confluent. Before use, cultures were visually examined for gaps using phase contrast microscopy and any with obvious gaps discarded.

To confirm that apparently confluent cultures were indeed confluent, the cells were stained with silver as described by de Bono (1976). Plate 10 shows the cell borders clearly and thus supports the phase contrast evidence which is slightly inconclusive since the cytoplasm of

PLATE 8

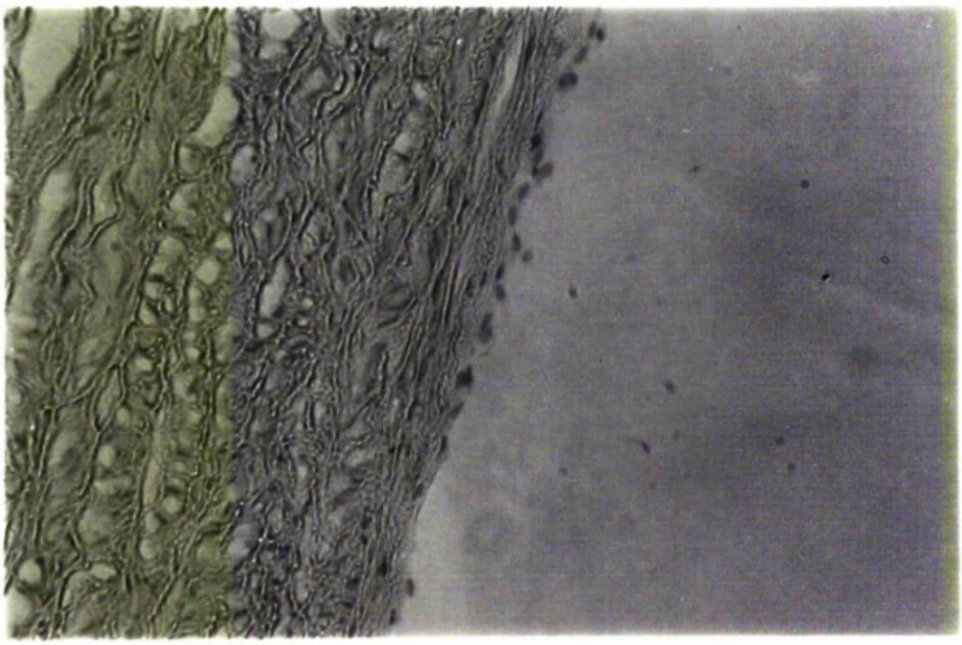
Aortic section before collagenase treatment.

(Haemotoxylin and eosin, x 265)

PLATE 9

Aortic section after collagenase treatment

(Haemotoxylin and eosin, x 425)



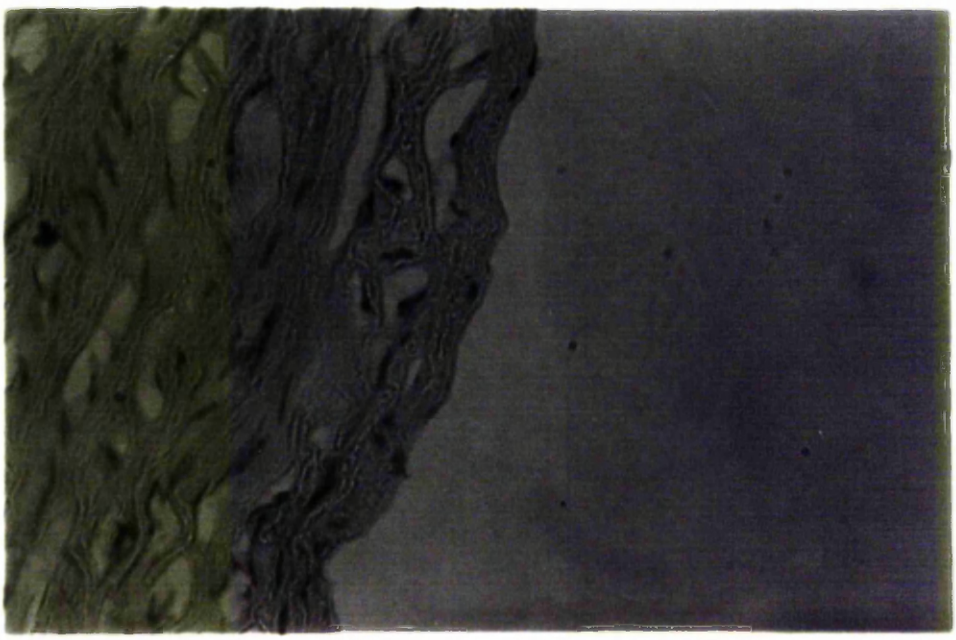
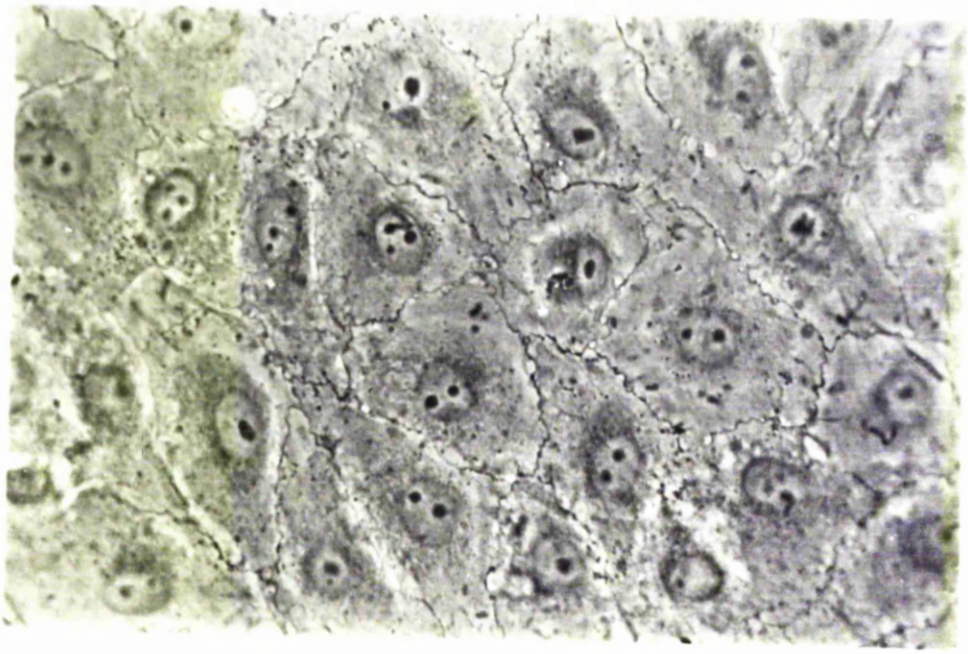


PLATE 10

Newly confluent endothelium stained with silver. (Phase contrast, x 660)



the cells is very thin and difficult to visualize. The silver staining of the cell borders provides support for the conclusion that the cells are endothelial.

CHAPTER 4

Adhesion of neutrophils to endothelial monolayers and serum coated glass.

Before attempting to modify neutrophil or endothelial adhesive behaviour, it was decided to confirm and extend the observations of Lackie and de Bono (1977) in order to more fully understand the behaviour of the cells when used in the adhesion assays.

Table 1 shows the results of such early experiments in which neutrophil adhesion to endothelial monolayers was assayed after 30 minutes in BSS containing 5% calf serum. Glass was also used as a reproducible, inert substratum in order to distinguish between effects on the adhesiveness of the neutrophils and endothelium. In the presence of serum in the medium, it is to a coat of this on the glass that the cells adhere. Such a coat forms almost instantly (Baier and Dutton, 1969). This coat prevents passive adsorption of the cells to the surface, a process which is independent of temperature, pH, cell integrity and cell fixation (Grinnell, 1978) and is therefore unlikely to be of biological significance. Serum is included in the assays merely to provide a 'more physiological' adhesive substratum for the neutrophils.

The most obvious feature of the results is the large day to day variation in the level of adhesion of neutrophils to both endothelial monolayers and glass. Over these particular experiments, adhesion to glass ranges from 7.8% - 40.4% and to endothelium from 1.2% - 27.4% of the added cells. The variation using glass as substratum suggests that the neutrophils' adhesiveness varies considerably as it is assumed that the adhesive characteristics of glass and the serum coat to which the cells adhere remain constant. This is a reasonable assumption especially since experiments were usually performed for several weeks using glass

TABLE 1 Adhesion of rabbit peritoneal neutrophils to glass and endothelial monolayers after 30 minutes (in the presence of 5% calf serum).

Expt.	Number of added neutrophils (x 10^{-6})	Percentage adhesion ± SD onto: Glass (n)	Endothelium (n)	Ratio of endothelium/glass
1	1.5	14.1 ± 0.6(3)	7.9 ± 0.4(3)	0.56
2	3.0	21.0 ± 2.1(3)	12.6 ± 0.1(2)	0.60
3	3.0	35.3 ± 2.6(3)	25.9 ± 1.8(3)	0.73
4	3.0	38.2 ± 2.6(3)	27.4 ± 5.2(3)	0.72
5	1.6	40.4 ± 2.7(3)	18.0 ± 4.3(3)	0.45
6	1.5	38.8 ± 5.3(3)	19.4 ± 2.2(3)	0.50
7	1.3	32.2 ± 2.4(3)	14.4 ± 1.5(3)	0.45
8	2.0	18.2 ± 0.2(2)	8.3 ± 1.0(3)	0.45
9	1.2	12.3 ± 2.2(3)	2.8 (2)	0.23
10	1.8	7.8 ± 1.5(3)	1.2 ± 0.5(2)	0.15

coverslips and serum from the same batches.

Over and above the variation in the adhesiveness of the neutrophils, there appears to be a variation in the adhesiveness of the endothelial monolayers as judged from the variation in the endothelium-to-glass ratio from 0.15-0.75. The variation is unlikely to be due to variations in experimental procedure since the replicates in any particular experiment generally gave good reproducibility.

The number of neutrophils adhering to endothelium was 48 ± 17% compared to glass. The mean difference in adhesion between endothelium and glass was 12.0 ± 1.8% (SE) ($T = 6.69$, $DF = 9$, $p<.001$). This is in contradiction to the report of Lackie and de Bono (1977) that significantly more neutrophils adhered to endothelium than to serum coated glass. The reason for this discrepancy is unknown but it may be that the slightly different conditions used for the adhesion assays account for these differences. The concentration of serum in the assays may be important in the adhesion to glass - a thicker coat may be formed in the presence of higher concentrations.

Consistent with this prediction, the results in Table 2 show that adhesion to glass is reduced in the presence of 10% serum. However, adhesion to endothelium was also reduced. The number of neutrophils adhering to endothelium was 38 ± 10.5% of that adhering to glass. Mean difference 7.6 ± 1.6 (SE) ($T = 4.83$, $DF = 6$, $p < .005$). As before, there was considerable variation in the level of adhesion from day to day. Plates 11 and 12 show typical preparations of neutrophils adhering to endothelium in the presence of serum. Most of the cells are rounded and appear to be on top of the endothelial monolayer.

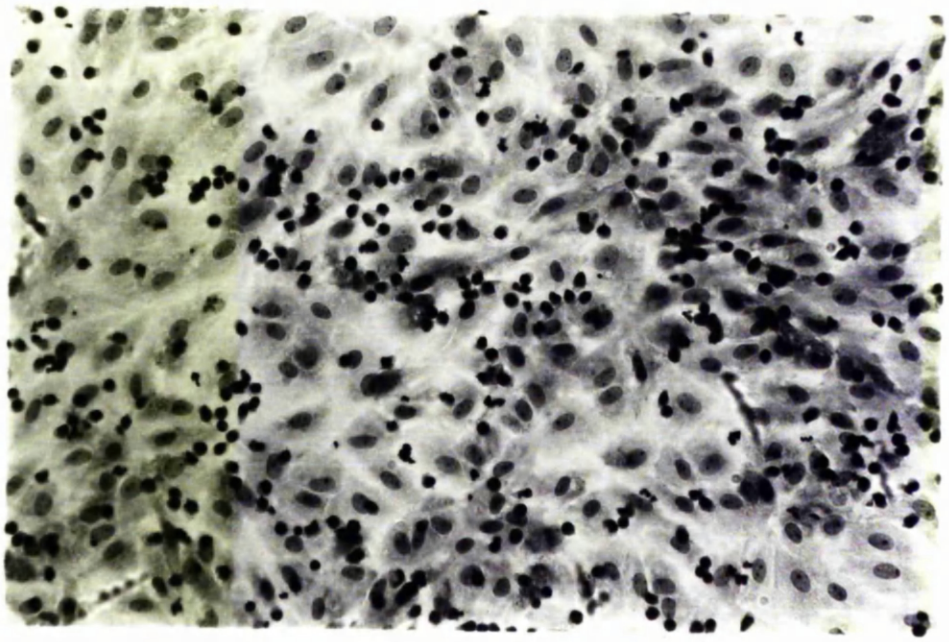
The reasons for the large variation in adhesion observed are not

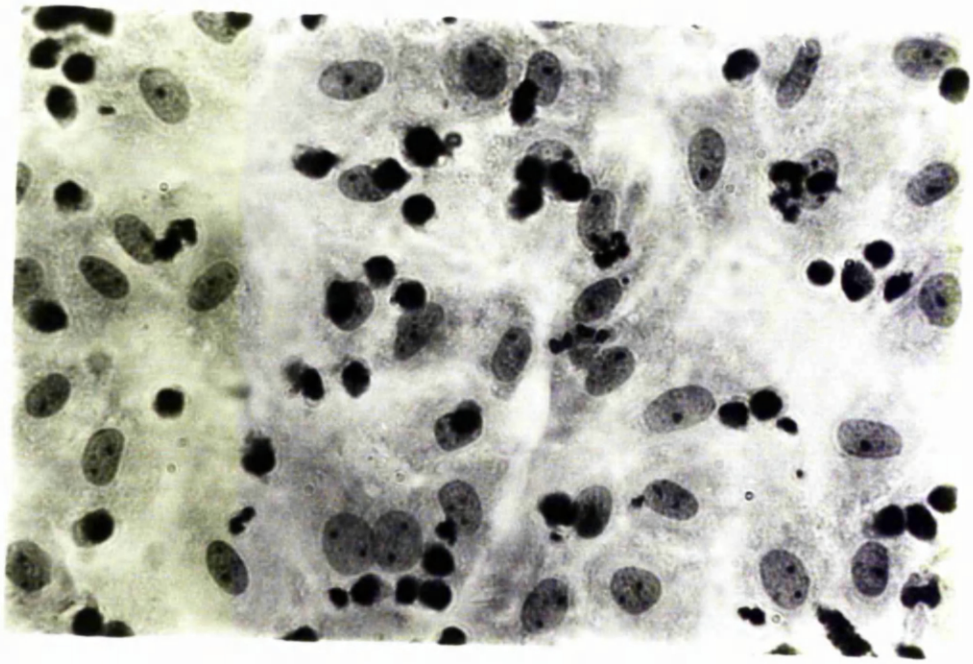
TABLE 2 Adhesion of rabbit peritoneal neutrophils to glass and endothelial monolayers after 30 minutes (in the presence of 10% calf serum).

Expt.	Number of added neutrophils (x 10^{-6})	Percentage adhesion ± SD onto: Glass (n)	Endothelium (n)	Ratio of endothelium/glass
1	1.0	16.1 ± 1.3(2)	7.2 ± 0.1(2)	0.44
2	1.2	7.4 ± 1.9(3)	2.3 ± 0.2(3)	0.31
3	1.1	5.6 ± 0.6(3)	1.4 ± 0.2(3)	0.26
4	1.8	3.8 ± 1.0(3)	1.2 ± 0.4(3)	0.31
5	1.1	11.4 ± 4.1(3)	4.0 ± 0.3(3)	0.35
6	2.3	17.1 ± 8.8(3)	7.1 (2)	0.42
7	2.0	33.7 ± 1.7(3)	18.8 ± 0.5(2)	0.57

PLATE 11 Neutrophils adhering to endothelial monolayer in presence of 10% calf serum. (Giemsa, x 265)

PLATE 12 As above. Note that majority of cells are above the monolayer as judged by difference in focal plane. (Giemsa, x 660)





known. Lackie (personal communication) found no correlation of adhesive behaviour with the rabbit source of the neutrophils, the time in storage or the number of times a particular rabbit had been used to provide peritoneal exudates. Although the data are insufficient for rigorous analysis, the results in Table 3 suggest that there is no simple relation between adhesion and rabbit source, time in culture of endothelium (between 24 - 72 hours) or number of subcultures of endothelium (from 1 - 3). During the course of these experiments, the impression was gained that endothelial cultures of high density were less adhesive than those which were only just confluent. Counting the number of adhering neutrophils and endothelial cell nuclei per unit area in these preparations revealed a negative correlation (Fig.1) between endothelial cell density and adhesion. Since more neutrophils adhere to glass than to endothelium, gaps in the monolayer may account for the higher levels of adhesion at low endothelial cell densities, if more neutrophils adhere there. Phase contrast microscopic examination before use and examination of preparations after fixation and staining did not reveal gaps in the monolayers and neutrophils were not noticed to be especially distributed at intercellular boundaries. The precise boundaries of the cells were difficult to visualize but silver staining (see Plate 9) of apparently confluent cultures confirmed their confluency. That differences in growth rate of high and low density cultures could not account for the difference (e.g. by limiting the amount of some surface component) seems to be the case since estimates of the mitotic indices of both types of culture were very similar (Table 4). It is tentatively concluded therefore that spread endothelial cells, just confluent, are a better adhesive substratum than

TABLE 3 Effects of factors which may contribute to day to day variation.

Source of neutrophils (rabbit)	Percentage adhesion to endothelium in different experiments.
6259	7.9, 2.8, 2.3, 1.4, 18.0
6260	19.4, 14.4, 8.3, 1.2, 7.2, 1.2, 4.0, 7.1, 36.8
6708	12.6, 25.9, 27.4, 18.8
Time in culture of endothelial cells (hours)	
24	7.9, 12.6, 25.9, 19.4, 14.4, 8.3, 2.8, 2.3, 18.8
36	1.2, 36.8, 7.2
48	27.4, 18.0, 1.2
72	12.5, 24.5, 1.4
Number of endothelial subcultures	
1	7.9, 12.6, 18.0, 19.4, 14.4, 1.4, 1.2, 4.0, 7.1, 36.8
2	25.9, 2.8, 7.2, 2.3, 18.8
3	27.4, 1.2, 8.3

FIGURE 1 Effect of varying endothelial cell density on neutrophil adhesion.

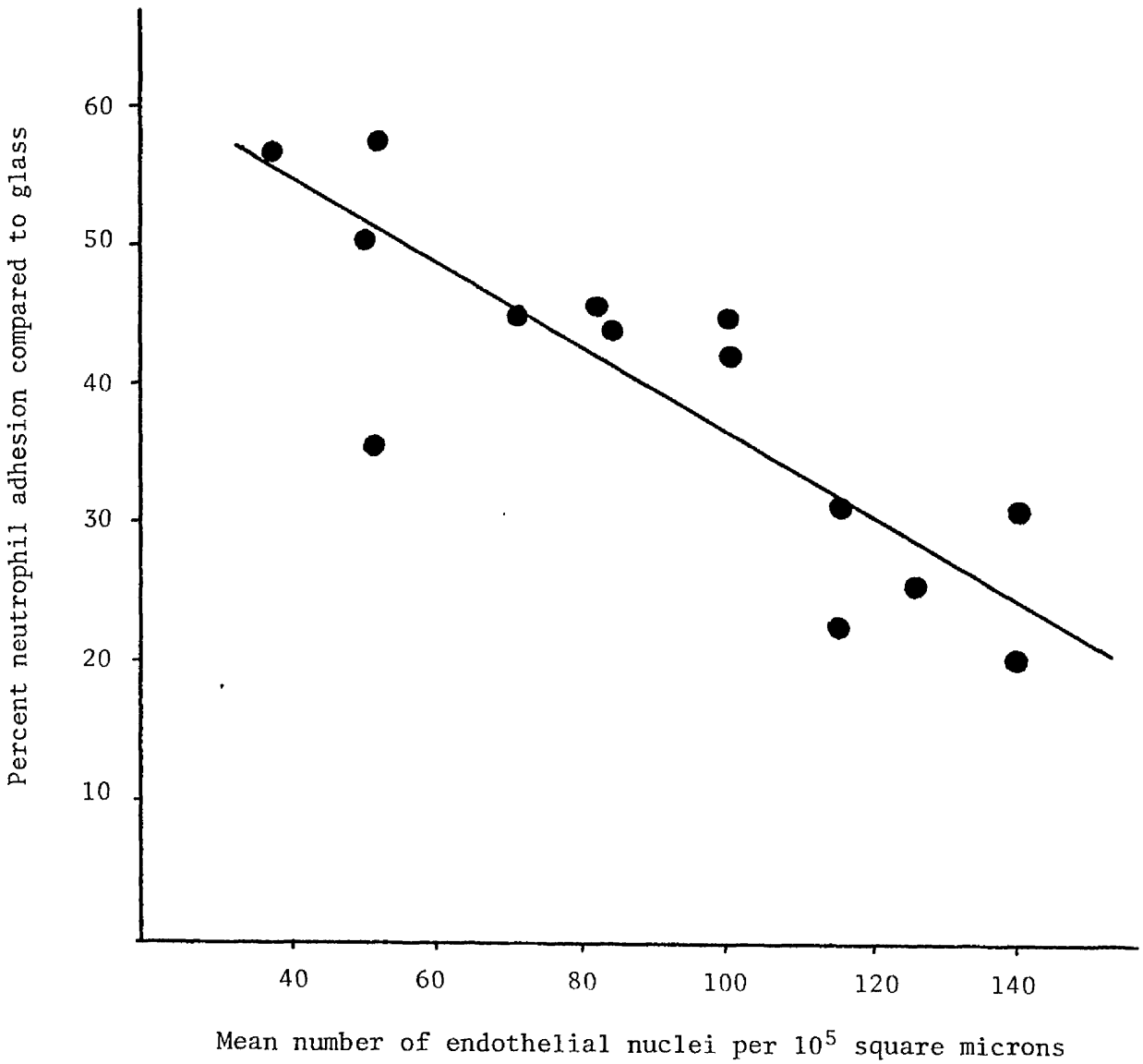


TABLE 4 Estimates of mitotic indices of confluent endothelial cultures at different cell densities.

Expt.	Mean no. of cells/field	Total no. of mitotic cells	Fields counted	Estimated % mitotic cells	
1	45	29	30	2.15	
2	49	58	35	3.37	2.54
3	57	36	30	2.09	
4	107	54	25	2.02	
5	132	57	17	2.54	2.59
6	128	62	15	3.22	

tightly packed cells. de Bono (1976) observed that there was an inverse relation between endothelial cell surface area and lymphocyte binding capacity when densely plated or contracted cells are used.

Since serum in the suspending medium inhibited neutrophil adhesion to both glass and endothelial monolayers, reducing the sensitivity of the assay and introducing another variable, adhesion was subsequently assayed in the absence of serum in the medium. Glass coverslips were coated with serum and rinsed with saline before use in assays. Adhesion to clean glass in the absence of serum is high (∿60%) but as this process has been shown to be independent of the need for metabolic energy or divalent cations (see introduction) which are generally required for cell-cell adhesion, it seems unlikely to be of biological significance. Typical values for adhesion in the absence of fluid phase serum are shown in Table 5. Day to day variation in adhesion was still evident. The levels of adhesion were much higher than in the presence of serum. In general the ratio of endothelial to glass was more reproducible, roughly equal numbers of neutrophils adhering to each substratum. It is interesting that in the absence of serum many neutrophils adhering to endothelium are very spread and at least some of these appear to be underlying the endothelial monolayer (Plates 13, 14). This may partially account for the increased number of adherent neutrophils.

The results using different serum concentrations are summarized in Fig.2. In two further experiments the presence of 10% calf serum in the medium reduced adhesion (67.5 ± 14%(n = 7)) compared to serum coats alone.

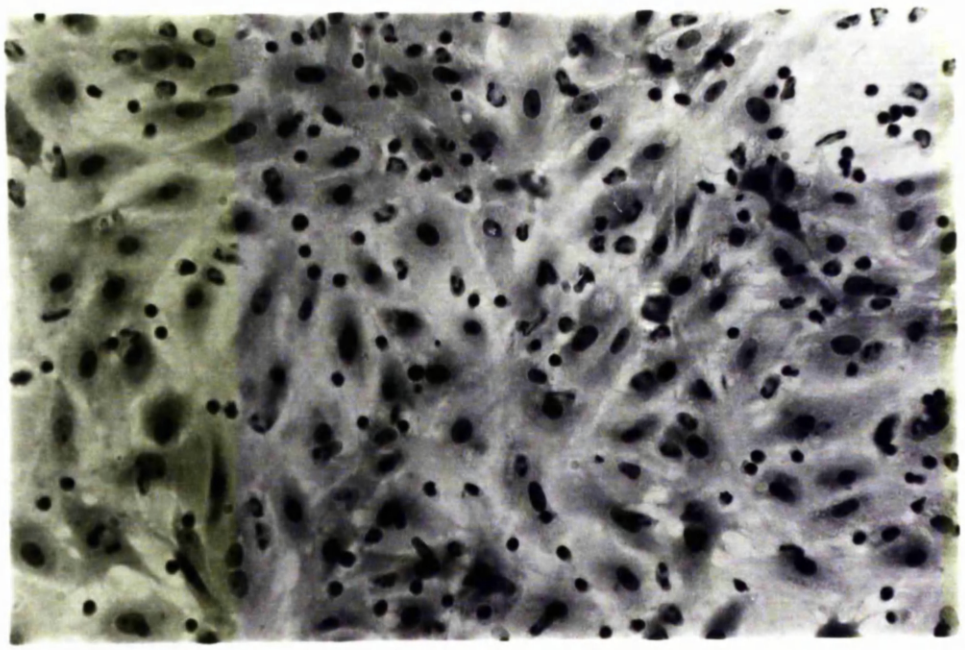
Why serum in the medium should reduce adhesion is not known. Since

TABLE 5 Adhesion of rabbit peritoneal neutrophils to serum coated glass and endothelial monolayers after 30 minutes (in the absence of fluid phase serum).

Expt.	Number of added neutrophils (x 10^{-6})	Percentage adhesion ± SD onto: Glass (n)	Endothelium (n)	Ratio of endothelium/glass
1	1.1	35.5 ± 2.2(3)	12.5 ± 0.55(3)	0.35
2	0.9	36.4 ± 3.8(3)	24.5 ± 2.4(3)	0.67
3	0.4	16.4 ± 3.3(2)	23.2 ± 2.0(2)	1.4
4	0.25	19.4 ± 2.6(4)	23.4 ± 4.2(4)	1.2
5	0.6	8.2 ± 1.2(6)	9.2 ± 1.5(6)	1.1
6	0.6	42.1 ± 2.3(4)	40.8 ± 7.1(4)	0.95
7	0.65	35.5 ± 3.0(2)	30.8 ± 6.3(3)	0.87
8	1.2	39.1 ± 4.3(2)	41.1 ± 3.9(4)	1.05

PLATE 13 Neutrophils adhering to endothelial monolayer in absence of fluid phase serum. Note large number of spread cells. (Giemsa, x 265)

PLATE 14 As above. Spread neutrophils appear to be below endothelium as judged by difference in focus from rounded neutrophils. (Giemsa, x 660)



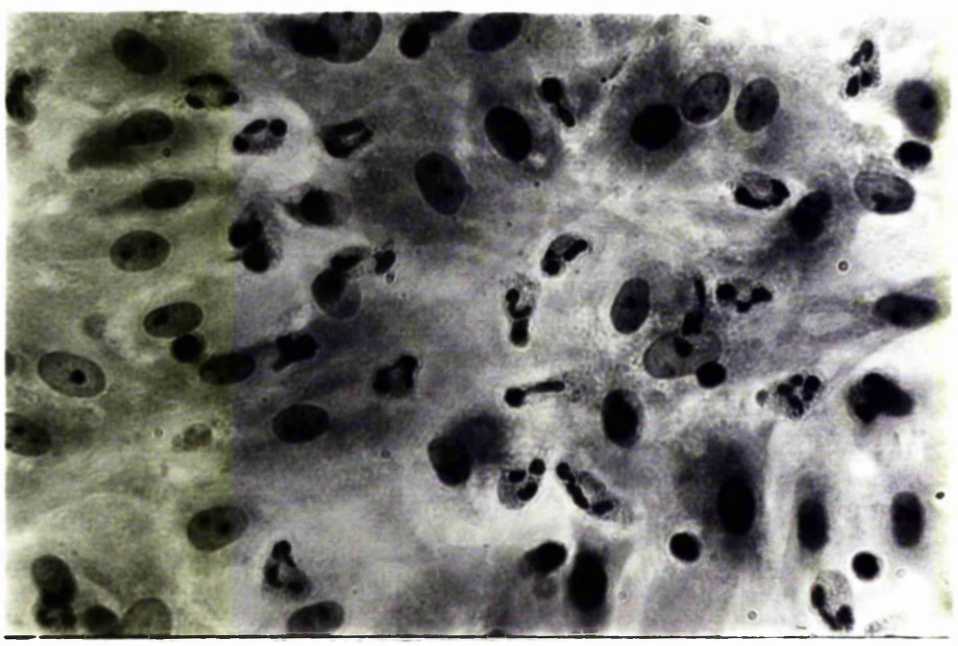
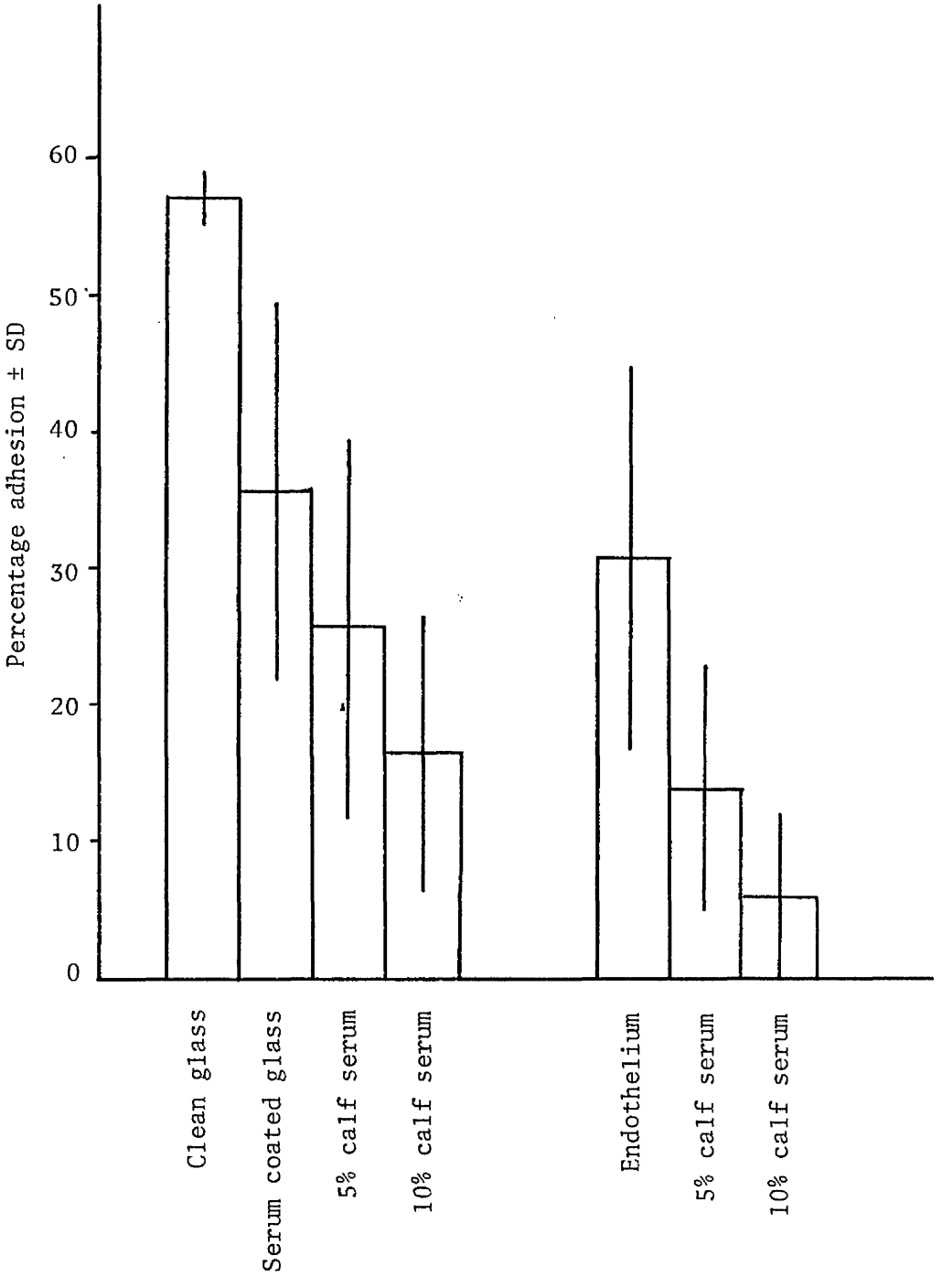


FIGURE 2 Summary of data in Tables 1, 2 and 3



the serum was only being used as a tool - to provide a suitable surface for adhesion - no further work was done on this question.

Time course of neutrophil adhesion to glass and endothelium

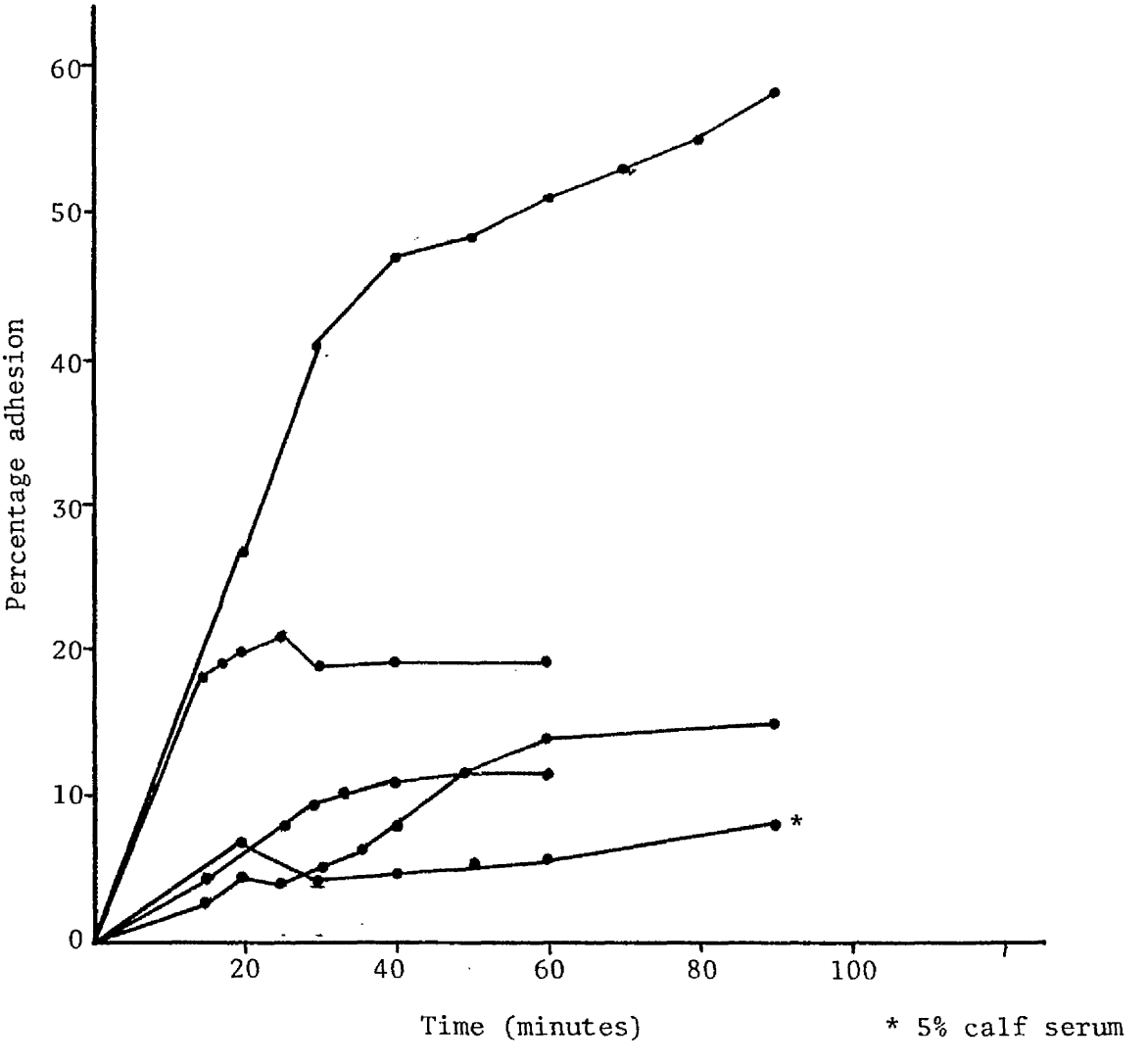
Armstrong and Lackie (1975), studying neutrophil adhesion to fibroblast monolayers and glass, reported that adhesion to glass was constant from 30 minutes to 6 hours. Adhesion to fibroblasts increased over 2 hours and then remained constant. Lackie found (unpublished results) that neutrophil adhesion to endothelial monolayers was essentially constant after 30 minutes.

Several time courses of adhesion to glass and endothelial monolayers were performed over periods from 0 - 90 minutes in the presence of 5%, 10% serum and serum coats. The results of these experiments are summarized in Figs.3 and 4.

A maximal level of adhesion was usually obtained in these experiments and this varied considerably in different experiments, confirming that the previously observed variation was partly a reflection of the cells' properties and not an artifact due to timing differences.

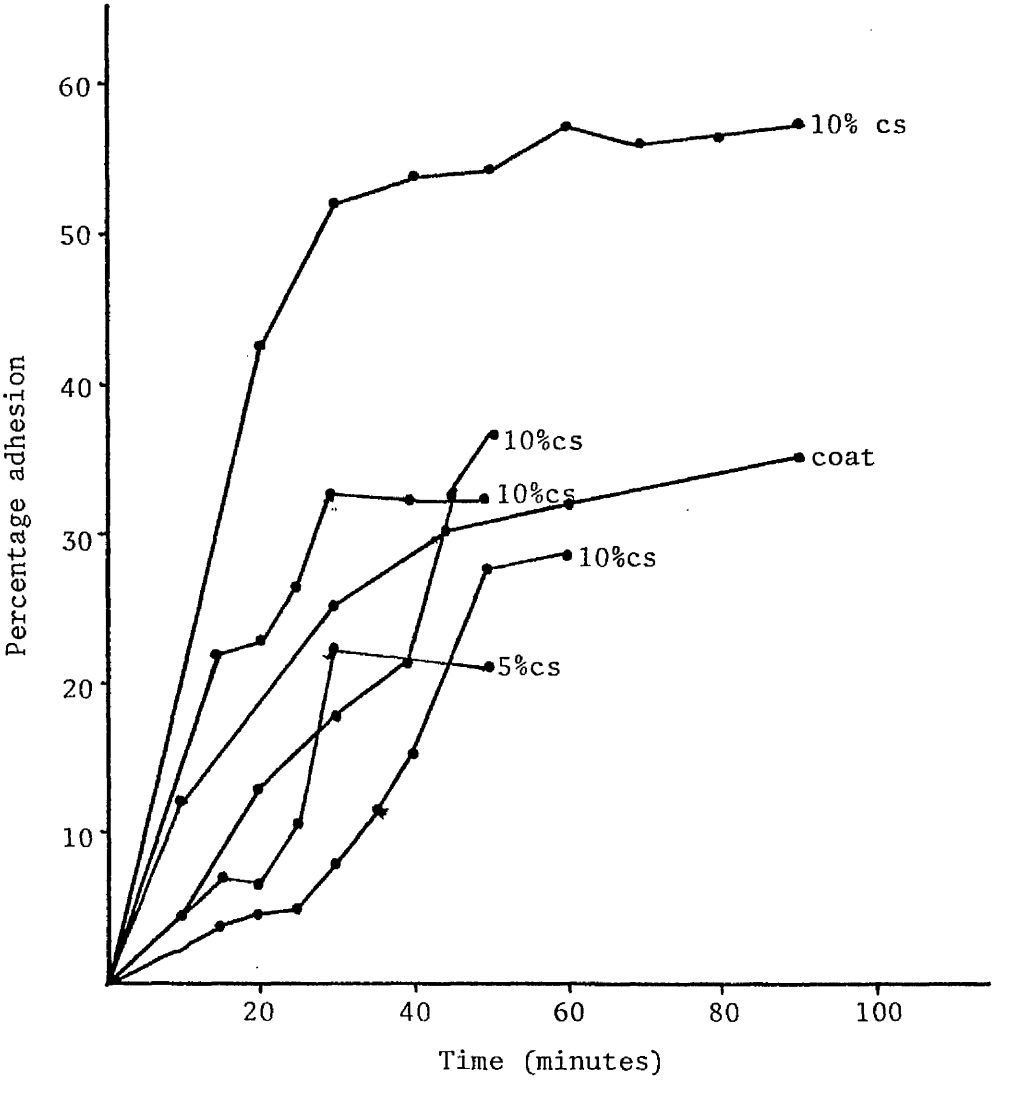
In general, the level of adhesion became constant after 30 minutes. In some experiments however, adhesion continued to rise for up to 60 minutes. In some of these experiments, large numbers ($3 - 4 \times 10^6$) of neutrophils had been used. It seemed possible that the difference in time course might be due to the high cell density and that a separate process was occurring to cause this difference. This does not explain the difference when lower ($\sim 10^6$) cell numbers were used. Thus it seems that part of the variation in the level of adhesion observed

FIGURE 3 Time courses of neutrophil adhesion to endothelium in presence of 10% calf serum.



Each point is the mean from at least 3 replicate coverslips.

FIGURE 4 Time courses of neutrophil adhesion to glass.



Each point is the mean from at least 3 replicate coverslips.

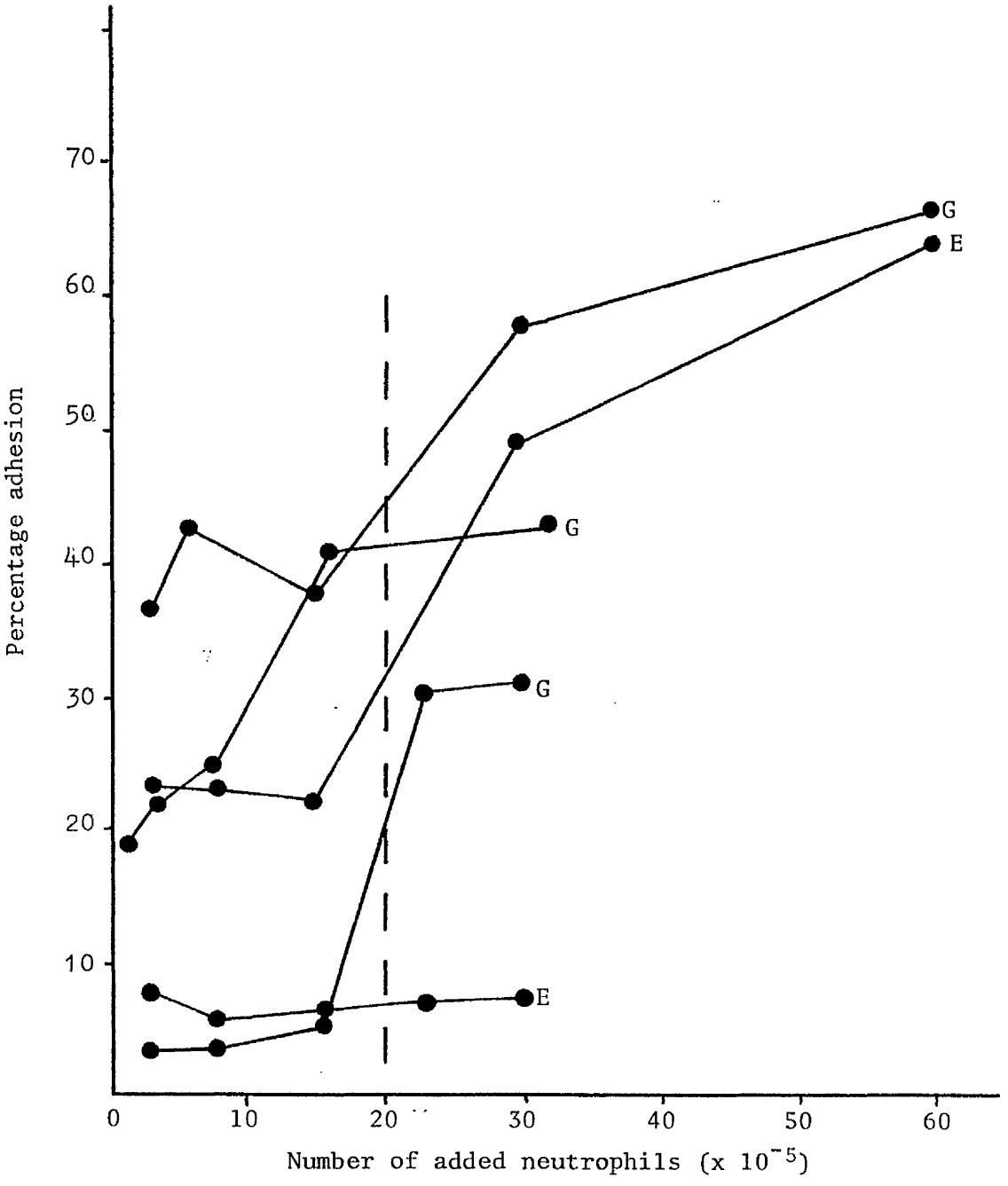
after 30 minutes is due to variation in the rate of adhesion.

Effect of variation in cell number on adhesion

To test the possibility that the presence of large numbers of cells in an adhesion assay altered the adhesive behaviour of the cells, experiments were performed using varying numbers of cells. The results of these are summarized in Fig.5. Up to added cell numbers of 1.5 - 2.0 x 10^6 neutrophils, the percentage adhesion was reasonably constant. Above 2 x 10^6 added cells there was often a sharp increase in the percentage adhesion although in one experiment, percentage adhesion remained constant up to 3 x 10^6 added neutrophils. Adhesion in this experiment was low.

One possible explanation for these results is that neutrophils release some adhesion promoting factor which may have a threshold concentration above which adhesion is stimulated. *In vivo*, if such a factor could be temporarily localized, such a mechanism could control the number of adhering neutrophils. This possibility was further examined in two ways. Firstly the fluid was removed from wells in which assays had been performed using high cell numbers giving high adhesion, and samples were added to a further set of assays, using low cell numbers. Table 6 shows the result of such an experiment. No increase in adhesion at low cell densities (0.9 and 1.8 x 10^6 cells) was observed when assays were performed in a 1:1 mixture of fresh BSS and BSS 'conditioned' by use in a previous assay. It was concluded that factors were not released from the neutrophils. This conclusion is not firm however since it is possible that a factor is rapidly broken down or was simply not present in the assays at a sufficiently high concentration. These possibilities were not further explored due to

FIGURE 5 Effect of varying number on neutrophil adhesion to endothelium (E) and glass (G).



Each point is the mean from at least 3 coverslips.

TABLE 6 Effect of 'conditioned' BSS (c BSS) on adhesion to serum coated glass.

Number of added neutrophils (x 10^{-5})	Percentage adhesion ± SD (n)		
	BSS[1]	BSS + cBSS (1:1)[2]	BSS[2]
36	47 ± 4(3)*	--	39(1)
18	21 ± 2(3)	22 ± 4(3)	19(1)
9	19 ± 3(3)	17 ± 2(3)	24(1)
4.5	23 ± 7(3)	--	17(1)

[1] These experiments performed first.

[2] These experiments then performed using cBSS from*.

another series of observations made concurrently. Microscopic examination of the coverslips during assays using high numbers of neutrophils revealed that at these cell densities, the neutrophils saturated the available surface area and in many areas were piled in clumps or multiple layers. Thus neutrophil-neutrophil adhesion was occurring to a considerable extent. It was discovered that at $\sim 2 \times 10^6$ neutrophils per well the entire available surface area was covered. It is above this number that the marked rise in adhesion often occurred. Examination of fixed and stained coverslips (Plate 15) suggested that cell-cell adhesion was occurring, leading to an apparently high level of cell-substratum adhesion.

It was concluded from these two sets of observations - the lack of effect of 'conditioned' BSS and the apparent cell-cell adhesion, that the increase in adhesion at high cell densities was most probably an artifact of the close packing of the cells during the assays.

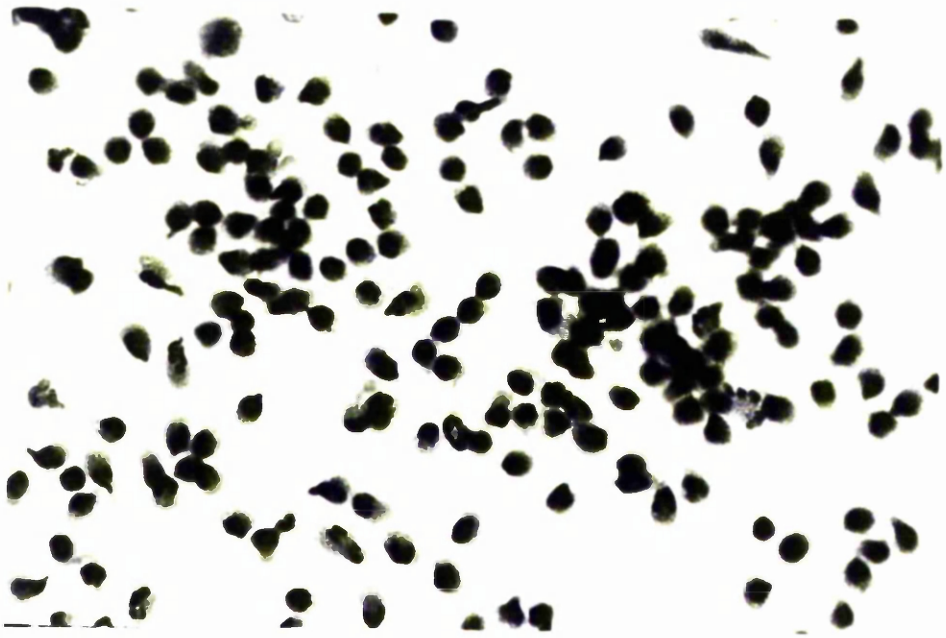
Adhesion of previously non-adherent cells

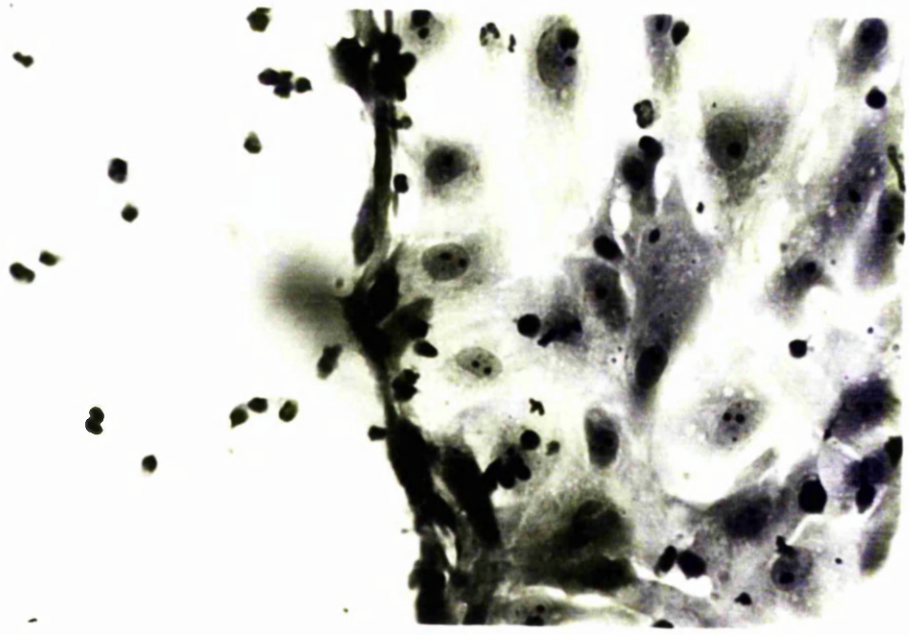
Since only a low percentage of the added cells adhere to the substrata in these assays, even after long periods of incubation, it was decided to examine the possibility that there were subpopulations of cells with widely differing adhesive characteristics. Experiments were performed in which cells which had not adhered in one set of adhesion assays were used in a second set immediately afterwards. Coverslips were removed from the incubation wells and rinsed in BSS which was then stored on ice. The medium in the wells was removed and added to the BSS. The cell suspensions were centrifuged and re-suspended at a cell density similar to that used in the first assays.

Fewer cells adhered in the second set of assays in both of such

PLATE 15 Neutrophils adhering to serum coated glass after addition of 3.10^6 cells to an assay. Note the many apparent cell-cell contacts. (Giemsa, x 670)

PLATE 16 Adhesion of neutrophils to endothelium damaged by scraping. No apparent increase in local adhesion. (Giemsa, X 425)





experiments (Table 7).

This suggests that some of the most adhesive cells have been removed from the population during the first assays, but does not support the concept of a discrete adhesive or non-adhesive population as some cell adhesion clearly still occurs in the second assays. Why cells should adhere in the second but not the first assays is not known, but it is possible that the processing of the cells for the second assays alters their behaviour. Because of this difficulty, it seems possible to conclude only that to some extent adhesion is a random process and that there is a distribution of adhesiveness throughout the cell population.

Adhesion to endothelial cells cultured in different media

The results of experiments in which adhesion of neutrophils was assayed using endothelial monolayers cultured in different media are shown in Table 8. Similar levels of adhesion were observed after growth in either medium.

Adhesion to endothelial cells 'in situ'

Several experiments were performed in which aortas were cut open, pinned flat and used for adhesion assays. Plastic tubes were pressed gently onto the surface of the aorta and filled with suspensions of neutrophils. After incubation the tubes were inverted, removed and the aorta rinsed with a stream of 0.9% saline. After fixation, staining, clearing in xylene and mounting, large numbers of adherent neutrophils were observed. No rigorous quantitation was attempted due to variations in the distribution of the adhering cells but it was estimated that $\sim$ 25% of the added cells had adhered.

TABLE 7 Adhesion of neutrophils that have failed to adhere in an adhesion assay.

Expt.	Percentage adhesion ± SD (n)	
	First assay	Second assay
1 (10% cs)	11.0 ± 1.2(6)	4.7 ± 0.4(6)
2 (5% cs)	27.1 ± 3.9(6)	15.3 ± 2.1(6)

TABLE 8 Adhesion of neutrophils to endothelium cultured in different media.

Expt.	Percentage adhesion ± SD (n) onto endothelium cultured in:	
	Eagles, 10% cs	Waymouths, 20% Fcs
1	18.0 ± 4.3(3)	19.4 ± 5.2(3)
2	14.4 ± 1.5(3)	11.9 ± 1.6(3)
3*	25.9 ± 1.8(3)	--
4*	--	21.2 ± 3.7(3)

* Performed using same source of neutrophils and endothelium but on different days.

Effect of fluid volume in assays

Observation of adhesion assays revealed that after 15 minutes, considerable numbers of cells were still in suspension above the coverslips and that very slight disturbances or convection currents considerably increased this number. It seemed possible therefore that the rate of settling of the cells could influence the apparent adhesiveness. To test this, experiments were performed using varying volumes of BSS in the assays. Fig.6 shows that adhesion decreased as volume increased. Neutrophil settling is thus a rate limiting step in these assays.

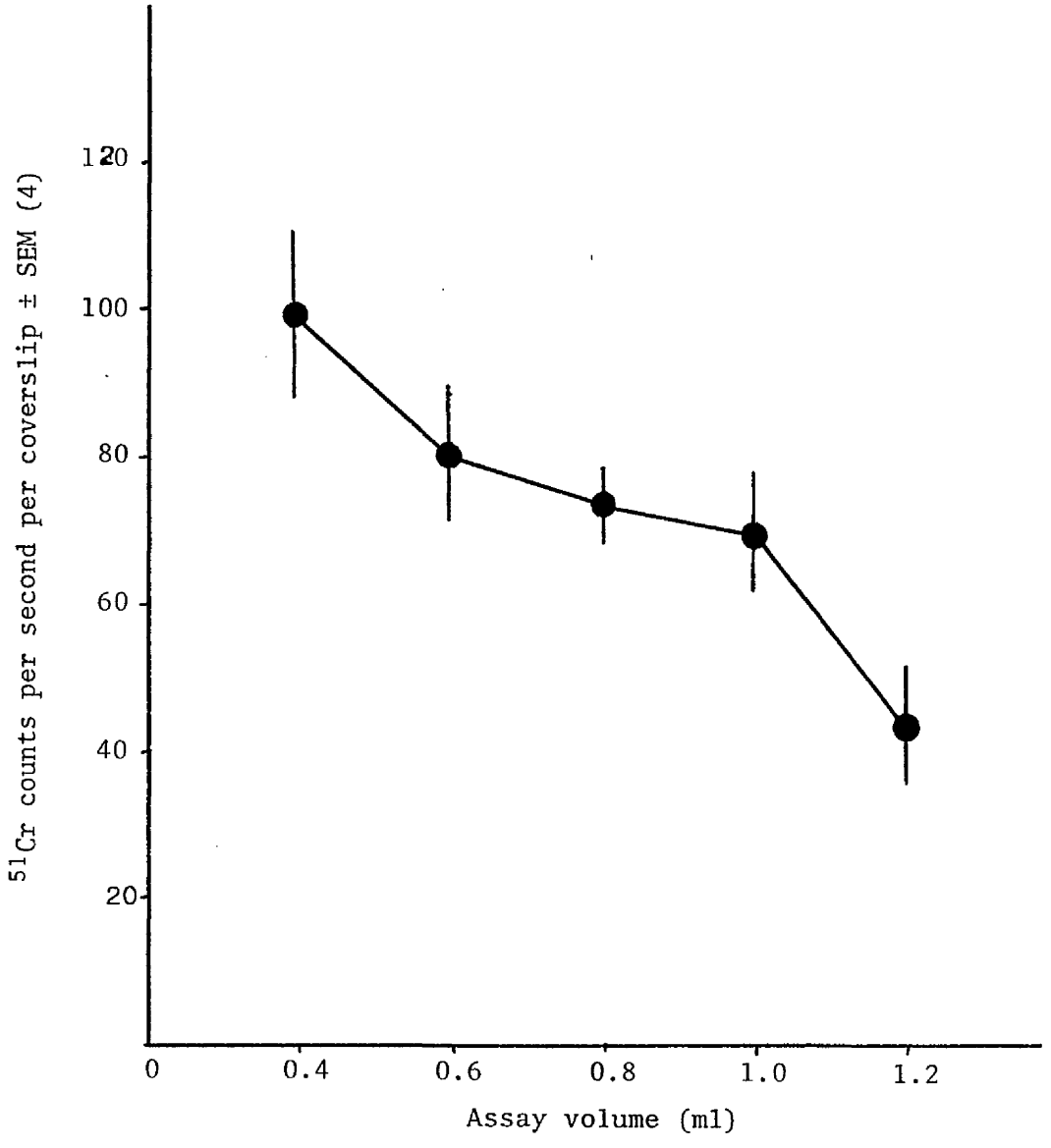
Experimental design

The day to day variation in the adhesiveness of both the neutrophils and the endothelium places some limit on the design of experiments and the comparison of results. Any experiment using endothelium has to be performed using monolayers from one aorta and cultured in an identical fashion. Similarly, any experiment must use only neutrophils from one batch that have been isolated, washed and resuspended together. No valid comparisons can usually be drawn between the absolute values for adhesion in different experiments unless, by chance, the control values are identical. To study the effect of any treatment or agent, an internal control is required in every experiment and the effect of the treatment compared to this. By this means the results of experiments on different days can be compared. This device however is not perfect if, for example, particular treatments are only effective in the presence of a low or high control level. The effect of this, however, will only be to dampen any effects on adhesion.

Presentation of results and statistics

Due to the limitations discussed above, adhesion results obtained

FIGURE 6 Effect of fluid volume on neutrophil adhesion.



from a number of experiments will often be presented in the form of a mean percentage of control adhesion (which is defined as 100%) as well as a mean percent adhesion for control and experimental assays. Significance of differences between experimental and control assays will usually be assessed by use of a paired sample T-test on the difference in adhesion of pairs of control and experimental coverslips.

CHAPTER 5

Inflammatory Mediators

A variety of substances are released or produced during an inflammatory response. These can be classified into two groups: those from the plasma and those from the tissues. In plasma, there are three interrelated mediator producing systems. 1) The kinin system (producing bradykinin, kallikrein, plasminogen activator), 2) the complement system (C3 fragments, C5 fragments, C567 complex etc). 3) The clotting system (fibrinopeptides, fibrin degradation products). Potential mediators released from tissues can be divided into several groups: a) vasoactive amines (histamine, 5-HT), b) acidic lipids (prostaglandins), c) lysosomal components, d) lymphocyte products. The production, distribution and activities of all of these are reviewed by Ryan and Majno (1977). Speculation about, rather than hard evidence for, exact functions of molecules is common and it is quite possible that some mediators may be involved in the initial adhesion of neutrophils to endothelium.

If a mediator could be found which increased the adhesiveness of either the neutrophils or the endothelium, a role for such a mediator in margination would be suggested. At another level, use of such a mediator to reliably alter the adhesiveness of the cells would open up pathways of investigation of the mechanism of adhesion by comparative studies of adhesive and non-adhesive cells. Accordingly, several mediators known to be involved in the inflammatory response were investigated for effects on adhesion. The results of these experiments are shown in Table 9.

Histamine had virtually no effect on either the adhesiveness of the endothelium or of the neutrophils though, if anything, a slight reduction in adhesion occurred. Five-hydroxy-tryptamine (5HT) also had no effect

TABLE 9 Effect of possible inflammatory mediators on neutrophil adhesion.

Agent	Mean percent adhesion to endothelium		Mean diff. ± SE	P	100 x $\frac{\text{Experimental}}{\text{control}}$ ± SD(n)
	BSS control ± SD(n)	Experimental ± SD(n)			
10^{-4}M histamine	10.0 ± 6.7(11)	9.0 ± 7.6(11)	1.0 ± 0.7	n.s.	81 ± 26(15)
10^{-4}M 5-HT	18.6 ± 7.4(7)	18.5 ± 7.5(7)	0.1 ± 1.3	n.s.	75 ± 25(13)
10^{-5}M 5-HT	12.6 ± 0.2(3)	12.1 ± 6.3(3)	0.5 ± 3.5	n.s.	83 ± 26(9)
10^{-5}M bradykinin	19.0 ± 7.7(4)	11.4 ± 3.8(4)	7.6 ± 3.3	n.s.	87 ± 41(7)
10^{-6}M "	17.3 ± 7.6(5)	10.0 ± 3.6(5)	7.3 ± 2.2	<.05	77 ± 30(7)
10^{-7}M "	19.0 ± 7.7(4)	12.3 ± 3.2(4)	6.6 ± 5.2	n.s.	82 ± 36(6)
	Mean percent adhesion to serum coated glass				
10^{-4}M histamine	16.0 ± 13.8(11)	14.5 ± 13.9(11)	1.5 ± 1.0	n.s.	82 ± 32(11)
*10^{-4}M histamine	20.7 ± 6.1(7)	14.6 ± 5.0(7)	6.1 ± 1.7	<.01	78 ± 20(11)
10^{-4}M 5-HT	22.3 ± 9.1(7)	19.7 ± 15.8(7)	2.6 ± 2.9	n.s.	86 ± 29(12)
10^{-5}M 5-HT	18.2 ± 1.5(5)	11.8 ± 0.8(5)	6.0 ± 1.2	<.02	74 ± 13(9)
10^{-5}M bradykinin	37.6 ± 11.6(5)	43.5 ± 22.1(5)	5.9 ± 9.6	n.s.	119 ± 64(5)
10^{-6}M "	37.6 ± 11.6(5)	31.2 ± 13.8(5)	6.4 ± 7.7	n.s.	87 ± 14(5)
10^{-7}M "	37.6 ± 11.6(5)	49.4 ± 10.6(5)	11.7 ± 7.1	n.s.	140 ± 51(5)

* ^{51}Cr labelled cells used.

on endothelial adhesion, though at 10^{-5}M an inhibition of neutrophil adhesion to glass was observed. Bradykinin appeared to have no marked effect except for a very slight reduction in endothelial adhesiveness. This agent was particularly variable in its effect, perhaps a reflection of its breakdown during the experiments.

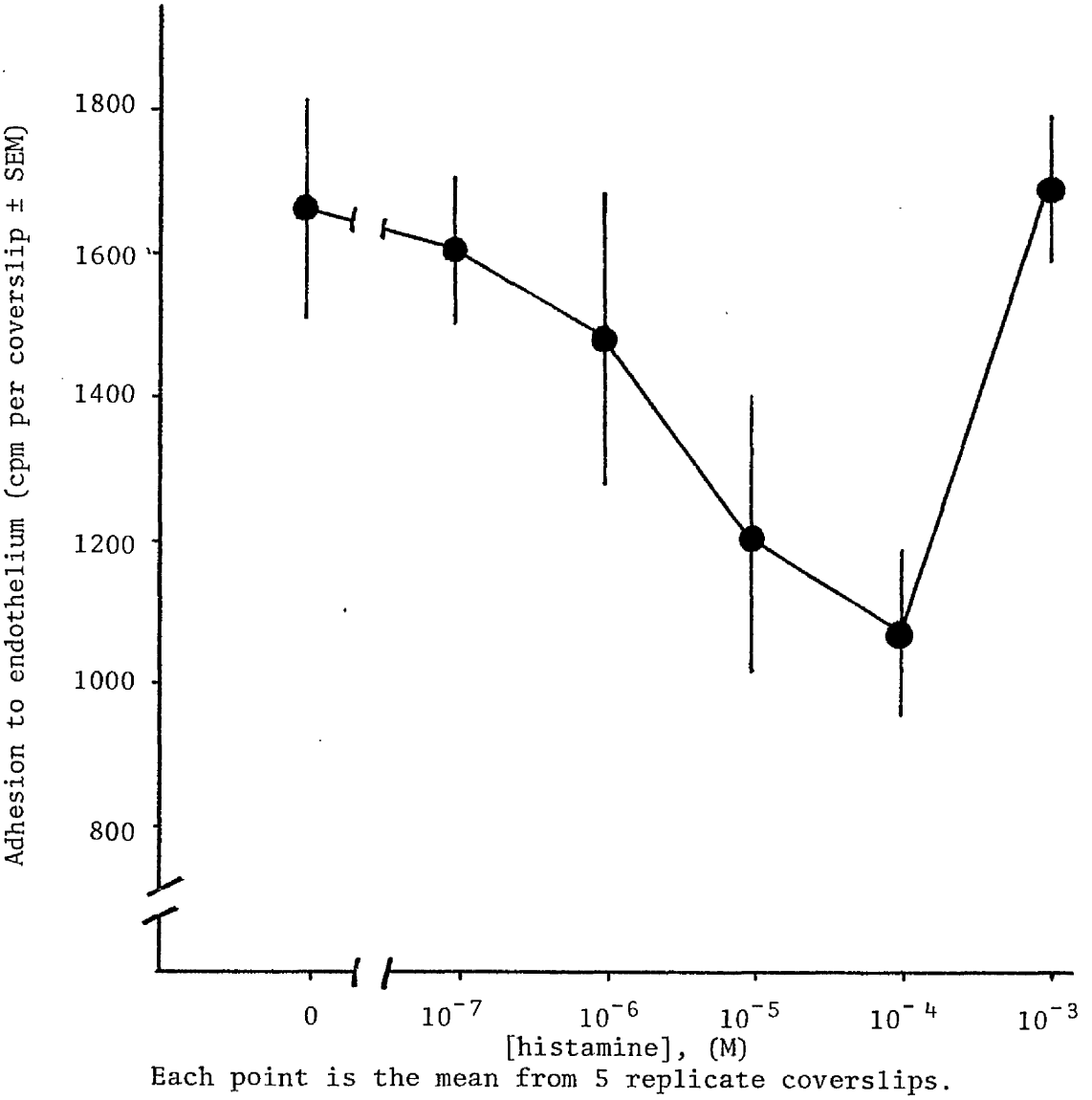
Dose response curves obtained for histamine and 5-HT (Figs.7 and 8) show that a concentration of 10^{-4}M produced the maximal response in each case. Since this high concentration produced only an inconsistant moderate (inhibitory) effect and no other effect was observed at lower concentrations it seems that neither of these agents mediates the neutrophil-endothelial adhesion. At a concentration of 10^{-3}M, both of these agents were less effective in reducing adhesion, possibly a result of cell damage.

No other mediators were examined directly. The possibility that endothelial cells may themselves release mediating substances was investigated by scraping the monolayer, measuring adhesion and examining the damaged area for any local effects. No changes in adhesion were observed, suggesting that damage releases no adhesion promoting substance although it is possible that control monolayers were also damaged in handling, thus masking any increase. Examination of damaged areas revealed no evidence of local adhesive change (Plate 16).

The exudate fluid in which the peritoneal neutrophils are obtained was tested for adhesion augmenting activity. Table 10 shows that for neutrophils purified from peripheral blood and peritoneal exudate neutrophils, no increase in adhesion was detected in the two experiments performed.

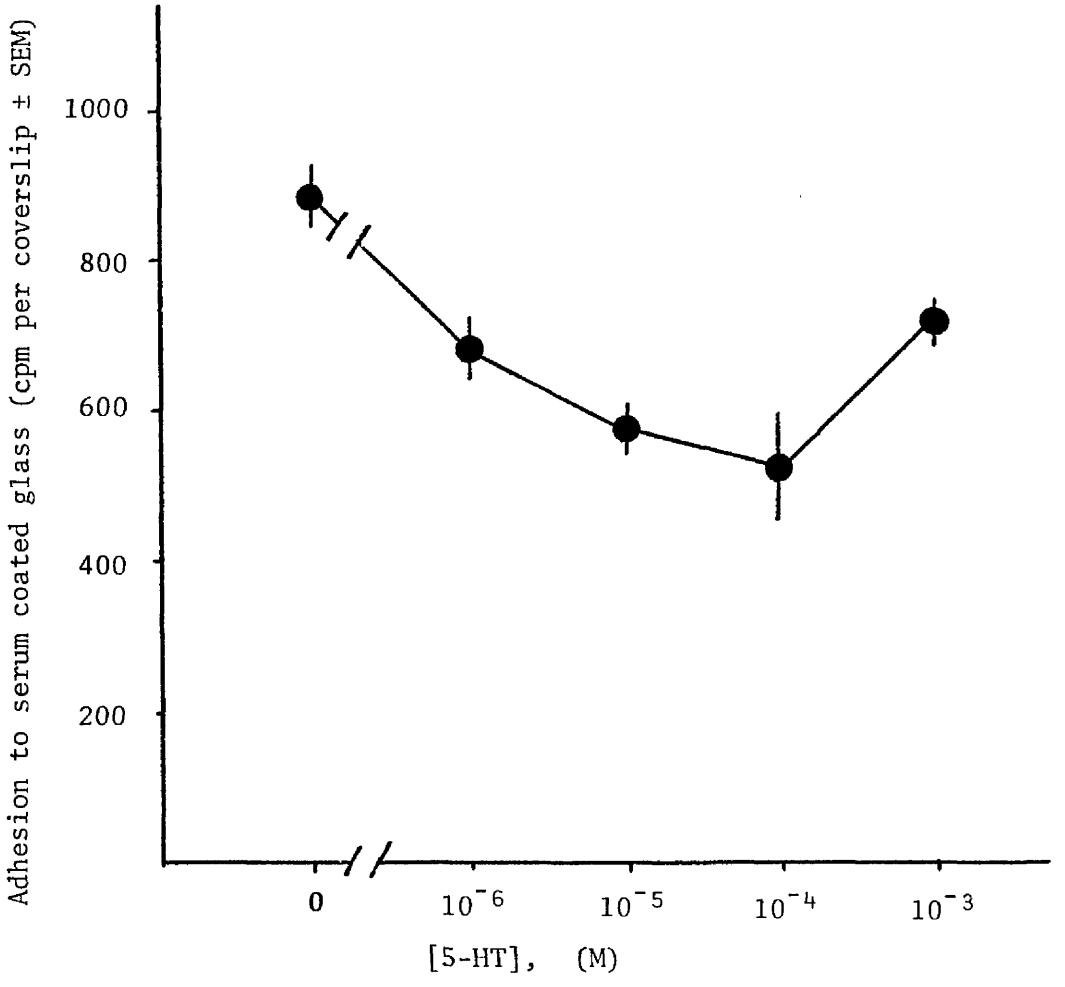
It was of interest to determine whether peripheral blood neutrophils

FIGURE 7 Dose response curve - histamine.



Each point is the mean from 5 replicate coverslips.

FIGURE 8 Dose-response curve - 5-HT



Each point is the mean from 4 replicate coverslips.

TABLE 10 Effect of peritoneal exudate fluid on neutrophil adhesion

		Mean per cent adhesion ± SD to:	
	Suspending medium	endothelium (n)	serum coated glass (n)
Expt. 1	BSS control	16.7 ± 2.1(3)	27.0 ± 1.0(3)
(Peripheral blood	0.1% glycogen	17.0 ± 2.0(3)	26.7 ± 2.1(3)
neutrophils)	exudate fluid	17.0 ± 2.6(3)	18.3 ± 0.6(3)
Expt. 2	BSS control	14.3 ± 2.1(3)	5.6 ± 0.8(3)
(Peritoneal exudate neutrophils)	exudate fluid	18.0 ± 3.6(3)	5.3 ± 0.3(3)

and peritoneal exudate cells (which have been through one inflammatory response) behaved differently. The result of three experiments, using cells from different rabbits, show that cells purified from blood adhered more to both endothelium and serum coated glass (Table 11). This unexpected result is confirmed by later experiments (Chapter 6) on the effects of chemotactic factors on blood and peritoneal cells.

Anti-inflammatory agents

The mechanism of action of these drugs in reducing inflammation is far from clear. One effect, however, that many anti-inflammatory drugs have is to reduce the accumulation of leucocytes in inflamed areas. This has been demonstrated many times *in vivo* and Di Rosa (1978) comprehensively reviews this field. The mechanisms involved in this reduction are not known but inhibition has been reported of margination (Grant *et al.*, 1962), locomotion of neutrophils (Ward, 1966), non-phagocytic discharge of lysosomal enzymes by neutrophils (Ignarro, 1977; Northover, 1977; Palmer and Weatherall, 1978), phagocytosis (Smith, 1977), adhesion to nylon wool (Macgregor, 1976) and chemotaxis (Rivkin *et al.*, 1976). Effects on the membrane potential of vascular endothelium *in vitro* have also been reported (Northover, 1975a; 1975b).

Since possible mediators had not enhanced adhesion, it seemed possible that the cells were already 'stimulated' and that anti-inflammatory drugs might reduce adhesion and provide an experimental tool. Several experiments were performed to investigate whether anti-inflammatory agents affected the adhesiveness of endothelial cells or neutrophils. Both non-steroidal and steroidal anti-inflammatory agents were tested (Table 12). The anti-protease E-amino-n-caproic acid (EACA) (used clinically as an anti-inflammatory agent), even at a

TABLE 11 Adhesion of peripheral blood and peritoneal exudate neutrophils

Expt.	Per cent adhesion to coverslips			
	Endothelium		Serum Coated Glass	
	Blood cells	Peritoneal cells	Blood cells	Peritoneal cells
A	36.7	32.6	34.4	34.1
	47.5	33.7	31.2	28.5
	38.8	32.8	35.6	34.9
	41.9	33.6	31.6	34.4
B*	19.0	11.0	27.0	14.0
	15.0	9.0	27.0	17.0
	16.0	8.0	27.0	20.0
C			12.7	6.6
			11.7	6.0
			12.7	4.9
			11.7	6.2

Endothelium: mean diff. = 7.7 ± 1.2 T = 6.7 $p < .001$

S.C. Glass: mean diff. = 5.0 ± 1.4 T = 3.6 $p < .005$

*^{51}Cr labelled cells

TABLE 12 Effect of anti-inflammatory agents on neutrophil adhesion

Agent	Mean percent adhesion to endothelium					
	BSS control ± SD(n)*	Experimental ± SD(n)*	Mean diff. ± SE	P	$\frac{100 \times \text{Experimental}}{\text{control}}$	± SD(n)*
10^{-5}M colchicine	22.5 ± 10.1(10)	10.7 ± 5.0(10)	11.9 ± 1.8	<.001	48	12(10)
10^{-4}M prednisolone	8.8 ± 4.9(11)	7.4 ± 5.4(11)	1.4 ± 0.8	n.s.	82	24(11)
10^{-3}M aspirin	17.2 ± 6.8(6)	10.4 ± 6.1(6)	6.8 ± 1.9	<.025	58	21(6)
10^{-4} M indomethacin	12.0 ± 6.0(9)	14.3 ± 7.6(9)	2.3 ± 0.9	<.025	115	19(9)
10^{-4}M hydrocortisone	20.9 ± 11.7(12)	13.6 ± 6.9(12)	7.3 ± 1.8	<.005	66	13(12)
10^{-5}M "	27.8 ± 12.0(6)	19.2 ± 7.6(6)	8.6 ± 3.2	<.05	66	12(6)
10^{-6}M "	27.8 ± 12.0(6)	24.2 ± 10.2(5)	6.0 ± 2.3	n.s.	80	17(5)
10^{-2}M EACA	21.1 ± 5.0(6)	21.7 ± 4.7(5)	0.6 ± 2.3	n.s.	107	33(4)
10^{-3}M "	22.4 ± 7.4(3)	22.1 ± 4.5(3)	0.3 ± 2.2	n.s.	110	30(3)

TABLE 12 continued

	Mean percent adhesion to serum coated glass				
10^{-5}M colchicine	15.0 ± 6.7(7)	12.9 ± 7.2(7)	2.1 ± 0.8	<0.05	82 ± 20(7)
10^{-4}M prednisolone	14.9 ± 11.1(8)	14.4 ± 11.9(8)	0.5 ± 1.0	n.s.	87 ± 18(22)
10^{-3}M aspirin	24.9 ± 9.6(8)	17.9 ± 9.1(8)	7.0 ± 1.6	<.005	72 ± 15(10)
10^{-4}M indomethicin	24.7 ± 12.9(8)	29.0 ± 16.7(8)	4.3 ± 2.1	n.s.	119 ± 25(8)
10^{-4}M hydrocortisone	27.6 ± 10.5(16)	22.9 ± 11.8(16)	4.7 ± 1.8	<.025	86 ± 20(37)
10^{-5}M "	24.0 ± 9.7(6)	18.8 ± 5.8(6)	5.2 ± 1.7	<.05	87 ± 18(26)
10^{-6}M "	24.0 ± 9.7(6)	24.1 ± 10.8(5)	0.1 ± 2.1	n.s.	94 ± 23(18)
10^{-2}M EACA	41.8 ± 2.6(4)	43.5 ± 4.0(4)	1.7 ± 2.9	n.s.	105 ± 14(4)
10^{-3}M "	41.4 ± 2.7(3)	45.8 ± 7.5(3)	0.4 ± 2.8	n.s.	110 ± 20(3)

* Since in some experiments absolute values of adhesion were not determined these numbers will not necessarily correspond.

concentration of 10^{-2}M had no effect on adhesion either to endothelium or serum coated glass. Indomethacin had a statistically significant, but very slight, enhancing effect on adhesion to endothelium. Aspirin reduced adhesion to both endothelium and glass but the effect was small. Colchicine reduced adhesion to endothelium by 11.9% and to glass by 2.1% suggesting that endothelium is also affected by this agent.

Of the steroidal drugs tested, hydrocortisone succinate (10^{-4}M) inhibited adhesion with slightly more effect on endothelium (7.28%) than glass (4.7%). Prednisolone succinate however had no significant effect on adhesion to either endothelium or serum coated glass. The concentrations used of these agents are all high compared with those found to be effective *in vivo*. Dose response curves (Figs.9, 10 and 11) did not reveal any other effects at lower concentrations.

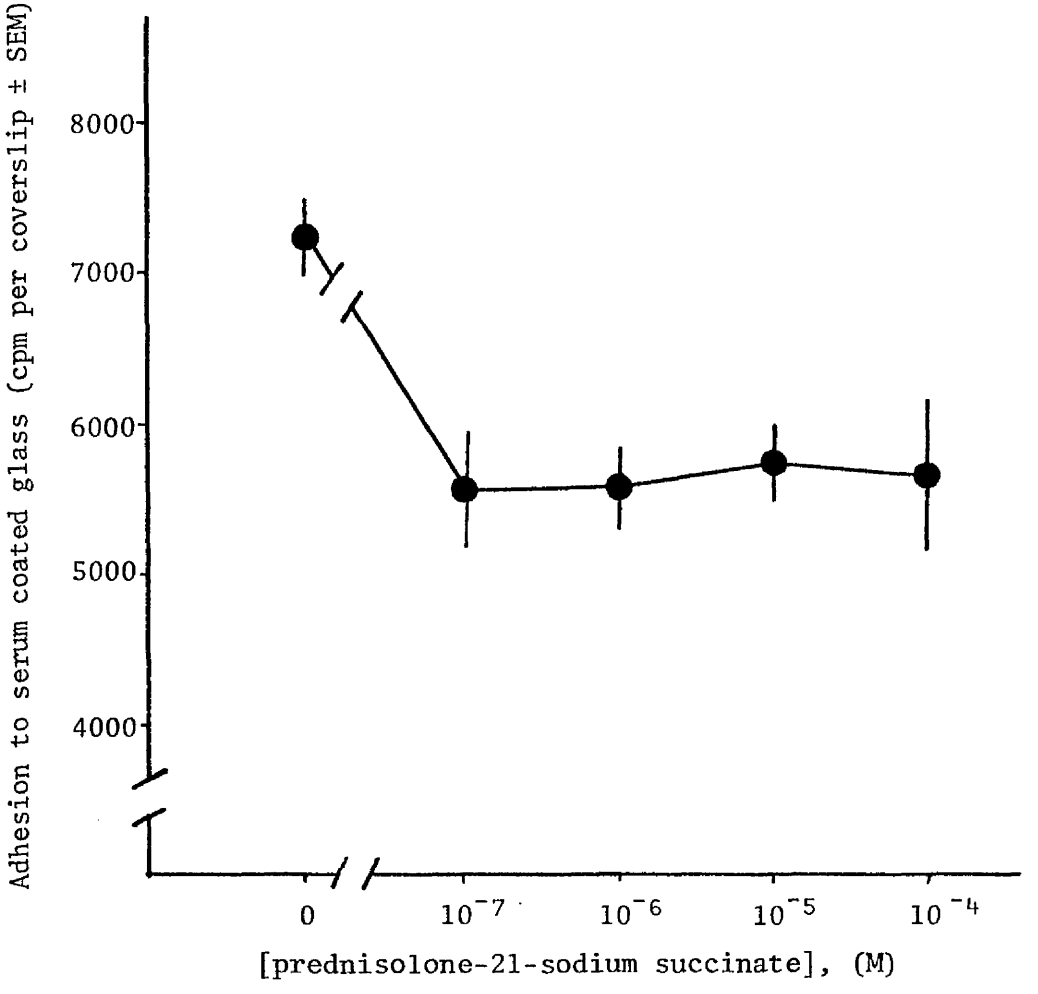
Miscellaneous agents

Table 13 shows the effect of fixation of endothelium on adhesion. This was to reduce neutrophil adhesion but only slightly and certainly not to the extent shown by fixation of the neutrophils which almost completely eliminated adhesion (qualitative observation only).

cAMP has been reported to possess anti-inflammatory activity (see Di Rosa, 1978). Aminophylline, a phosphodiesterase inhibitor which should lead to an increase in intracellular cAMP levels, reduced adhesion to both endothelium and serum coated glass. Heparin had no effect on adhesion to either glass or endothelium.

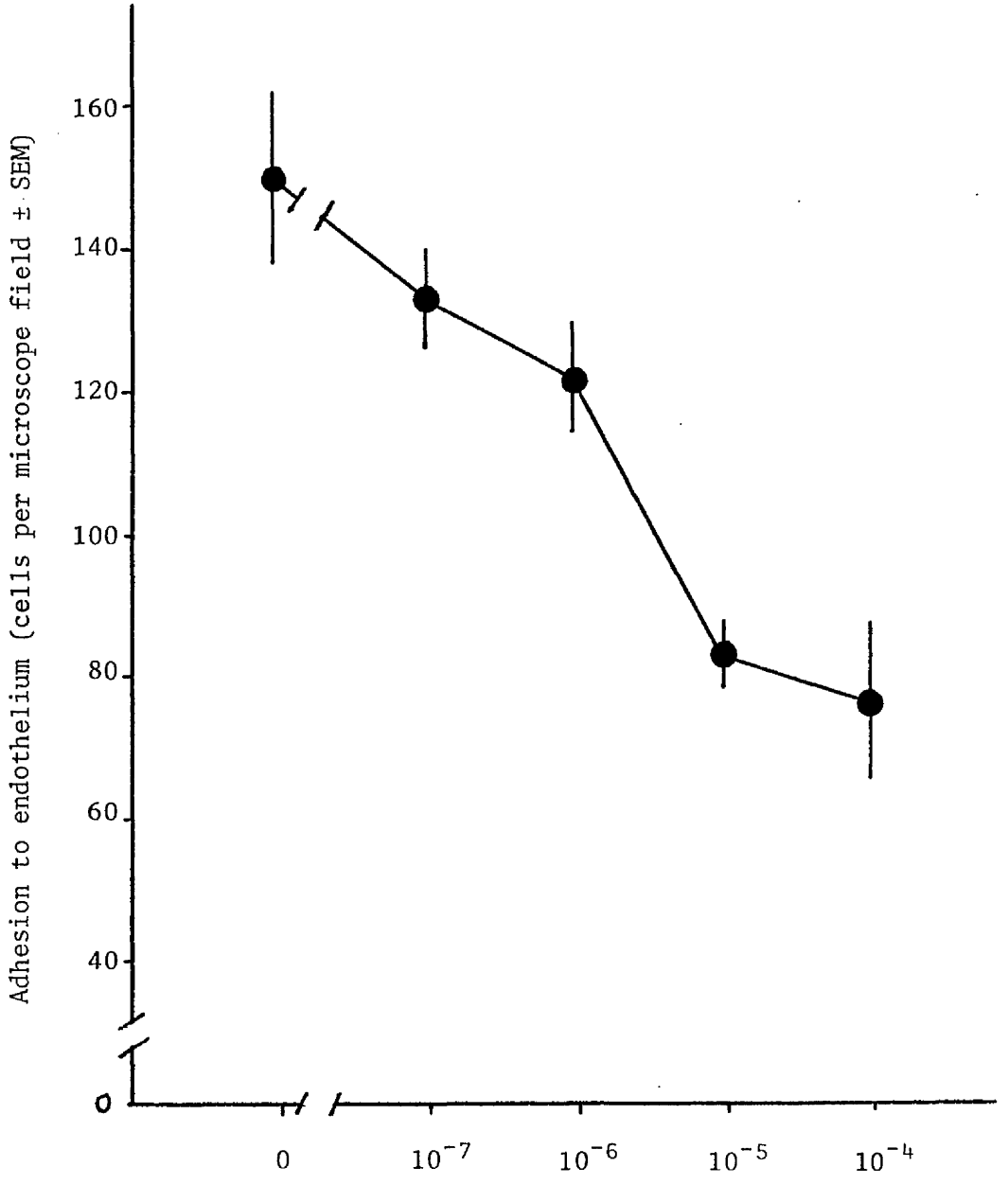
A variety of sugars, which might block lectin-like carbohydrate binding sites involved in adhesion had no effect when tested at a concentration of 10mM (Table 14) except that α-lactose increased adhesion.

FIGURE 9 Dose-response curve. Prednisolone-21-sodium succinate.



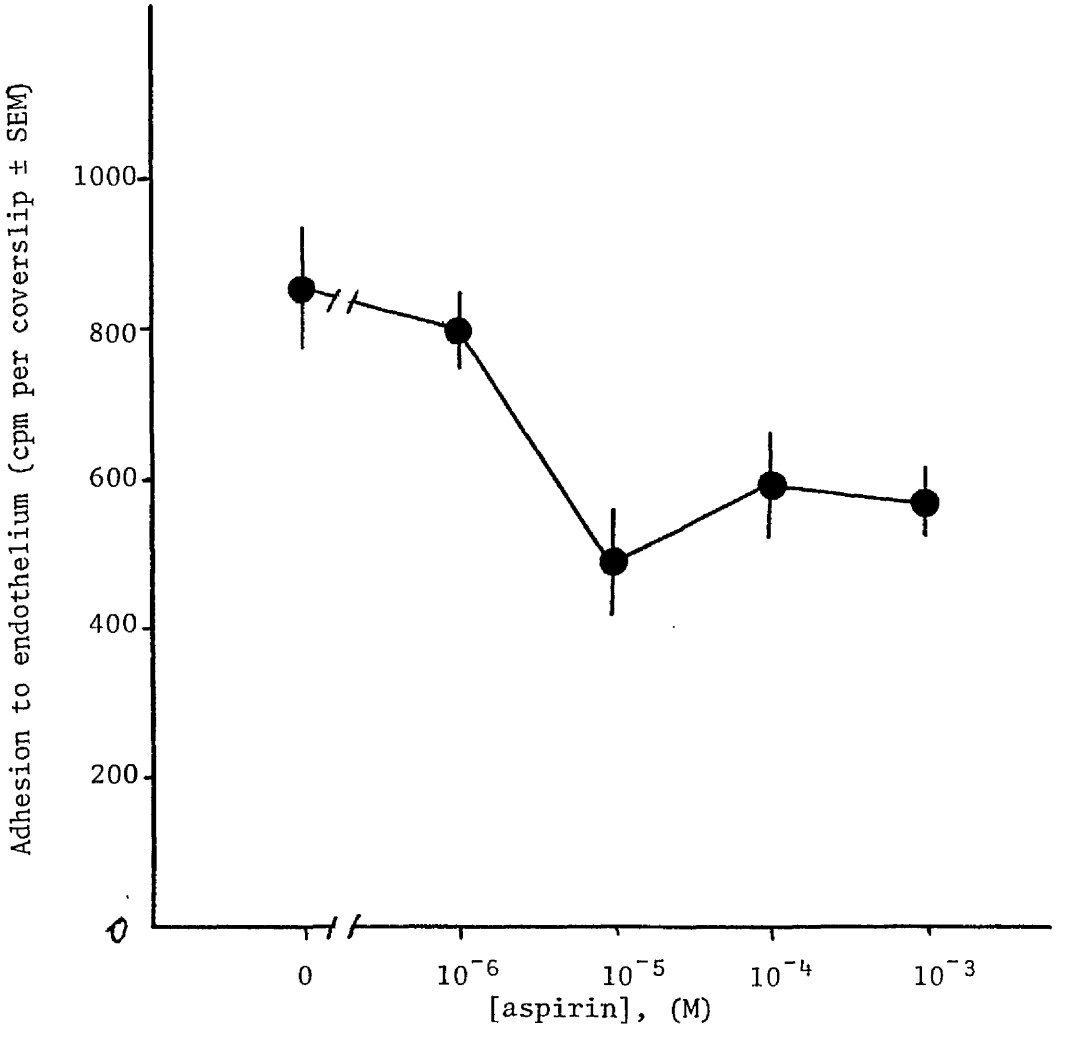
Each point is the mean from 4 replicate coverslips.

FIGURE 10 Dose response curve - hydrocortisone-21-sodium succinate.



Each point is the mean from 4 replicate coverslips.

FIGURE 11 Dose-response curve-aspirin.



Each point is the mean from 4 replicate coverslips.

TABLE 13 Effect of miscellaneous agents on neutrophil adhesion

Agent	Mean percent adhesion to endothelium				Mean diff.	P	$100 \times \frac{\text{Experimental}}{\text{control}} \pm SD(n)$
	BSS control	SD(n)	Experimental	SD(n)	± SE		
Fixation with							
Formalin, 10%	33.5 ± 3.6	(3)	27.8 ± 8.6	(3)	5.6 ± 3.1	n.s.	67 ± 20(7)
IOUml^{-1} heparin	23.8 ± 3.9	(5)	22.7 ± 6.8	(5)	1.0 ± 2.1	n.s.	95 ± 22(5)
10^{-3}M aminophylline	19.4 ± 15.4	(8)	14.7 ± 11.5	(8)	4.7 ± 1.9	.05	100 ± 67(11)
10^{-4} "	24.9 ± 2.9	(3)	13.4 ± 4.7	(3)	11.5 ± 4.1	n.s.	68 ± 17(6)
	Mean percent adhesion to serum coated glass						
10Uml^{-1} heparin	43.0 ± 5.9	(6)	40.9 ± 4.9	(6)	2.1 ± 2.4	n.s.	96 ± 12(6)
10^{-3}M aminophylline	20.7 ± 13.0	(9)	16.8 ± 8.2	(9)	3.9 ± 1.8	n.s.	99 ± 25(12)
10^{-4}M "	26.0 ± 7.7	(10)	20.4 ± 5.2	(10)	5.6 ± 1.4	.005	79 ± 17(13)

TABLE 14 Effects of various sugars on neutrophil adhesion to endothelium

Sugar (10mM)	cpm per coverslip ± SD(n)
control - BSS alone	814 ± 57(5)
mannose-6-phosphate	935 ± 25(3)
α-D-mannoside	835 ± 76(3)
D-mannose	837 ± 37(3)
n-acetyl-glucosamine	923 ± 94(3)
D-galactose	829 ± 55(3)
α-lactose	1168,1161(2)

Conclusions

None of the possible inflammatory mediators tested increased the adhesiveness of either the neutrophils or the endothelium, suggesting that they play no direct role in margination.

Several anti-inflammatory agents decreased the adhesiveness of neutrophils but others had no effect. Hydrocortisone also slightly affected the endothelial adhesiveness.

Since the effects were small and yielded no useful clues to the mechanism of margination, no further experiments of this type were performed.

CHAPTER 6

Introduction

The migration of neutrophils once in the tissues is generally accepted as being by a chemotactic mechanism and many substances have now been reported to be chemotactic *in vitro*. An attractive hypothesis is that as well as directing the locomotion of neutrophils, chemotactic factors influence the initial margination either by affecting the adhesiveness of the endothelial cells or the neutrophils. Application of chemotactic factors *in vivo* has been shown to increase margination (Atherton and Born, 1972) but it is not clear from these experiments whether the chemotactic factors act directly on the endothelium, the neutrophils or on plasma components to cause this effect. Nor is it absolutely clear whether any adhesive change occurred, since changes in blood flow or vessel diameter may have occurred. Accordingly, the effect of chemotactic factors on *in vitro* adhesion was studied.

Adhesion - to - substratum experiments

Initial experiments were performed using endothelial monolayers and serum coated glass as substrata, with the chemotactic factors present during the period of the assay (30 minutes). All the factors studied were initially used at concentrations at or near their chemotactically optimal concentrations. The results of these experiments (Table 15) show that all the factors substantially reduced the number of adherent cells. In general, adhesion of 50-60% of control levels was observed. Since in these assays 20-40% of the added cells generally adhere in the controls, the effect of chemotactic factors is to reduce this to 10-20% of added cells adhering. There seemed no obvious relation between the efficacy of an active chemotactic factor at its optimal concentration for chemotaxis and its effect on adhesion. Also shown in Table 15 are

TABLE 15(a) Effect of chemotactic factors on neutrophil adhesion

Chemotactic factor	Concentration (M)	Mean percent adhesion to endothelium BSS control ± SD(n)	Experimental ± SD(n)	Mean diff. ± SE	P	$100 \times \frac{\text{Experimental}}{\text{control}}$ ± SE
f-met-phe	10^{-5}	41.0 ± 9.3(14)	12.1 ± 6.6(14)	28.1 ± 1.7	<.001	42 ± 17(30)
f-tri-tyr	10^{-9}	31.0 ± 4.1(4)	15.2 ± 6.4(4)	15.8 ± 1.4	<.005	63 ± 22(11)
FMLP	10^{-8}	13.1 ± 3.9(7)	11.4 ± 2.0(7)	1.7 ± 1.2	n.s.	85 ± 23(11)
	10^{-9}	28.5 ± 22.9(7)	10.5 ± 8.4(7)	18.0 ± 5.7	<.02	46 ± 16(22)
	10^{-10}	13.1 ± 3.9(7)	9.7 ± 2.1(7)	3.4 ± 1.8	n.s.	56 ± 14(11)
α_s casein	4.2×10^{-5}	15.1 ± 3.2(6)	6.4 ± 1.2(6)	8.7 ± 1.4	<.005	43 ± 13(6)
β casein	4.2×10^{-5}	31.0 ± 5.4(8)	17.1 ± 3.8(8)	13.9 ± 1.0	<.001	63 ± 12(13)
Alkali denatured HSA	1.4×10^{-5}	25.1 ± 11.9(9)	12.5 ± 7.8(9)	12.6 ± 2.5	<.001	56 ± 26(12)
(1) native HSA	1.4×10^{-5}	23.7 ± 8.7(10)	32.0 ± 9.7(10)	8.3 ± 0.8	<.001	136 ± 28(14)
(2) met-phe	10^{-5}	n.d.	n.d.			*84 ± 26(13)

TABLE 15 (a) continued

		Mean per cent adhesion to serum coated glass				
f-met-phe	10^{-5}	45.8 ± 12.2(17)	21.3 ± 8.1(17)	25.5 ± 2.2	<.001	55 ± 22(34)
f-tri-tyr	10^{-9}	44.0 ± 17.9(7)	26.1 ± 8.9(7)	17.9 ± 5.2	<.01	60 ± 20(15)
FMLP	10^{-8}	34.5 ± 21.9(10)	28.3 ± 16.0(10)	6.2 ± 2.9	n.s.	94 ± 31(22)
	10^{-9}	28.9 ± 18.0(43)	16.4 ± 10.8(43)	12.5 ± 1.8	<.001	59 ± 20(65)
	10^{-10}	31.6 ± 21.1(15)	21.5 ± 11.1(15)	10.1 ± 3.3	<.01	74 ± 34(27)
α_s casein	4.2×10^{-5}	35.4 ± 3.8(3)	22.0 ± 0.2(3)	13.4 ± 2.1	<.02	62 ± 6(3)
β casein	4.2×10^{-5}	28.1 ± 9.1(6)	14.2 ± 7.3(6)	14.0 ± 2.3	<.005	49 ± 15(6)
Alkali denatured HSA	1.4×10^{-5}	19.6 ± 15.4(5)	14.5 ± 17.9(5)	5.0 ± 3.1	n.s.	52 ± 42(5)
(1) native HSA	1.4×10^{-5}	34.6 ± 2.2(8)	49.6 ± 11.4(8)	15.0 ± 3.6	<.005	143 ± 29(8)
(2) met-phe	10^{-5}	n.d.	n.d.			*83 ± 15(12)

(1) not chemotactic (2) weakly chemotactic

* no absolute values determined for this factor. Raw data (cpm) shown in (b)

TABLE 15(b) Raw data for adhesion in presence of met-phe

Expt.	BSS control	10^{-5}M f-met-phe	10^{-5}M met-phe
	Adhesion to endothelium (cpm per coverslip)		
1	6910	3095	5310
	5330	3204	4426
	5243	2244	2276
	4364	3015	3254
2	1367	755	1002
	1188	565	966
	1292	608	941
	1000	596	896
	1191	--	875
3	1860	1101	1701
	2377	1393	2266
	1900	1671	2507
	2092	1358	2124
	Adhesion to serum coated glass (cpm per coverslip)		
1	6326	4420	3109
	5184	4271	5713
	4879	4361	4773
2	1371	786	1015
	1216	559	1099
	1141	659	1020
	1243	626	1080
	1378	690	1013
3	2177	2237	2343
	2370	1984	2117
	2241	1537	2080
	2099	967	1751

the effects of two other compounds. The non-formylated dipeptide, met-phe, which is only feebly chemotactic, inhibited adhesiveness very slightly compared to f-met-phe: native HSA, which is not chemotactic, actually increased adhesion in contrast to the reduction observed in the presence of denatured HSA.

Since the inhibition of adhesion occurred with either endothelium or serum coated glass as substratum, it appeared that the effect of the chemotactic factors was on the neutrophils, rather than the endothelium.

Neutrophil viability was frequently checked by the ability to exclude nigrosine and was consistently found to be greater than 95% after 30 minutes incubation in BSS alone or in BSS containing chemotactic factor. Thus cell death cannot explain this unexpected reduction in adhesion.

Neutrophil swelling has been reported to occur upon exposure to chemotactic factors (Hsu and Becker, 1975). It is possible that the increase in size may, by itself or as a result of concomitant density changes, decrease the rate of settling of the neutrophils onto the substratum, which is known to be a rate limiting step in these assays (see Chapter 4). To test the possibility that different settling rates may produce an apparent reduction in adhesion, coverslips were photographed at various times during the assays onto serum coated glass for counting of the number of cells per unit area from the photographs. The coverslips were then rinsed in the usual way and the number of adherent cells counted. The results of these experiments (Table 16) show that in the presence of chemotactic factor the number of settled cells was not significantly different from controls.

Cell spreading almost certainly contributes to the strength of adhesion formed between a cell and a surface and the level of adhesion

TABLE 16 Effect of FMLP on neutrophil settling and adhesion

Expt.	Time (mins)	Settled cells per unit area ± SEM(n)		Percent adhesion ± SEM	
		BSS control	10^{-9}M FMLP	BSS control	10^{-9}M FMLP
1	15	530 ± 90(4)	544 ± 102(4)	43.7 ± 3.9	37.5 ± 5.0
	30	775 ± 189(4)	674 ± 100(4)		
2	15	199 ± 20(8)	195 ± 23(8)	10.3 ± 1.4	8.08 ± 1.7
	30	249 ± 43(8)	263 ± 40(8)		
3	15	71 ± 22(4)	57 ± 22(4)	21.9 ± 7.6	13.5 ± 3.8

as measured in an adhesion - to - substratum type of assay may include a component due to spreading. To ascertain whether chemotactic factors affected spreading, neutrophils were allowed to adhere and spread on serum coated glass, photographed using differential interference-contrast (Nomarski) optics which allows clear visualization of the cell edges, and the mean cell areas compared by projecting the negatives onto 'Bristol board' of uniform thickness, cutting out the outlines and weighing the card. The results (Table 17) show that no significant difference in the areas of the cells was detected in the presence of a chemotactic factor which reduced adhesion.

It was concluded therefore, that the reduction in the number of cells adhering to substrata in the presence of chemotactic factors was due to an adhesive change.

Pretreatment experiments

It is possible that chemotactic factors bind to surfaces and alter their adhesive characteristics. Dierich *et al* (1977) suggested that neutrophil chemotaxis may be due to binding to surface bound chemotactic factor. Binding of factors to endothelial cells or serum coats might reduce the adhesiveness of these surfaces. In fact in most models of cell adhesion, it is possible to hypothesize that binding of chemotactic factor to either of these surfaces or the neutrophils may reduce adhesion. To determine which component(s) of the adhesive interaction were being affected pretreatment experiments were performed in which neutrophils, endothelium or serum coated glass were incubated in the presence of chemotactic factor, washed and then used in adhesion assays.

The results of these experiments (Table 18) show that pre-exposure

TABLE 17 Effect of FMLP on neutrophil spreading

Expt.	Time (mins)	Mean weight of card (g)	
		BSS(n)	10^{-9}M FMLP(n)
1	30	0.089(30)	.089(30)
2	30	0.137(40)	.138(40)
3	30	0.107(33)	.110(32)

TABLE 18 Effect of pretreatment of neutrophils, endothelium or serum coated glass on adhesion.

Chemotactic factor	Adhesion to:	BSS control ± SD (n)	Chemotactic factor present ± SD (n)
a) Neutrophil pretreatment			
10^{-5}M f-met-phe	endothelium	46.4 ± 6.9(7)	15.7 ± 7.7(7)
"	serum coated glass	52.9 ± 9.9(10)	25.2 ± 7.9(10)
10^{-9}M f-FMLP	endothelium	50.0 ± 2.6(4)	19.7 ± 3.9(4)
"	serum coated glass	52.8 ± 4.4(4)	21.4 ± 3.7(4)
b) Endothelial pretreatment			
10^{-5}M f-met-phe	endothelium	35.2 ± 7.3(7)	8.7 ± 2.5(7)
10^{-9}M FMLP	"	55.5 ± 6.9(4)	22.1 ± 3.4(4)
4.2×10^{-5}M β-casein	"	31.8 ± 6.5(7)	22.7 ± 7.5(7)
c) Serum coated glass pretreatment			
10^{-5}M f-met-phe	serum coated glass	35.6 ± 6.7(7)	15.7 ± 4.0(7)
10^{-9}M FMLP	"	56.4 ± 4.7(4)	27.9 ± 6.6(4)
4.2×10^{-5}M β-casein	"	28.5 ± 11.2(7)	11.4 ± 3.9(6)

Mean percent adhesion				
Mean diff. from control ± SE	P	Pretreatment with chemotactic factor ± SD (n)	Mean diff. from control ± SE	P
30.7 ± 1.4	<.001	23.9 ± 5.1(7)	22.6 ± 2.0	<.001
27.8 ± 2.7	<.001	27.0 ± 5.5(10)	25.9 ± 1.9	<.001
30.3 ± 2.9	<.005	36.9 ± 3.5(4)	13.2 ± 2.8	<.02
31.4 ± 3.2	<.005	31.2 ± 2.1(4)	21.6 ± 2.4	<.005
26.5 ± 3.0	<.001	29.5 ± 10.6(7)	5.7 ± 2.3	<.05
33.4 ± 5.1	<.01	49.3 ± 7.1(4)	6.2 ± 5.5	n.s.
9.1 ± 1.0	<.001	29.5 ± 6.1(7)	2.1 ± 0.9	n.s.
19.9 ± 3.1	<.001	30.5 ± 10.2(7)	5.1 ± 3.1	n.s.
28.5 ± 2.9	<.005	55.1 ± 5.1(4)	1.3 ± 3.6	n.s.
19.1 ± 3.1	<.005	29.5 ± 13.1(7)	0.9 ± 1.9	n.s.

of neutrophils, but not endothelium or serum coated glass to a chemotactic factor led to a reduction in adhesion similar to that observed in the presence of the chemotactic factor. This result confirms that the effect on adhesion is on the neutrophils rather than the endothelium or serum coated glass.

It has been reported, however, that exposure to chemotactic factors increases the adhesiveness of endothelial cells (Hoover *et al* 1978). Several variations to the pretreatment experiments using endothelium were therefore performed to investigate whether adhesive changes became apparent after incubation for different times or in different concentrations.

The results in Table 19 show that after pretreatment for one hour in 10^{-9}M FMLP there was no significant difference in the level of adhesion observed when assayed after 30 or 60 minutes. Pretreatment with 10^{-7}M FMLP appeared to decrease adhesion at both times compared with controls. This result may be due to residual amount of chemotactic factor present on the coverslips. As 10^{-10}- 10^{-9}M FMLP reduces adhesion markedly, only 0.1 - 1% of the original 10^{-7}M would be required to produce this effect. It is clear though that pretreatment does not increase adhesion. Interestingly, 60 minutes incubation of endothelium in BSS reduced the adhesion of neutrophils. Pretreatment for 30 minutes in 10^{-9}M and 10^{-7}M FMLP (Table 20) also had no effect when adhesion was assayed at 30 and 60 minutes.

It was concluded that FMLP, f-met-phe, β-casein and probably other chemotactic factors have no effect on endothelial cell adhesiveness.

TABLE 19 Effect of 60 minutes pretreatment of endothelium with FMLP on neutrophil adhesion at 30 and 60 minutes

Endothelium pretreatment	FMLP in assay	Mean adhesion (cpm per coverslip) ± SEM	
		30 minutes (n)	60 minutes (n)
None	none	1300 ± 63(4)	1494 ± 109(3)
None	10^{-9}M	237 ± 16(4)	420 ± 8(3)
None	10^{-7}M	654 ± 140(4)	1430 ± 90(3)
BSS, 60 mins	none	717 ± 87(4)	1008 ± 44(3)
10^{-9}M, 60 mins	none	620 ± 59	1244 ± 59(3)
10^{-7}M, 60 mins	none	258 ± 22(4)	416 ± 25(3)

TABLE 20 Effect of 30 minutes pretreatment of endothelium with FMLP on neutrophil adhesion at 30 and 60 minutes

	Endothelial pretreatment	FMLP in assay	Mean adhesion (cpm per coverslip) ± SEM	
			30 minutes (n)	60 minutes (n)
Expt.1	BSS, 30 mins	none	974 ± 43(4)	1608 ± 39(4)
	BSS, 30 mins	10^{-9}M	608 ± 38(4)	1071 ± 47(4)
	BSS, 30 mins	10^{-7}M	934 ± 35(4)	1576 ± 119(4)
	10^{-9}M, 30 mins	none	1038 ± 16(4)	1496 ± 111(4)
	10^{-7}M, 30 mins	none	776 ± 45(4)	1518 ± 30(4)
Expt.2	BSS, 30 mins	none	1150 ± 107(4)	2340 ± 118(4)
	10^{-7}M, 30 mins	none	853 ± 125(4)	2007 ± 141(4)
	BSS, 30 mins	10^{-7}M	883 ± 233(4)	1804 ± 210(4)

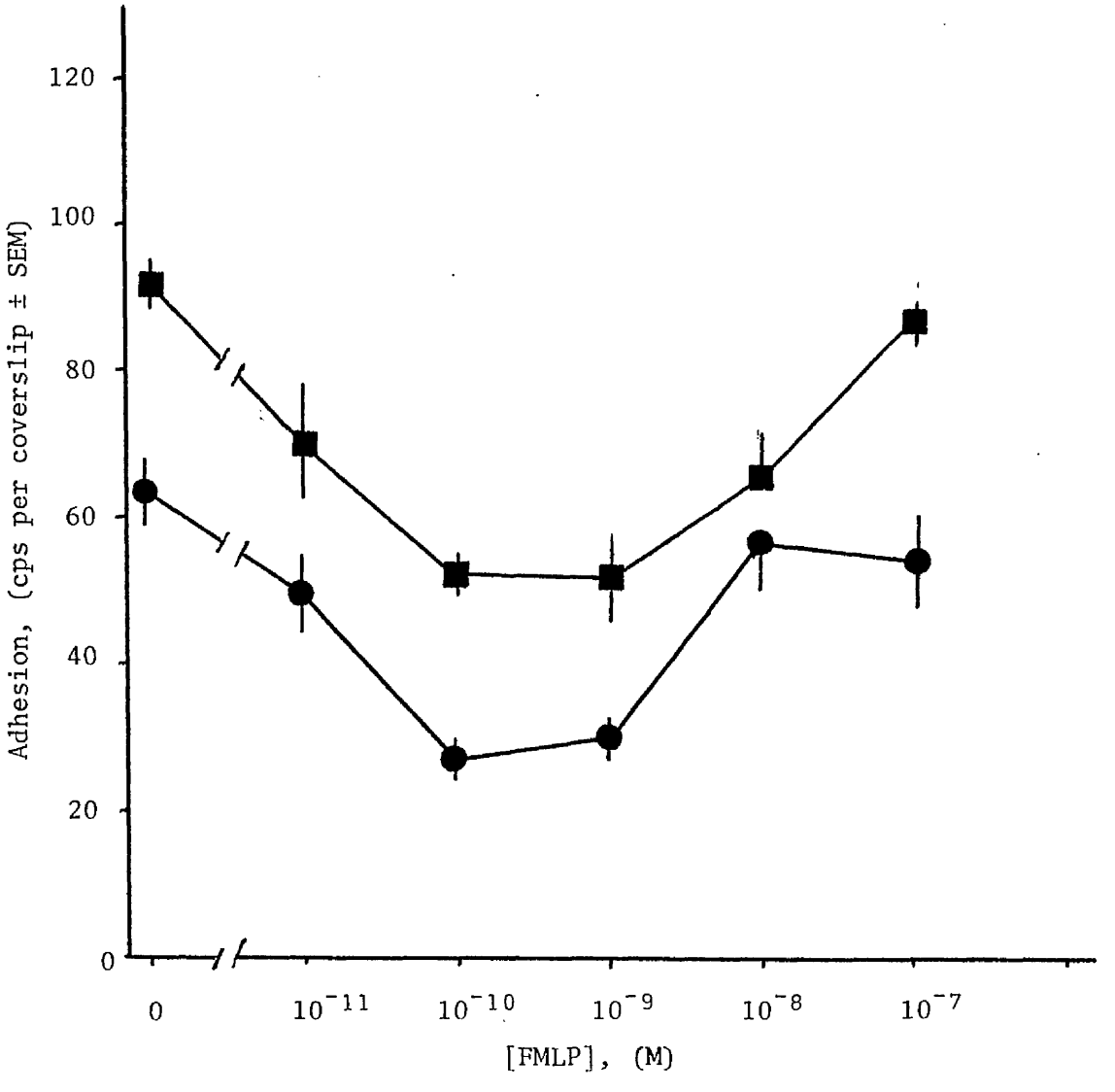
Effects of different concentrations of chemotactic factor on adhesion

To investigate the effects of chemotactic factors on neutrophil adhesion further, the dose response relationships were determined for several chemotactic agents. These are shown in Figs. 12, 13, 14 and 15. These results confirm the initial observations that the effect of chemotactic factors at optimal chemotactic concentrations is to reduce adhesion. Lower concentrations have a less marked effect. However, using FMLP to study the effect of concentrations above the chemotactic optimum the inhibition of adhesion was diminished and adhesion often returned to control levels. This result was consistently obtained. The actual minimum varied between 10^{-10} - 10^{-9}M, possibly reflecting changes in the cells sensitivity as well as dilution inaccuracies. This observation appears to rule out any explanation of the adhesive change which involves simple binding of the chemotactic factor to the cell surface.

Effects of chemotactic factors on neutrophil - neutrophil adhesion

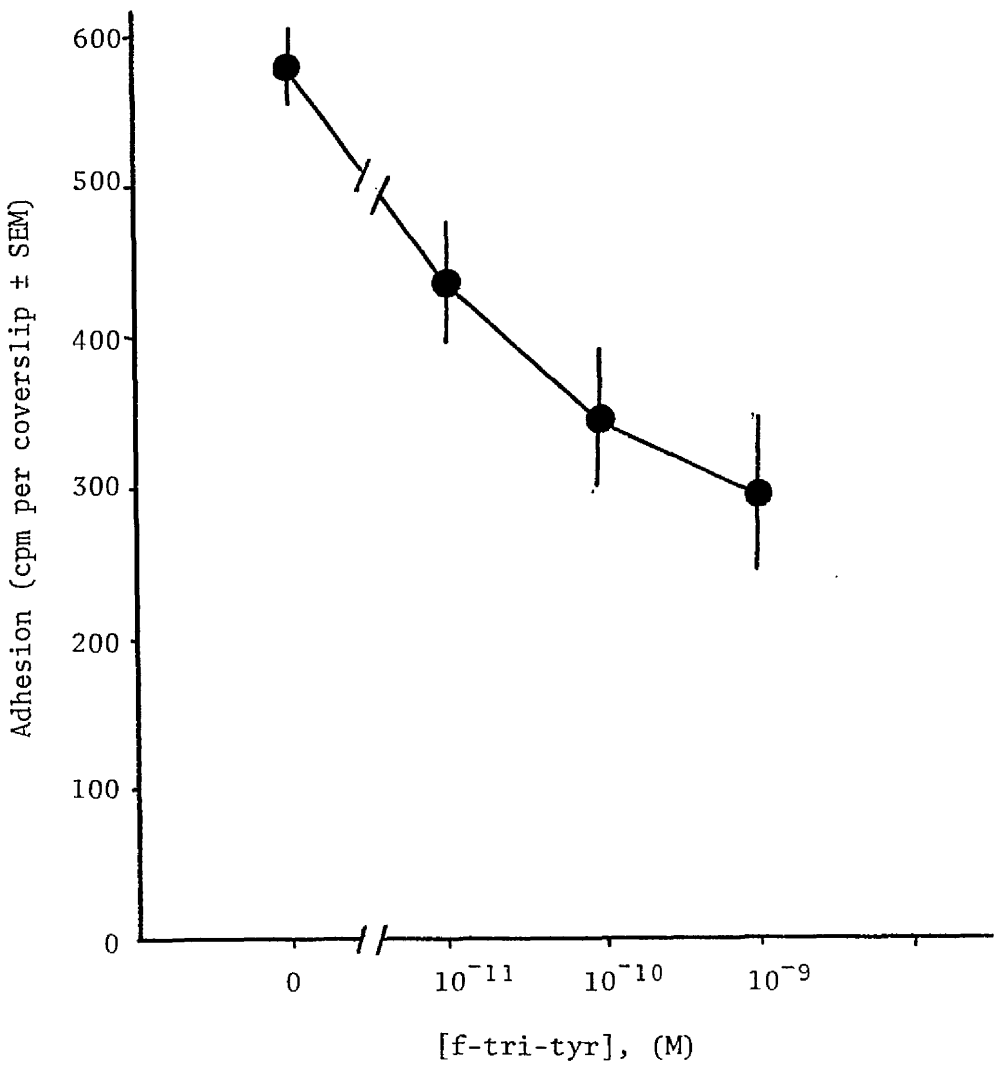
When suspended in BSS and agitated, neutrophils will rapidly and irreversibly aggregate (Lackie, 1974). The extent of aggregation gives a sensitive measure of cell adhesiveness and yields information about the neutrophils themselves. The results in Table 21 show the effect of the presence of chemotactic factors on this aggregation. All the factors tested had effects on adhesion as measured in this assay. The caseins both inhibited aggregation (as had been expected from the adhesion-to-substratum assay results) but the formyl-peptides tested enhanced aggregation at both chemotactically optimal and higher concentrations. A typical aggregation does-response curve (for FMLP) is shown in Fig.16. Little or no effect on aggregation is apparent up

FIG.12 Dose-response for neutrophil adhesion. FMLP



] For adhesion, each point is mean from 4 replicate coverslips.

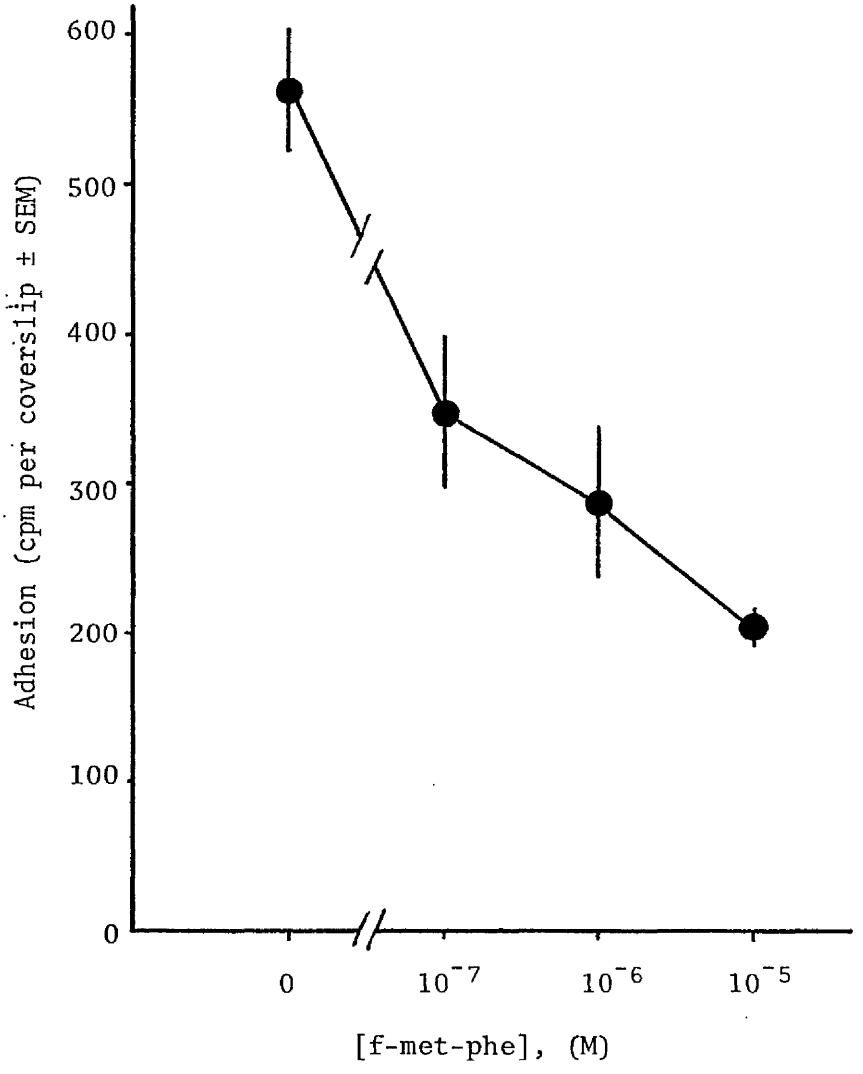
FIG.13 Adhesion of neutrophils to endothelium.
Effect of f-tri-tyr.



Each point is the mean from 3 replicate coverslips.

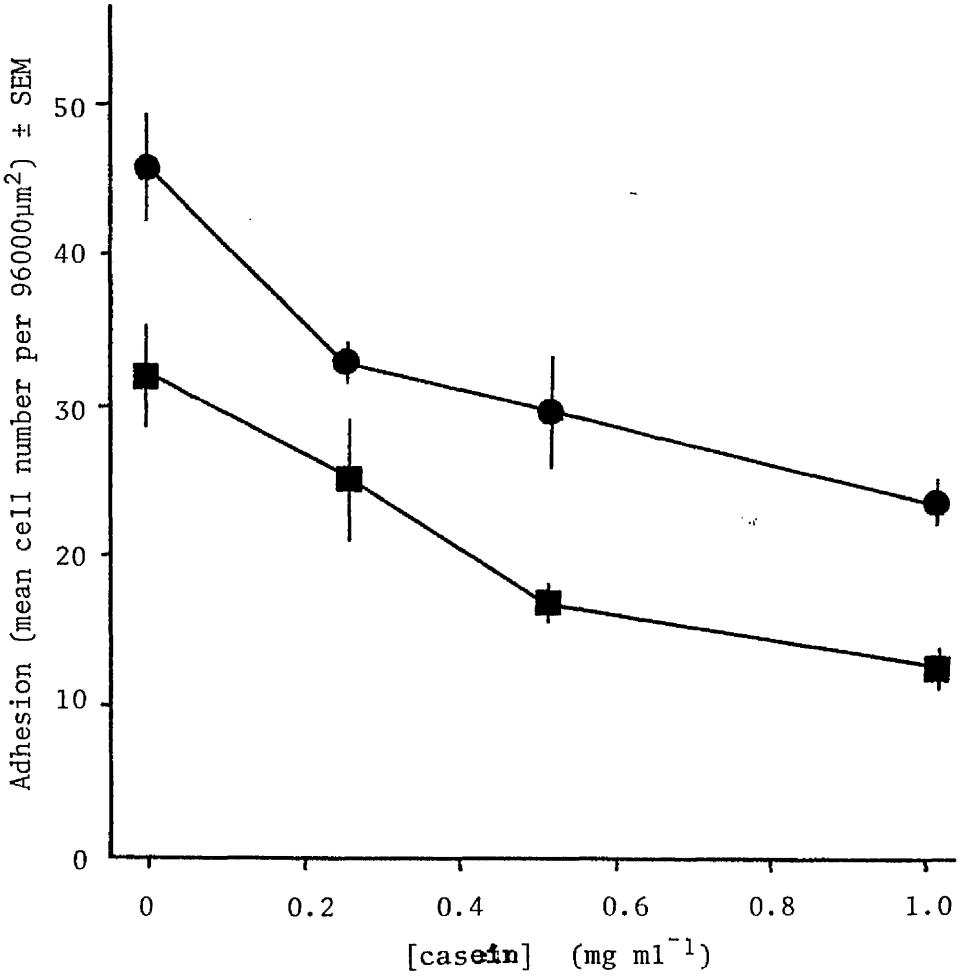
FIG.14 Adhesion of neutrophils to endothelium.

Effect of f-met-phe.



Each point is the mean from 4 replicate coverslips.

FIG.15 Adhesion of neutrophils to endothelium and serum coated glass. Dose-response for α_s-casein.



Each point is the mean from 3 replicate coverslips.

■—■ Serum coated glass

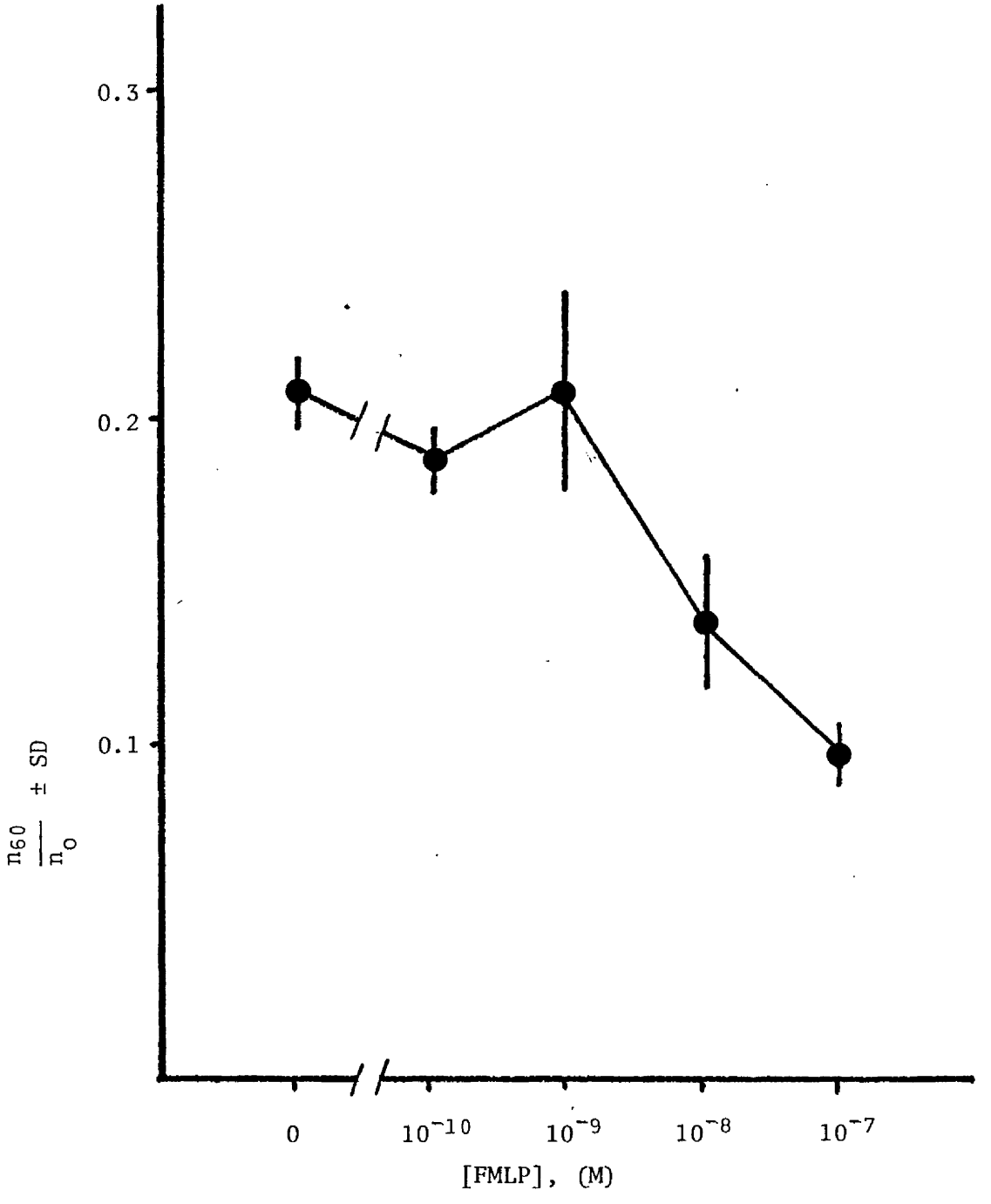
●—● endothelium

TABLE 21 Effect of chemotactic factors on the aggregation of neutrophil granulocytes after 60 minutes at 37°C.

Chemotactic factor	Concentration (M)	Percentage of control (= 100%) particle number ± SEM (n)
α_s-casein	4.2×10^{-5}	147.6 ± 3.4(18)
β-casein	4.2×10^{-5}	143.3 ± 6.4(24)
f-met-phe	10^{-5}	86.2 ± 1.2(24)
FMLP	10^{-9}	87.2 ± 5.2(11)
	10^{-7}	36.3 ± 7.0(15)

n - total number of replicate assays.

FIG.16 Dose response for aggregation, FMLP



Each point is the mean from 6 replicate assays.

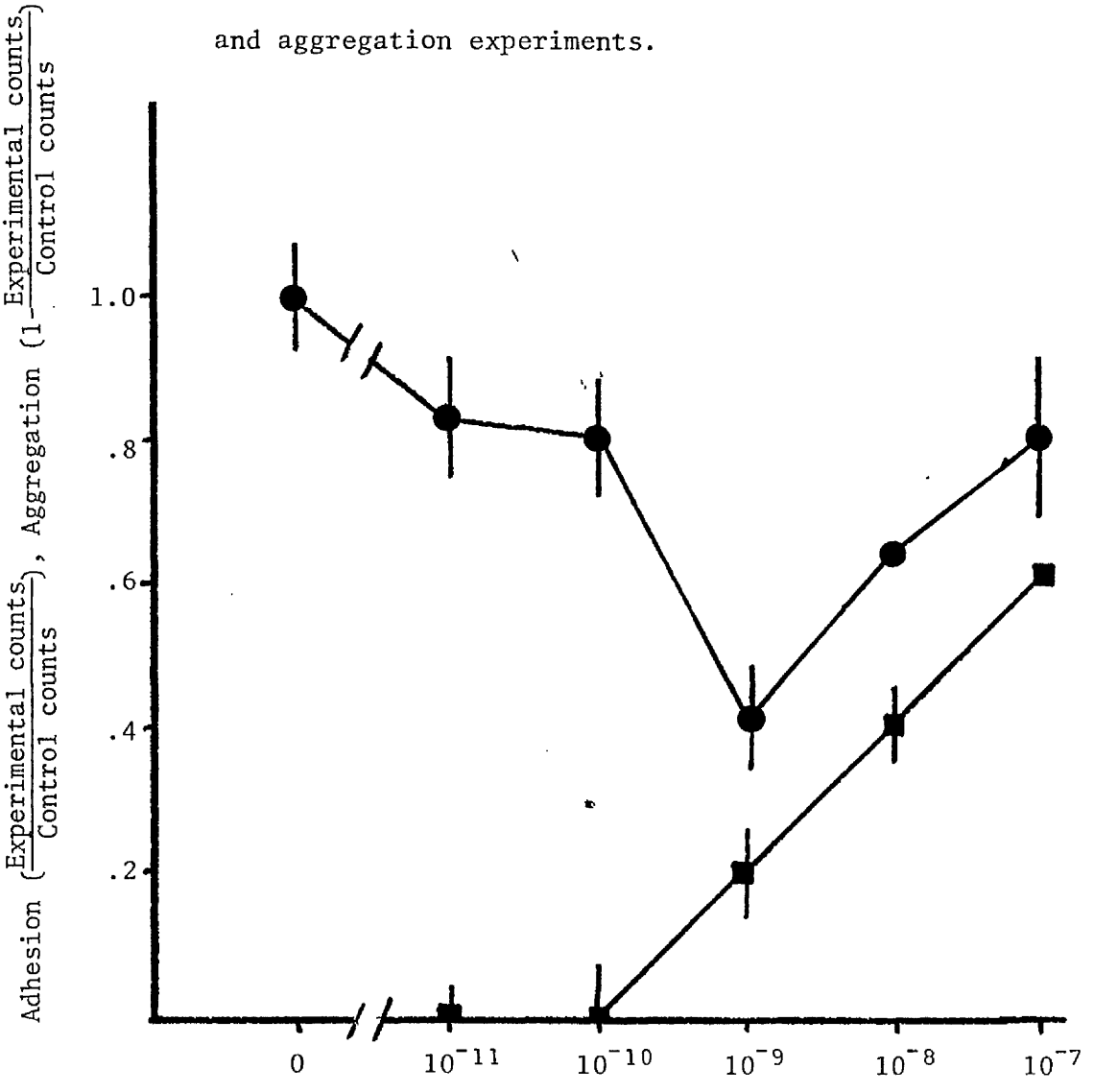
to concentrations of 10^{-9}M, at which concentration slight enhancement generally occurred. Above this concentration, aggregation was markedly enhanced. The dose-response relationships for aggregation and adhesion-to-substrata are therefore quite different. This is confirmed by the results of an experiment in which adhesion and aggregation assays were performed in parallel using identical cell suspensions, solutions and FMLP stocks (Fig.17).

The effects of FMLP on neutrophil aggregation were also studied using the Couette viscometers (Curtis, 1969). Total particle number in the cell suspension was counted at time intervals of 7 minutes from 0-28 minutes. Three experiments were performed, each on different days and with different cells. Neutrophils aggregated well in these experiments although some variability was obvious (Fig.18). FMLP inhibited aggregation in these experiments, in contradiction to the enhancement observed in the shaking water bath aggregation assay. Examination of the time courses of these experiments suggested that aggregation was more inhibited at early times and that at 28 minutes, in FMLP, particle number was still decreasing, while in BSS, the number was levelling off. It seemed possible that the different sampling times of the two aggregation assays may explain the different effects of FMLP.

Time courses of adhesive change

The courses of the aggregation response were performed at various concentrations of FMLP. The results are shown in Fig.19. At 10 minutes aggregation was inhibited in a dose dependent fashion. At 20 and 30 minutes, inhibition of aggregation showed a maximum at 10^{-9}M. At 60 minutes, little effect was apparent at concentrations of 10^{-9}M or lower but above this

FIG.17 Behaviour of neutrophils in parallel adhesion and aggregation experiments.



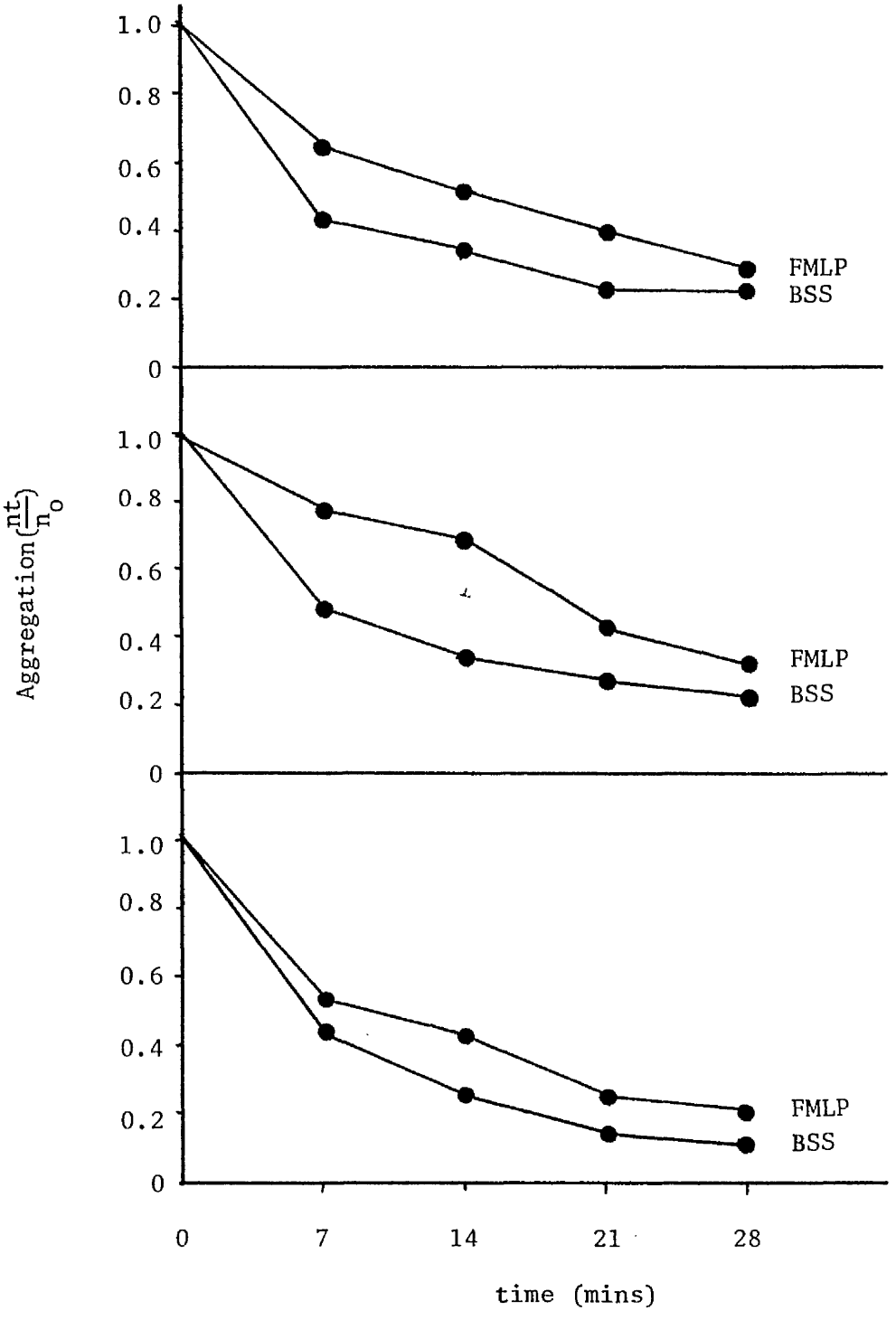
For adhesion to serum coated glass, each point is the mean from 4 replicate coverslip pairs.

For aggregation, each point is mean from 6 replicate assays.

■—■ Aggregation

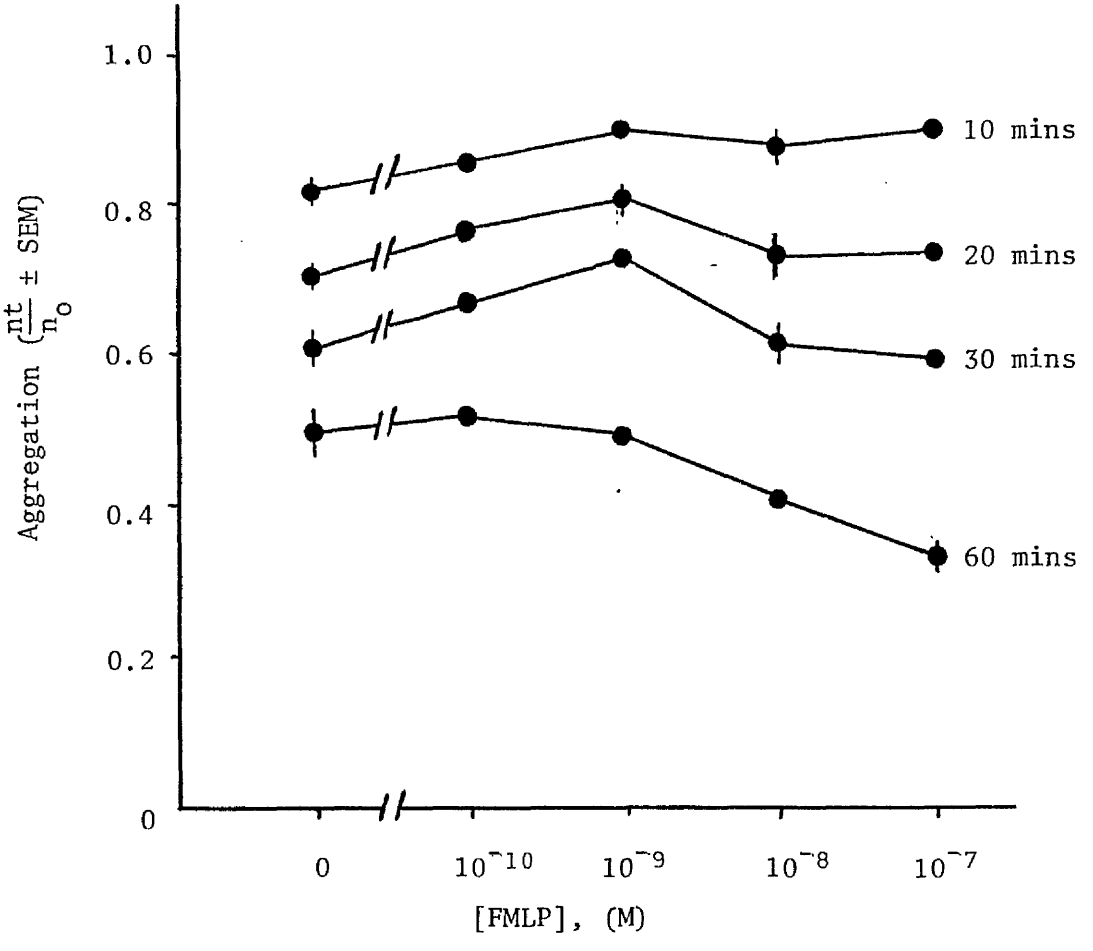
●—● Adhesion

FIG.18 Aggregation of neutrophils in couette Viscometers - effect of FMLP (10^{-9}M). (3 experiments)



Each point is the mean from 6 haemocytometer fields.

FIG.19 Time course - dose response for aggregation in presence of FMLP.



Each point is the mean from 3 replicate assays.

concentration, aggregation was enhanced. This confirms that the differences in effect of FMLP seen in the two types of aggregation assay were due to time changes.

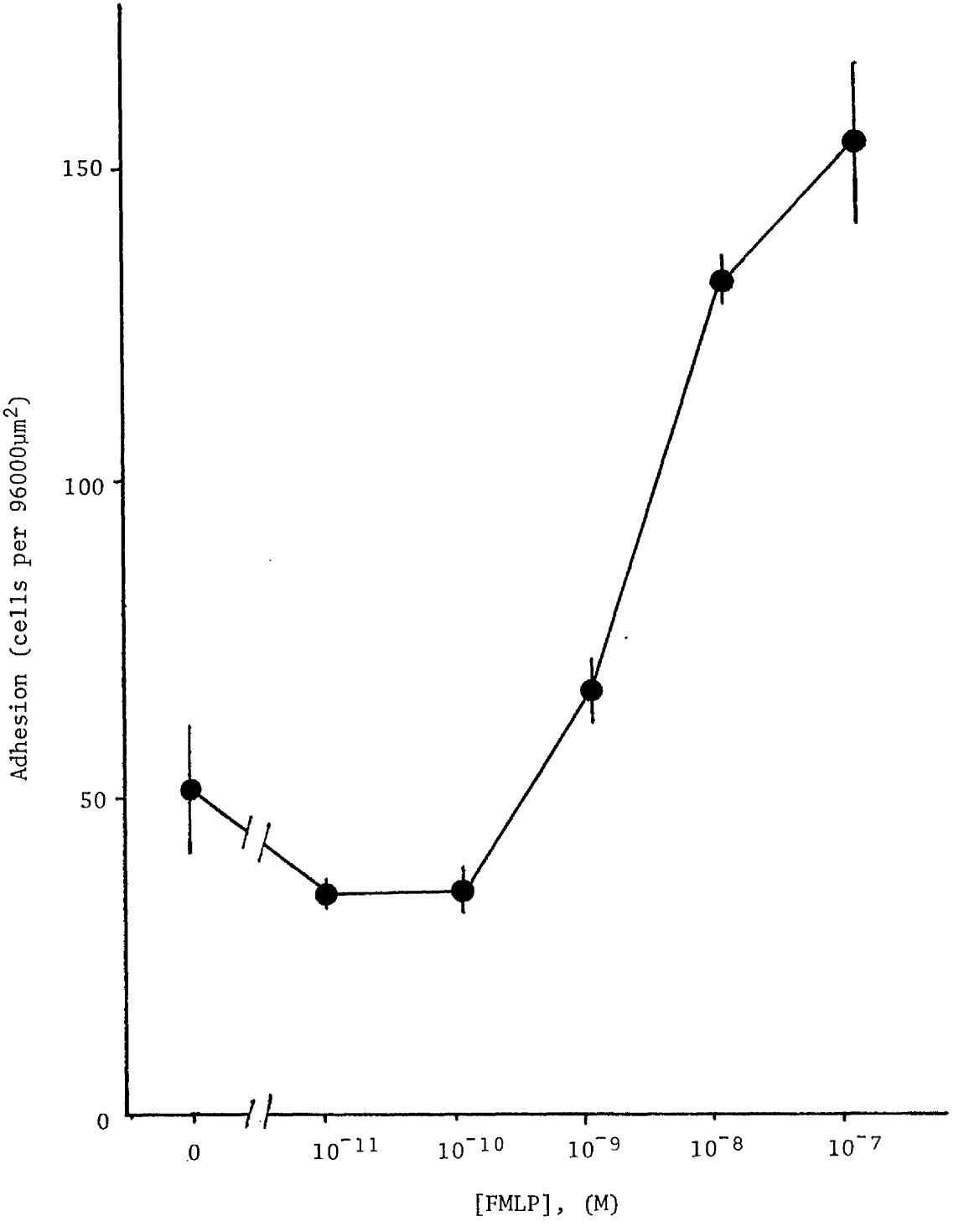
Adhesion-to-substratum assays were then performed at 60 minutes. Fig.20 shows a dose-response curve for such an experiment using FMLP. At high concentrations (10^{-8}- 10^{-7}M) adhesion is clearly enhanced while little or no effect is evident at lower concentrations. This is qualitatively the same result as that obtained from aggregation assays.

From both types of assay, then, it is concluded that FMLP induces in neutrophils a series of time and concentration dependent adhesive changes starting with an inhibition at early times, especially at low concentrations and leading to an enhancement at later times at high concentrations.

To determine more precisely when the change from lesser to greater (than control) adhesiveness occurs, a series of aggregation time courses were performed in various concentrations of FMLP. Typical curves are shown in Fig.21. The point at which the slope of the curve for FMLP at the same particle number, was steeper than the control slope, was taken as an estimate of the time of the adhesive change. These times are plotted in Fig.22. A clear concentration dependence on the timing of the change is apparent. Day-to-day variation in both the timing and the overall degree of aggregation make it difficult to obtain exact times for the transition, but times of approximately 0, 10 and 20 minutes were estimated for

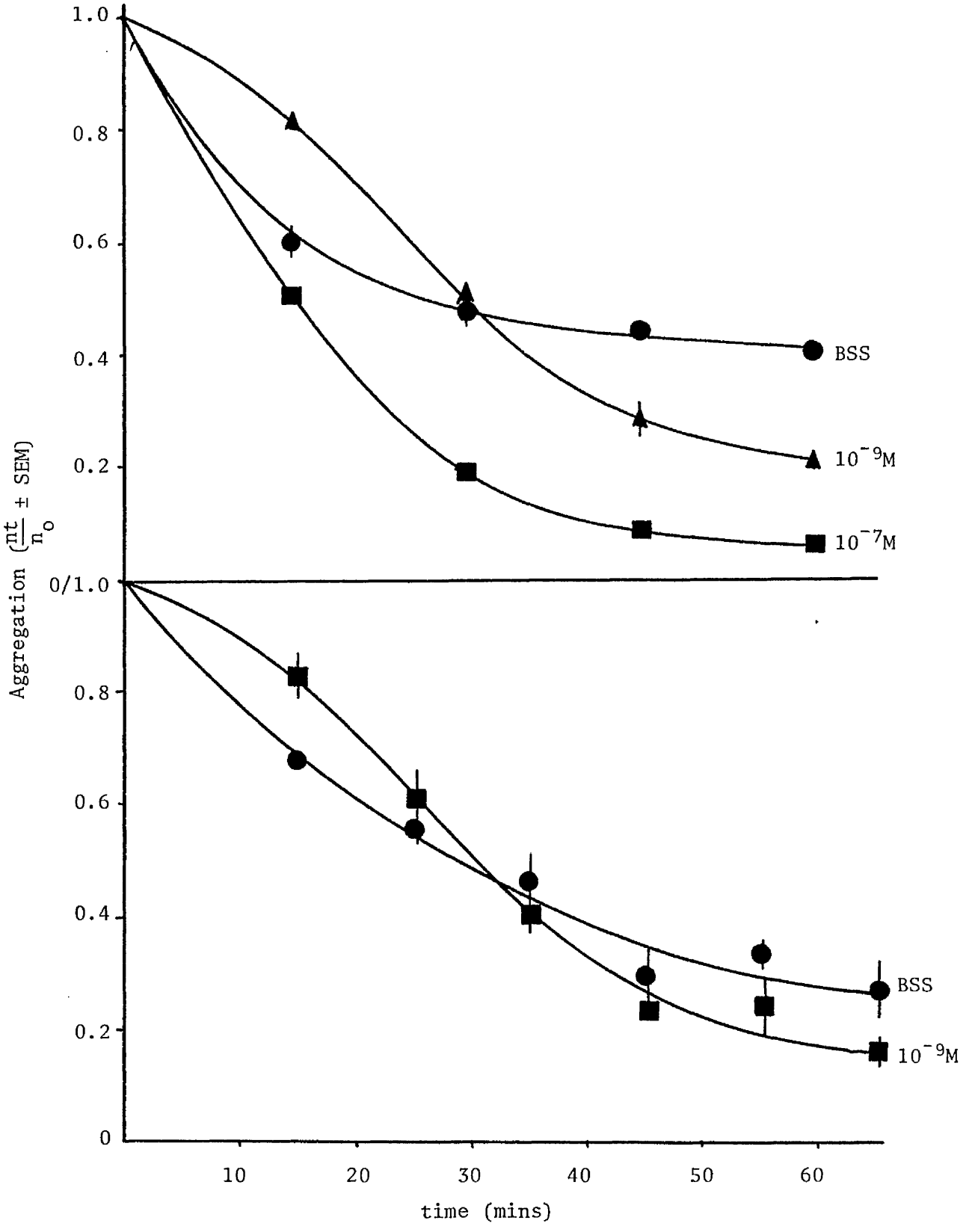
FIG.20 Adhesion of neutrophils to serum coated glass.

Effect of FMLP.



Each point is the mean from four replicate coverslips.

FIG.21 Time courses of neutrophil aggregation in the presence of FMLP at various concentrations.



Each point is the mean from 3 replicate assays.

FIG.21 (cont.)

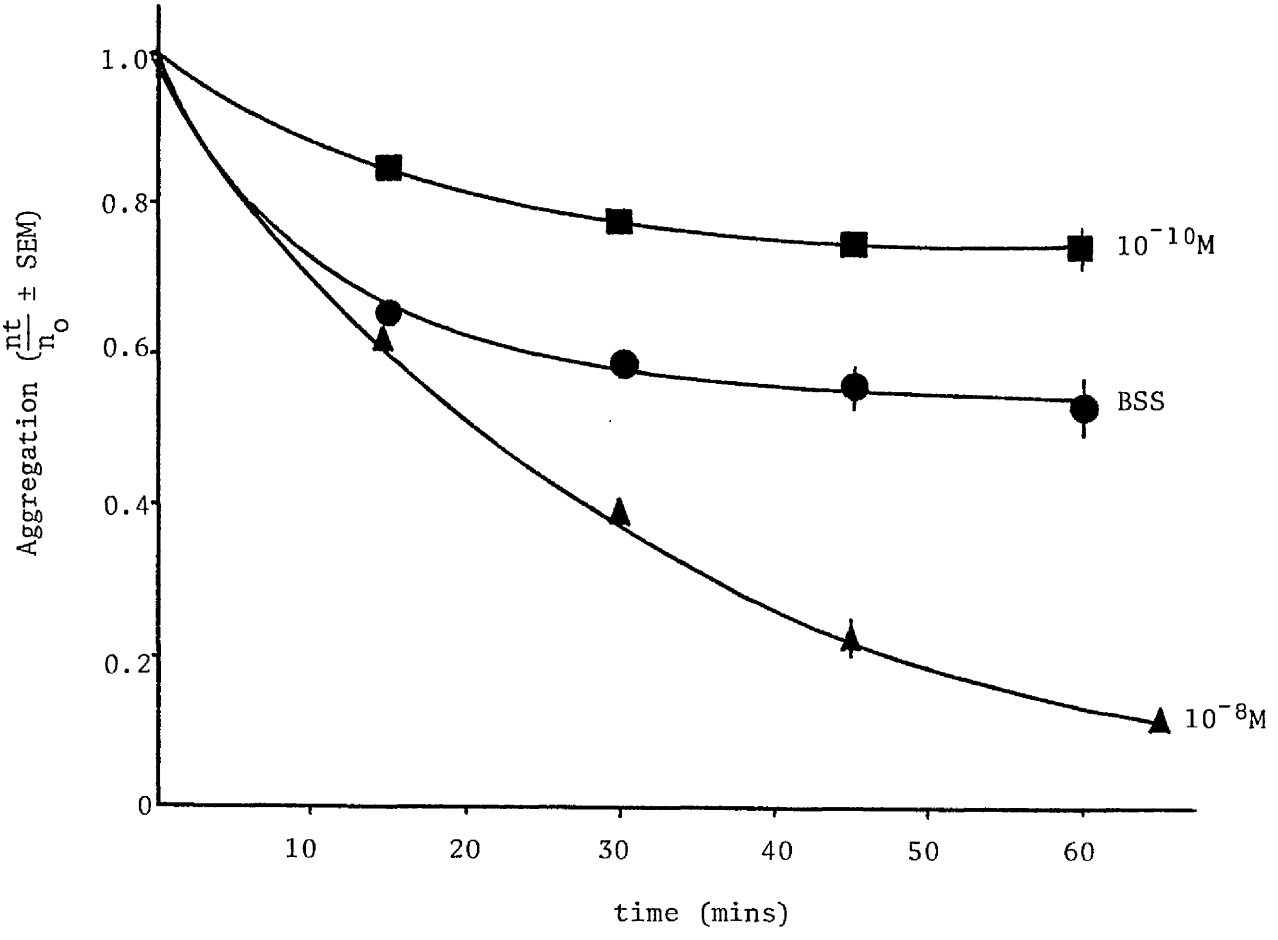
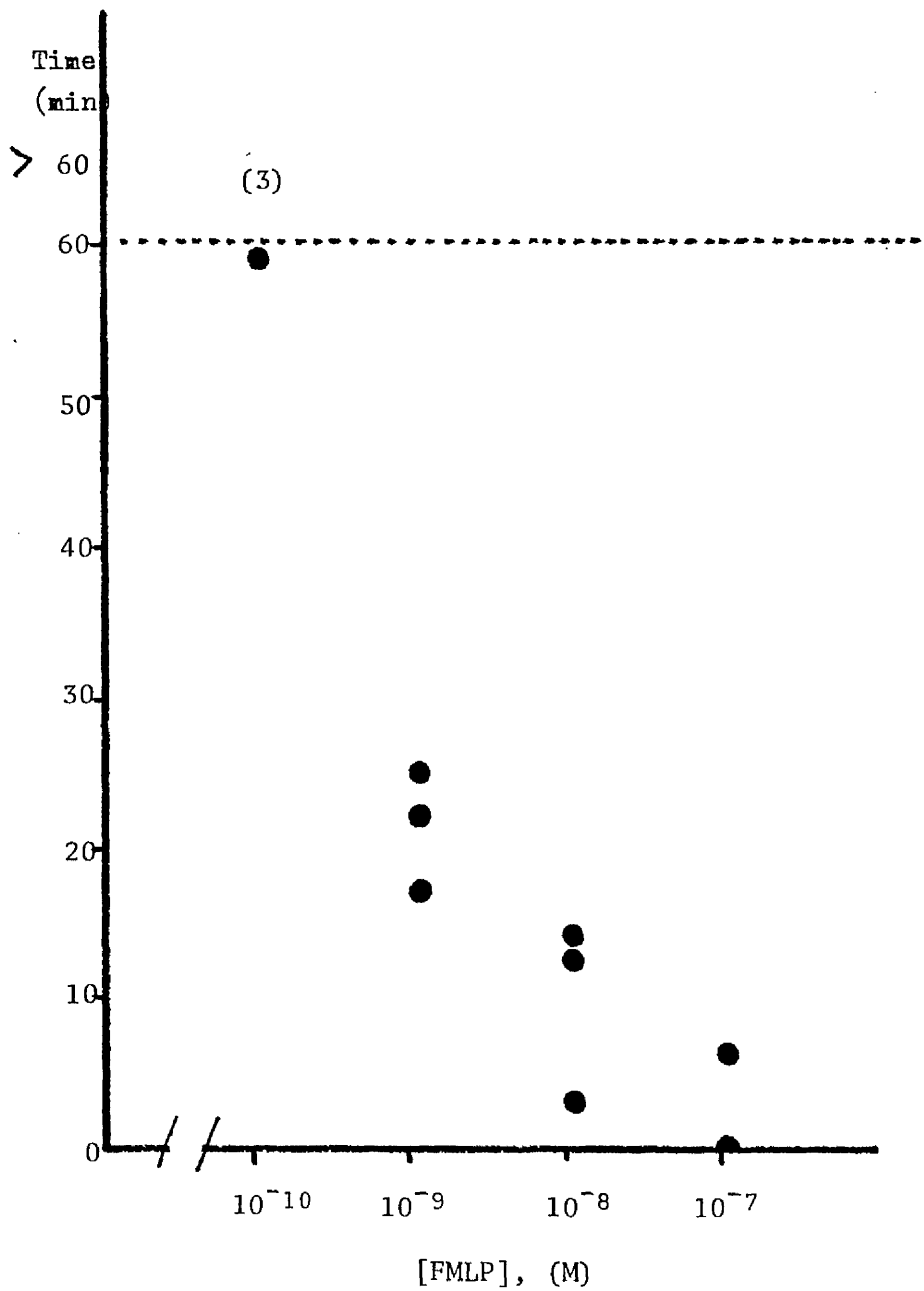

1.0
0.8
0.6
0.4
0.2
0
Aggregation ($\frac{nt}{n_o}$ ± SEM)
10
20
30
40
50
60
time (mins)
10^{-10}M
BSS
10^{-8}M

FIG.22 Time of change from lesser to greater than control adhesiveness.



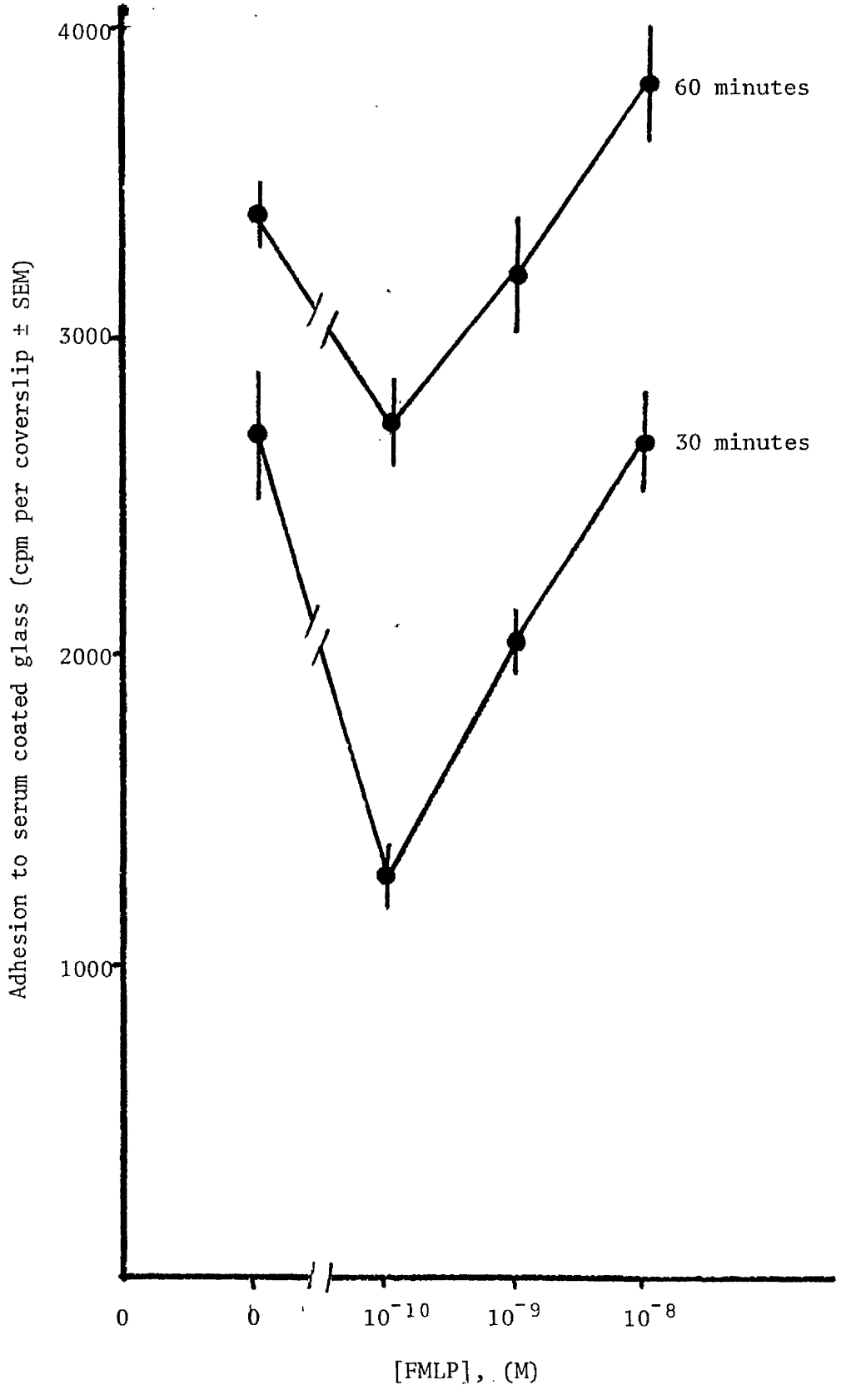
10^{-7}M, 10^{-8}M and 10^{-9}M FMLP respectively.

Adhesion to substrate assays were performed for different lengths of time. Fig.23 shows adhesion after 30 and 60 minutes. These results are qualitatively very similar to those obtained using aggregation assays. The aggregation assays, however, seem to be more sensitive at indicating increased adhesion, since the stimulation of adhesion to substrates at 60 minutes by high concentrations was often not very large.

Comparison of peripheral blood and peritoneal neutrophils

All of the effects of chemotactic factors reported so far have been observed using peritoneal exudate neutrophils. These have already migrated from blood towards an inflammatory site and thus may not behave in an identical fashion to circulating cells. Further, in the exudate, the cells have been exposed, presumably, to a variety of chemotactic factors (although in earlier experiments, no effect on adhesion of exudate fluid was detected). The effects observed using these cells may be the net result of these and the experimentally applied chemotactic factors. The effects of chemotactic factors on the adhesion of purified blood neutrophils were therefore studied. The results (Table 22) show that blood neutrophils responded in a similar fashion to FMLP compared with peritoneal neutrophils. After 30 minutes in 10^{-9}M FMLP adhesion of blood neutrophils was reduced by 17.9% compared to 15.1% for peritoneal cells. On a proportional basis this is a slightly smaller effect than on the peritoneal cells, however, since more blood neutrophils adhere in the controls. At 60 minutes in 10^{-7}M FMLP, blood cells show only a very small adhesive increase (5.4%) compared to peritoneal neutrophils (13.9%). Thus it appears that

FIG.23 Dose-response for adhesion to glass, FMLP.



Each point is mean from 4 replicate coverslips.

TABLE 22 Adhesion of blood and peritoneal neutrophils to serum coated glass in the presence of FMLP.

Cell source	[FMLP] (M)	Time (mins)	Mean percent adhesion ± SD (n) BSS control	Experimental	Mean diff. ± SE	P
Peritoneal	10^{-9}	30	29.8 ± 14.9(15)	14.7 ± 9.0(15)	15.1 ± 2.6	<.001
Blood	10^{-9}	30	45.0 ± 15.7(13)	27.2 ± 12.4(13)	17.9 ± 3.1	<.001
Peritoneal	10^{-7}	60	22.0 ± 12.1(15)	35.8 ± 14.8(15)	13.9 ± 1.9	<.001
Blood	10^{-7}	60	38.0 ± 8.5(12)	43.4 ± 12.8(12)	5.4 ± 2.3	<.05

TABLE 23 Adhesion of neutrophils. Effect of a methylated tri-peptide (one experiment).

	Adhesion (cpm per coverslip) ± SD (n)	
	Endothelium	Serum coated glass
BSS control	2529 ± 356(8)	2652 ± 413(8)
10^{-9}M f-tri-tyr	2061 ± 201(4)	1475 ± 304(4)
10^{-9}M f-tri-tyr-CH_3	1734 ± 565(4)	1933 ± 404(4)
10^{-7}M f-tri-tyr-CH_3	1652 ± 396(4)	1793 ± 247(4)

blood cells are less responsive. However it must be borne in mind that the blood cell suspensions are on average 69% pure. Binding of chemotactic factors by other cell types may affect the result. Also, the blood neutrophils already adhere better than the peritoneal cells. If there is an upper limit to the adhesion possible (either due to some property of the cells or artifact of the assay) then an adhesive increase may be masked.

Effect of a blocked carboxyl group on the efficacy of a chemotactic factor

Table 23 shows the results of a single experiment in which the methyl ester of f-tri-tyr was compared with ftri-tyr. A reduction in adhesion was observed with both endothelium and serum coated glass as substratum. In this experiment no significant difference was found between the methylated and non-methylated peptides. This result seems to eliminate the simple explanation that the reduction in adhesion is caused by increased negative surface charge due to binding of a peptide which has a free carboxyl group.

CHAPTER 7

Introduction

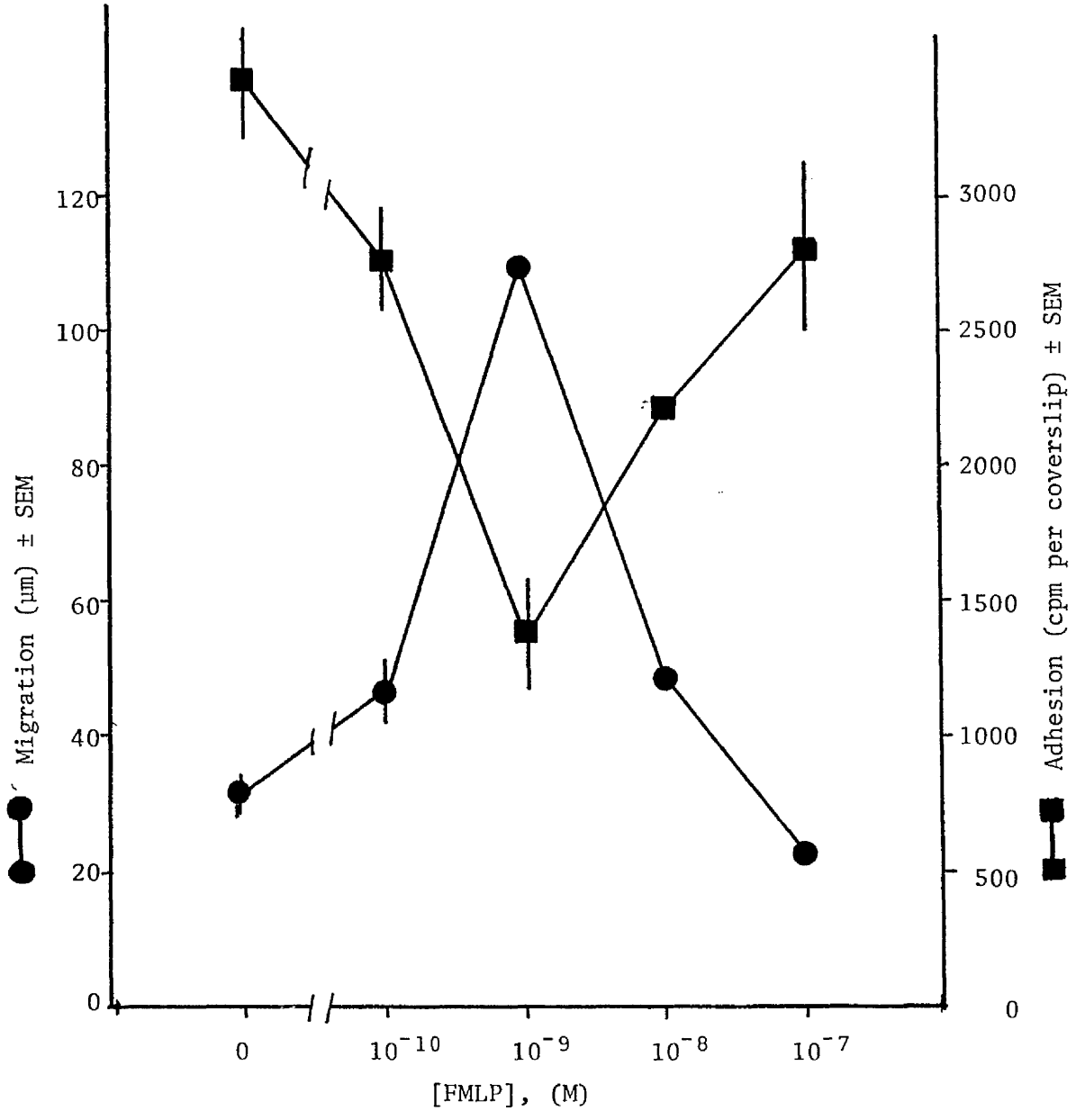
The results shown in the previous chapter led to the conclusion that although chemotactic factors had a range of effects on neutrophil adhesion, none of these appeared to be related to the process of margination. What then is the significance of these changes? Chemotactic factors were first defined by their effects on cell locomotion. Since cell adhesion and cell locomotion have often been related (e.g. Carter, 1965; Harris, 1973; Gail and Boone, 1972), it seemed possible that the adhesive changes in the neutrophils might contribute to their locomotory behaviour.

Locomotion

The dose-response curves for adhesion using FMLP and α-casein as chemotactic factors were found to be inversely related to the dose-response curves obtained previously, using human neutrophils, for chemotaxis, (Smith, Lackie and Wilkinson, 1979). When adhesion and chemotaxis were assayed in parallel using rabbit cells, a similar inverse relation was found (Fig.24). When locomotion into filters in the absence of a chemotactic gradient was assayed, a very similar result was found (Fig.25) showing that the inhibition of chemotaxis by high levels of chemotactic factor is due to a change in the locomotory rate rather than direction. Thus it seemed that these concentrations of chemotactic factor which stimulated locomotion, reduced adhesion and *vice versa*.

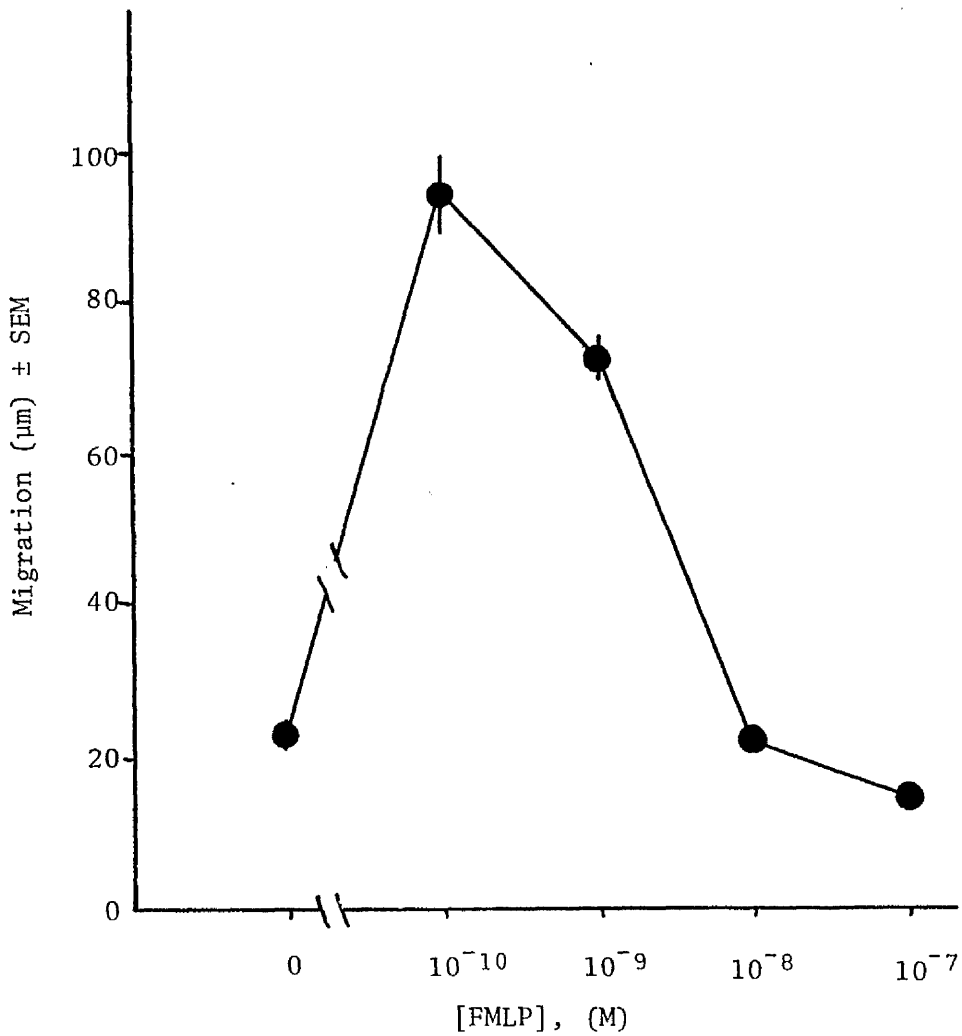
If a close link exists between neutrophil locomotion and adhesion (as these results suggest) then it should be possible to demonstrate locomotory changes with time since it has previously been shown that chemotactic factors induce a series of time dependent adhesive changes. Fig.26 shows that the rate of locomotion at different times in different

FIG.24 Migration of neutrophils into filters towards FMLP and adhesion to serum coated glass. Dose-response curves.



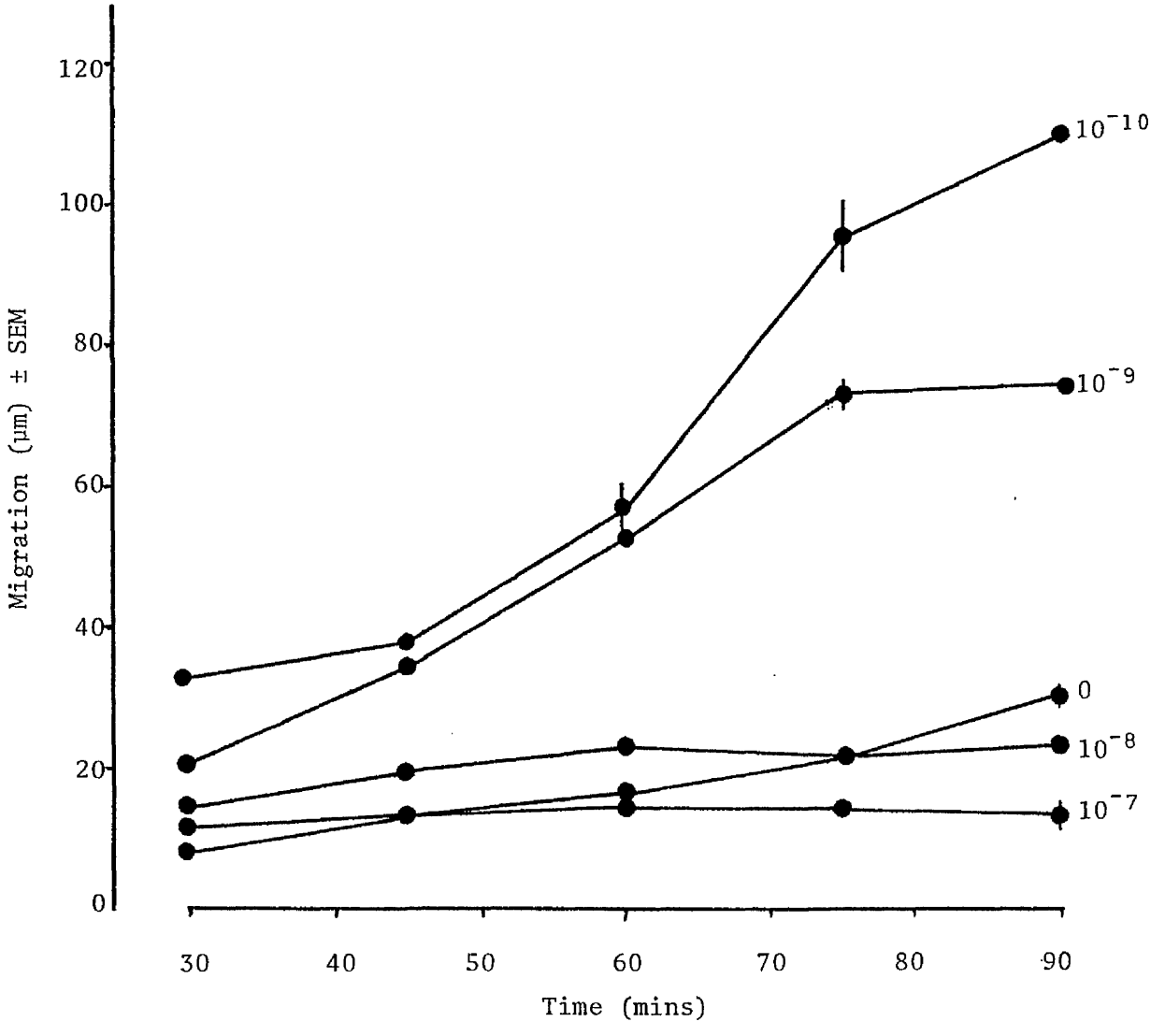
For adhesion, each point is mean from 4 replicate coverslips. For migration, each point is mean from 5 measurements on duplicate filters.

FIG.25 Neutrophil migration into millipore filters. Dose-response for locomotion in FMLP.



Each point is the mean from 5 measurements on duplicate filters.

FIG.26 Migration of neutrophils into millipore filters in the presence of FMLP at different concentrations (M).



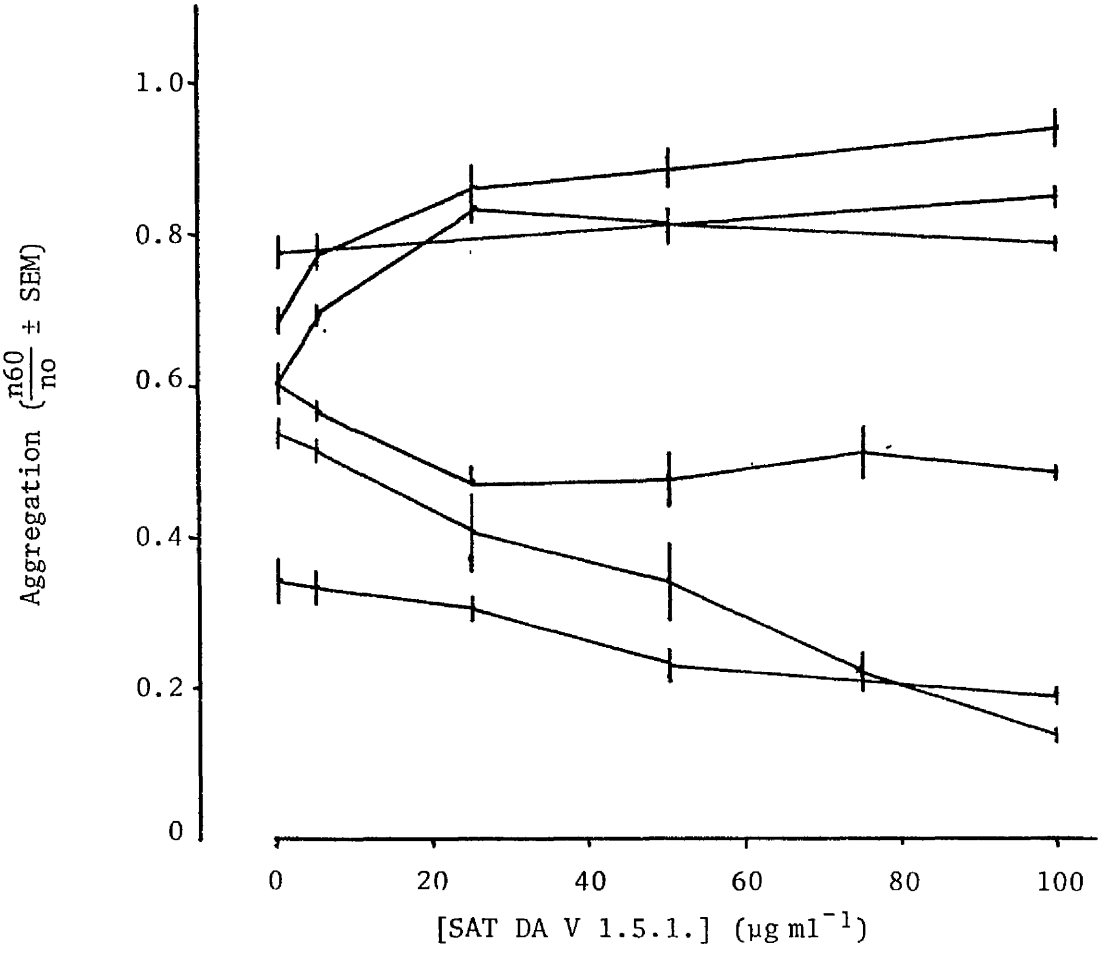
Each point is mean from 5 measurements on each of 4 replicate filters.

times in different concentrations of FMLP shows a pattern of locomotion inversely related to adhesion. Thus in 10^{-10}M FMLP a rate of locomotion is maintained throughout the time course of the experiment. In 10^{-9}M FMLP, enhanced locomotion occurs but at late times, locomotion diminishes. In 10^{-8}M and 10^{-7}M FMLP the cells barely move. This pattern of locomotion would be predicted from the adhesive behaviour of these cells if locomotion was enhanced by lowered adhesion and inhibited by raised adhesion. It must be stressed however that no such causal relationship has been demonstrated and that the reverse relationship, that chemotactic stimulation leads to adhesive change, is quite possible.

Most chemotactic factors stimulate the rate of neutrophil locomotion (i.e. they are also chemokinetic). Some, however, have been reported which possess only chemotactic activity. Keller, Wissler, Hess and Cottier (1978) found that various peptide fractions (Wissler, 1972) possessed this quality. Since the chemotactic factors which affected the rate of locomotion had affects on adhesion, it was of interest to discover if those which did not affect the rate of locomotion had any effects on adhesion. Aggregation dose-responses over the reported chemotactic range of concentrations were performed on a variety of occasions. The results were variable and effects not very large (Fig. 27). Thus it is impossible at present to ascribe to this factor any consistent adhesive change.

Preliminary experiments were performed using levamisole, a compound which has been reported to stimulate neutrophil locomotion (Anderson, Glover, Koornhof and Rabson, 1976) and to prevent inhibition of chemotaxis induced by pre-exposure to a chemotactic factor (Wilkinson, 1979). If the inhibition of chemotaxis towards a particular chemotactic factor

FIG.27 Summary of all dose-response results for aggregation of neutrophils in presence of peptide fraction SAT DA V 1.5.1. (Wissler, 1972).



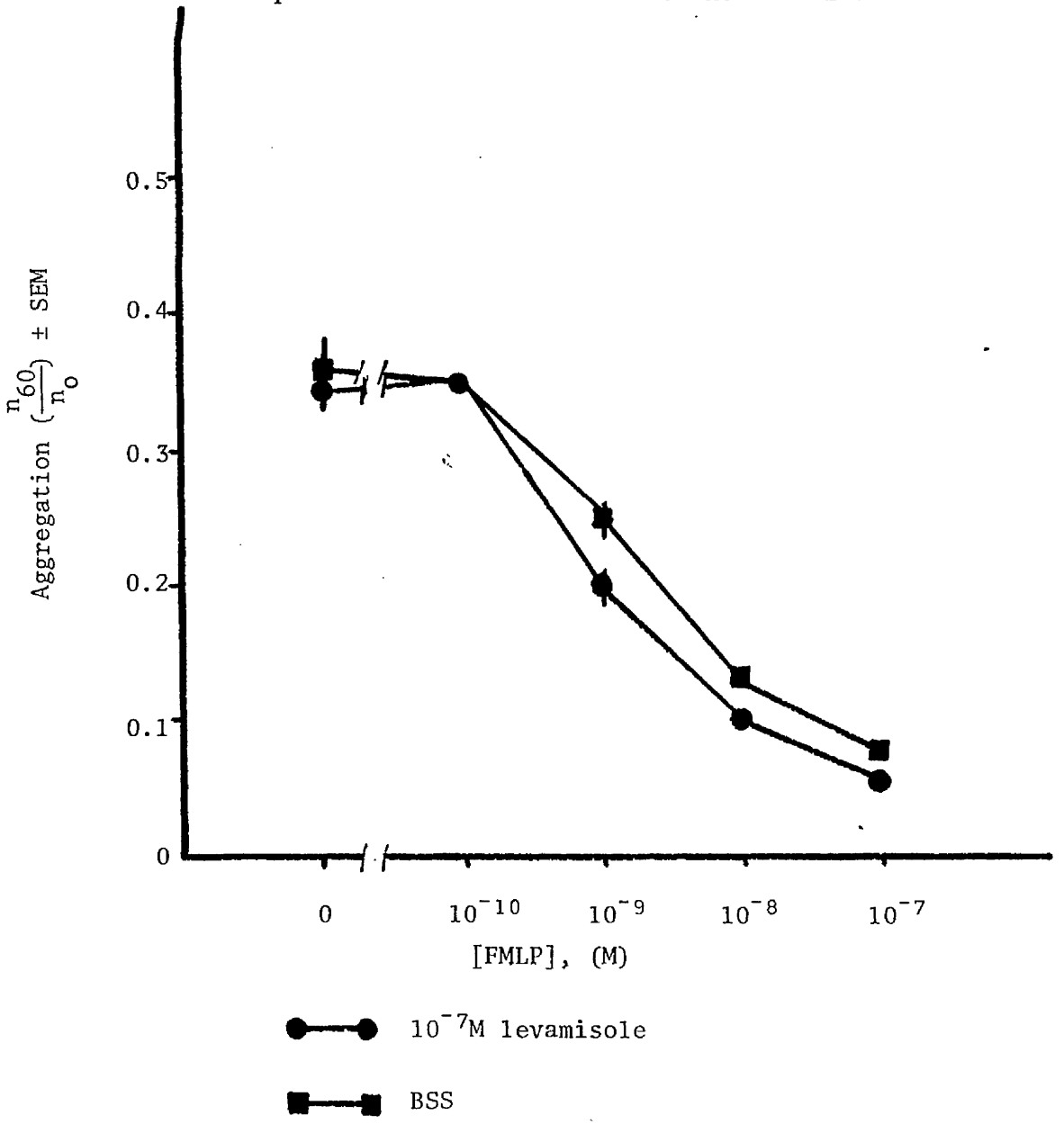
Each point is the mean from at least 3 replicate assays.

caused by prior incubation of neutrophils in that chemotactic factor is due to an adhesive increase, then it would be predicted that an agent which prevented this inhibition of chemotaxis would also prevent the adhesive increase. Fig.28 shows that levamisole (10^{-7}M) had no effect on the aggregation induced by FMLP over the range 10^{-10} - 10^{-7}M. Nor did a range of levamisole concentrations have any effect on the aggregation of neutrophils in 10^{-8}M or 10^{-9}M FMLP (Fig.29). Fig.30 shows that pretreatment of neutrophils in 10^{-7}M levamisole for 30 minutes and aggregation in 10^{-7}M levamisole had no effect on aggregation. Pretreatment of neutrophils for 30 minutes in 10^{-8}M FMLP led to an enhancement of aggregation which was not affected by the presence of levamisole during both the pretreatment and the aggregation. This result suggests that levamisole cannot prevent the FMLP-induced adhesive increase and thus argues against the simple interpretation of previous results that locomotory inhibition is due to enhanced adhesion. However, only one experiment was performed and no positive controls (to show that levamisole does prevent inhibition of chemotaxis) were performed so that as yet no firm conclusions can be drawn.

Secretion

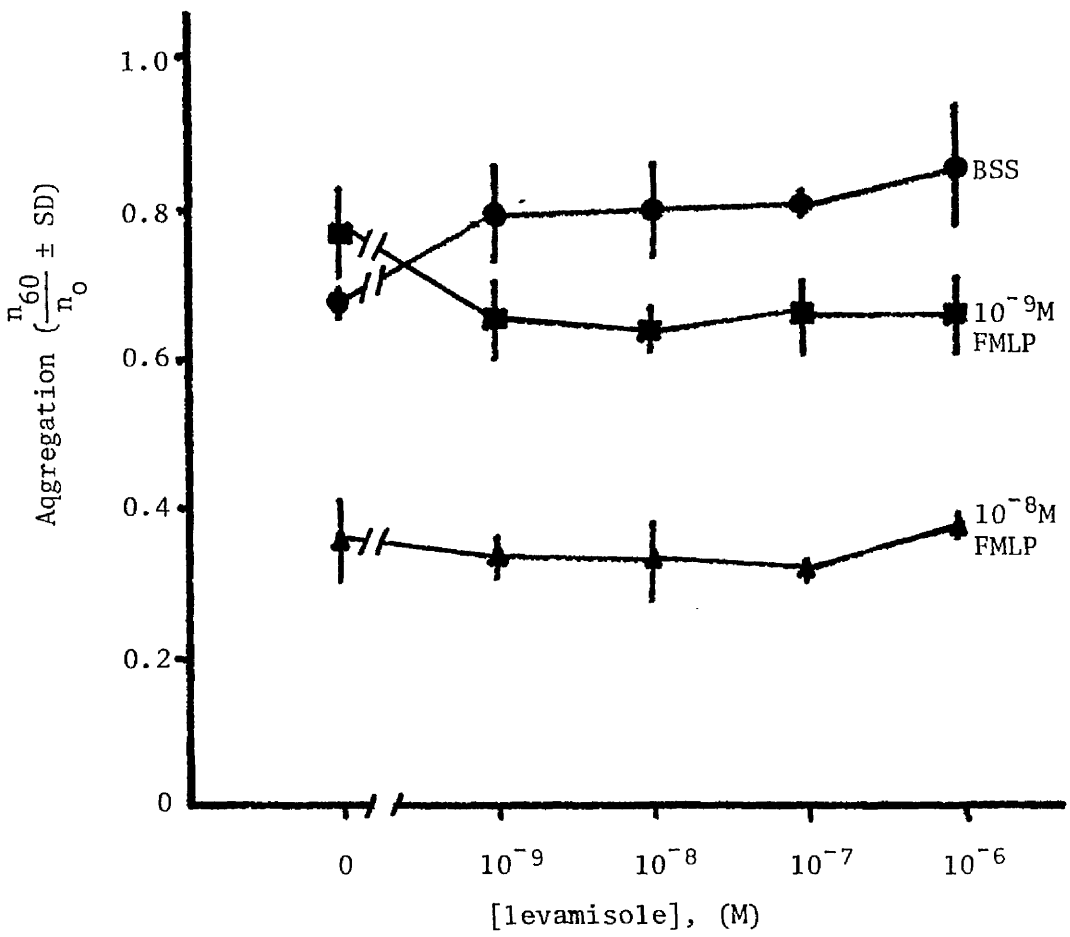
Chemotactic agents are known to induce the secretion of lysosomal enzymes from neutrophils *in vitro*, either in the presence of cytochalasin B (Showell *et al.*, 1976) or when the cells are on an appropriate surface (Becker, Showell, Henson and Hsu, 1974). This chemotactic factor-induced secretion can be inhibited by exposure to anti-inflammatory agents (Palmer and Weatherall, 1978). Since secretion and adhesion have been correlated (Lackie, 1977), it seemed possible that anti-inflammatory agents, by reducing secretion, might also reduce the

FIG.28 Effect of levamisole (10^{-7}M) on aggregation of neutrophils at various concentrations of FMLP.



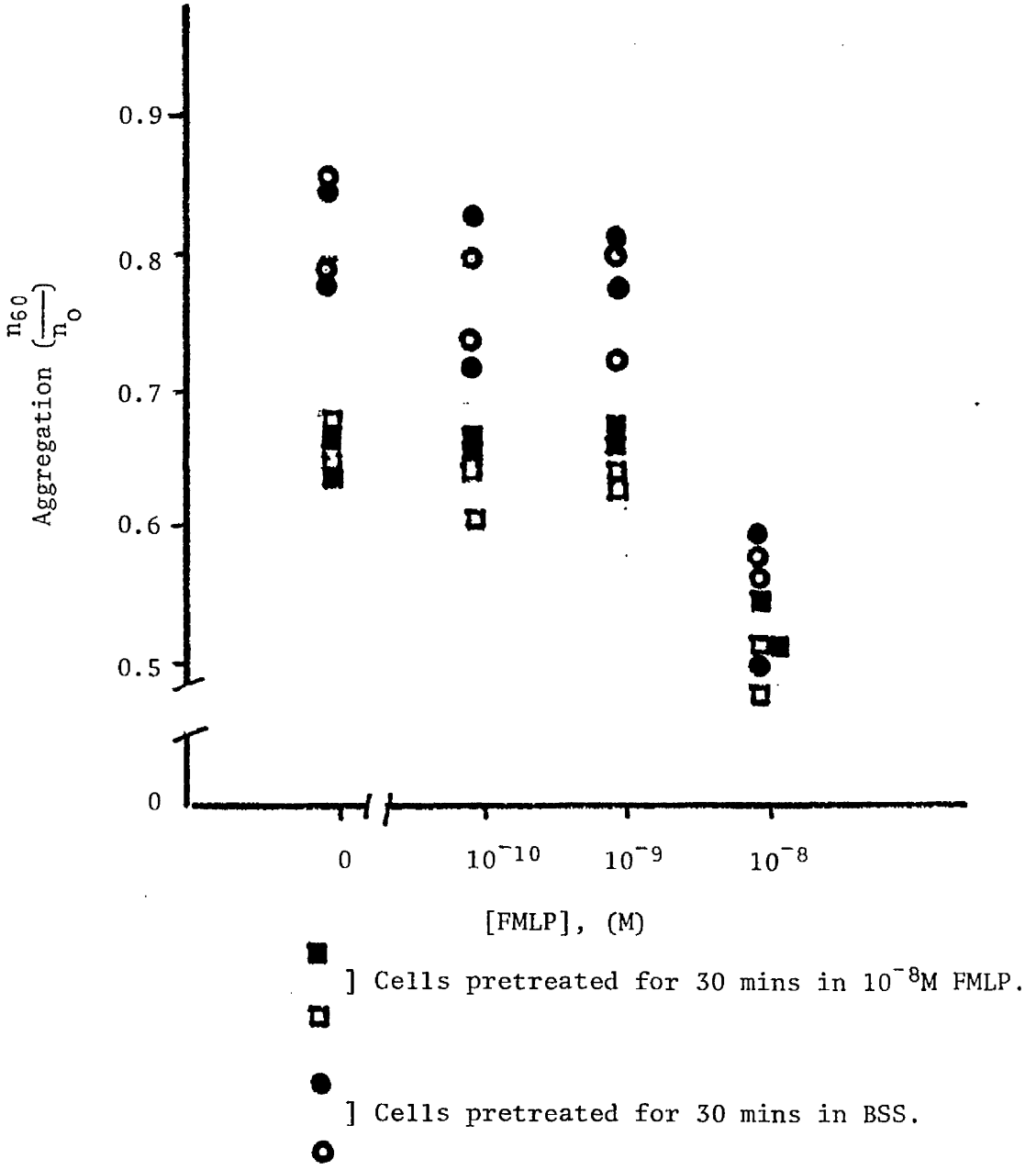
Each point is the mean from 3 replicate assays.

FIG.29 Effect of levamisole on aggregation of neutrophils induced by FMLP.



Each point is the mean from 3 replicate assays.

FIG.30 Effect of 10^{-7}M levamisole on aggregation following pre-exposure to 10^{-8}M FMLP.



Closed symbols - pretreatment and aggregation performed in presence of 10^{-7}M levamisole.

adhesive increase induced in neutrophils by chemotactic factors.

Aggregation experiments were performed to test this hypothesis using FMLP as chemotactic factor and hydrocortisone sodium succinate as anti-inflammatory agent. Assay of the supernatant after aggregation revealed that under the conditions used in these assays, FMLP induced enzyme secretion and that secretion and aggregation were related (Table 24). Hydrocortisone inhibited the chemotactic factor-induced secretion by about 50%. Fig.31 shows that hydrocortisone up to 10^{-3}M had no effect on the aggregation induced by 10^{-7}M FMLP. Aggregation induced by lower concentrations of FMLP than this could be inhibited however (Fig.32). This effect was more apparent when 10^{-2}M hydrocortisone was used, (Fig.33), although when the cells were more adhesive (Fig.34) less inhibition was noted. No further effect was observed at other times (Fig.35).

It appears therefore that hydrocortisone inhibits in parallel the chemotactic factor induced secretion of lysosomal enzymes and adhesive increase. This strengthens the link between these two phenomena. The prevention of the adhesive change may contribute to the anti-inflammatory activity of hydrocortisone.

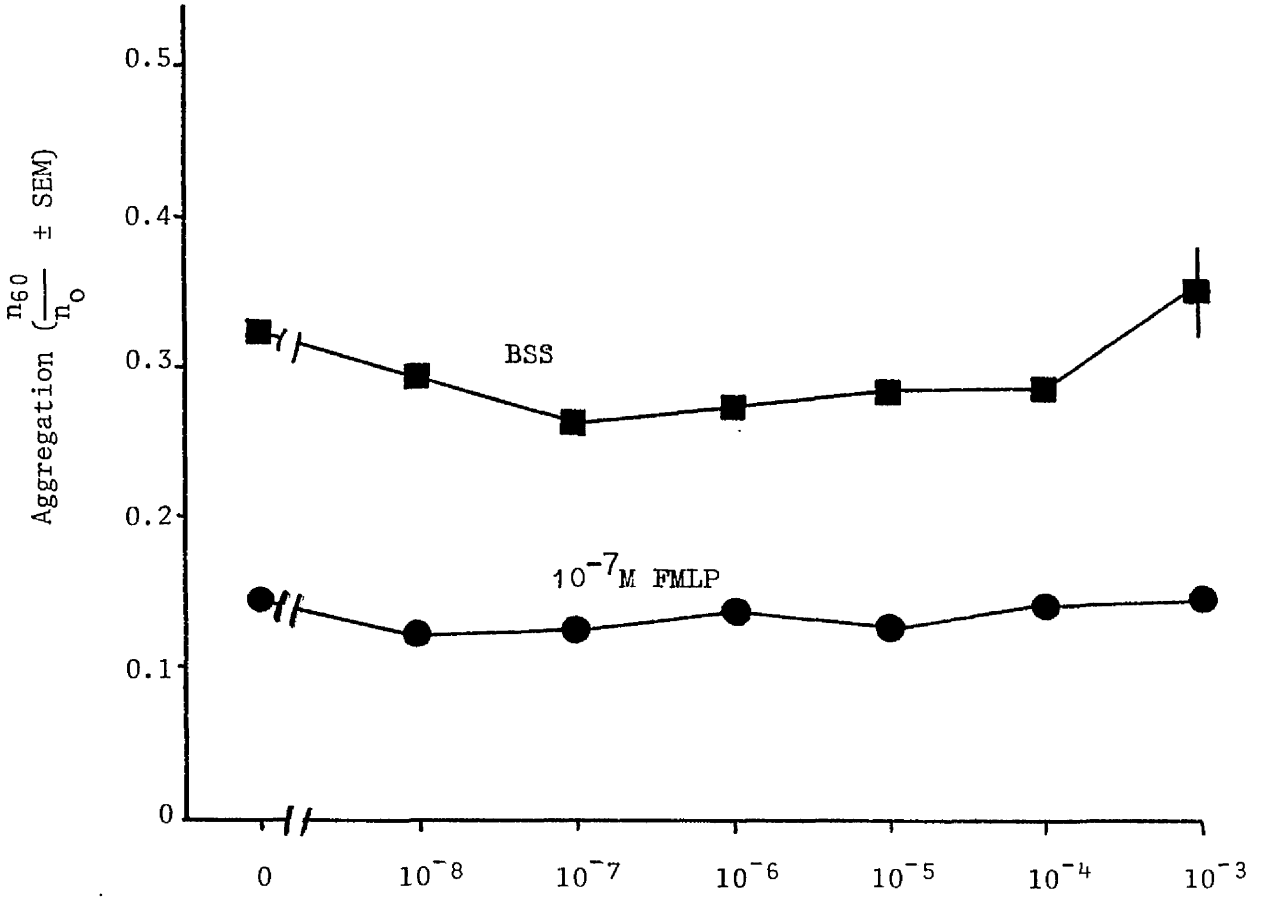
TABLE 24

EFFECT OF CHEMOTACTIC FACTOR ON NEUTROPHIL AGGREGATION AND ENZYME SECRETION

Treatment	Aggregation $(\frac{n_{60}}{n_o})$	% Total Cellular Release		Experiment
		LDH	Lysozyme	
BSS	0.95	2.2	6.1	1
BSS	0.92	3.1	5.8	
10^{-7}M FMLP	0.13	1.9	17.5	
10^{-7}M FMLP	0.09	1.9	17.5	
BSS	0.34	5.5	13.3	2
BSS	0.28	5.6	15.6	
10^{-8}M FMLP	0.11	5.5	24.6	
10^{-8}M FMLP	0.10	6.5	25.3	
10^{-3}M HC*	0.30	5.0	13.0	2
10^{-3}M HC	0.38	4.8	14.9	
Both	0.20	6.1	19.4	
Both	0.19	6.1	19.0	
BSS	0.45	2.1	15.5	3
BSS	0.46	2.4	17.7	
10^{-7}M FMLP	0.02	2.2	35.1	
10^{-7}M FMLP	0.03	1.6	36.3	

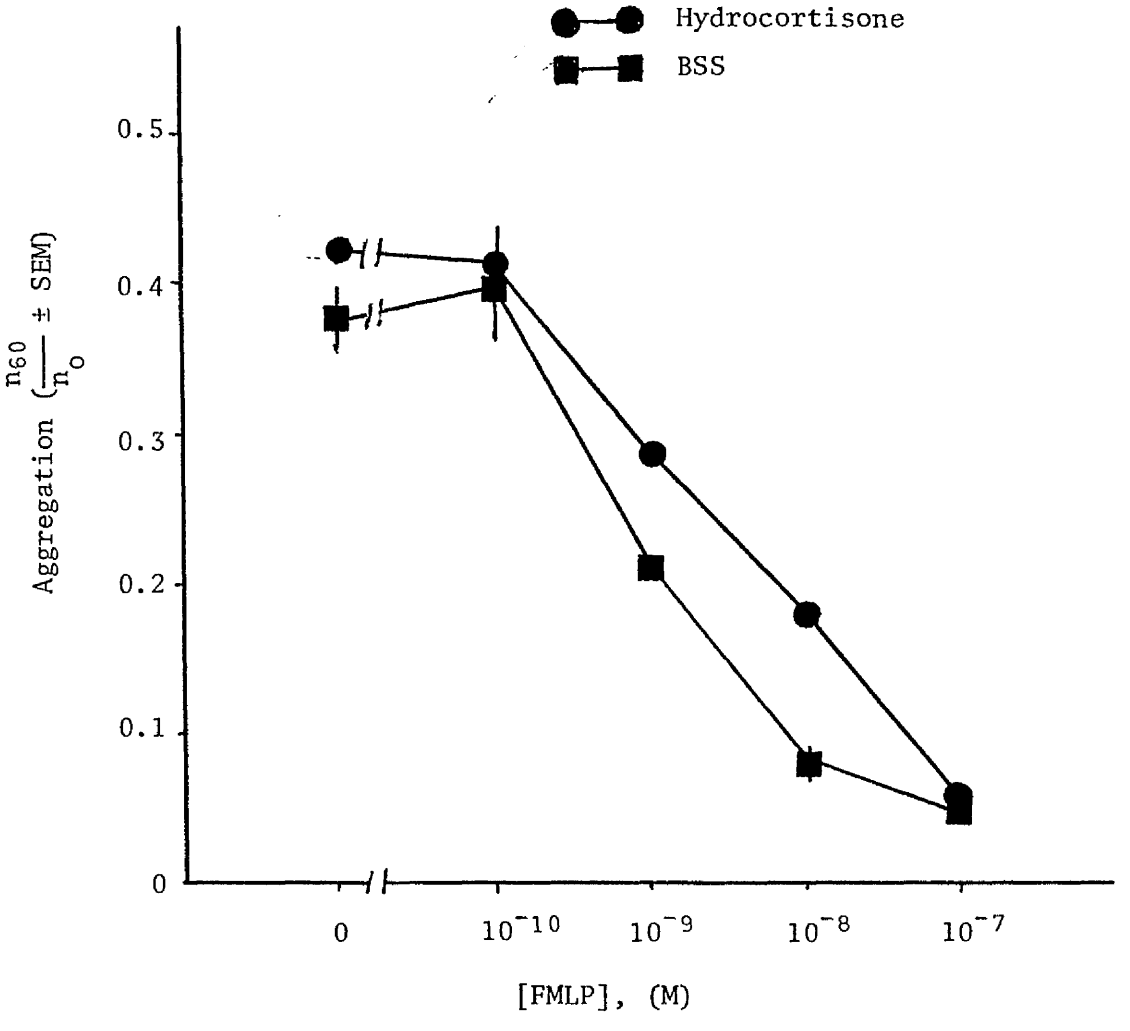
*HC - Hydrocortisone-21-sodium succinate.

FIG.31 Aggregation dose-response for Hydrocortisone sodium succinate.



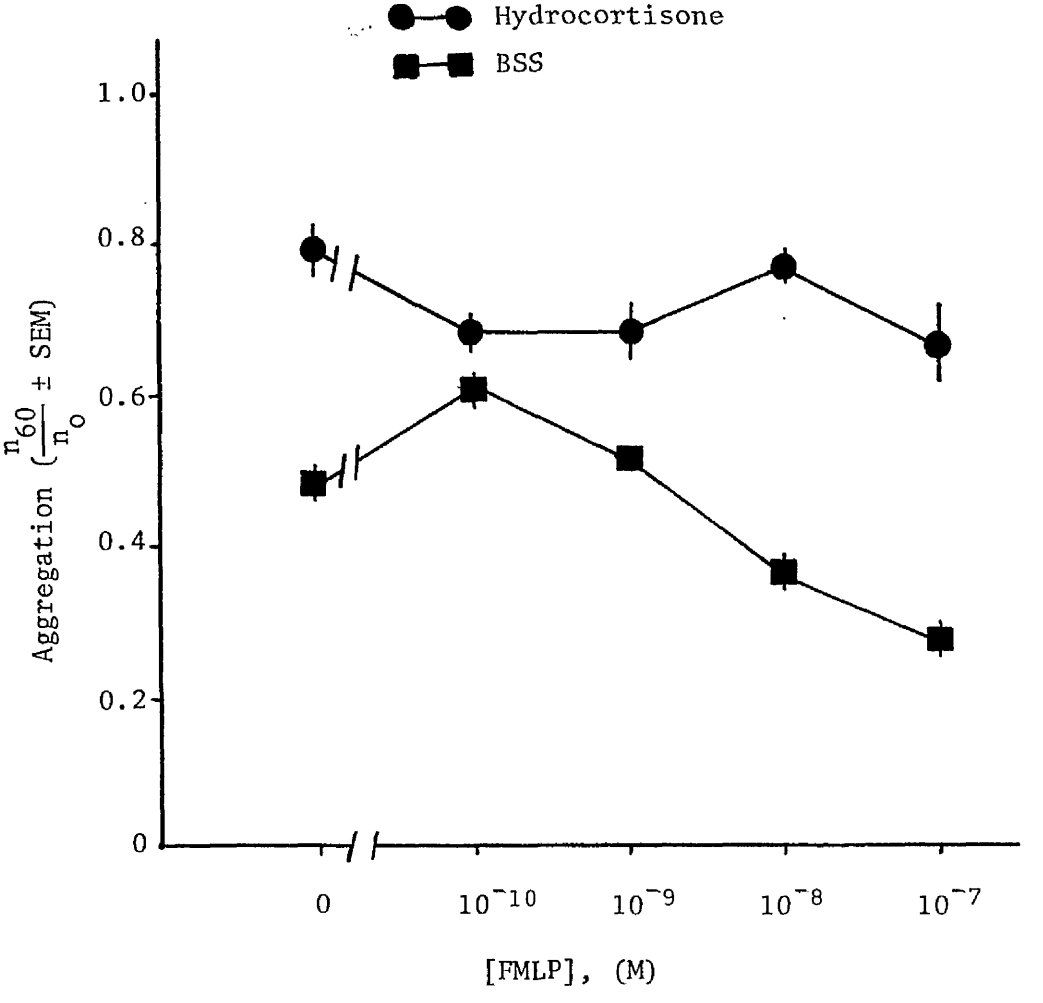
Each point is the mean from 3 replicate assays.

FIG.32 Aggregation dose-response for FMLP. Effect of 10^{-3}M Hydrocortisone succinate.



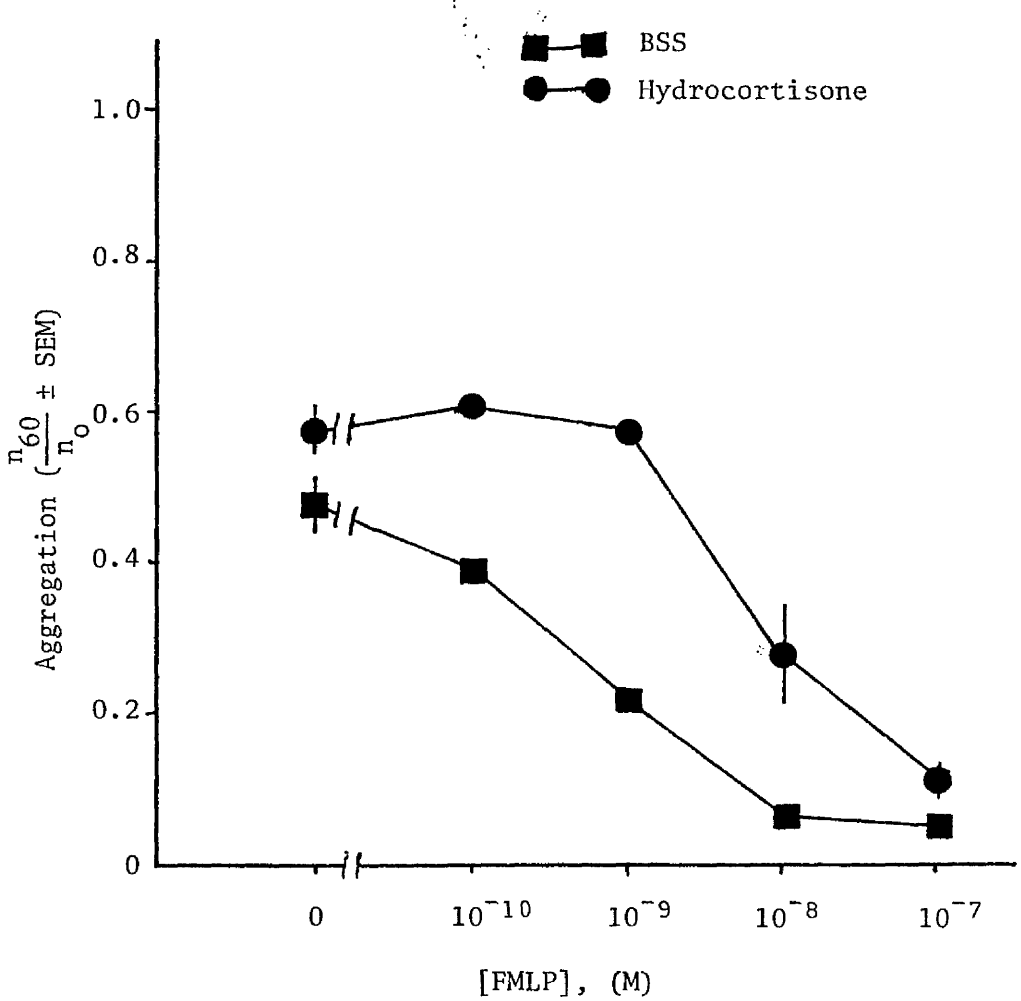
Each point is mean from 3 replicate assays.

FIG.33 Aggregation dose-response for FMLP. Effect of 10^{-2}M hydrocortisone succinate.



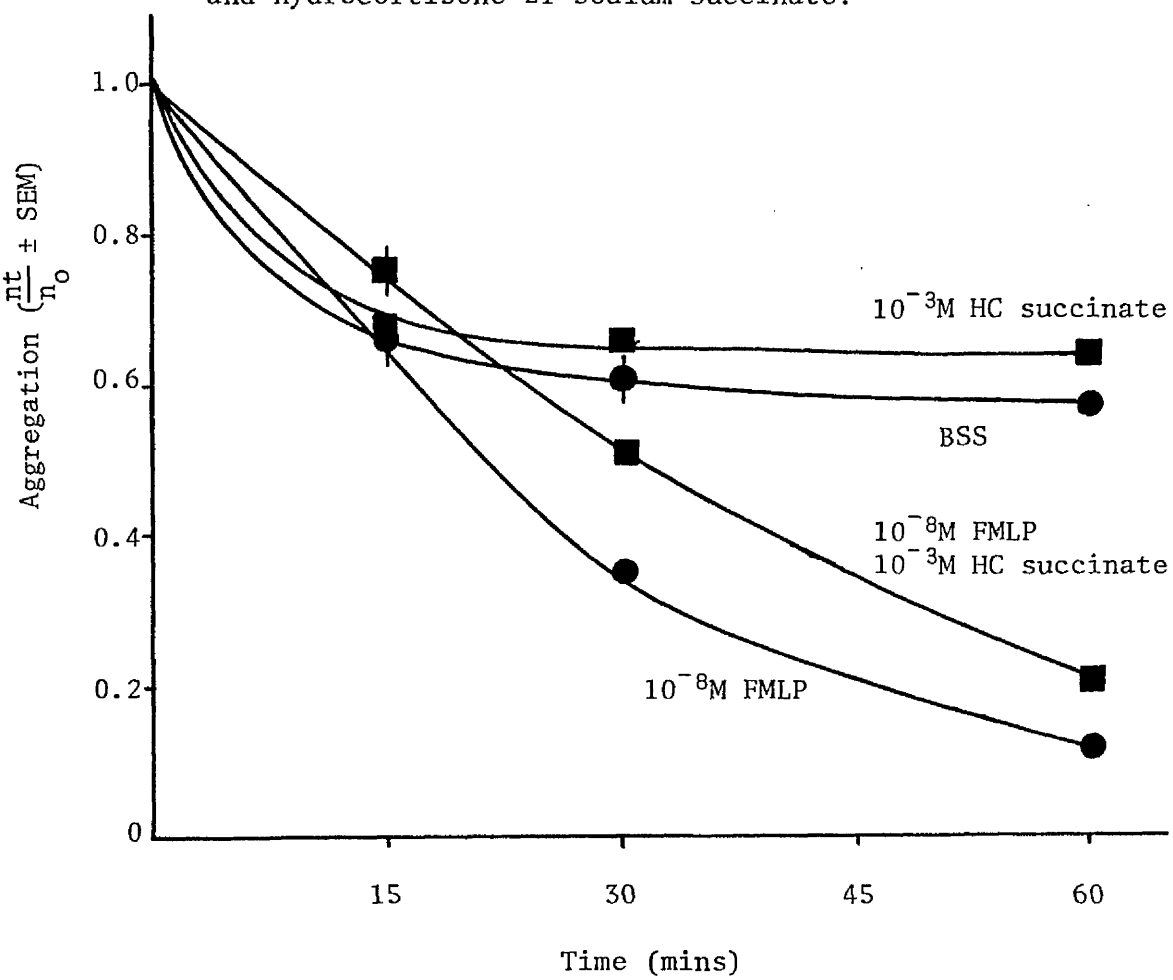
Each point is mean from 3 replicate assays.

FIG.34 Aggregation dose-response for FMLP. Effect of 10^{-2}M hydrocortisone succinate.



Each point is the mean from 3 replicate assays.

FIG.35 Time course of neutrophil aggregation. Effect of FMLP and hydrocortisone-21-sodium succinate.



Each point is the mean from 3 replicate assays.

CHAPTER 8

Introduction

The rationale behind this work was that by studying and modifying *in vitro* the adhesive behaviour of the cells involved in the early events of the inflammatory response, clues to the nature and control of the adhesive changes occuring during neutrophil emigration might be found.

The main findings of the work can be summarized as follows. Firstly, none of the inflammatory mediators tested increased the adhesiveness of either the neutrophils or the endothelial cells. Secondly, several (but not all) anti-inflammatory agents reduced the adhesiveness of the neutrophils but had little or no effect on the endothelium. Thirdly, chemotactic factors had no effect on endothelial cell adhesiveness but diverse effects on the adhesiveness of neutrophils. Briefly, neutrophil adhesiveness was reduced at early times using chemotactically optimal concentrations and increased after a variable time by higher concentrations of a chemotactic peptide. High molecular weight protein chemotactic factors reduced adhesion in all circumstances examined.

Some discussion of results has already been given in previous chapters. This discussion will centre on two main areas. Firstly, the general lack of marked adhesive change in the endothelial cells; secondly the effects of chemotactic factors. Inflammatory mediators and anti-inflammatory drugs will be briefly discussed. Finally, a brief attempt will be made to draw parallels with the results and conclusions from other experimental systems where essentially the same process, directed cell movement, takes place.

Endothelial cell adhesiveness

Work *in vivo* has suggested that adhesive changes occur in endothelial cells during inflammation (Chapter 1). It was therefore surprising to find that almost none of the treatments or agents studied had any effect in stimulating the adhesiveness of the cultured endothelial cells used in the assay system here. There are two basic interpretations of this finding. Either the assay system is inadequate or the finding is a true reflection of events occurring *in vivo*. These possibilities will be discussed in turn.

a) The In vitro assay

Many features of this assay could account for the lack of any observed effect on endothelial adhesion.

The use of cells from different species (and growth of endothelium in serum from a third species) may, if the neutrophil-endothelial adhesion is species specific, eliminate any chance of detection of the endothelial cells' response to an adhesion promoting stimulus. Criticism of this complication of the assay, rather arbitarily introduced for experimental convenience, cannot be directly answered, but some evidence suggests that it may not be a major problem. The results of Lackie and de Bono (1977), Hoover *et al.*, (1978) and Macgregor (1978) showing a high level of neutrophil-endothelial adhesion, support the idea that the interaction of neutrophils and endothelium is a cell specific rather than species specific one. These results can be used to argue against the possibility that rabbit neutrophil adhesion to pig endothelium is the result of non-specific recognition of a foreign surface by the neutrophils. However, a high level of adhesion of neutrophils to endothelium does not in itself indicate that any putative

effect of inflammation on the endothelial cells will be recognized by the neutrophils. The finding of Pearson *et al.*, (1979) of only small adhesive changes using a similar assay with pig neutrophils and endothelium suggests that species differences may not be relevant to the lack of marked effects observed here.

In vivo adhesion is primarily to endothelial cells in the post-capillary venules. The use of aortic endothelial cells may be a problem if the cells of the post capillary venules are specialized in some way for adhesion; several observations argue against the existence of such specialization. Adhesion can occur in arterioles (Mayrovitz *et al.*, 1977: Goodman *et al.*, 1979) and in large veins (Stewart, Ritchie and Lynch, 1974). Large numbers of neutrophils adhere to aortic endothelium *in vitro* when assays are performed using opened vessels (Chapter 4). Beesley *et al.*, (1978) found comparable levels of adhesion of pig neutrophils to aortic and venous endothelium. These results all show that adhesion can occur to other than post-capillary venule endothelium. Differences in endothelial cells from different sources have however been detected. Blose and Chacko (1974, 1975) studied the *in vitro* behaviour of guinea pig thoracic aorta and portal vein endothelial cells. Venous cells had a much greater mitotic potential, a lesser affinity for the substratum, did not accumulate lipid and were very tightly packed. *In vivo* differences have also been detected. Simionescu, Simionescu and Palade (1976) prepared serial EM sections from vessels in the mouse diaphragm known to be arterioles, venules and capillaries. The loading, transit and discharge of vesicles was found to proceed faster at the venule end of the capillary. Endothelial junctions in capilliaries were 'tight'

and impermeable to horseradish peroxidase, whereas in the venules the loose junctions were peroxidase permeable. While there is no evidence to suggest that endothelial cells vary in their adhesive properties and while none of these differences immediately suggests that they might, the existence of differences indicates that a range of endothelial cell types exist.

Another possible criticism of the assay is its use of peritoneal exudate neutrophils. These have already migrated in response to an inflammatory stimulus and may be activated in some manner, possibly preventing recognition of adhesive endothelium. In general, peritoneal and blood neutrophils show very similar behaviour in vitro. Only a few direct comparisons have been performed however. Sabioncello, Dekaris, Veselic and Silobrcic (1976) found that blood and peritoneal exudate cells behaved similarly in migration inhibition tests. Walker, Smith and Ford-Hutchinson (1976) found that the chemotactic responses of blood and peritoneal neutrophils to prostaglandin E, were different. Cunningham, Smith, Ford-Hutchinson and Walker (1979) further showed that the chemotactic response of rat peritoneal neutrophils to activated serum was significantly reduced when compared to cells isolated from peripheral blood. When re-injected, peritoneal cells also showed a greatly diminished ability to accumulate in inflammatory exudates in implanted sponges and also blocked the migration of peripheral blood neutrophils. Peritoneal cells exhibited normal random migration and phagocytosis in vitro compared with blood cells. My results (Chapters 4 and 6) revealed a difference in the levels of adhesion of peritoneal and blood cells, with rather more blood cells than peritoneal adhering. There also may be a difference in their responses to chemotactic factors since no significant increase in adhesion of blood cells at 60 minutes in high concentrations (10^{-7} M) of FMLP was evident. Differences

in the handling of the cells from the two sources may account for the observed differences in adhesive behaviour.

Other, much simpler reasons may account for the lack of marked effect on endothelial adhesion. The assay system may be insufficiently sensitive to reveal differences. For example, if the adhesive change is slight and the shear force during rinsing is large compared to the force of adhesion, then such changes will never be detected. Simple incubation in BSS in the presence of agents being tested may be insufficient treatment to elicit any effect. Blood components may be essential for the endothelial response. The number of hypothetical possibilities is large and consideration of these is not an obviously fruitful task. Consideration of the alternative interpretation of the lack of change seems more useful.

b) Relation of *in vitro* to *in vivo* endothelial behaviour

It is possible that the lack of observed endothelial adhesive change *in vitro* is a true reflection of events *in vivo*. In other words, during inflammation, an adhesive change in the endothelium does not occur. Although endothelial adhesive change is an obvious and attractive hypothesis to explain margination, the evidence for such a change in inflammation is not extensive and alternative explanations have not been eliminated. It is possible that rheologic factors, perhaps in combination with an increase in neutrophil adhesiveness could induce very localized margination. My results are consistent with this hypothesis. The finding that the adhesion of neutrophils to formaldehyde fixed endothelium is not dramatically reduced compared to live endothelium suggests that endothelium plays a passive role in neutrophil adhesion. A similar passive role of endothelium in

lymphocyte adhesion was suggested by de Bono (1976).

In vivo, however, damaged endothelium is apparently more adhesive than normal (Grant, 1973). It was disappointing therefore not to observe any adhesive increase *in vitro* when endothelial monolayers were damaged by scraping.

That it is possible to alter the adhesiveness of endothelial cells *in vitro* has been shown by the results of other groups of workers. Increased adhesion of neutrophils to endothelium, but not to serum coated glass, was observed by Pearson *et al.*, (1979) In the presence of a one hundred fold excess of purified erythrocytes and a decrease was observed in the presence of platelet rich (but not cell free) plasma. The presence of platelets apparently reduced the adhesiveness of the endothelial cells. This observation is consistent with a role for platelets in 'supporting' the endothelium that has been suggested by Gimbrone, *et al.*, (1969) who found that *in vitro* perfusion of organs with platelet rich medium preserved vascular integrity. Pearson *et al.*, (1979) also found that prostaglandins increased adhesion and the parallel between variations in adhesion and the levels of prostaglandin synthesis by cultured cells led to the suggestion that prostaglandins are partly responsible for the modulation of granulocyte adhesion by endothelial cells. The majority of agents tested for effects on adhesion by Macgregor *et al.*, (1978) seemed to affect the neutrophils since adhesion to nylon wool columns was affected similarly to adhesion to endothelial cells. Treatment of endothelial cells with trypsin however led to a decrease in neutrophil adhesion.

At present it seems that the adhesiveness of both neutrophils and endothelium can be modified but that treatments which affect the neutro-

phils have been found more commonly. It is not clear whether this is a reflection of events *in vivo* or merely the choice of agents so far investigated.

The role of inflammatory mediators in adhesion and emigration

None of the inflammatory mediators tested, histamine, 5-HT or bradykinin increased neutrophil adhesion to either serum coated glass or endothelium. Histamine and 5-HT, in fact, reduced adhesion. It seems likely that these agents are not directly involved in *in vivo* adhesion. The effects of these agents on neutrophil adhesion have been studied by other workers. Penny *et al.*, (1966) found bradykinin to reduce adhesion to glass bead columns. Banks and Mitchell (1973c) reported that 5-HT increased leucocyte adhesion to glass, but using a platelet rich medium. Neutrophil adhesion may be secondary to platelet aggregation or adhesion. Bryant and Sutcliffe (1974) found histamine to reduce adhesion to serum coated glass.

Several *in vivo* observations have been reported. Graham *et al.*, (1965) reported that bradykinin induced an increase in the number of leucocytes rolling along vessel walls. Atherton and Born (1972) reported that histamine had no effect on the number of rolling leucocytes. The possibility that all three of the agents may be involved in adhesion and emigration is suggested by the observations of Spector and Willoughby (1964) that all three promote the emigration of neutrophils. However, their effect was only slight and the lack of further clear evidence either *in vivo* or *in vitro* argues against such a role.

The effects of anti-inflammatory drugs

Since little effect had been observed using inflammatory mediators or after damaging the endothelium it was felt that the cultured endo-

thelial cells might be stimulated or 'inflamed' in some way as a result of their isolation and growth *in vitro*. Several anti-inflammatory agents were studied in an attempt to modify endothelial adhesion. That these agents might reduce adhesion was suggested by observations that a wide range of anti-inflammatory agents have been shown to reduce the number of neutrophils in inflammatory exudates (Borel and Feurer, 1978: Di Rosa, 1978) and also to increase the number of circulating leucocytes following administration of the drugs (Boggs, Athens, Cartwright and Wintrobe, 1964; Fruhman, 1962; Thompson and Van Furth, 1970), consistent with a reduction in the number of adherent cells.

Of the drugs tested for effects on adhesion indomethacin, EACA, and prednisolone-21-sodium succinate had virtually no effect on adhesion though in some experiments prednisolone reduced adhesion. The other drugs all reduced adhesion to both endothelium and serum coated glass. Colchicine appeared to alter the adhesiveness of the endothelial cells. Hydrocortisone similarly appeared to slightly reduce endothelial adhesiveness. It is possible that these agents owe their effectiveness *in vivo* at least partly to an inhibition of adhesion but this does not seem to be a general feature of anti-inflammatory agents.

Other workers, however, have reported reductions in neutrophil adhesion in the presence of anti-inflammatory agents. Allison and Lancaster (1964) found that EACA inhibited clumping of rabbit neutrophils in the presence of bacteria. The concentrations used were high (0.3 - 0.5M) and it is unlikely that the effect had any biological significance. In an earlier report, Allison *et al.*, (1963) found EACA to have no effect on *in vivo* leucocyte-endothelial adhesion. Macgregor (1976) studied the adhesion of granulocytes from patients receiving

aspirin, indomethacin, phenylbutazone, colchicine or prednisone. Adhesion to nylon wool columns was significantly less than control granulocytes from untreated volunteers. However the addition of glucocorticoids and salicylates to blood *in vitro* had no effect on adherence. Plasma from volunteer subjects treated with either drug contained a factor which inhibited the adhesion of normal granulocytes, This suggests that these anti-inflammatory agents exert their effect on adhesion indirectly through some plasma component. The need for a plasma factor is in contradiction to my results, showing direct effects on adhesion caused by some anti-inflammatory agents, arguing, perhaps, that the assays I have used are more sensitive to small changes in adhesiveness. Effects of anti-inflammatory agents in adhesion in the absence of plasma have also been reported by Hammerschmidt, White, Craddock and Jacob (1979) who found that methyl-prednisolone and hydrocortisone inhibited complement induced neutrophil aggregation. This supports my conclusion that anti-inflammatory agents can exert a direct effect on neutrophil adhesion. Hydrocortisone-21-sodium succinate was also found (Chapter 7) to inhibit the chemotactic factor FMLP induced increase in aggregation. This is a very similar finding to that of Hammerschmidt *et al.*, (1979). It seems possible then that anti-inflammatory drugs interfere with the neutrophils' chemotactic response, possibly preventing their adhesive increase and reducing the number of emigrating cells. However, in my hands the adhesive increase observed in the presence of chemotactic factor occurred in high concentrations or after a time lag in low (chemotactically optimal) concentrations. It is difficult to imagine how such adhesive changes could be involved in margination (see later discussion).

Anti-inflammatory agents have been reported to inhibit locomotion and chemotaxis (Phelps, 1969; Phelps and McCarty 1967; Higgs, McCall and Youlten, 1975; Rivkin, Foschi and Rosen 1976) and also the secretion of lysosomal enzymes (Northover, 1977; Palmer and Weatherall 1978; Ignarro, 1977; Smith, 1977) and it is possible that these effects account for the inhibition of migration of neutrophils.

The inhibition of secretion is of interest since secretion and adhesion have been shown to be correlated (Lackie, 1977; Gallin *et al.*, 1978). Since secretion caused by chemotactic factors occurs in the presence of high concentrations of these factors, the adhesive decrease caused by anti-inflammatory agents may be a result of inhibition of secretion. If this is so then this argues further that the adhesive increase observed in the presence of chemotactic factor is unrelated to margination since secretion is most likely to occur at or near the focus of an inflamed area.

The effects of chemotactic factors on adhesion

Chemotactic factors were studied for effects on adhesion with the expectation that they might increase the adhesiveness of either or both the endothelial cells and neutrophils. Such an increase was observed but only in concentrations of chemotactic factor much higher than the chemotactic optimum or at chemotactically optimal concentrations after a variable time lag.

It is difficult to imagine how margination could be induced by chemotactic factors since their first observed effect in my hands is to reduce adhesion. Since chemotactic factors were found to have no effect on the adhesiveness of endothelial cells, their involvement in margination through an effect on these cells also seems unlikely.

Other workers, however, have reported *in vitro* results on adhesion consistent with a role for chemotactic factors in margination.

Hoover *et al.*, (1978) showed that treatment of both endothelial cells and neutrophils before use in adhesion assays led to an increase in adhesion. My results are in direct contradiction to these observations. Some of the differences, however, may be resolved.

The concentrations at which the chemotactic factors were used by Hoover *et al.*, (1978) may be above the chemotactic optimum. In this case, an immediate adhesive increase when neutrophils are treated would be consistent with my observations since I found that pretreatment of neutrophils with 10^{-8}M FMLP led to an enhancement in aggregation (see Fig.3 o Chapter 7). This cannot explain the result for the chemotactic factor f-met-ala, which was used at 10^{-4}M, the chemotactic optimum, (Schiffmann *et al.*, 1975). However, since the chemotaxis dose-response curve for f-met-ala shows a very rapid drop above 10^{-4}M and since the activities reported for chemotactic peptides such as this vary tremendously in the literature, parallel dose response experiments for adhesion and chemotaxis are essential to clarify the effect of the peptide.

The times after which the assays were carried out were not given by Hoover *et al.*, (1978). If the assays had been performed after incubation for one hour or more an increase in adhesion at all but very low concentrations of chemotactic factor would be consistent with my results if pretreatment with chemotactic factors induces a similar series of adhesive changes as in the presence of the factors.

Neither of the above possibilities, however can account for the

differences between the effects of chemotactic factors on the endothelial cells reported by Hoover et al., (1978) and myself. For this reason, considerable effort was expended confirming my results and performing pretreatment experiments on different batches of endothelium, for different times and in different concentrations. Adhesion was also assayed at different times of incubation following pretreatment. Since no adhesive increase was ever detected, this difference between my results and those of Hoover et al., (1978) remains unexplained at present.

The effects of chemotactic factors on neutrophil adhesion have been extensively studied by other workers. Several results have been reported which appear to be in contradiction to those I have obtained.

Using an aggregation assay with electronic particle counting on a Coulter Counter, O'Flaherty, Kreutzer and Ward (1977a, b) reported that several chemotactically agents (C3fr, C5fr, FMLP and formyl-methionyl-methionyl-methionine, FMMM) induced rabbit neutrophils to aggregate reversibly and to slowly swell. Aggregation reached a peak at one minute after addition of the chemotactic factor and then declined rapidly, total particle number returning to pre-addition values by eight minutes at the latest. Using FMLP the effect increased with dose over the range 5.10^{-11}M - 10^{-6}M. In parallel with this aggregation response a transient neutropenia could be demonstrated in rabbits following infusion of the chemotactic factor (O'Flaherty, Showell and Ward, 1977a). These results support the hypothesis that in vivo during inflammation release of chemotactic factors stimulates neutrophil adhesion to endothelium. The authors, however, concentrate on the argument that in vivo aggregation may underlie the neutropenia

which follows the infusion of the agents into rabbits.

These effects on neutrophil aggregation are strikingly different from any of my observations.

The first point of difference is the lack of any aggregation in controls observed by O'Flaherty, Kreutzer and Ward (1977a, b) over the entire 15 minute observation period. Thesuspensions used by these workers were agitated by the "moderately rapid" rotations of a magnetic stirring bar. Less rapidly stirred or unstirred suspensions did aggregate spontaneously. This latter observation is much more in agreement with my and other (Lackie, 1974; 1977) observations on the behaviour of neutrophils. Presumably the lack of aggregation in the absence of chemotactic factors observed by O'Flaherty, Kreutzer and Ward (1977a, b) was due to the high shear forces present in the assay they use.

O'Flaherty, Kreutzer and Ward,(1977a, b) observed an immediate, transient increase in aggregation following addition of chemotactic factor over the entire concentration range studied. Comparison of the extent of aggregation observed by O'Flaherty, Kreutzer and Ward (1977a, b) is complicated by the fact that the effects they observe are directly proportional to $\log_{10}$ of the number of added cells. However, one can calculate from their data that using 10^6 cells ml^{-1} (a figure comparable to that which I use) the maximum effect observed using FMLP would be 70%, 40% and 0% drop in total particle number in 10^{-7}M, 10^{-8}M and 10^{-9}M FMLP respectively. The effects would have disappeared in 2 minutes. Since I start sampling cell suspensions after 3 minutes at the earliest, it is possible that the cellular changes observed by O'Flaherty *et al.*, (1977) are over before I start assaying aggregation.

O'Flaherty, Kreutzer and Ward (1977a, b) did not observe any inhibitory effect of chemotactic factors on aggregation. Since in the absence of chemotactic factors no aggregation occurs in their assay, such an effect cannot be detected.

There are some peculiarities about the results of O'Flaherty, Kreutzer and Ward (1977a, b). The timing of the response depends on the number of cells present. Thus after 60 seconds in the presence of 5.10^{-10}M FMLP cells are maximally aggregated at a density of 3 - 4 x 10^6 ml^{-1} but totally unaggregated at a concentration of 1 - 2 x 10^{-5} ml^{-1}. In parallel with particle number, particle volume is measured. There is some discrepancy between these two sets of figures which is not explained. For example in conditions shere a 70% fall in particle number occurs, mean particle volume rises only 60% (should be $\sim$ 300%). This may reflect a lack of sensitivity in the volume measurements or it may be that part of the particle number decrease is due to adhesion to the vessel walls. This latter possibility may not be a serious problem, however, since increased adhesiveness is probably also involved in wall adhesion.

Further studies (O'Flaherty, Kreutzer, Showell and Ward, 1977) using rabbit peritoneal neutrophils and a partially purified bacterial factor obtained by butanol extraction of *Escherichia coli* culture filtrates showed that this chemotactic factor also induced neutrophil aggregation. At 37°C the pattern of aggregation was similar to that described previously for other factors except that aggregation induced by this factor occurred irreversibly. Aggregation was found to be inhibited by low temperature (4°C) and inhibitors of glycolysis.

Sodium cyanide, dinitrophenol, cycloheximide, colchicine and vinblastine had no effect on aggregation. Cytochalasin B enhanced aggregation which increased over the entire observation period when macroscopic clumps were visible. In the absence of chemotactic factor, cytochalasin B had no effect. Lackie (1974; 1977) has shown both colchicine and cytochalasin B to have clear effects on neutrophil aggregation, suggesting that the assay method he used is more sensitive to adhesive changes, presumably due to the lower shear forces present. In other respects, e.g. divalent cation dependence, metabolic requirements, the results of O'Flaherty *et al.*, (1977) are generally in agreement with previous observations.

Studies using a reversible inhibitor of binding of synthetic f-met-peptides (carbobenzoxy-phenylalanyl-methionine, CBZ-phe-met) (O'Flaherty, Showell, Kreutzer, Ward and Becker, 1977) suggested that interaction of f-met-peptides with a single cell surface receptor induced chemotaxis, enzyme secretion, aggregation and *in vivo* induction of neutropenia, suggesting a very close interrelation between these phenomena.

Detailed studies of the effects of divalent cations (O'Flaherty, Showell and Ward, 1977b; 1978) showed that the concentration of both of these ions affected the degree of aggregation induced by chemotactic factors.

Similar studies have been performed using human leucocytes (O'Flaherty, Kreutzer and Ward, 1978; 1979a; O'Flaherty, Showell, Becker and Ward, 1978; O'Flaherty, Kreutzer, Showell, Vitkauskas, Becker and Ward, 1979). In general human cells behaved similarly to rabbit cells although aggregation was much less marked and the reaction

was apparently irreversible. It was suggested that this was due to increased adhesion to the vessel walls and they produced evidence in support of this. Very much higher concentrations of chemotactic factor were required to aggregate human cells. Using FMLP, for example, 5×10^{-6}M was the concentration normally used to elicit the aggregation response. This is a concentration almost five orders of magnitude higher than the published ED_{50} for FMLP (Showell, Freer, Zigmond *et al.*, 1976). The relevance to chemotaxis of any effects caused by this concentration may be therefore doubtful. Experiments in which the order and timing of addition to the aggregation vials of the chemotactic factors and the divalent cations Mg++ and Ca++ showed that addition of chemotactic factor before divalent cations led to a reduction in response, a phenomenon termed desensitization. Study of this led to the suggestion that opening and closing of calcium 'gates' in the neutrophil membrane were important in triggering aggregation. Most of this work has been recently reviewed by the authors (O'Flaherty and Ward, 1979).

Granulocyte aggregation has also been studied by another group of workers. Using an aggregometer, Craddock *et al.*, (1977) reported that C5a induced reversible aggregation of human neutrophils. Pseudopod formation invariably preceded aggregation and it was considered that this might be related to aggregation. Craddock, White and Jacob (1978) showed that aggregation was potentiated by cytochalasin B. The effect of cytochalasin B was dose dependent and irreversible. However, cytochalasin B treated cells failed to develop plasma membrane pseudopods, showing that these are not always necessary for aggregation.

Hammerschmidt, White, Craddock and Jacob (1979) found that aspirin up to 100μg ml^{-1} (.57mM) had no effect on aggregation while corticosteroids (methyl-prednisolone, 1.7mM and hydrocortisone, 5mM) markedly impaired aggregation.

The qualitative pattern of aggregation is very similar to that reported by O'Flaherty and co-workers, although it is not possible to compare the two sets of data directly since no quantitation of the optical density changes is given by Craddock's group. Of interest is the finding of Hammerschmidt *et al.*, (1979) that the degree of aggregation is greater at lower stirring speeds. At 300rpm the increase in light transmission induced by the addition of zymosan activated plasma to cells was 2.5 times as great as at 1200rpm, again showing that greater sensitivity can be obtained under lower shear conditions.

All the results from these two groups of workers indicate that a transient, reversible adhesive increase occurs in neutrophils in response to chemotactic factor (in the absence of cytochalasin B). Using different assay methods, other workers have reported irreversible changes in adhesiveness.

Smith, Hollers, Patrick and Hasset (1979) studied the attachment of human neutrophils to protein coated glass. Several chemotactic factors, f-met-phe, zymosan activated serum, C5a and a bacterial chemotactic factor caused a significant increase in attachment, measured after 12½ - 15 minutes. For all but the bacterial chemotactic factor, high adhesion was still observed after 45 minutes incubation in the absence of chemotactic factor. These experiments were performed over a range of concentrations with the same result. The reason for this

apparent contradiction with my results is not known. Interestingly, these workers also noted a shape change from spherical to polarized when neutrophils in suspension were exposed to chemotactic factors. This shape change was virtually complete by 1 - 2 minutes after the addition of chemotactic factor.

One other (final) group of workers have studied the effects of chemotactic factors on neutrophil adhesion. Fehr and Dahinden (1978) studying adhesion to petri dishes found that FMLP induced minimal adherence when used at a concentration of 10^{-9}M and maximal adherence at a concentration of 10^{-7}M, apparently in very close agreement with my observations. These changes were also irreversible.

The results of studies of the effects of chemotactic factors fall into two classes. One group of results, using high shear aggregation assays indicates that chemotactic factors induce a transient adhesive increase. A second group using lower shear rate aggregation or adhesion-to-substratum assays indicates an irreversible increase in adhesion with various dose and time effects and some adhesive decreases. The differences between these two groups of results may be nothing more than artifacts of the methods used to obtain them. However another possibility is that they indicate the existence of two separate phenomena.

The transient adhesive increases follow a time course very similar to the shape changes reported by Smith *et al.*, (1979) and to the locomotory changes recently reported by Zigmond and Sullivan (1979). It is possible that the cell surface events contributing to these changes cause or are caused by the transient adhesive change and either that once this phase of activity is over, the cells' adhesion returns

to normal, or in the high shear assays this initial adhesion is rapidly broken again and aggregation declines.

The majority of the results where an irreversible increase has been shown to occur have been obtained under less vigorous shear conditions. It is possible that following contact under these circumstances, some cell surface change (either external or internal e.g. cytoskeletal) occurs leading to the formation of a more permanent adhesion. In the high shear systems a sufficient degree of contact or spreading may not be possible thus preventing the appearance of this putative secondary adhesion. In this respect, it is interesting that high shear assays performed in the presence of cytochalasin B, which might be expected to render the cells more deformable, leads to the formation of irreversible aggregates (Craddock *et al.*, 1978).

Similar proposals have been advanced to account for certain aspects of platelet aggregation. In response to ADP (a potent aggregating stimulus) platelets change from a smooth discoid shape to one with spiky pseudopodia and small blebs (Born, 1970). The shape change appears to be obligatory for the subsequent aggregation (Boyle-Kay and Fudenberg, 1973). Normally there is a strong electrostatic repulsion between resting platelets which have a high negative surface charge due to the presence of sialic acid; it has been suggested that the long thin pseudopodia formed during the shape change allows this repulsion to be overcome and "zipper" together the platelets into loose aggregates (Willis, 1978). Willis (1978) further suggested that such events occur during the "first phase" of aggregation induced by ADP, explaining why disaggregation occurs so readily. Under appropriate conditions,

the aggregates become consolidated and fuse into large masses (see Willis, 1978) in the "second phase" of aggregation.

Chemotactic factors are known to induce the formation of pseudopodia in neutrophils (Craddock *et al.*, 1977) and their proposals to explain neutrophil aggregation are very similar to those outlined above for platelets. The finding by Craddock *et al.*, (1978) that aggregation occurred in the absence of pseudopod formation indicates that another mechanism of increased adhesiveness exists. It is perhaps via this second mechanism that the irreversible adhesive changes observed by other workers occur. This potential sequence of events is not an original proposal. As long ago as 1960, Bangham and Pethica (1960) suggested that the small radius of curvature of cellular processes would enable them to overcome the electrostatic barrier between cells. Curtis (1973) however noted that there is also evidence that cell extensions such as microvilli, reduce cell adhesion.

Adhesion and the mechanism of chemotaxis

It was concluded in an earlier section that the effects of chemotactically optimal concentrations of chemotactic factors, since initially adhesion was reduced, were not related to margination. The finding of an inverse relation between adhesion and locomotion (Chapter 7), however, suggests another possible role for adhesive changes in neutrophil behaviour.

Adhesion to the substratum is essential for cell locomotion to occur and it is possible that the degree of adhesion may affect the rate of locomotion. It is evident that at adhesive extremes, locomotion will not be possible due to either too much or too little adhesion. Evidence exists, however, for a more subtle effect on

locomotion. Carter (1965, 1967) produced adhesive gradients experimentally by vacuum evaporation of palladium onto cellulose acetate. He found that cells moved in the direction of increasing adhesiveness and that the rate increased with greater adhesion. Gail and Boone (1972) found that the motility of Balb/3T3 fibroblasts varied inversely with cell-substrate adhesion when the behaviour of cells on pyrex and cellulose acetate was compared. Harris (1973) showed that cells accumulated preferentially on more wettable substrata and suggested that substratum adhesiveness was the determining factor. Weiss and Glaves (1975) reported an inhibition of macrophage migration associated with a decreased cell detachment from the substratum. Keller, Hess and Cottier (1977) reported that increased neutrophil locomotion and decreased adhesion to the substratum occurred in parallel. On the other hand, however, Curtis and Büültjens (1973) found no relation between cell adhesion changes and cell locomotion rate over a fairly wide range of adhesion.

On the whole though, these results indicate that the degree of adhesion to the substratum can influence the locomotory behaviour of cells. An attractive hypothesis suggested by Carter (1967) is that adhesion changes induced in neutrophils by chemotactic factors modulate their locomotion. It is possible that the reduction in adhesion at chemotactically optimal concentrations brings the adhesive strength to an optimum for locomotion. Most chemotactic factors stimulate the rate of locomotion and it is plausible that a reduction in cell adhesiveness could account for this. Locomotion in high concentrations of chemotactic factor is often less than optimal (Showell *et al.*, 1976). Since these concentrations increase adhesion, the high adhesiveness of the

cells under these conditions may account for this reduced locomotion. In vivo such a mechanism may underlie the neutrophils' behaviour in tissues. In low concentrations, the cells being less adhesive, move rapidly, in high concentrations, the cells slow down and stop moving. It is presumably in areas of high concentration of chemotactic factor that it is desirable for cells to localize.

Adhesion, chemotaxis and locomotion have been linked by other workers. Smith et al., (1979) found that preincubation of neutrophils in the presence of chemotactic factor led to a decrease in locomotion. Such preincubation leads to "chemotactic deactivation", i.e. that after exposure to a factor and washing, the cells no longer respond to that factor. Since Smith et al., (1979) also found adhesiveness to be in increased under these conditions they suggested that increased adhesion accounted for the inhibition of chemotaxis. These workers also found that simple incubation of the cells in the presence of chemotactic factor increased neutrophil adhesiveness. If increased adhesion does reduce locomotion then this result seems paradoxical, since chemotactic factors by definition affect locomotion. Fehr and Dahinden (1978) found chemotaxis to be optimal towards 10^{-9}M FMLP while towards concentrations greater than 10^{-7}M, it fell below random motility. This decrease in locomotion was paralleled by an increase in cell adhesion. These authors concluded that chemotactic deactivation and inhibition of chemotaxis were due to high cell adhesiveness. These results and the interpretation placed on them by these workers correspond closely to the results and interpretations discussed in this thesis.

Locomotion and adhesion of neutrophils have also recently been studied under a variety of conditions (Keller, Barandun, Kistler and Ploem, 1979). These workers found that HSA, fibrinogen and acid treated gamma globulin stimulated neutrophil locomotion and reduced adhesion to glass. Using interference reflexion microscopy to study

the pattern of cell-substratum adhesions. They concluded that it was the type of adhesion, rather than the absolute level of cell adhesiveness that correlated best with the locomotory behaviour of the neutrophils.

Gallin and Wright (1978) found that agents which induced enzyme secretion from neutrophils reduced migration *in vitro* and increased cell adhesion, thus indirectly linking adhesive increase with inhibition of locomotion. These workers also found that cytochalasin B in low concentrations reduced adhesion to petri dishes. Such concentrations of cytochalasin B have been shown previously to enhance chemotaxis (Gallin and Rosenthal, 1974; Becker, Davis, Estensen and Quie, 1972).

The effects on adhesion of chemotactic factors may have more complex effects on neutrophil locomotion.

Carter (1965, 1967) and Harris (1973) have shown that moving cells tend to accumulate on more adhesive substrata, and such a mechanism has been suggested to explain the action of the chemotactic factors, casein (Dierich, Wilhelmi and Till, 1977). These workers suggested that neutrophils moved up a chemotactic gradient by adhesion to surface bound chemotactic factor. Wilkinson and Allan (1978) extended these observations to show that neutrophils could respond chemotactically to gradients of substratum bound chemotactic factor, in the absence of fluid phase factor. They further observed that chemotactic peptides did not bind appreciably to the surface of the micropore filters, arguing against a role in altering the adhesiveness of the substratum. However, if instead of a gradient of substratum adhesiveness, there existed a gradient of cellular adhesiveness, then the end result should be the same, i.e. that cells accumulate where they are most adhesive.

For FMLP, such a gradient exists at 30 minutes above concentrations of 10^{-10}M and more clearly so at 60 minutes (see adhesion dose-responses-Chapter 6). In such a gradient, one end of a neutrophil may be more adhesive than the other. Although the *in vitro* locomotion results indicate that locomotion occurs less rapidly in increasing concentrations of chemotactic factor, this interpretation of the results is perfectly feasible since adhesion *in vitro* is arbitarily determined by the substratum and *in vivo* may be much weaker so that more adhesive cells move more rapidly.

Implicit so far in this discussion of adhesion and the mechanism of chemotaxis has been the assumption that the adhesive change and the locomotory behaviour are causally linked. No such causal link, but only a correlation, has been demonstrated experimentally. The two phenomena could be independent or causally linked in the in the opposite direction, i.e. that the locomotory events cause the adhesive change. As mentioned earlier, chemotactic factors are known to induce the formation of ruffled membrane andpseudopodia (Craddock *et al.*, 1977) and this gross alteration of the cell surface may render the cell more or less adhesive.

Such shape chamges are most probably caused by contraction of the cytoskeleton. Actin and myosin have been demonstrated in neutrophils in association with the plasma membrane (Boxer, Hedley-Whyte and Stossel, 1974; Rikihisa and Mizumo, 1977; Senda, Tamura, Shibata *et al.*, 1975). Since chemotactic factors stimulate locomotion at least partly by an action on the contractile apparatus of the cells and since filaments may be involved in cell adhesion (Rees *et al.*, 1977), it is perhaps not surprising that chemotactic factors have effects on adhesion/

as well as locomotion. That filaments are involved in neutrophil adhesion has been recently demonstrated by Boyles and Bainton (1979) who showed that contact with an adhesive substratum rapidly triggered the formation of a subplasmalemmal three dimensional interlocking network of globular projections and radiating microfilaments.

In a sense this amounts to saying that the measurements of adhesion and chemotaxis are merely different assays of the same cellular response, which would of course very simply account for the close parallels in the dose-response and time relationships.

Mechanism of adhesive change induced by chemotactic factors

Consideration of possible mechanisms which might explain the adhesive changes induced by chemotactic factors is complicated by the fact that different effects have been reported by different workers and by the occurrence of other cellular processes (locomotion, secretion) induced by the same factors. Disentangling these phenomena is an extremely difficult task, but some clues have been suggested by several experimental results.

That shape changes may cause adhesive changes has already been suggested. This hypothesis is very attractive in the light of the recent results of Zigmond and Sullivan (1979). The rounding up and ruffling of neutrophils reported by these workers follow a strikingly similar time course to the transient aggregation response described by O'Flaherty & Ward (1979) and Craddock *et al.*, (1977) in their reports. Of course the cause and effect may be in the opposite direction.

The finding by Craddock *et al.*, (1978) that massive aggregation in the presence of cytochalasin B occurs without the formation of ruffles or pseudopodia indicates that other surface changes can lead to

increased adhesion. Gallin, Durocher and Kaplan (1975) reported that C5a induced a reduction in net surface negative charge. Thorne, Oliver and Lackie (1977) reported the appearance of increased amounts of cell surface protein associated with adhesive increase induced by exposure to bacterial toxins. Both of these processes may occur during the response of neutrophils to chemotactic factors.

Any mechanism to account for the adhesive changes must take account of the complex dose - and time - responses of the cells. Neutrophils possess specific receptors for chemotactic peptides (Aswanikumar, Corcoran, Schiffmann *et al.*, 1977; Williams, Snyderman, Pike and Lefkowitz, 1977). Simple binding of the factors to the receptors could not in itself produce such complex responses, if the response to binding is a direct adhesive change. The possible existence of a two site receptor (Schiffmann, Corcoran and Aswanikumar, 1978) could allow complex dose - and time - responses but I feel that it is more likely that a series of cellular changes occur which yield adhesive changes and that it is the interactions between these processes that produce the net adhesive changes observed.

Adhesion and movement in other experimental systems

The main finding of this work is that neutrophil chemotactic factors which stimulate locomotion also have a range of effects on neutrophil adhesion. However these two sets of phenomena are related and whatever their mechanisms, the phenomena occur.

The observations have many features in common with the behaviour of lymphocytes and other cells reported by Curtis (1978). The essence of what has been termed the "Interaction-Modulation Theory" to explain cell positioning (which must involve cell movement) is that a diffusible

substance is produced by one cell type that diminishes the adhesiveness of another cell type. In theory the existence of such substances can easily explain such phenomena as sorting out or chemotaxis of cells. Curtis and De Sousa (1973, 1975) described interaction modulation factors (IMFs) from murine lymphocytes. These factors specifically diminish the adhesion of lymphocytes of the opposite type. This T-IMF diminishes the adhesion of B-lymphocytes and *vice versa*. Curtis (1978) and Curtis, Davis and Wilkinson (1979) presented evidence that T-IMF could play an important role in lymphocyte circulation and positioning *in vivo*. Very interestingly, Curtis *et al.*, (1979) found that T-IMF had chemokinetic effects on B-lymphocytes. Thus in this system, blood cell adhesion and locomotion may be linked.

Since adhesive mechanisms have often been proposed to explain the phenomenon of sorting out of cells (see e.g. Steinberg, 1963; Moscona, 1962; Curtis, 1974) the report of Matsumaka and Durston (1979) that sorting out of cells of *Dictyostelium discoideum* occurs by a chemotactic mechanism suggests that in this system also, chemotaxis and adhesion are linked.

Concluding comments

This project was started with the expectation that adhesive changes would be stimulated in either neutrophils or endothelium and that characterization of these changes and study of their mechanisms would advance the understanding of the process of adhesion of neutrophils to endothelium in the early stanges of inflammation.

The only class of compounds found to consistently elicit marked adhesive changes in neutrophils were the chemotactic factors; their initial (and in some cases only) effect was to reduce adhesiveness, and

only later did they increase adhesiveness. This finding seems difficult to reconcile with neutrophil behaviour *in vivo* during margination.

No marked increases in endothelial cell adhesiveness were ever detected. It may be that *in vivo* such changes do not occur and that margination occurs by another mechanism.

The finding that neutrophil locomotion and adhesion were inversely related suggested a role in inflammation for the chemotactic factor induced adhesive changes. Thus it seems possible that adhesive changes do affect neutrophil behaviour *in vivo* by affecting their movement in the tissues rather than by affecting margination.

Further discussion of results in this thesis appears in the attached article " Interactions of leukocytes and endothelium ".

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