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EFFECTS OF CHEMOTACTIC FACTORS AND Ca^{++} ON
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ADHESIVENESS OF HUMAN NEUTROPHILS: EFFECTS OF CHEMOTACTIC FACTORS AND Ca^{++} ON REDISTRIBUTION OF ADHERENCE SITES

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ABSTRACT

ADHESIVENESS OF HUMAN NEUTROPHILS:
EFFECTS OF CHEMOTACTIC FACTORS AND Ca^{++} ON
REDISTRIBUTION OF ADHERENCE SITES

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Human peripheral blood neutrophils obtained from healthy adults were examined in vitro. The effects of sequential stepwise increases in the concentration of chemotactic dipeptide N-formyl-L-methionyl-L-phenylalanine (f-Met-Phe) and the fifth component of complement (C5a) on neutrophil attachment to serum-coated glass, and the distribution of binding sites for albumin-coated latex beads (ACLB) on the cell surface were assessed in a Ca^{++}-free medium and complete medium.

The initial exposure to f-Met-Phe or C5a resulted in increased adhesiveness and binding sites for ACLB in a random pattern over the cell membrane. The second exposure to either of these chemotactic factors resulted in decreased adherence and movement of binding sites for ACLB to the uropod. The effects of the second stimulus were significantly inhibited in Ca^{++}-free medium.

The results support the concept that chemotactic factors modulate the distribution of adhesion sites on the neutrophil surface.

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INTRODUCTION

Neutrophils are phagocytic wandering cells that play a central role in the mediation of inflammatory tissue injury, including injury associated with bacterial infection, antibody complex formation or trauma. The neutrophils, in performing their function of defending against microbial infection in the tissue, adhere to the wall of the finer blood vessels, migrate through the blood vessels and move through the tissue in response to a chemotactic signal emanating from the site of inflammation. Once at the site, they engulf the offending microorganism into a phagosome, and the lysosomal granules fuse with the phagosome, secreting their contents into contact with the microorganism. This lysosomal action together with products such as O^{2-}, H_2O_2, and OH^- resulting from the respiratory burst kill the engulfed microorganism.

The mechanisms of neutrophil locomotion have been studied extensively in the past. An event which accompanies neutrophil locomotion is attachment to a substratum. Surface adhesiveness has been shown to influence adherence of neutrophils as well as locomotion (9, 60). Albumin decrease neutrophil adherence to glass (60). The presence of albumin also is important in obtaining an effective chemotactic response in the Boyden assay (60). Chemotactic factors are agents that influence the rate and direction of migration. Chemotactic factors have a proven effect on neutrophil locomotion

in vitro and have been shown to increase neutrophil adherence to protein coated glass and plastic (48, 60). The cell seems to detect a concentration difference of chemotactic factor across its diameter and migrates toward the source of the gradient (71, 74). Exposure of human neutrophils to a high concentration of certain chemotactic factors result in chemotactic deactivation (60, 64). Chemotactic factors cause a significant increase in neutrophil attachment to serum-coated glass. This enhanced adhesiveness is not reversible upon removal of the chemotactic factor. Cells treated under these conditions also exhibit significantly reduced translocation, but have not lost the ability to sense a chemotactic stimulus (50). Human neutrophil adhesiveness is a Mg^{++}-dependent process (9, 34) while the proposed mechanical work elements (microfilament and microtubules) are Ca^{++}-dependent (3, 22, 30). Chemotactic factors interact with specific receptor on the neutrophil surface inducing a graded displacement of intracellular bound Ca^{++} from membranous stores into the neutrophil cytoplasm. In addition they increase the membrane permeability to Ca^{++} (4, 5). Calcium may play a role in the control of microtubule assembly/disassembly processes (43).

This paper describes an investigation of the effects of exposure of neutrophils to stepwise increases in the concentration of chemotactic factors (i.e., f-Met-Phe and C5a). Adherence of neutrophils to serum-coated glass was assessed upon single and double exposure to f-Met-Phe and C5a. The appearance of adhesion-sites for albumin-coated latex beads on the neutrophil surface and the pattern of movement of latex beads was assessed upon restimulation with higher

concentrations of f-Met-Phe or C5a. Cells were fixed in glutaraldehyde and observed for morphological patterns. The experiment was also performed utilizing Ca^{++}-free medium.

The data here indicate that interaction of chemotactic factor (i.e., f-Met-Phe or C5a) with neutrophil surface receptors results in an increase in adhesion sites and induction of "internal signal(s)" which cause the adhesion sites to move to the uropod upon restimulation with a higher concentration of chemotactic factor. The phenomenon appears nonspecific in terms of chemotactic factors (at least for f-Met-Phe and C5a), and is Ca^{++}-dependent.

LITERATURE REVIEW

Adherence, a cell property by which non-adhesive circulating neutrophils stick to vessel walls and emigrate into the inflamed tissues, has been known for over a century (14, 18, 42). Adherence is of obvious interest since it may affect granulocyte margination, diapedesis, chemotaxis, opsonization and phagocytosis of microorganisms. Neutrophil adherence, an important but poorly understood phenomenon, has received much attention because of its possible involvement in the inflammatory response.

Neutrophil adherence and motility appear essential in the development of acute inflammatory reactions. Abnormal adherence has been reported in patients with recurrent infections, diabetes mellitus and after injection of corticosteroids. It also has been reported in various isolated cases. Numerous reports have described disease conditions associated with impaired directed migration of neutrophils. These include lazy leukocyte syndrome, Chediak-Higashi syndrome, rheumatoid arthritis, neoplasms, Job's syndrome, diabetes mellitus, viral infections and various acute bacterial and yeast infections (52).

TECHNIQUES USED TO ASSESS NEUTROPHIL ADHERENCE

Historical Development

Although it has been known for nearly 100 years that neutrophils have a role in the inflammatory process, it is only during the past two decades that the development of techniques and reagents has greatly advanced our understanding of the biology and biochemistry of neutrophil function. It was described by Ebert and Grant (18) that in 1824 Dutrochet studied the vasculature during inflammation. He observed that, in the small blood vessels of the tail of the frog tadpole, leukocytes adhered to the walls of the finer blood vessels and emigrated through the blood vessels to the inflamed tissue. Accurate studies on the margination and emigration of leukocytes in inflammation were done between 1833 and 1850 by several investigators. Ebert and Grant (18) reported that the amoeboid locomotion of leukocytes was studied by Metchinkoff and coworkers at the end of the 19th century. Metchinkoff et al. found that leukocytes were attracted to living or dead bacteria that had been injected into the peritoneal cavity. Later, Metchinkoff noted that leukocytes had the ability to "sense" the chemical signals which are produced during inflammation. The name of Metchinkoff is associated with leukocyte phagocytosis and chemotaxis and his descriptions still form the basis of most chemotactic research.

In 1908, it was demonstrated that leukocytes would adhere to glass, but little quantitative study was done on this until 1961 when Garvin used a glass bead column method to study white cell adhesiveness (26). In 1969 and 1977 Kvarstein and Lorente et al. respectively also studied leukocyte adhesiveness by using a different

type of bead column (33, 38). The column consisted of a siliconized glass tube with a thin pad of unsiliconized glass wool in the bottom, and unsiliconized glass beads on top. The blood, collected in a siliconized glass tube, was delivered into the bead column. The rate of flow through the column during the adhesiveness assay was mechanically regulated. One milliliter of blood was collected in a siliconized glass tube from the outlet tube. Total counts were made before and after blood passage through the column. The difference between the two counts was defined as leukocyte adhesiveness and calculated as percent adhesiveness.

Of several glass bead column methods which have been used to measure neutrophil adhesiveness, each has certain disadvantages and sources of error. Those include a need for constant-temperature water jackets, infusion pumps, preparation of the beads, control of shearing forces and the unpredictability of the number of sequestered non-adherent cells remaining in the columns.

Nylon fiber columns have been used by MacGregor et al., and Schiffer et al. (40, 59). Scrubbed nylon fibers were weighed and packed into Pasteur pipettes. They reported that the column length and tip opening are critical measurements. When columns are packed more tightly or when the tip apertures are narrower, increased adherence occurs. The percentage of granulocyte adhesiveness is calculated by using the total count obtained in the original and in the effluent blood .

In 1973, Banks and Mitchell measured the adherence and aggregation of neutrophils on the walls of modified Payling-Wright rotator flasks (1).

The rotating flask method was first developed by Payling Wright in 1941 for studying platelet adhesiveness. A volume of heparinized whole blood or cell suspension is added to a pyrex conical flask held horizontally on a wheel in an incubator at 37 C. The total leukocyte number is estimated before and at various times after rotation. These counts are then expressed as the percentage of the initial count and plotted against time. Aggregation methods have recently begun to be used by several groups, though the methods of bringing about collisions between the cells vary (35, 48).

In 1977, Lackie and smith used static coverslips in wells for studying neutrophil adhesiveness (36). Adhesion experiments were carried out in Linbro tissue culture plates with confluent endothelium, confluent fibroblasts or glass as the collecting substrate. A suspension of neutrophils was added to each well. The neutrophils were allowed to settle and adhere at 37C for 30 minutes. The coverslips were then removed, washed by dipping through an air-medium interface, fixed and stained. The mean number of neutrophils per unit area was calculated for each coverslip. In this technique neutrophils can be distinguished easily from endothelial cells or fibroblasts. The similarity of neutrophil adherence to nylon fiber and to endothelial monolayers in vitro was reported by MacGregor et al.(41). They suggested that results with the nylon fiber assay could reflect in vivo neutrophil-endothelium interaction. Furthermore, the endothelial monolayer offers an opportunity for studying this cell-cell relationship in vitro. However, there is some variability in the data from different groups. Some indicate that an endothelial

substrate is more adhesive than serum-coated glass or plastic, while others suggest that endothelium, although a good substrate is not quite as good as serum-coated glass. Some believe that cultured endothelial cells are "inflamed" by the conditions of their isolation and that such experiments reflect stimulated adhesion (36).

Most of the in vitro techniques allow for neutrophil attachment to a surface. the number of cells that detach as external forces are applied reflects neutrophil adhesiveness. The forces used to detach the cells include centrifugation, elution off a column, washing, shaking and rotation. The external forces promote cell shearing, destruction and aggregation thereby confusing evaluations of adhesiveness. Comparison of the results with different assay systems is often difficult. In 1979, Smith et al. described a technique to measure neutrophil adhesiveness in slide chambers without using external forces (60). The chambers are filled with a suspension of neutrophils, and cells settling onto the glass surface are observed using an inverted phase contrast microscope. The chamber is then inverted and the unattached cells allowed to fall off the surface. After a period of time, cells remaining attached to the glass surface are again counted.

Neutrophil adhesiveness in vivo is often correlated with the total numbers of circulating neutrophils. Factors decreasing the numbers of circulating neutrophils increase the numbers of neutrophils in the marginal pool. This shift is caused by an increased in neutrophil adhesiveness.

Despite the diversity of the assay systems employed, the results obtained for neutrophil function in adherence are generally in agreement. However, the importance of these results derived from *in vitro* techniques may not apply to neutrophil adhesiveness *in vivo* since the substrates used, either glass or endothelial monolayers, are foreign to the neutrophil and the testing is done under artificial conditions.

THE MECHANISMS OF ADHERENCE

Despite intensive investigations, the detailed mechanisms of cell adhesion remain obscure. Local damage, of whatever kind, leads to an inflammatory response. Damage leads to changes in blood flow in the adjacent microvasculature which are followed by the adhesion of leukocytes to the vessel walls (margination). It is hypothesized that margination is a function of stickiness. Margination may continue untill the walls of the vessels are completely lined with leukocytes and is shortly followed by the emigration of leukocytes from the vessel wall towards the site of damage or infection (13, 18, 19, 66). In 1974, Eberts' associates damaged the vascular endothelium with laser beams and produced margination, suggesting that the damaged endothelial cells become "sticky" (18). Several groups have reported that chemotactic factors increased neutrophil adhesiveness (7, 21, 46, 47, 48, 60). Smith et al. have shown that the initial exposure of neutrophils to a high concentration of chemotactic factors irreversibly increased cellular adherence (60). Albumin has been found to decrease neutrophil adherence to

glass (32, 60). Several investigators have demonstrated that a deficiency of Mg^{++} in the culture medium significantly reduced neutrophil adhesiveness to protein-coated glass (34, 60). It has been shown that the anti-inflammatory agents, cyclic AMP and prostaglandins decrease neutrophil adherence (35, 40). The surface charge of neutrophils might affect adherence. Gallin et al., have reported that chemotactic factors cause a small yet significant decrease in neutrophil negative surface charge which might be related to increased neutrophil adhesiveness and aggregation (21,22). Smith and Hollers demonstrated that the initial exposure to chemotactic factor(s) resulted in increased adhesiveness and binding sites for albumin-coated surfaces in a random pattern over the cell membrane. Yet, the second exposure to chemotactic factor(s) resulted in decreased adherence and movement of binding sites to the tail or uropod of the cell. These binding sites gradually disappear from the uropod (or become nonfunctional) but appear again at the front of the cell or lamellipodia when exposed to a third chemotactic stimulus (61).

It appears that the substrate or cellular adhesiveness may be affected by various factors, but the mechanisms by which these factors interact with neutrophils and influence adherence remain vague. In addition, the importance of results derived from <u>in vitro</u> experiments may not apply to neutrophil adherence <u>in vivo</u>.

CHEMOTACTIC FACTORS

The development of the Boyden technique helped investigators to find many different agents that exert chemotactic activity. with few exceptions, the agents producing chemotactic activity have not been isolated in pure form or identified chemically. Chemotactic factors have been shown to stimulate the motility and adhesiveness of responsive cell in vitro. However, the majority of substances assessed for chemotactic activity have been implicated in inflammatory responses, and their importance in vivo is vague. Certain agents appear to influence neutrophil responsiveness directly. Other agents induce chemotactic activity in serum, plasma or other biological fluids by activating enzymes in the complement, coagulation, fibrinolytic and kinin generating systems (18). Many of these chemotactic factors are of host origin and commonly exist in a precursor form that requires enzymatic activation. Complement components are the most important biological substrates for the formation of chemotactic factors. A small molecular weight, heat stable fragment of the fifth component of complement (C5a) released during the activation of complement by either the classical or alternate pathway was partially purified by several investigators (23, 24, 49). This fragment was designated as C5a. It has been reported that other complement components are also chemotactic for neutrophils (i.e., C3a and activated $\overline{C567}$ complex) although the evidence shown has not been universally accepted (2, 44, 70). Fernandez et al. have reported that purified human C3a appears not to be a chemotactic

stimulus for neutrophils. They have suggested that previous observations of C3a activity may have resulted from minor contamination of the C3a with C5a, or a C3 fragment other than C3a may be responsible for the activity (20). The native molecule (C3a, C5a) possesses classical anaphylatoxic properties, i.e., it causes smooth muscle contraction, histamine release from mast cells and enhanced vascular permeability,effects that have been termed spasmogenic (11). Once formed in human serum, the potent C5a is rapidly converted to a des Arg 74 derivative (C5a-des-Arg) by a carboxypeptidase B-like enzyme (11). Human C5a-des-Arg is a form of C5a that is inactive as an anaphylatoxin. Therefore, it is the C5a-des-Arg that is believed to represent the major physiological end product in man. Fernandez et al., have found that pure C5a acting alone exhibited chemotactic activity over a concentration range of 0.04 to 1.7 X 10^{-8} M. In contrast, homogeneous C5a-des-Arg, when employed alone in the Boyden assay, was found to be devoid of chemotactic activity. However, C5a-des-Arg added to small amounts of nonactivated human serum largely regained its chemotactic activity, suggesting that a specific serum "helper factor" was required for expression of C5a-des-Arg chemotactic activity in <u>that particular assay system</u> (20). Subsequently, Perez et al., purified from normal human serum a heat stable, anionic polypeptide of approximately 22,000 molecular weight which they termed "helper factor" (51). They have suggested that complexes of C5a-des-Arg and helper factor account for the bulk of chemotactic activity generated in whole serum by activation of the complement system. Conversely, Chenoweth

et a., have reported that both C5a and C5a des Arg showed activity in the absence of serum proteins when they measured cellular migration on a solid support under agarose (12).

Numerous investigators have reported that complement activators such as aggregated IgG, antigen-antibody complexes, bacterial endotoxin, microbial cell walls, damaged tissue and cellular enzymes can generate complement-related chemotactic factors.

Various cells and tissues contain chemoattractants or substances capable of generating this activity. Neutrophil homogenates have a limited chemotactic activity that, under certain conditions, may be released extracellularly (68, 70). In addition, the granules of neutrophils contain substances which are capable of splitting a chemotactic factor from C5 directly or by activating complement (68, 69). The transfer factor extracted from lymphocytes is chemotactic for neutrophils (25). It has also been found that the supernatant fractions of virally infected tissue culture cells have chemotactic activity. Chemotactic activity has been found in a variety of denatured or degraded products of proteins such as albumin, hemoglobin, immunoglobin and collagen (65, 67). Therefore, it is obvious that many types of tissue injury may lead to the generation of chemotactic activity.

Numerous investigators have found products of bacterial growth to be chemotactic. This activity appears to be related to two groups of compounds: N-formyl-methionyl-oligopeptides and oxidized lipids (56, 57, 58). The oxidized lipids have not been structurally identified. Trace amounts of these substances have shown chemotactic

activity. It has been sggested that lipid substances might also be generated from human cells. Prokaryocytic cells such as bacteria initiate their protein synthesis with an N-terminal formyl-methionyl group. Eukaryocytic cells do not synthesize these formylated peptides. Schiffmann and Wahl examined the possibility that proteins and peptides with an N-terminal formyl-methionyl group might be chemotactic agents and attract phagocytic cells. This reasoning led them to the discovery of a number of N-formyl-methionyl peptides which are in fact chemotactically active, some at concentrations as low as 10^{-11}M. (57). The formylated peptides and C5a are the only relatively purified chemotactic factors available for studying chemotaxis. The formylated peptides can be synthesized in large quantities in pure form in the laboratory. The diversity of chemotactic factors is immense, and the individual attractants vary widely in physiochemical properties. Amino acids, peptides, proteins and lipids can possess chemotactic activity. Some chemotactic factors have a net anionic charge, e.g., the lipid and chemotactic oligopeptides; others have a net cationic charge e.g., the fragments of C5 and C3. However, many of these factors do possess one common feature, an exposed hydrophobic residue. This property may be an important clue to the nature of neutrophil-chemotactic factor binding.

Chemotactic activity has been evaluated primarily by use of the Boyden technique. The presence of a low concentration of serum or albumin is required in the cell medium in order to observe enhanced chemotactic migration. Some substances may have chemokinetic activity that might be misinterpreted as chemotactic activity. Chemotactic

factors are agents that influence both the rate and direction of cell migration; chemokinetic factors are those that influence only the rate of migration. In 1973, Zigmond and Hirsch differentiated chemotactic activity from chemokinetic activity using the leading front technique with varying concentrations of test substances below and above the filter (70). It was observed that in the absence of a test agent, neutrophils migrated randomly into the filter. The distance of migration were similar to results of random diffusion of molecules from a front. Chemokinetic factors stimulated cell motility and the distances of migration were greater than predicted by the diffusion theory. This occurred equally when the test agents were below or above the filter. Chemotactic factors also stimulated cell motility when above or below the filter. However, the distances of migration were significantly greater when the concentration of the agent was higher below the filter. Apparently only chemotactic factors are chemoattractants that orient migration of the cells towards increasing concentration gradients. Although high concentrations of chemotactic factors inhibited cell migration, this did not occur with chemokinetic factors. In general, all chemotactic factors were chemokinetic and enhanced the rate of cell migration. However, not all chemokinetic factors were chemotactic, since orientation and direction of migration were not affected.

The mechanism by which chemotactic or chemokinetic substances interact with the neutrophil is not understood. Many groups have attempted to demonstrate the presence of chemotactic receptors on the neutrophil membrane. In 1978, Zigmond (71) noted that neutrophils sense

chemotactic factors across their diameter, thus enabling the cell to orient to a gradient of chemotactic factors. Ward and Becker found that when neutrophils were exposed to serine esterase inhibitors, they did not respond to chemotactic factors (64). They suggested that serine esterase receptors on the neutrophil membrane may be receptors for chemotactic factors. Thus, serine esterase was referred as the "activatable esterase". Apparently, saturation of activatable esterase receptors with high concentrations of chemotactic factors may have "exhausted" the cell and resulted in a state of "deactivation". Deactivated neutrophils showed apparently decreased migration when compared to untreated cells and were unresponsive to other chemotactic factors. In 1979, Smith et al. studied the response of deactivated neutrophils to different chemotactic factors, e.g., C5a; f-Met-Phe; Zymosan activated serum (ZAS); and bacterial chemotactic factor (BCF) (60). Their observations were similar to those of Ward and Becker (64). Pretreatment of cells with BCF did not reduce the cell migration in the Boyden assay. Deactivated neutrophils were also able to change shape in response to a second chemotactic stimulus. However, although neutrophils pretreated with f-Met-Phe were specifically unresponsive to restimulation with f-Met-Phe, they were quite responsive to the other chemotactic factors. Under these conditions neutrophils showed a sustained enhancement of adherence that was not observed in cells pretreated with BCF. They concluded that deactivated cells retain the ability to sense chemotactic factors, though they show reduced migration. This reduced migration may be due both to saturation of receptor sites and nonspecific changes in the

mechanisms of cellular locomotion (60). Neutrophils may have multiple receptors for various chemotactic factors. Although it has been suggested that the neutrophil receptors for formyl-methionyl peptides are distinct from those for the C5a Goetzl and Mehta provided an alternate hypothesis (27). They suggested that formyl-methionyl peptides may interact with the same surface receptors on neutrophils as C5a, but that the formyl-methionyl peptides interact with one or more amino groups in the receptor (27). In 1979, Spilberg and Mehta have demonstrated the presence of neutrophil receptors for a cell-derived chemotactic factor (63). These binding sites are saturable, time-dependent, present both on intact cell surfaces and on disrupted cells and are not displaced by either formyl-methionyl peptides or complement-activated plasma. Therefore, multiple receptors may exist on the neutrophil membranes for different chemotactic substances.

Chemotactic factors have a profound and extensive influence on the neutrophils. These include altering cell morphology, enhancing Na^{+}/K^{+} ATPase activity and Ca^{++} fluxes, assembly of microtubules and microfilaments, stimulating cellular adhesiveness and locomotion. The relation of these activities to cellular adhesiveness and locomotion remains unexplained, and is actively being investigated.

TECHNIQUES FOR ASSESSING NEUTROPHIL CHEMOTAXIS

Chemotaxis, the unidirectional migration of cells in response to a gradient of specific chemical substances, has fascinated biologists for many years (71). Ebert and Grant (18) described that in 1884,

Peffer coined the name "chemotaxis"to describe the attraction of fern sparmatozoid by malic acid. Thease authors also noteted that the first *in vivo* chemotaxis experiments involving leukocytes were performed in 1888. A noxious agent was injected into the cornea of rabbit's eye and the accumulation of neutrophils in the surrounding cappillaries was observed.

Since most leukocyte chemotactic factors increase the rate of cell locomotion at moderate concentrations and inhibit the rate at high concentrations, careful consideration of assay systems is essential. Two basic types of systems exist to detect and measure chemotaxis. In the first, one measures the migration and distribution of a sample of a cell population. In the second, one measures the locomotion of individual cells (67, 70).

The most popular method for measuring the migration and distribution of a population of cells uses a micropore filter. It was first described by Boyden and has since been used to identify a number of chemotactic factors (8). In 1962, Boyden used a chamber that consisted of an upper and lower half separated by a porous filter (with 0.65 to 5 μm pores). Like the endothelial wall of the vessel, the diameter of the openings in the filter are smaller than the cell diameter. A stimulus is placed below the filter and, as the stimulus diffuses into the filter, a concentration gradient of the agent is established. It should be emphasized that the presence of a gradient is a prerequisite for chemotaxis. Neutrophils are placed above the filter and migrate into it in response to stimulus. One can then evaluate the redistribution of the cell population by measuring a) the distance that cells have migrated into the filter

or b) the number of cells which have migrated a set distance into or completely across the filter after a period of time (28, 31). If either measure shows an increase when a test substance replaces buffer beneath the filter, the substance has often been considered to be chemotactic. However, this assay is not definitive for chemotaxis since it evaluates cell migration that could be due either to specific stimulation (chemotaxis), to nonspecific stimulation (chemokinesis) or both. This assay can be adapted to be a true test of chemotaxis if the chemokinetic effects of a test agent are first determined and then corrected for when evaluating the chemotactic response (70).

Observation and evaluation of the locomotion of individual cells yields detailed information on a variety of parameters of locomotion including the variation among the cells of the population or in a given cell at different times (72). One can analyze such aspects as the rate of locomotion, the frequency or magnitude of turns and the orientation of movement relative to the gradient. Interpretation and evaluation of results with this technique are straightforward. Much of what we know about the behavior of cells that exhibit chemotaxis has come from such studies.

Another common technique for studying chemotaxis of neutrophils uses agarose. This method is based upon migration of cells under agarose gel in response to chemotactic factors diffusing through the gel. This technique allows measurements to be made of both population and individual cell behaviors (45).

Few significant *in vivo* techniques have been developed. The skin window technique which was developed by Rebuck and Crowley in 1955 is the most common one (53). A circular abrasion is made on the human forearm and test agents are added to the abrasion. The site is then covered with a sterile glass coverslip and the accumulation of leukocytes in response to the trauma or test agent is observed. The skin window is still a popular technique which is used by a number of groups in clinical studies.

The establishment of a chemotactic gradient has never been demonstrated and no technique has shown that neutrophils recognize, orient or move directionally toward chemotactic agents *in vivo*. Cell accumulation may be in direct response to test agents yet whether these agents slectively attract the neutrophil remains to be determined. Most of the information about chemotaxis has come from *in vitro* techniques.

Conclusions derived from *in vitro* techniques may not apply to neutrophil movement in vivo since the substrates used, either glass or micropore filters, are foreign to the neutrophil. Studies are currently under way assessing neutrophil responsiveness on endothelial cells

RELATIONSHIP OF NEUTROPHIL ADHERENCE TO MOTILITY

Leukocyte margination near inflammatory sites was first studied in 1824 (18). During an acute inflammatory response, neutrophils adhere to the endothelial cells which line the vessel walls and migrate between the endothelial cells towards the site

of damage or infection. Numerous investigators have focused on studies of neutrophil adhesiveness since adhesion is an essential prerequisite for cell movement. Other groups have noted the importance of the substratum on which these cells move. In 1961 several investigators showed that amoeboid movement is characterized by contact between the cell and the surface on which it will crawl. The propulsive action of the cell is maintained by the reaction between the posterior end of the cell and the surface (16). It has been reported that filopodia at the front of the nerve cell adhere to other objects and, by contracting, are able to draw the cell body towards such objects (16). In 1972, Ramsey reviewed cell movement on glass surfaces (54). As observed in Boyden assays, the presence of serum or albumin was required for neutrophil movement, since the cells tended to flatten on the plane surface of glass. In the presence of serum or albumin, neutrophils attach to the surface, sending out pseudopodia or lamellipodia which also attach to the glass. The cell elongates as the lamellipodium spreads forward and the cytoplasmic contents stream forward into the front part of the cell. The regions of adherence to the substrate are under the lamellipodia, cell body, tail and tips of retraction fibers. In 1980, Smith and Hollers observed that detachment occurred first at the front part of cells, i.e., the region of the lamellipodia with the result that cells hung by their uropods, and eventually dropped off the glass surface (61). In 1977, Dierich et al., studied the role of surface-bound chemoattractants in leukocyte migration. They concluded that leukocytes migrating <u>in vivo</u> would be crawling along

tissue surfaces coated with chemoattractants. This movement would only be possible if unoccupied receptors could be made available continuously at the leading edge of the cell or on the protrusions actively reaching out from the cell body searching for sites of attachment. Therefore, the cell must either be able to synthesise new receptor sites or to re-use the already existing ones (17). Smith and Hollers hypothesized that as neutrophils sense an increasing concentration of chemotactic factors, the binding sites move to the tail and new binding sites appear at the front of the cell (61). Harris reported that the surface proteins in moving cells continuously circulate within the membrane from the front of the cell to its trailing end, then become internalized. After a hypothetical passage through the cytoplasm, these proteins become incorporated again into the front section of the cell (29). It is possible that proteolytic activity might be involved in freeing the receptors from the bound substances (17). Marchesi and Florey observed that neutrophils moving through the vasculature also formed pseudopods between endothelial cells and that the cell contents streamed from the tail to the pseudopod (42). It appears that adhesion provides the frictional forces required for locomotion and is crucial to cell movement.

MATERIALS AND METHODS

REAGENTS

All reagents were reagent grade and adjusted to pH 7.4. Ficoll, glutaraldehyde, latex beads, and bovine serum albumin (BSA) fraction V powder were obtained from Sigma Chemical Company, St. Louis, Missouri. Hypaque was purchased from Winthrop Laboratories, New York. N-formyl-L-methionyl-L-phenylalanine (f-Met-Phe) was obtained from Andeulis Research Corporation, Bethesda, Maryland, and zymosan was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Hanks balanced salt solution (HBSS) was obtained from Gibco, Grand Island, New York. The C5a was prepared by myself in the Department of Anatomy, Michigan State University, East Lansing, Michigan by the method of Gallin and Rosenthal (23). The 3-μm-pore size micropore filters were purchased from Schleicher and Schuell, Keene, New Hampshire. The blind-well chambers were purchased from Neuro Probe, Incorporated, Bethesda, Maryland.

PREPARATION OF CHEMOTACTIC SOLUTIONS

N-formyl-L-methionyl-L-phenylalanine (f-Met-Phe) was dissolved in HBSS to a concentration of 1 mM and diluted further with HBSS to the desired concetrations.

The chemotactic fragment of the fifth component of complement (C5a) was partially purified by a minor modification of the method

described by Gallin and Rosenthal (23). Blood from normal adult volunteers was allowed to clot in glass tubes at room temperature and the serum was collected by centrifugation. A 10 ml portion of serum was incubated with 10 mg of zymosa at 37 C for 1 hour and residual complement was then inactivated by heating at 56 C for 30 minutes. After rapid cooling, zymosan particles were removed by centrifugation at approximately 600 G for 10 minutes and the activated serum was then layered on a Sephadex G-75 column (100 X 52 Cm). The column was equilibrated with Ca^{++} and Mg^{++}-free HBSS at pH 7.4, and the serum fractions were eluted with Ca^{++} and Mg^{++}-free HBSS by descending flow at 4 C. The column was calibrated with the following substances: Blue dextran (2 X 10^6 mol. wt.), ribonuclease A (13,700 mol. wt.), chymotrypsinogen (25,000 mol. wt.) and ovalbumin (45,000 mol. wt.). Fractions in the 10,000 to 20,000 mol. wt. range were tested in modified Boyden chambers by using a modified Lichtman method for chemotactic activity. Those fractions showing chemotactic activity were pooled and frozen at -70 C. The pool containing chemotactic activity had 35 µg of protein/ml. A modified Folin method was used for protein determination (39). This protein will be referred to as C5a.

Human serum albumin and BSA were dissolved in HBSS and all solutions were adjusted to pH 7.4 with sodium hydroxide.

ISOLATION OF HUMAN NEUTROPHILS

Blood samples were obtained from healthy adult volunteers. Blood was collected in plastic syringes and heparin (10 Units/ml)

was added. A 1 ml portion of 6% dextran was mixed with 10 ml of sample to enhance red blood cell sedimentation. After approximately 1 hour at 18 to 20 C the leukocyte-rich plasma was removed into a 15 X 100 mm siliconized tube. The leukocyte-rich plasma was diluted with an equal volume of HBSS. After centrifugation at 200 G for 10 minutes at 10 C, plasma and HBSS were removed by suction. The cell button was resuspended in 4 ml of HBSS and centrifuged at 800 G at 10 C for 30 minutes on a Ficoll-Hypaque cushion consisting of 10 parts of 33.9% Hypaque and 24 parts of 9% Ficoll. This solution provided a density gradient to separate granulocytes from platelets and other leukocytes in the cell suspension. The Ficoll-Hypaque solution and HBSS were removed by suction and the cell button was resuspended in HBSS. The granulocytes were counted by an electronic cell counter (Coulter Counter, model B). This cell suspension contained greater than 90% granulocytes, of which approximately 95% were neutrophils. No platelets were seen in the preparations. The erythrocyte (RBC)-to-neutrophil ratio was consistently less than 2:1. The neutrophil viability was greater than 98% as determined by eosin exclusion. The neutrophils were resuspended at 10^7/ml in HBSS and immediately refrigerated at 4 C.

ASSESSMENT OF NEUTROPHIL MOTILITY

Neutrophil motility into micropore filters was tested by a modified Boyden technique using a blind well chamber (Figure 1). The chambers were prepared by placing cells (2 X 10^4 neutrophils/mm^2 of exposed filter surface) in the upper compartment and chemotactic

factors or control solution in the lower compartment. The concentration differences of reagents in the two compartments established a concentration gradient through the filter. The chambers were then incubated at 37 C in an atmosphere of 5% CO^2 and high humidity for 60 minutes. The experiment was terminated after the incubation by removing the filter, fixing the cells present in the filter with absolute methanol and staining with hematoxylin. Hematoxylin stained the cell nuclei but not the filter. The filters were then made transparent by soaking them inxylene and mounting on a glass slide. The depth of migration was measured microscopically using an image analyzer (Optimax). Each experiment was duplicated and the counts averaged. The migrational behavior of neutrophils was evaluated by 2 methods as previously described by Zigmond and Hirsch (70, 74). 1) Briefly, the distance of neutrophil penetration was evaluated by measuring the distance (μm) from the cell origin (top of the filter) to the depth at which only two cells remained in focus ("leading front"). The leading front determined by averaging 4 measurements for each of duplicate filters in each experiment 2) The distribution of cells in the filter was determined by counting the number of cells/20X microscopic field at 10 μm intervals through the filter. At least 4 determinations were made for each of duplicate filters and results were averaged for each experiment.

The migrational behavior of neutrophils was evaluated with varying concentration of chemotactic factors or control solutions placed below the filter.

Figure 1: Vertical cross section of a blind well chamber.

Figure 2: Vertical cross section of a slide chamber.

SCHEMATIC CROSS SECTION OF A BLIND WELL CHAMBER

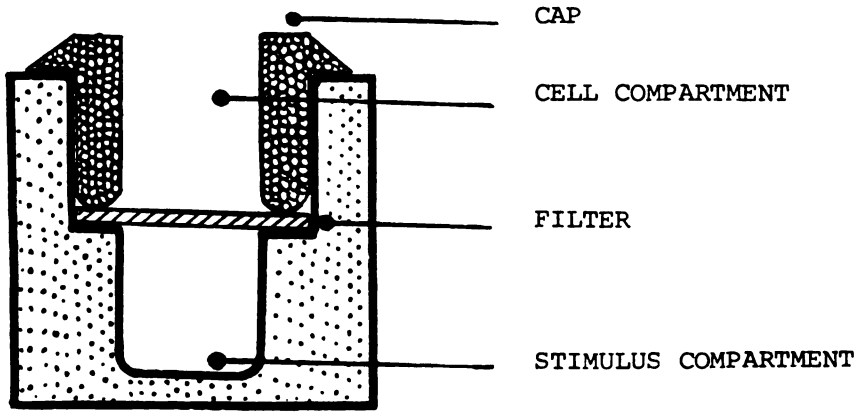


Figure 1.

SCHEMATIC CROSS SECTION OF A SLIDE CHAMBER

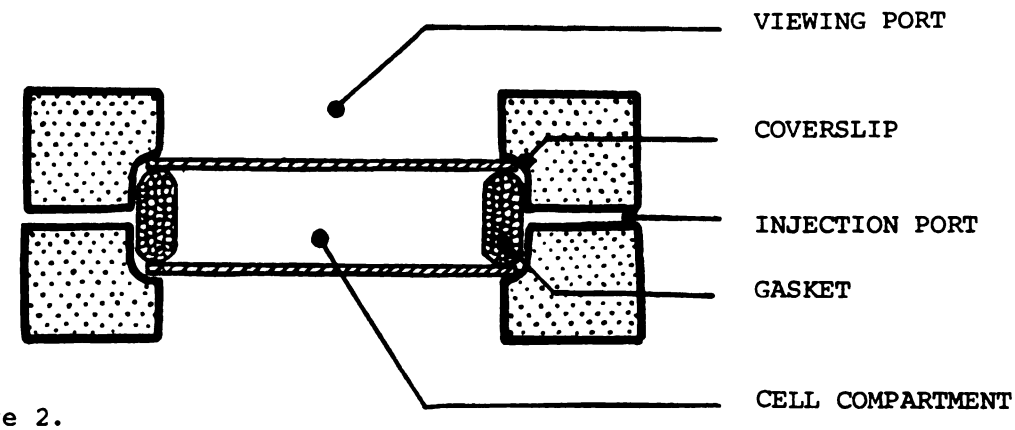


Figure 2.

ASSESSMENT OF NEUTROPHIL ADHESIVENESS

The interaction of neutrophils with glass surface was evaluated by using slide chambers (Figure 2). The chambers were filled with suspensions of neutrophils (1 X 10^6/ml) in various reagents. The chambers were immediately placed on the stage of an inverted phase-contrast microscope and cells settling onto the glass surface were observed at room temperature with a 50X oil-immersion objective. The numbers of neutrophils on the surface were counted in five randomly selected 50X microscopic fields between 350-500 seconds after injecting the cells into the chamber. The chambers were then inverted for 400 seconds to allow the unattached cells to fall off the surface. The number of cells remaining attached to the glass surface were again counted in five 50X fields. The ratio of counts of cells remaining attached to the glass surface to the initial cell count was expressed as percent adherent cells. Adherence was assessed when the cells were in non-stimulatory and stimulatory reagents.

The glass coverslips used in this chamber were incubated in 10% human serum for 2 minutes, removed and washed in 2 changes of HBSS.

ASSESSMENT OF CHANGES IN NEUTROPHIL SHAPE

A modification of the method of Lichtman et al., (37) was used. Neutrophils (1 X 10^6/ml of reagents) were incubated with various reagents. At the appropriate time the cell suspension was added dropwise to 10 ml of cold (4 C) 1% glutaraldehyde. The

glutaraldehyde solution was mixed constantly while the cells were being added. After remaining in the cold glutaraldehyde solution for 1 hour, the cells were washed and resuspended in one drop of HBSS. The neutrophils were examined with a 100X phase-contrast objective and were classified either as round or motile. The shapes observed under phase-contrast were confirmed with scanning electron microscopy (Figure 3).

ASSESSMENT OF BINDING OF LATEX BEADS

Latex beads were washed and dispersed into a solution of 2% human albumin in HBSS. After a 2 minute incubation at room temperature, the beads were washed and resuspended in HBSS. Cells suspended in various reagents were exposed to a 1% suspension of beads for 2 minutes at room temperature. Cells were then fixed in glutaraldehyde as described above and were separated from unbound latex beads by centrifugation on Ficoll-Hypaque cushions (Sp. gr., 1.077 at 25 C).

SCANNING ELECTRON MICROSCOPY

Cells in suspension were fixed in cold 1% glutaraldehyde for 2 hours. One drop of cell suspension was added onto a glass coverslip pretreated with polylysine (1% in water) for 5 minutes at room temperature. After the cells settled onto the glass coverslip, they were dehydrated in a graded ethanol series. Cells were dried using a Bomar critical-point-drier (Bomar Company, Tacoma, Washington) with CO^2 as the carrier gas. The glass coverslips were

Figure 3. Change in shpe of neutrophils exposed to chemotactic factors. (a) Neutrophils were suspended in HBSS (10^6 cells/ml) at room temperature and then fixed in suspension in cold (4 C), buffered glutaraldehyde for 1 hour. (b and c) Neutrophils were exposed to 10^{-8} M f-Met-Phe or 4 μg protein/ml C5a for 10 minutes before fixing in glutaraldehyde. (d, e, and f) Neutrophils were exposed to 10^{-6}M f-Met-Phe or 18 μg protein/ml C5a for 10 minutes and then fixed in glutaraldehyde. Scanning electron microscopy, X5,000.

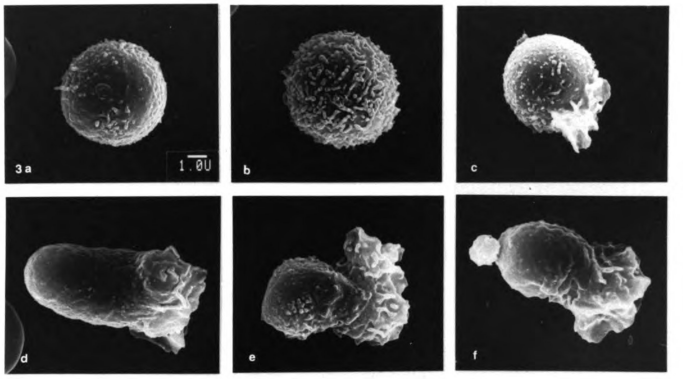


Figure 3.

then mounted on aluminum stubs, sputter-coated with 20-30 nm of gold, and examined in an ISI super III scanning electron microscope (International Scientific Instruments, Incorporated, Santa Clare, California).

PRESENTATION AND ANALYSIS OF DATA

The data are expressed in terms of mean $\pm$ standard error of the mean; n represents the number of separate experiments and, in most cases, the number of separate donors. Each experiment contained duplicate determinations. The student's t test was used to assess significance.

RESULTS

ASSESSMENT OF C5a FOR CHEMOTACTIC ACTIVITY IN BLIND WELL CHAMBERS

A significant response was noted with a high concentration of C5a (18 μg protein/ml). At a low concentration of C5a (4 μg protein/ml) the chemotactic response was not significant (Table 1). The distribution of cells responding to chemotactic solutions is shown in figure 4.

EFFECT OF C5a ON CELL SHAPE

Figure 3 shows the appearance of neutrophils fixed in suspension with buffered glutaraldehyde. At room temperature (i.e., 22 C) before fixation, the cells were mostly round, occasionally with a slightly ruffled membrane on a small portion of the surface (Figure 3a,b,c). The addition of C5a to the cell suspension resulted in a change in neutrophil shape (Figure 3d, e, and f). In an effort to quantify this effect of C5a on neutrophils, cells were categorized as spherical or nonspherical. The spherical category included round cells and cells whose shape was generally round with some ruffled membrane (Figure 3a,b, and c). The nonspherical category included cells whose overall form was oval to elongated (Figure 3d, e, and f). Neutrophils incubated for 10 minutes in C5a (i.e., 18 μg protein/ml) formed a bipolar shape. However, they remained spherical when C5a was preincubated

Tablel 1. The effects of a gradient of chemotactic factors on neutrophil migration in blind well chambers.

Filter * pretreatment	Cell compartment	Stimulus compartment	Distance** migrated ($\mu m \pm SEM$)	n	P
BSA (20 mg/ml)	HBSS	HBSS	20 ± 2	3	
"	"	"	f-Met-Phe ($10^{-6}M$)	46 ± 3	"	***
"	"	"	C5a (18 μg protein/ml)	55 ± 4	"	***
"	"	"	C5a (4 μg protein/ml)	24 ± 3	"	

* Filters were pretreated with BSA (20 mg/ml) for 2 minutes and washed twice in HBSS. Abbreviations of reagents used are: bovine serum albumin (BSA), and Hanks'buffer (HBSS).

** The distance of migration were determined by the leading front technique; incubation, 60 minutes.

*** Significantly different ($P < 0.02 > 0.01$) compared to control values for migration.

with antibody to human C5 prepared in goat (anti-C5). The antibody was dialysed against HBSS for 24 hours. The C5a preparation was preincubated with a 1% and a 10% solution of anti-C5 for 45 minutes at room temperature, the cells were then incubated in the media. The percentage of bipolar cells is shown in Table 2. These results indicate that the chemotactic activity which has been isolated was related to the fifth component of complement.

Table 2. Effect of various reagents on neutrophil morphology *.

Solution in Cell Suspension	Round (%±SEM)	Bipolar (%±SEM)
1- HBSS	98	2
HBSS + 10% anti C5	80	20
HBSS + 1% anti C5	95	5
2- f-Met-Phe (10^{-6}M)	7	93
" " + 10% anti C5	5	95
" " + 1% anti C5	8	92
3- C5a (18 μg protein/ml)	7 ± 3	92 ± 4
" " + 10% anti C5	88 ± 4	12 ± 4
" " + 1% anti C5	58 ± 2	42 ± 2

* The morphology of the neutrophils in suspension was determined microscopically (100X oil objective after fixing the cells with glutaraldehyde; cells were classified as round or, when pseudopods and uropod were present, as bipolar.

Figure 4. The distribution of neutrophils responding to C5a or f-Met-Phe in the stimulus compartment of the blind well chambers. The filters were pretreated with BSA (2%) for 2 minutes and washed twice in HBSS. The numbers of cells in a 20X microscopic field were counted at 10 μm intervals through the filter using an image analyzer (Optimax). Three field cores were counted on each of duplicate filters and averaged.

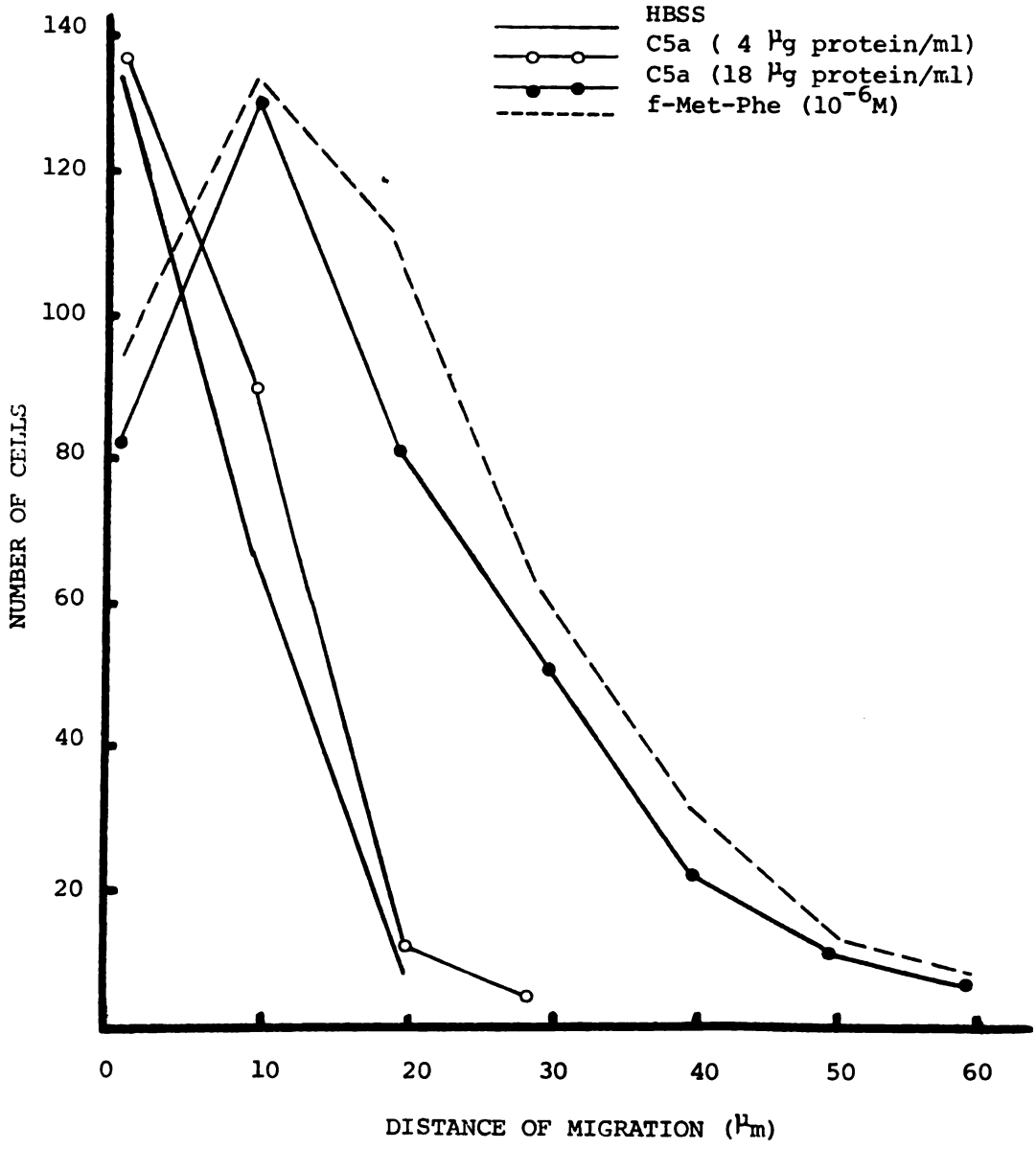

HBSS
C5a (4 μg protein/ml)
C5a (18 μg protein/ml)
f-Met-Phe (10^{-6}M)
NUMBER OF CELLS
140
120
100
80
60
40
20
0
10
20
30
40
50
60
DISTANCE OF MIGRATION (μm)

Figure 4.

EFFECT OF C5a AND f-Met-Phe ON NEUTROPHIL ADHERENCE

As the neutrophils suspended in HBSS settled onto the lower surface of the adherence chamber, most appeared round and essentially the same as cells fixed in suspension (Figures 3a, b, and c). The addition of C5a or f-Met-Phe significantly increased attachment of neutrophils to serum-coated glass (Table 3).

RESTIMULATION OF NEUTROPHIL: EFFECTS ON CELL ADHESIVENESS

Previous experiments have shown that under carefully controlled conditions, restimulation of neutrophils with a higher concentration of f-Met-Phe causes a significant drop in adhesiveness (61). Similar results were observed using C5a. As shown in Table 4 levels of adhesiveness were significantly decreased after restimulation with higher concentrations of stimuli.

The experiment was also carried out in the absence of Ca^{++} ion in the medium. The Mg-EGTA (i.e., ethyleneglycol-bis-(beta-aminoethyl ether) N,N'-tetraacetic acid) complex (i.e., 4×10^{-8} M) was added to Ca^{++}-free HBSS, C5a, and f-Met-Phe to chelate the trace amount of Ca^{++} ion possibly contaminating these preparations. Neutrophils, washed twice in Ca^{++}-free HBSS and incubated in Ca^{++}-free HBSS containing f-Met-Phe or C5a for 5 minutes, gave the same degree of adherence when Ca^{++} was present in the medium. However, when adhesiveness of neutrophils was examined upon restimulation with an increased stimulatory dose in Ca^{++}-free medium, there was a significant difference in adhesiveness. In contrast, restimulation of neutrophils with a higher concetration of Ca^{++}-free C5a and

f-Met-Phe did not result in decreased adherence (Figure 5 and Table 4).

Table 3. Effect of f-Met-Phe and C5a on adhesiveness of neutrophils in the slide chamber

Neutrophils * suspended in	Glass ** pretreatment	Attachment *** (% ± SEM)	n	P****
HBSS	Human serum (10%)	36 ± 2	5	
f-Met-Phe (10^{-10} M)	" " "	43 ± 1	"	< .01
" (10^{-9} M)	" " "	45 ± 2	"	< .05
" (10^{-8} M)	" " "	46 ± 2	"	< .025
" (10^{-7} M)	" " "	57 ± 4	"	< .02
" (10^{-6} M)	" " "	74 ± 3	"	< .005
" (10^{-5} M)	" " "	79 ± 3	"	< .001
C5a (4 μg protein/ml)	" " "	57 ± 3	"	< .005
" (10 " ")	" " "	67 ± 3	"	< .001
" (18 " ")	" " "	75 ± 3	"	< .001
" (25 " ")	" " "	79 ± 2	"	< .001
" (30 " ")	" " "	80 ± 2	"	< .001

* Cells suspended in various reagents one minute before injection of cells into chambers.

** The glass coverslip in the slide chamber was pretreated by exposure to serum (10%) for 2 minutes, and washed in two exchanges of HBSS.

*** Mean percent ± SEM remaining attached to the glass.

**** Compared with control values.

Table 4. Adhesiveness of human neutrophils upon restimulation with an increased dose of f-Met-Phe and C5a

First incubation (5 minutes)	Second incubation (10 minutes)	n	Attachment* (% ± SEM)	P**
HBSS	--	5	31 ± 1	--
f-Met-Phe (10^{-8}M)	--	"	45 ± 1	--
f-Met-Phe (10^{-6}M)	--	"	66 ± 5	--
C5a (4 μg protein/ml)	--	"	52 ± 3	--
C5a (18 " ")	--	"	63 ± 4	--
f-Met-Phe (10^{-8}M)	f-Met-Phe (10^{-6}M)	"	37 ± 4	< .001
C5a (4 μg protein/ml)	" "	"	38 ± 3	< .005
" " "	C5a(18 μg protein/ml)		41 ± 3	< .005
f-Met-Phe (10^{-8}M)	" " "	"	43 ± 2	< .005
	Ca^{++}-Free ***			
HBSS	--	"	33 ± 3	--
f-Met-Phe (10^{-8}M)	--	"	44 ± 5	--
f-Met-Phe (10^{-6}M)	--	"	70 ± 4	--
C5a (4 μg protein/ml)	--	"	46 ± 4	--
C5a (18 " ")	--	"	69 ± 4	--
F-Met-Phe (10^{-8}M)	f-Met-Phe (10^{-6}M)	"	64 ± 4	< .2 > .1
C5a (4 μg protein/ml)	" "	"	67 ± 5	< .4 > .3
" " "	C5a(18 μg protein/ml)		62 ± 5	< .1 > .05
f-Met-Phe (10^{-8}M)	" " "	"	63 ± 5	< .2 > .1

* Mean percent ± SEM remaining attached to the surface.

** Compared with adhesiveness of neutrophils exposed to a single stimulus (i.e., f-Met-Phe 10^{-6}M, and C5a 18 μg protein/ml).

*** This experiment was carried out in Ca^{++}-free medium containing 4 x 10^{-8}M Mg-EGTA complex.

BINDING OF ALBUMIN-COATED LATEX BEADS TO NEUTROPHILS: EFFECT OF RESTIMULATION WITH f-Met-Phe AND C5a

Neutrophils were preincubated for 5 minutes at 22 C in HBSS, f-Met-Phe (10^{-8} M) and C5a (4 μg protein/ml). Cells were centrifuged, the supernatant was discarded and cells were resuspended in a higher concentration of f-Met-Phe (10^{-6} M) or C5a (18 μg protein/ml) for 15 minutes at 22 C. The cells were then exposed to albumin-coated latex beads for 2 minutes and fixed in suspension with glutaraldehyde. A significant difference was observed in the distribution pattern of beads over the surface of the cells (Table 5). Control cells, which were incubated only in HBSS, had a round configuration with few beads (i.e., 3 ± 1) attached randomly to their surface (i.e. 46 ± 4 %, Figure 6a). Approximately 80% of the cells which were exposed to low concentration of a single stimulus were round, but more beads (i.e. 8 ± 2) were randomly distributed over the entire cell (Figure 6b). The cells which were exposed to a high concentration of a single stimulus had a polarized configuration (i.e. 97 ± 3 %), and beads were evenly distributed over the entire cell surface (Figure 6c). A low percentage of cells (i.e. 5 ± 2 %) had clusters of beads on the uropod (Figure 6d). In contrast, cells pretreated in f-Met-Phe or C5a had a polarized configuration with a high percentage showing beads bound on their uropods (i.e. 67 ± 3 %) (Figure 6d). Only a low percentage of cells (i.e. 25 ± 3 %) had beads distributed randomly over the cell surface. These beads were principally towards the uropod.

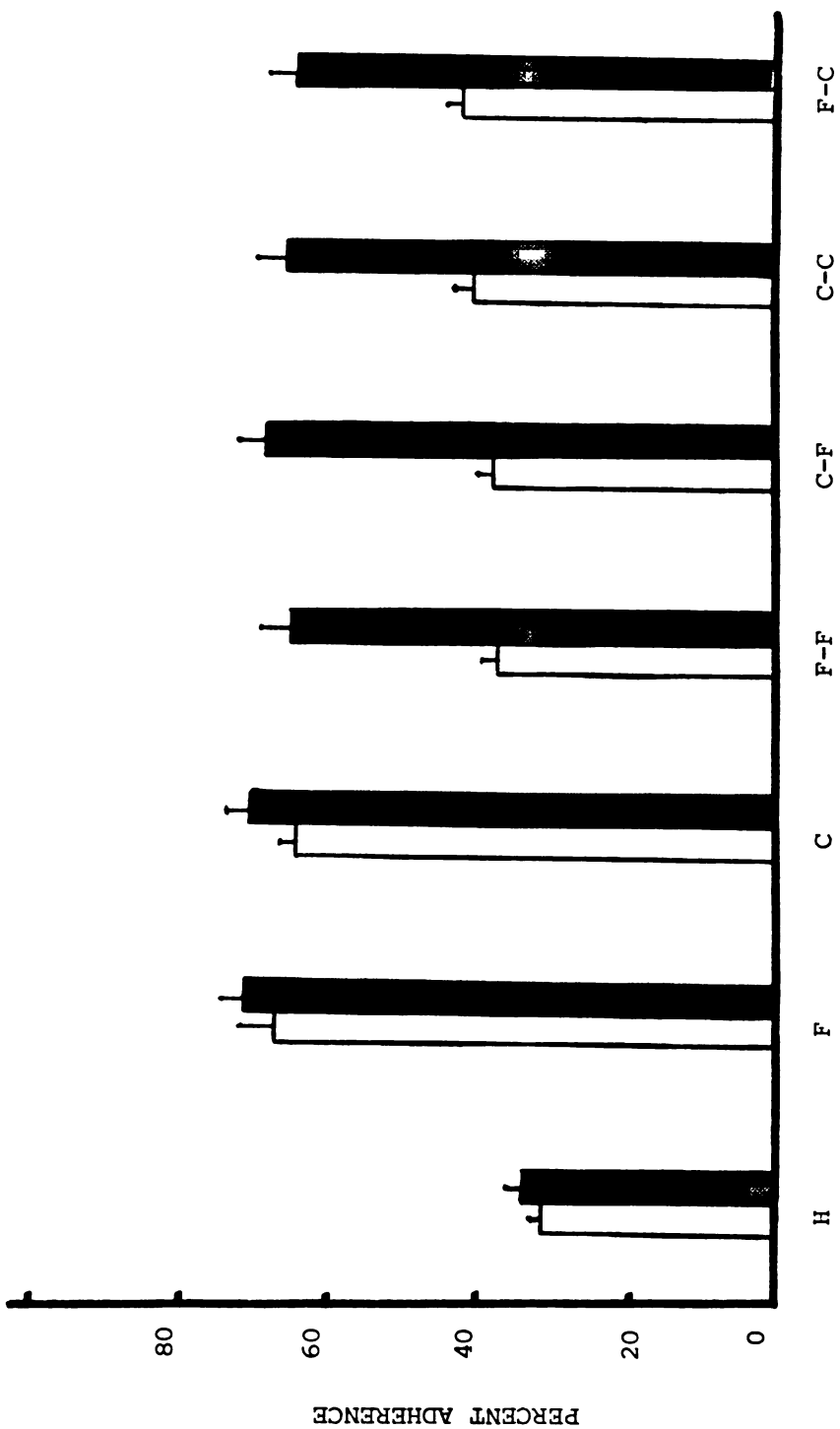
Neutrophils incubated in Ca^{++}-free medium containing C5a or

Figure 5. Adhesiveness of human neutrophils to serum-treated glass.

The results of three experimental conditions are given: neutrophils suspended in HBSS; neutrophils incubated for 5 minutes in HBSS containing C5a (18 μg protein/ml) or f-Met-Phe (10^{-6}M); and neutrophils preincubated for 5 minutes at 22 C with HBSS containing C5a (4 μg protein/ml) or f-Met-Phe (10^{-8}M). The cells were then reincubated with higher concentration of C5a (18 μg protein/ml) or f-Met-Phe (10^{-6}M) for 5 minutes. Adhesiveness was then assessed.

Solid lines show neutrophil responses in Ca^{++}-free medium. Percent refers to the percentage of neutrophils remaining attached to the serum-treated glass. The vertical lines represent $\pm$ SEM.

Abbreviations: HBSS (H); f-Met-Phe (F); C5a (C); first stimulus f-Met-Phe, second stimulus f-Met-Phe (F-F); first stimulu C5a, second stimulus f-Met-Phe (C-F); first stimulus C5a, second stimulus C5a (C-C); and first stimulus f-Met-Phe, second stimulus C5a (F-C).



Figre 5.

f-Met-Phe exhibited the same pattern as when they were incubated in complete medium. However, a significant difference was observed when cells were restimulated with C5a or f-Met-Phe (Table 5). Cells had a bipolar configuration and most (i.e. 76 $\pm$ 4 %) had beads randomly distributed over the entire surface (Figure 6c). A low percentage of cells (i.e. 20 $\pm$ 3 %) had clusters of beads on the uropod. No significant difference was observed in terms of the number of beads attached to cells treated in the absence or presence of Ca^{++} ion in the medium. Figure 7 shows a comparison of the level of beads binding to the uropods of neutrophils treated in the presence or absence of Ca^{++} ion in the medium.

The results show that the effect of the double stimulus is not dependent on chemotactic factors. C5a caused unipolar binding of beads on f-Met-Phe pretreated cells and f-Met-Phe caused this effect on C5a pretreated cells.

EFFECTS OF A THIRD EXPOSURE TO f-Met-Phe AND C5a

Previous experiments have shown that exposure of neutrophils to a third stimulus containing latex beads resulted in binding of the beads in the region of the lamellipodia (61). This event will occur only if the concentration of the third stimulus is higher than the second one. Ten minutes after the second exposure, cells were exposed to albumin-coated latex beads, 0.6 μm in diameter for 2 minutes, then washed to remove excess beads. Larger beads (i.e. 1.1 μm diameter) were included with the third stimulus (i.e. C5a or f-Met-Phe) and cells were fixed in suspension with glutaraldehyde

30 seconds later. The concentration of the third stimulus was higher than that of the second stimulus. Small beads were clustered at the uropod, larger beads were attached most frequently to the lamellipodia (Figure 8). When cells were allowed to incubate for a longer period (i.e. 3-5 minutes), most of the larger beads were found closer to the small beads on the uropod. In this experiment C5a and f-Met-Phe was used as stimuli for the first, second and third stimulation alternately.

Table 5. Binding of albumin-coated latex beads to human neutrophils. Effect of restimulation with C5a and f-Met-Phe

* Spherical cells with beads distributed randomly over the entire surface (Figure 6a, and b).

** Polarized cells with beads distributed randomly over the entire surface (Figure 6c).

*** Polarized cells with beads clustered only at the uropod (Figure 6d).

**** Compared with the level of uropod binding of neutrophils stimulated with a single stimulus (i.e. f-Met-Phe 10^{-6}M, and C5a 18 μg protein/ml).

***** Compared with the level of uropod binding of neutrophils examined in the presence of Ca^{++} ion in the medium.

****** This experiment was carried out in Ca^{++}-free medium.

Table 5. Binding of albumin-coated latex beads to human neutrophils. Effects of restimulation with C5a and f-Met-Phe.

Preincubation	Restimulation	# beads/ cell	Sphere* binding (%±SEM)	Bipolar** binding (%±SEM)	Uropod*** binding (%±SEM)	n	P****
HBSS	HBSS	3 ± 1	46 ± 6	3 ± 1	--	5	--
HBSS	C5a (4 μg protein/ml)	8 ± 2	69 ± 3	16 ± 2	--	"	--
HBSS	C5a (18 " ")	13 ± 3	8 ± 2	83 ± 3	6 ± 2	"	--
HBSS	f-Met-Phe (10^{-8}M)	8 ± 2	75 ± 2	14 ± 1	--	"	--
HBSS	" " (10^{-6}M)	13 ± 3	5 ± 1	87 ± 2	4 ± 1	"	--
f-Met-Phe (10^{-8}M)	" " (")	" "	6 ± 1	30 ± 3	64 ± 3	"	< .001
C5a (4 μg protein/ml)	" " (")	" "	4 ± 1	26 ± 3	69 ± 4	"	< .001
C5a (" " ")	C5a (18 μg protein/ml)	" "	3 ± 1	27 ± 2	70 ± 3	"	< .001
f-Met-Phe (10^{-8}M)	C5a (" " ")	" "	3 ± 1	30 ± 1	68 ± 2	"	< .001
		Ca^{++}-Free ******					P*****
HBSS	HBSS	3 ± 1	46 ± 7	3 ± 1	--	"	--
HBSS	C5a (4 μg protein/ml)	8 ± 2	73 ± 2	11 ± 2	--	"	--
HBSS	C5a (18 " ")	13 ± 3	13 ± 4	78 ± 5	4 ± 1	"	--
HBSS	f-Met-Phe (10^{-8}M)	8 ± 2	79 ± 1	12 ± 1	--	"	--
HBSS	" " (10^{-6}M)	13 ± 3	14 ± 3	80 ± 2	2 ± 1	"	--
f-Met-Phe (10^{-8}M)	" " (")	" "	7 ± 1	76 ± 1	17 ± 2	"	< .001
C5a (4 μg protein/ml)	" " (")	" "	5 ± 2	73 ± 2	22 ± 3	"	< .005
C5a (" " ")	C5a (18 μg protein/ml)	" "	6 ± 1	71 ± 2	23 ± 2	"	< .001
f-Met-Phe (10^{-8}M)	C5a (" " ")	" "	6 ± 1	72 ± 2	22 ± 1	"	< .001

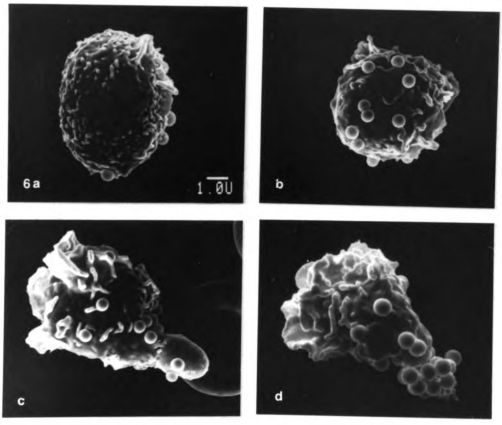


Figure 6. Binding of albumin-coated latex beads to neutrophils in suspension: (a) Neutrophils suspended in HBSS exposed to beads for 2 minutes and then fixed in glutaraldehyde; (b) Neutrophils were exposed to 10^{-8}M f-Met-Phe or 4 μg protein/ml C5a for 10 minutes, exposed to beads for 2 minutes and then fixed in glutaraldehyde; (c) Neutrophils were exposed to 10^{-6}M f-Met-Phe or 18 μg protein/ml C5a for 10 minutes, exposed to beads for 2 minutes and then fixed in glutaraldehyde; (d) Neutrophils were exposed to 10^{-8}M f-Met-Phe or 4 μg protein/ml for 5 minutes, washed, exposed to 10^{-6}M f-Met-Phe or 18 μg protein/ml for 10 minutes and then exposed to beads for 2 minutes without changing the concentration of the second chemotactic stimulus. Scanning electron microscopy, X5,200.

Figure 7. Binding of albumin-latex beads to the uropod upon stimulation and restimulation of neutrophils with C5a and f-Met-Phe in the presence and absence of Ca^{++} in the medium.

The results of three experimental conditions are given: neutrophils suspended in HBSS exposed to latex beads for 2 minutes; neutrophils exposed to 10^{-6}M f-Met-Phe or 18 μg protein/ml C5a for 15 minutes and then exposed to latex beads for 2 minutes; neutrophils were exposed to 10^{-8}M f-Met-Phe or 4 μg protein/ml C5a for 5 minutes, washed, then exposed to 18 μg protein/ml C5a or 10^{-6}M f-Met-Phe for 15 minutes and then exposed to latex beads for 2 minutes.

Solid lines show neutrophil responses in Ca^{++}-free medium. Percent refers to the percentage of cells with clusters of beads at the uropod. The vertical lines represent ± SEM.

Abbreviations: HBSS (H); f-Met-Phe (F); C5a (C); first stimulus f-Met-Phe, second stimulus f-Met-Phe (F-F); first stimulus C5a, second stimulus f-Met-Phe (C-F); first stimulus C5a, second stimulus C5a (C-C); and first stimulus f-Met-Phe, second stimulus C5a (F-C).

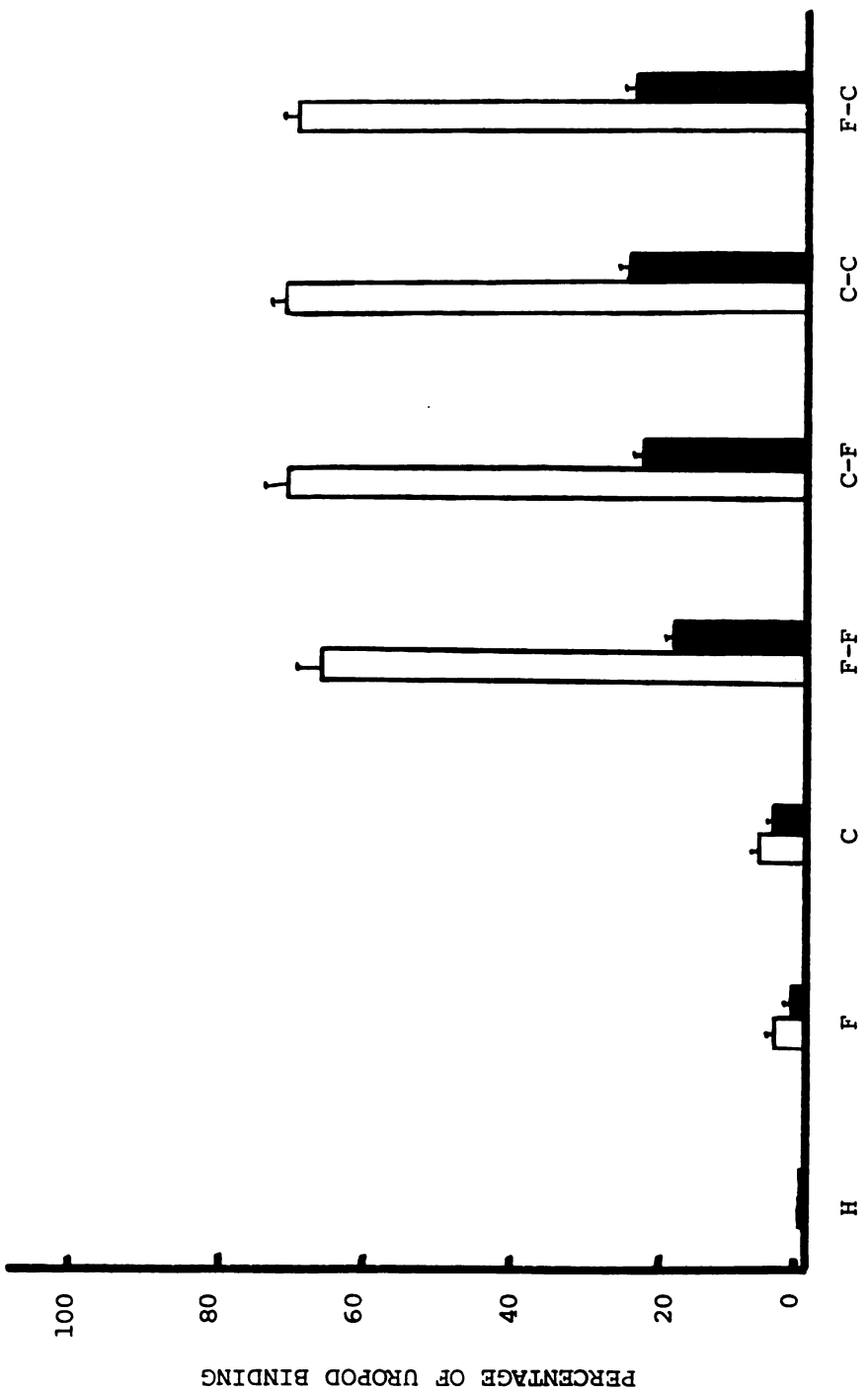


Figure 7.

Figure 8. Effect of a third exposure to f-Met-Phe or C5a on the binding of albumin-coated latex beads to human neutrophils in suspension. Neutrophils were exposed to 10^{-8} M f-Met-Phe or 4 μg protein/ml C5a for 5 minutes, washed and then exposed to 10^{-7}M f-Met-Phe or 10 μg protein/ml C5a for 10 minutes. Cells were collected and exposed for 2 minutes to a solution of 10^{-7}M f-Met-Phe or 10 μg protein/ml C5a containing latex beads (0.6 μm Diameter). This allowed binding of beads to the uropod. Cells were then washed in HBSS containing 10^{-7}M f-Met-Phe or 10 μg protein/ml C5a to remove excess beads and to prevent cells from rounding up. Pelleted cells were then resuspended in HBSS containing 10^{-6} M f-Met-Phe or 18 μg protein/ml C5a and 1.1-μm diameter beads for 30 seconds before fixing in glutaraldehyde. Scanning electron microscopy, X6,000.

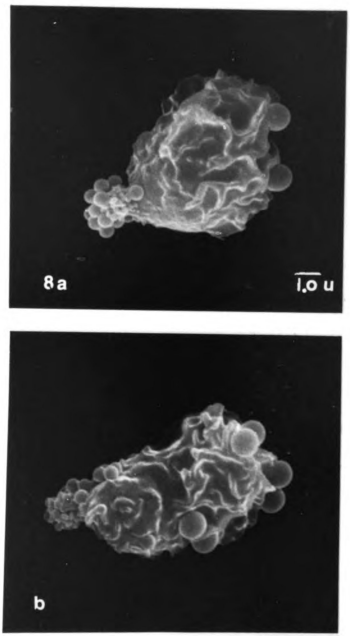


Figure 8.

DISCUSSION

CHEMOTACTIC ACTIVITY OF COMPLEMENT COMPONENT (C5a)

A low-molecular-weight component of activate human serum was shown to have chemotactic activity. Neutrophils exposed to this material exhibited increased motility in micropore filters and increased their attachment to serum-coated glass. Exposure of neutrophils to this fraction in the absence of a gradient also resulted in a change in cellular shape from a spherical to a bipolar configuration. This activity was ablated with anti-C5. These results indicate that the isolated protein from human serum is antigenically related to the fifth component of complement, and is most likely C5a. The results are also consistent with previous reports (15, 23, 24, 49).

EFFECTS OF CHEMOTACTIC FACTORS ON CELLULAR ADHESIVENESS

Exposure of previously unstimulated human neutrophils to f-Met-Phe and C5a resulted in enhanced adhesiveness in a dose-dependent fashion and binding of albumin-coated latex beads in a random pattern over the cell surface. However, under carefully controlled conditions, restimulation of neutrophils with f-Met-Phe or C5a resulted in a significant drop in adhesiveness, and movement of binding sites for latex beads to the uropod. These results are

consistent with those of Smith and Hollers who used only one type of chemotactic factor (i.e. f-Met-Phe) for both the initial exposure and the second exposure (61).

Since separate receptors for C5a and formyl-methionyl peptides have been reported (27), it seems that either of these chemotactic factors is able to prime or induce "internal signal(s)" which cause(s) the adhesion sites for albumin-coated surfaces to move to the tail of the cell upon restimulation with higher concentration of these factors. The mechanism involved in the induction of "internal signal(s)" is not clearly understood at present.

It is important to note that the concentration of the initial and second stimuli is very crucial. In addition, Smith et al. have shown that timing of the second stimulus had a significant effect on adhesiveness (61). They incubated cells with a low concentration of f-Met-Phe for 2, 3, 4, and 5 minutes at which time the concentration of f-Met-Phe was abruptly increased. They observed adhesiveness which remained high when the f-Met-Phe concentration was increased after 2 and 3 minutes. When increased at 4 or 5 minutes, the adhesiveness was significantly decreased.

Previous experiments have shown that exposure of rabbit and human neutrophils to a very high concentration of certain chemotactic factors results in chemotactic deactivation (60, 64). Cells treated under this condition (i.e. deactivation) exhibited significantly reduced motility on glass and in micropore filters. However, these cells have exhibited a sustained enhanced adhesiveness

upon restimulation with chemotactic factors (60).

The observation here seem consistent with those of Smith et al. (60, 61) who investigated neutrophil attachment and spreading on serum-coated coverglass upon reexposure to f-Met-Phe. Neutrophils were incubated in HBSS or f-Met-Phe (10^{-8}M) for 5 minutes and allowed them to adhere to the upper coverglass of a slide chamber. The adherent cells were washed and then exposed to f-Met-Phe (10^{-6}M). Cell spreading occurred rapidly after infusion of f-Met-Phe into the chamber. The cells gradually assumed a polarized shape typical of motile neutrophils (i.e., lamellipodia at one end and a distinct tail or uropod at the other). This occurred maximally between 300-350 seconds and seemed unaffected by pretreatment in f-Met-Phe. However, cells pretreated in f-Met-Phe became detached from the coverglass in greater numbers than control cells, i.e., those preincubated in HBSS. Detachment occurred first at the anterior end of the cells in the region of the lamellipodia resulting in a high percentage of cells hanging by the uropod. Many of these cells eventually dropped off the glass surface. The above experiment was modified by addition of high concentration of albumin-coated latex beads to the cell suspensions after the initial incubation. Initially the cells were round, with latex beads attached randomly on their surfaces. Infusion of 10^{-7}M f-Met-Phe into the chamber resulted in a polarization of cells. The beads then began to move toward the uropod, forming a cluster of latex beads on the uropod.

Smith and Hollers have hypothesized that the decreased adhesiveness is the result of the movement of adhesion sites to a small area of

the cell tail (61). The transport of surface-bound substances to the tail of the cells has been previously observed in neutrophils migrating in a chemotactic gradient (55) and in cells treated with colchicine (6). The results here seem to be consistent with previous reports and support the concept that in human neutrophils a similar transport is activated by an increasing concentration of chemotactic stimulus and the process can occur without binding to a substratum or particle. It also has been reported that formation of a polarized cellular configuration is separable from this transport process (61). The observations here also indicate that transport of the adhesion site for albumin-coated surfaces is a nonspecific process in terms of chemotactic factors (i.e., at least for two of them, C5a and f-Met-Phe).

Under different conditions, neutrophils have exhibited different reactions upon exposure to the chemotactic factors. Careful study of neutrophil behaviors might introduce some possible role of factors which seem to be involved in the transport of adhesion sites. Smith and Hollers have observed that detachment of cells from the coverglass in the slide chamber after exposure to a second stimulus occurred in a consistent fashion, proceeding from the region of the lamellipodia and progressing to the tail (61). However, TLCK (N-alpha-P-tosyl-L-lysine chloromethyl ketone, an active site inhibitor of trypsin-like enzyme) treatment of cells after the initial stimulation with f-Met-Phe inhibited the detachment of cells from the coverglass and the movement of latex beads to the uropod. N-alpha-P-tosyl-L-lysine chloromethyl ketone at this

concentration (0.2 mM) did not reduce the change in cellular shape caused by f-Met-Phe (61). Neutrophils exposed to a single dose of stimulus developed a polarized configuration with beads distributed in a random pattern over their surfaces and showed increased adherence. In contrast, colchicine treated cells had a polarized configuration with a high percentage showing beads clustered on their uropod (61). Colchicine is an alkaloid which binds with tubulin and causes the rapid and complete disassembly of cytoplasmic microtubules in all mammalian cells (50). Cytochalasin B-treated cells which were spherical exhibited increased adhesiveness upon exposure to a second f-Met-Phe stimulus and had beads distributed over the surface. Cytochalasin B, a fungal metabolite usually causes structural disorganization of microfilaments in mammalian cells. It also inhibits the active transport of sugar in neutrophils at lower concentrations than those required to affect microfilament organization (50). Thus, microtubules and microfilaments might have an important role in the transport of adhesion sites.

The induction of centriole-associated microtubules in response to surface binding events has previously been reported (50). It has been demonstrated that a concanavalin A (Con-A)-receptor complex maintains a uniform surface distribution on human neutrophils possessing an intact microtubule system. However, if microtubule assembly is prevented by colchicine or other drugs, Con-A moves to one pole of the cell to form a cap (50). Capping has been defined as a phenomenon that follows microtubule disassembly. The recruitment of microfilaments to cytoplasm subtending regions of

ligand-membrane interaction has also been previously demonstrated (50). It has been considered that the microfilament distribution is significantly regulated by microtubules. Oliver has suggested that surface ligands such as Con-A and phagocytic particles are distributed to regions of the leukocyte membrane that are enriched for associated microfilaments. Cells containing microtubules show a regulated, uniform recruitment of microfilaments and bind Con-A and particles over their entire surface. Cells lacking microtubules lose the capacity to restrain or direct the distribution of microfilaments. Hence, the filaments aggregate at one pole of the cell, which becomes the site of ligand concentration. Oliver et al. have concluded that microfilament recruitment is associated with specific changes in membrane properties at sites of ligand binding. Microtubules may regulate these membrane changes and so indirectly regulate the distribution of microfilaments. Therefore, if adhesion sites for Con-A and albumin-coated surfaces exhibit analogous behavior, it can be assumed that movement of adhesion sites for latex beads is due to disassembly of microtubules. However, further study is needed to support this assumption.

The results show that in the absence of Ca^{++} in the medium, exposure of previously stimulated neutrophils to f-Met-Phe or C5a did not result in decreased adherence or significant movement of binding sites for albumin-coated latex beads to the uropod. It seems that the presence of Ca^{++} in cell media is crucial for the transport process. Most investigators agree that divalent cations, a source of energy and some cellular contractile elements

(microtubules and microfilaments) are responsible for the translocation of a chemoelectrical signal into mechanical work (23). Previous studies have demonstrated that human neutrophil adhesiveness is a Mg^{++}-dependent process (9, 34) while the proposed mechanical work element (microtubules and microfilaments) is Ca^{++}-dependent (3, 30). It has been suggested that Ca^{++} may play a role in the control of microtubule assembly/disassembly processes. Marcum et al. have shown that Ca^{++} can cause the depolymerization of microtubules in vitro (43). Becker and Stossel have reported that chemotactic factors interact with specific receptors on the neutrophil surface inducing a graded displacement of intracellular bound Ca^{++} from membranous stores into the neutrophil cytoplasm. In addition, they increase the membrane permeability to Ca^{++}. This, in the presence of extracellular Ca^{++}, causes an influx of Ca^{++} into the cell. These two prcesses raise the free Ca^{++} in the cytoplasm. The increased cytoplasmic Ca^{++}, by a complex series of presently undefined reactions, leads to the various neutrophil functions (4, 5).

Increasing the concentration of chemotactic stimulus in a stepwise manner not only induces movement of surface binding sites, but appears to promote appearance of binding sites for albumin-coated latex beads at the front of the cell. This process seems nonspecific in terms of chemotactic factors. Previous work by Smith and Hollers has yielded similar observation (61). These workers used only f-Met-Phe as a chemotactic stimulus and observed that exposure of neutrophils to a third and higher f-Met-Phe concentration for 30 seconds led to binding of latex beads in the region of the lamellipodia, while

longer incubation led to predominant binding of beads on the uropod.

In conclusion, the results reported here raise the possibility that interaction of chemotactic factors (i.e., f-Met-Phe and C5a) with neutrophil surface receptors results in an increase in adhesion sites and induction of "internal signal(s)" which cause the adhesion sites to move to the uropod upon restimulation with higher concentration of chemotactic factors (i.e., f-Met-Phe and C5a). It also seems that this phenomenon is nonspecific in terms of chemotactic factors (at least for C5a and f-Met-Phe), is Ca^{++} dependent and requires microfilament function.

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