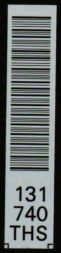

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EFFECTS OF NEUTROPHIL ADHERENCE TO MICROPORE FILTERS ON MIGRATION ASSESSMENTS IN VITRO

presented by

Thomas L. York

has been accepted towards fulfillment
of the requirements for

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Kathryn Colando
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ABSTRACT

EFFECTS OF HUMAN NEUTROPHIL ADHERENCE TO
MICROPORE FILTERS ON MIGRATION
ASSESSMENTS IN VITRO

By

Thomas L. York

The degree of neutrophil adhesiveness to a surface may influence locomotion. To investigate this relationship *in vitro*, adherence and motility were assessed by a new modification of the Boyden assay. Decreasing adhesiveness to the filter surface by coating the filters with albumin was necessary for random and chemotactic migration of neutrophils *in vitro*. Neutrophils preincubated with high concentrations of fMetPhe or a chemotactic factor from activated serum (C5a), reduced their locomotive responsiveness and significantly enhanced adherence to albumin coated filters. If the adhesiveness reduced, then the locomotive responsiveness returned to control levels. Other chemotactic solutions affected neutrophil adhesiveness differently when used to coat the filter or when placed with the cells. Apparently, agents existed in these solutions that affected both the filter surface (decreasing adherence like albumin), and the neutrophils (increasing adherence like C5a and fMetPhe).

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INTRODUCTION

Leukocyte migration to inflammatory sites is a fundamental event in mammalian homeostasis. The mechanisms of cell locomotion have been studied extensively in the past. Since Boyden developed a technique to assess leukocyte migration through micropore filters, much data has accumulated about neutrophil locomotion in response to a gradient of various biological agents *in vitro*.[5] Chemokinetic factors are agents that influence the rate of migration and chemotactic factors are agents that influence the rate and direction of migration. Chemotactic and chemokinetic factors have been shown to affect many cellular mechanisms, but the mechanism controlling locomotion remain unclear.

Surface adhesiveness has been shown to influence the rate and direction of locomotion of certain tissue cells *in vitro*.[7,18] Several observations made in the past indicate that adherence of neutrophils to a surface may influence locomotion as well. 1) Albumin has a chemokinetic effect in the Boyden assay and has been found by many investigators to be important in obtaining an effective chemotactic response.[3,4] Albumin also decreased neutrophil adherence to glass.[19,30] 2) Chemotactic factors have a proven effect on neutrophil migration *in vitro* and have been shown to increase neutrophil adherence to protein coated glass and plastic.[26,30] 3) Certain drugs which increase intracellular cyclic adenosine monophosphate decrease neutrophil migration

and adherence to glass.[2,6] 4) Deficiency of magnesium ions in the culture medium significantly reduced neutrophil migration in the Boyden assay and adhesiveness to protein coated glass.[3,12] 5) Chemotactic solutions of casein bound to micropore filters increase neutrophil migration in the Boyden assay.[10]

This paper describes an investigation of the possible relationship between neutrophil adherence and migration. Neutrophils, or the substratum on which they migrate, were exposed to chemotactic or chemokinetic solutions and the effects of these solutions on adherence and motility were assessed by a new modification of the Boyden assay. Although the techniques used ot assess migration by Boyden assays have been used extensively in the past, they were modified slightly to investigate the possibility that alterations to the filter surface may influence migration. The adherence technique was unique and designed to assess adherence in the blind well chamber. This technique allows for comparison of neutrophil adherence with motility since assessments were made using the same experimental conditions. Adherence to glass was also measured. In addition, cellular responses to various agents were measured by incubation of neutrophils with the agents. fixing the cells in glutaraldehyde and observing for morphological alterations.

Our results indicate that conditions which affect neutrophil adherence significantly affect neutrophil migration into and through micropore filters. This was observed when adherence was altered either by treating the cells or the substratum with various chemotactic and chemokinetic factors. Neutrophil migration in response to these agents <u>in vitro</u> may reflect changes in adherence as well as changes in other cellular mechanisms of locomotion.

REVIEW OF THE LITERATURE

Chemotaxis, a phenomenon commonly described as the directional movement of cells towards a gradient of specific chemical substances[2,11,13,34] has fascinated biologists for over 100 years. Chemotaxis is of obvious interest, since it may play a role in nutrition, the development of cellular organization, reproduction, the recognition of noxious agents, and inflammation. Several types of motile cells exhibit chemotaxis, but due to its possible involvement in the inflammatory response, neutrophil chemotaxis has received much attention.

Neutrophil mobility appears essential for the migration of cells from the vasculature to sites of inflamaation. Adequate numbers of neutrophils, functional locomotor mechanisms, directed cell locomotion, and adherence appear necessary for host defense.[13] Abnormal adherence has been reported in patients with recurrent bacterial infections and after injection of corticosteroids. Intrinsic defects of cellular locomotion have been observed with recurrent infections in patients described as having the "lazy leukocyte" syndrome. Numerous reports have described disease conditions with defects of directed locomotion such as Job's Syndrome, diabetes, neoplasms, rheumatoid arthritis, viral infections, and various acute bacterial and yeast infections.

Techniques Used to Assess Neutrophil Chemotaxis
Historical Development

Chemotactic investigations have been limited by inadequate techniques, and only in the past 20 years have the technical abilities developed to answer questions asked over 100 years ago. In 1882, Cohnheim studied the vasculature during inflammation.[11] He observed neutrophils emigrating through the endothelial cells that lined the vessel wall, and their subsequent migration to inflammed tissues. In 1884, Pfeiffer introduced the term chemotaxis and in 1888, Leber performed the first *in vivo* leukocyte chemotaxis experiment, by injecting noxious agents into the cornea of rabbits and observing neutrophils accumulating in the surrounding capillaries.[11] Metchnikoff and coworkers found, in 1890, that leukocytes were attracted to living or dead bacteria that had been injected into the peritoneal cavity. Later, Metchnikoff theorized that chemical signals were produced during inflamation and leukocytes had the ability to "sense" these signals. The observations and theories of Metchnikoff on leukocyte pagocytosis and chemotaxis, printed in the late 1800's, are the foundation of chemotactic research today.[25]

Few significant *in vivo* techniques were developed after Leber's initial experiment. Clark and Clark, in 1920, did use a unique animal model, the tadpole.[8] Substances were injected into the tail of the tadpole, and neutrophil margination, emigration, and accumulation were easily seen. Some have questioned the validity of this type of technique, citing that cellular damage and trauma resulting from the injections may influence the results. Another *in vivo* technique, the skin window, developed by Rebuck and collegues in 1955, has been used

extensively.[29] A circular abrasion was made on the forearm of a human subject and test substances added to the abrasion. The site was covered with a sterile glass coverslip and the accumulation of leukocytes in response to the trauma or test substances was observed. The skin window is still a popular technique used by a number of groups in clinical studies.

The establishment of a chemotactic gradient has never been demonstrated and no technique has demonstrated that neutrophils recognize, orient, or directionally migrate to chemotactic agents in vivo. Cell accumulation may be a direct result of test substances yet whether these substances selectively attract the neutrophil remains to be determined. Most of the information about chemotaxis was determined by in vitro techniques.

Although in vitro chemotactic experiments were documented as early as 1899, the techniques were crude and unreliable. In 1954, Harris reviewed and dismissed all prior data.[17] Today 2 basic systems exist to measure chemotaxis and most of the evidence for chemotaxis has been derived from these techniques. The first deals with the movement of individual cells on a flat surface. Harris layered neutrophils, in autologous serum, on glass slides and photographed migration in response to various stimuli. Lengthening exposure times or overlapping the time lapse exposures on a single negative allowed for visualization of the path of migration or "tracks" of neutrophils. Neutrophils turned frequently and randomly in the absence of suitable stimuli. When bacteria were added, the neutrophils migrated directionally towards them. In 1972, Ramsey modified this technique to observe neutrophil locomotion on a surface.[28] Recently, Zigmond

modified Harris's technique to evaluate the frequency in which neutrophils turn, their rates of migration, and orientation towards suitable stimuli.[35] Modifications of Harris's technique have produced a great deal of information about neutrophil mobility on glass.

The development of the Boyden technique in 1962 produced a simple, accurate, and reproducible method of evaluating chemotaxis.[5] Virtually all of the recent work on chemotaxis stems from Boyden's original paper which has been universally accepted among workers. This was the first technique allowing for the assessment of soluble chemotactic substances. Boyden used a chamber that consisted of an upper and lower half separated by a porous filter. Like the endothelial wall of the vessel, the diameter of the openings in the filter are smaller than the diameter of the cell. A stimulus was placed below the filter and, as the stimulus diffused into the filter, a chemical concentration gradient was established (later verified by Keller and Sorkin).[11] Neutrophils placed above the filter migrated into the filter in response to the stimulus. Migration was evaluated by counting the number of cells on the bottom surface of the filter (i.e., migrated completely through the filter). in 1966, Cornely demonstrated that neutrophils migrated towards increasing concentrations of chemotactic factors and reversing the gradient reversed the direction of migration.[9]

Boyden's technique has undergone many modifications, most of which were designed to eliminate errors in evaluating neutrophil migrational responsiveness. Keller demonstrated that as many as 50% of the cells getting through the filter detached from the filter surface.[13] He developed a double filter technique to prevent

neutrophils from falling off. Gallin and coworkers labelled the cells with radioactive chromium, enabling more effective counting with a scintillation counter.[13] Zigmond and Hirsh shortened the incubation times, preventing the neutrophils from migrating compeltely through the filter, and measured the distance of penetration into the filter ("leading front").[36]

The importance of results derived from in vitro techniques may not apply to neutrophil locomotion in vivo.[22] The substrates used, either glass or micropore filters, are foreign to the neutrophil. Studies are currently underway assessing neturophil responsiveness on endothelial cells.

Identification and Recognition of Chemotactic and Chemokinetic Factors

The development of the Boyden assay enabled workers to identify many chemotactic substances. With few exceptions, the components producing chemotactic activity have not been isolated or chemically identified. The majority of substances assessed for chemotactic activity have been implicated in inflammatory responses, but their effects were demonstrated in vitro, usually by the Boyden assay, and their importance in vivo is unproven. Certain substances appear to affect neutrophil chemotaxis directly, whereas other substances generate chemotactic activity in serum, plasma, or other biological fluids by activating enzymes in the complement, coagulation, fibrinolytic and kinin generating systems.[11] The activated proteolytic enzymes cleave substrates, yielding chemotactic fragments. While most of the fragments are poorly characterized, a small molecular weight, heat stable fragment cleaved from the 5th component of the complement system was

partially purified by Ward, Gallin, and Snyderman.[12,13] This fragment was designated as C5a. Synderman et al. have contended that C5a was important for in vivo chemotaxis since neutrophil accumulation, occurring in response to inflammatory stimuli, was not effectively demonstrated in C5a deficient mice.[31] However, the mice were not prone to infection. Becker and Ward have suggested that other complement components are chemotactic for neutrophils (i.e., C3a and C567 complex,[2,25] although the evidence presented has not been universally accepted.[36]

The "activation" of serum or plasma to produce chemotactic activity, presumably by activating enzymes of complement or other systems, has been performed by numerous techniques. The addition of yeast cell walls or zymosan, antigen-antibody complexes, endotoxin, proteolytic enzymes (i.e., plasmin, cobra venom factor, trypsin, and kallikrein), or damaged tissues to serum or plasma generates chemotactic activity.

Numerous investigators have found bacteria to be chemotactic. In addition to secretions that may activate complement (i.e., endotoxin, antigen-antibody reactions), other soluble substances released from bacteria directly affect neutrophil chemotaxis.[25] Bacteria initiate protein synthesis with formylated methionine, unlike humans, and Shiffmann has synthesized several formylated peptides that are chemotactic for neutorphils.[35] The formylated peptides and C5a are the only relatively purified chemotactic components available for chemotaxis experiments.

Wilkinson and coworkers have demonstrated that denatured proteins and casein, a major milk protein, are also chemotactic.[13] Additional reports have indicated that fibrin split products, damaged

cells, enzyme digested collagen, prostaglandins, lipids, and cyclic-adenosine monophosphate are chemotactic.[2,6,13] In addition, Zigmond demonstrated that soluble substances release from neutrophils activated by chemotactic factors or phagocytosis are chemotactic.[35] The diversity of the chemotactic factors is immense, yet most are proteins, and the isolation and identification of common chemical sequences is an active field of investigation.

Chemotactic activity has been evaluated primarily by Boyden assays. Since conception of the assay, serum or albumin have been required in the cell medium to observe enhanced chemotactic migration, although an explantion of this requirement is lacking.[20] Early investigations of substances enhancing neutrophil migration did not account for agents producing chemokinetic affects and many substances were probably misinterpreted as chemotactic. In 1973, Zigmong used the leading front technique with varying concentrations of test substances above and below the filter to differentiate chemotaxis from chemokinesis. These data are often presented as a "grid."[36] In the absence of a test substance, neutrophils penetrated randomly into the filter and the distances of migration were similar to results derived with equations used to predict random diffusion of molecules from a front. Chemokinetic factors stimulated motility and the distances of migration were greater than predicted by the diffusion theory. This occurred equally well when the test substances were above or below the filter. Chemotactic factors also stimulated motility above or below the filter, but the distances migrated were significantly increased when the concentration of the chemotactic factor was greater below the filter. Apparently only chemotactic factors are

chemoattractants, and orient migration towards increasing concentration gradients. High concentrations of chemotactic solutions inhibited cell migration, a phenomenon not produced by chemokinetic factors. All chemotactic factors were chemokinetic and enhanced the rate of neutrophil migration. However, all chemokinetic substances were not chemotactic, since orientation and direction of migration were not affected. Thus, there appears to be 2 types of chemical messages presented to the cell, one influences orientation and another the motile mechanism. Using Zigmond's definitions, Wilkinson demonstrated that human and boving serum albumin were chemokinetic, but the requirement for chemokinetic factors in the medium to observe chemotaxis in Boyden assays remains unexplained.[19,34,35]

The mechanism by which chemotactic or chemokinetic factors interact with the neutrophils remains undetermined and a fertile field of investigation. Numerous investigators have unsuccessfully attempted to demonstrate the presence of chemotactic receptors on the cell membrane of meutrophils. Ward and Becker found when neutrophils were incubated with serine esterase inhibitors, they were unable to give a chemotactic response to chemotactic factors.[32] They concluded that there were serine esterase receptors on the neutrophil that may be receptors for the chemotactic factors. One such identified serine esterase was termed the "activatable esterase." Saturation of activatable esterase receptors with high concentrations of chemotactic factors appeared to "exhaust" the cell and produce a state of "deactivation." Deactivated neutrophils were less motile than untreated neutrophils and were unresponsive to other chemotactic factors. Multiple receptors may exist for various chemotactic factors. Zigmond

reviewed this concept, noting that C5a did not compete with formylated peptide binding.[35] Therefore, there may be at least 2 types of chemotactic receptors. Zigmond also contended that neutrophils sense chemotactic receptors across their dimensions, thus enabling the cell to orient to a gradient of chemotactic factors. Therefore, the cell membrane must contain numerous receptors encompassing the neutrophil.

Chemotactic factors specifically activate several neutrophil activities other than enhancing locomotion. These include: promoting lysosomal enzyme release, altering the cell's morphology, assembly of microtubules and microfilaments, increasing metabolic activities, and cellular adhesiveness.[13,25,30] The involvement of these activities with locomotion remain unexplained, and obscure interpretations of measurements of cellular responses to chemotactic factors such as cation fluxes, net surface charges, membrane potential, and the concentration of cyclic nucleotides.

Control of Chemotactic Response

So far, only mechanisms that tend to increase the chemotactic response, with the exception of deactivation, have been discussed. In 1974, Ward reviewed the clinical importance of chemotactic inactivations for turning off the inflammatory process.[13] Again, most of the evidence for inhibiting neutrophil locomotion was derived fron *in vitro* observations and scattered clinical abnormalities. Inhibition may be accomplished by affecting cellular mechanisms of locomotion or by preventing the activation of chemotactic factors. As suggested by Ward and Becker, deactivation by incubating neutrophils with high

concentrations of chemotactic factors inhibits the ability of the cell to detect and respond to other chemotactic factors.[32,35] This may be a biological control, preventing cells from leaving the site of inflammation. Goetzel and Austen described a neutrophil immobilizing factor released after phagocytosis which may inhibit the ability of the cell to migrate.[15] Gallin observed the same effect with lysosomal enzyme release.[13] They suggested the possibility of internal cell controls for directed cell locomotion. Others have described patients with a variety of disorders having serum inhibitors that directly impair neutrophil locomotion, but the nature of these inhibitors remains unclear.[11,25] Other researchers have used various chemicals to inhibit neutrophil locomotion. The specificity of these chemicals on specific components within the cell are questionable, but may provide evidence for the mechanisms of cell locomotion. Malawiste, although not supported by others, has reported that colchicine, which prevented microtuble assembly, inhibited random locomotion.[25] Becker reported that cytochalasin B, which prevented microfilament assemble, inhibited random and chemotactic neutrophil locomotion.[2] Various chemical inhibitors of metabolism, membrane ATPase activity, and protein synthesis also inhibit neutrophil locomotion.[13,25] Several inhibitors of chemotactic factor activation may be an important control of chemotaxis. C5 inactivator, C1 inhibitor, and alpha-2-macroglobulin inhibit the chemotactic activity generated by C5, C1, and kallikrein or plasminogen activator respectively <u>in vitro</u>.[11,13,25]

Relationship of Neutrophil Adherence to Motility

In the early 1800's, Dutrochet described leukocyte margination near inflammatory sites.[11] Numerous investigators have focused on investigations of neutrophil adhesiveness since adhesion to the endothelial lining of the vessel may be a critical event in the inflammatory response. Other investigators have noted the importance of cell attachment to a surface on which these cells crawl. In 1972, Ramsey reviewed locomotion on glass.[28] As observed in Boyden assays, serum or albumin were required for neutrophil locomotion on glass, since the cells just flattened out on the glass surface without serum or albumin. In a protein environment, neutrophils attach to the surface, sending out pseudopods which also attach to the glass. The intracellular contents were observed flowing from the tail process or uropod to the pseudopods. The uropod was then released, or borken off, drawing the cell to the attached pseudopod.[4] DeBruyn, in 1946, showed neutrophils to be more adherent at the pseudopod than the uropod.[11] Machesi noted neutrophils migrating through the vasculature also sent pseudopods between the endothelial cells and the cell contents flowed from the uropod to the pseudopod.[11] It appears that adhesion provides the frictional forces required for translocation and is a vital characteristic of neutrophils.

Techniques Assessing Neutrophil Adherence

In vivo assessments of neutrophil adherence have relied upon direct observations of marginated neutrophils or upon counting the numbers of circulating neutrophils. The assumption was made that factors decreasing the numbers of circulating neutrophils increased

the numbers of neutrophils in the marginal pool due to increased neutrophil adhesiveness. Several in vitro techniques have developed to assess neutrophil adherence to glass and nylon.[13] Most of the in vitro techniques allow for neutrophil attachment to the surface and the numbers of cells that detach as external forces were applied reflect neutrophil adhesiveness. The forces used to detach the cells include centrifugation, elution off a column, shaking, and rotation. The external forces may promote cell shearing, destruction and aggregation, thereby confusing evaluations of adhesiveness. Smith et al. have recently described a technique to assess neutrophil adherence to glass without using external forces.[30] O'Flaherty and coworkers have developed a technique to assess cell to cell adhesion or aggregation by counting the numbers of cells passing through a sizing aperture or by using a platelet aggregometer.[26,27] The experimental conditions of the adherence techniques are extremely different from the conditions used to evaluate neutrophil motility (i.e., substrate, cell distribution, external forces, chemotactic gradient). Therefore, correlations of effects on adherence to motility may have little meaning.

Control of Adherence Mechanisms

Very little is understood about the mechanism of adherence or agents that influence this mechanism. The nature of the binding site to glass also remains speculative. Carter (1965) and Harris (1973) demonstrated that substratum adhesiveness was a determining factor for translocation.[7,18] Grant and Epstein (1974) damaged the vascular endothelium with laser beams and produced margination, suggesting that the damaged endothelial cells became "sticky."[11] However,

several workers have implied that chemotactic factors increased neutrophil adhesiveness.[26,27,30] Smith et al. (1978) have demonstrated that incubation of neutrophils with high concentrations of chemotactic factors irreversibly increased cellular adherence.[30] Keller and Smith demonstrated that albumin reduced neutrophil adherence to glass.[19,30] McGregor has shown that anti-inflammatory drugs decrease adherence and a plasma factor augments adherence.[13,23] Bryant et al. have shown that c-AMP and prostaglandins decreased neutrophil adherence.[6] Kvarstein and Smith have demonstrated that neutrophil adherence was dependent on magnesium ions.[21,30]

It appears that the substrate or cellular adhesiveness may be affected by various agents. Not surprising is the fact that many of the substances shown to influence adherence also affected neutrophil translocation. It is surprising, however, that no technique exists to assess neutrophil adhesiveness to micropore filters when the bulk of information about neutrophil locomotion has been derived from Boyden assays.

MATERIALS AND METHODS

Reagents

All reagents were reagent grade and adjusted to pH 7.3. Ficoll, glutaraldehyde, cacodylic acid, sodium heparin sulfate, casein, human serum albumin (HSA), crystallized 1 time and bovine serum albumin (BSA) crystallized 4 times were obtained from Sigma Chemical Company, St. Louis, Missouri. Hypaque was purchased from Winthrop Laboratories, New York, New York. The blind well chambers were purchased from Neuroprobe Incorporated, Bethesda, Maryland. The micropore filters and Swinny adaptors (18mm. diameter) were obtained from Millipore Corporation, Bedford, Massachusetts. Zymosan was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, and fMetPhe from Andeulis Research Corporation, Bethesda, Maryland. Hanks balanced salt solution (HBSS) was obtained from Gibco, Grand Island, New York. The C5a was a generous gift from Dr. Richard A. Patrick, prepared by Clare Hassett in the Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan.

Preparation of Chemotactic and Chemokinetic Solutions

Zymosan activated serum (ZAS) was prepared by incubating 10 mg of zymosan with 1 ml of fresh human serum for 30 minutes at 27C and then diluting this mixture 10 fold with HBSS. The zymosan particles were removed by centrifugation.

The formylated peptides were dissolved in HBSS to a concentration

of 10^{-3}M and diluted further with HBSS to desired concentrations.

Casein was dissolved in 1N NaOH, diluted to desired concentrations with HBSS, and adjusted to pH 7.3 with HCl.

A low molecular weight chemotactic factor was prepared from activated human serum by the method of Gallin, et al.[12] Briefly, human serum was incubated with 0.1 mg/ml E. coli lipopolysaccharide at 37C for 1 hour. The serum was then heated at 56C for 30 minutes. After rapid cooling, the activated serum was layered on a Sephadex G-75 column (90 x 5 mm). The column was equlibrated with calcium and magnesium free HBSS by descending flow at 4C. The column was calibrated with the following substances: blue dextran, molecular weight (MW) 2 X 10^6; cytochrome C, MW 12,384; ribonuclease A, MW 13,700; chymotrypsinogen, MW 25,000; and ovalbumin, MW 45,000. Fractions in the 20,000 to 10,000 MW range were assayed in modified Boyden chambers and those showing chemotactic activity were pooled and frozen at -70C. The pool containing chemotactic activity had 40 µg of protein/ml. This reagent will be referred to as C5a.

Human serum albumin and BSA were dissolved in HBSS and all solutions were adjusted to pH 7.3 with NaOH.

Isolation of Human Neutrophils

Blood samples were obtained from 6 healthy adult volunteers (3 men and 3 women). Blood was collected in plastic syringes and placed in heparinized (15 units/ml blood) tubes. Dextran (6%) was mixed with the samples to enhance red blood cell sedimentation. After approximately 45 minutes at room temperature, the leukocyte rich plasma was removed and placed in 17 mm siliconized tubes. The

leukocyte rich plasma was diluted with an equal volume of HBSS, centrifuged at 500 G, and the plasma and HBSS removed by suction. The cell button was resuspended in 4 ml of HBSS and centrifuged at 800 G for 30 minutes on a Ficoll-Hypaque solution (4 ml) 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. When the Ficoll-Hypaque solution and HBSS were removed by suction, the cells in the cell button were resuspended in HBSS and differentially counted. This cell suspension contained greater than 98% polymorphonuclear leukocytes of which approximately 95% were neutrophils. No platelets were seen in the preparations and the red blood cell to neutrophil ratio was consistently less than 2:1. Neutrophil viability was greater than 98% as determined by eosin exclusion.

Assessment of Neutrophil Motility

Neutrophil motility was tested by a modified Boyden technique using blind well chambers (Figure 1a). The chambers were prepared by placing cells (2×10^4 neutrophils/mm^2 of exposed filter) suspended in various reagents in the cell or upper compartment. The cells then settled onto a micropore filter (3 μm pore size) which separated the cell compartment from the stimulus compartment. Various reagents were placed into the stimulus compartment, and the concentration differences of reagents in the two compartments established a concentration gradient in the filter. After the chambers were prepared, they were incubated at 37C in an atmosphere of 5% CO_2 and high humidity. Incubation times were controlled for each experiment such that cells

Figure 1a: Vertical cross section of a blind well chamber. Magnification 5X.

Figure 1b: Vertical cross section of a slide chamber. Magnification 12X.

SCHEMATIC CROSS SECTION OF A
BLIND WELL CHAMBER.

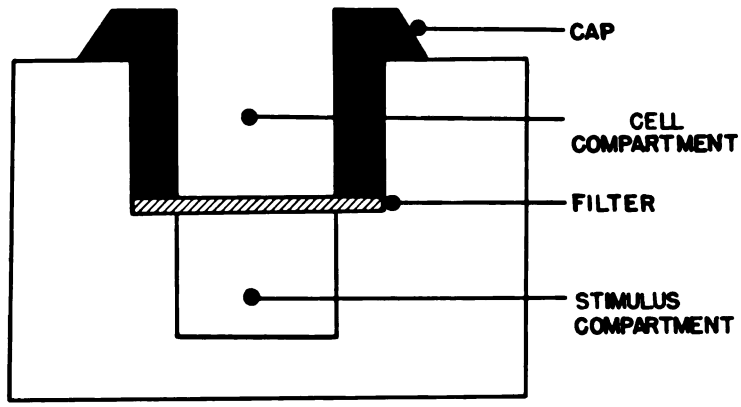

Figure 1a.

SCHEMATIC CROSS SECTION OF A
SLIDE CHAMBER.

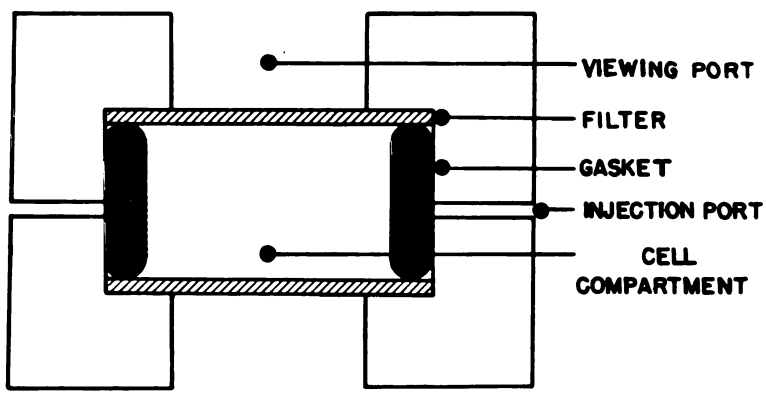

Figure 1b.

migrated into or completely through the filters. (Migration from the top of the filter to the bottom of the filter). These time intervals varied and are specified for each experiment. The experiment was terminated after the incubation period by removing the filter, rinsing it in absolute methanol to fix the cells present and staining it with hematoxylin. Hematoxylin stained the cells, but not the filter. The filters were then soaked in xylene to make them transparent. Migrational behavior in the filters was assessed as follows. The depth of migration was determined microscopically. When neutrophils migrated completely through the filters, the cells in 10 randomly chosen 40X microscopic fields on the bottom surface of the filter were counted. Each experiment was duplicated and counts averaged. When neutrophils did not migrate completely through the filter, migration was assessed by 2 methods as previously described by Zigmond and Hirsch.[35,36] 1) Briefly, the distance of neutrophil penetration was determined by measuring the distance (μm) from the cell origin (top of the filter) to which only 2 cells remained in focus ("leading front"). In each experiment, the leading front was determined by averaging 5 measurements for each of duplicate filters. 2) The distribution of cells in the filter was determined by counting the number of cells/40X microscopic field at 20 μm intervals through the filter. At least 3 determinations were made for each of duplicate filters and averaged for each experiment.

Neutrophil migration was assessed when varying concentrations of chemotactic or chemokinetic solutions were placed above and below the filter or used to pretreat the neutrophils or filters.

Assessment of Neutrophil Adhesiveness

The interaction of neutrophils with 2 surfaces, glass and micropore filters, were evaluated using 2 techniques. 1) Slide chambers (Figure 1b) similar to those reported by Lichtman et al.[24] 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 with a 50X oil immersion objective at room temperature. The numbers of neutrophils on the surface were counted in 5 randomly selected 50X microscopic fields 350 to 500 seconds after injecting cells into the chamber. The chamber was then inverted and the unattached cells allowed to fall off the surface. After 1000 seconds, cells remaining attached to the glass surface were again counted. Each experiment was performed in duplicate. The comparison of counts of cells remaining attached to the glass surface with the initial cell count was expressed as percent adherent cells. Adherence was assessed when reagents were in the cell suspension, used to pretreat the cells, or used to pretreat the glass coverslips. 2) Blind well chambers were filled with 0.5 ml of cell suspension (5 X 10^5 neutrophils/ml of various reagents) in the cell compartment and HBSS or various reagents in the stimulus compartment. The chambers were incubated for 5 minutes at room temperature as the neutrophils settled onto cell impermeable micropore filters (0.22 um pore size). Hanks' balanced salt solution was gently added over the cell suspension until the cell compartment was completely filled. An 18 mm glass coverslip was placed over the cell suspension (insuring that no air bubbles were introduced) to seal the cell compartment. After an additional 15

minute incubation at room temperature, the chambers were inverted, allowing the unattached cells to fall off the filter. The filter was removed after 20 minutes, and the cells attached to the surface counted. The cells in five 50X oil objective fields were counted on each of duplicate filters for each experiment. The counts of cells remaining attached under various conditions were compared to counts in HBSS remaining attached to untreated filters. Since virtually 100% of the neutrophils remained attached to untreated filters when in HBSS, this comparison was expressed as the percentage of adherent cells. Adherence was assessed when reagents were in a gradient, used to pretreat the cells, or used to pretreat the filters.

Assessment of Changes in Neutrophil Shape

A modification of the method of Lichtman et al.[24] was used. Neutrophils (10^6 neutrophils/ml of reagent) were exposed to various reagents. The pH was adjusted to 7.3 using 5% CO_2 in air. After a 30 minute incubation time with these reagents, the cells were added dropwise to 10 ml of cold (4C) glutaraldehyde (1%) in 0.1M cacodylic acid. 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 0.1 ml of distilled water. The neutrophils were examined by using a 100X phase contrast objective and they were classified either as round or motile (Figure 2).

Pretreatment of Micropore Filters and Glass Coverslips

Glass coverslips used in the slide chamber were pretreated with various agents. Unless otherwise specified, the coverslips were

Figure 2: The morphological alterations of neutrophils when exposed to various solution. Magnification 1000X. Cells similar to those in picture (a) were classified as round, while those similar to the neutrophil in picture (b) as motile.

Figure 2(a).

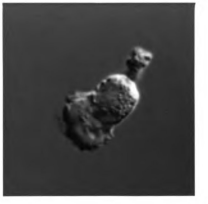

Figure 2(b).

incubated in the pretreated solution for 2 minutes, removed and washed in 3 changes of HBSS. If 2 solutions were used in pretreatment, the coverslips were washed after each incubation. Filters used in the blind well chamber were also pretreated. The filters were incubated in various solutions for 4 minutes (2 minutes/side), placed in a Swinny adaptor, and washed with 20 ml of HBSS. If 2 pretreatment solutions were used, the filters were rinsed in HBSS after the initial incubation and washed after the second incubation.

Pretreatment of Neutrophils with Chemotactic Factors

Neutrophil suspensions were placed in glass tubes coated with serum and centrifuged at 500 G for 10 minutes. The supernate was removed by suction. Chemotactic or control solutions were added to the cell button, mixed, and subsequently incubated at 37C for 30 minutes. After incubation, the treated neutrophils were washed 3 times with 5 ml HBSS and resuspended in HBSS to the desired working concentration. The concentrations of chemotactic factors used to treat the neutrophils were 0.1 ml of 10^{-5}M fMetPhe or 40 ug of protein/ml of C5a per 10^{6} neutrophils.

Presentation and Analysis of Data

The data are expressed in terms of the mean ± standard error of the mean; n represents the number of duplicate experiments. Students' t test was used to assess significance.

Zigmond and Hirsch have presented leading front data in the form of a "grid." This presentation was used to differentiate random chemotactic, and chemokinetic migration, as defined by them.[35,36]

RESULTS

Agents Assessed for Chemokinetic and Chemotactic Activity

Chemotactic and chemokinetic activity was assessed by placing varying concentrations of the reagents in the cell and stimulus compartment of the blind well chamber and assessing neutrophil migration by grid analysis of the leading front data. Albumin solutions (BSA and HSA) were chemokinetic, since the albumin solutions enhanced migration to the same extent whether above or below the filter (Table 1). Zymosan activated serum, C5a, and fMetPhe were chemotactic, producing results similar to those presented for solutions of casein (Table 2). Enhanced migration was more pronounced when casein was below the filter.

Smith, et al. have reported that neutrophils form motile configurations after exposure to chemotactic factors (C5a, ZAS, or fMetPhe), but do not do so after exposure to chemokinetic BSA.[30] Human serum albumin did not significantly affect the cellular morphology whereas solutions of casein did (Table 3).

Effect of Albumin on Neutrophil Adherence and Migration

Albumin is often required in the medium while assessing random or chemotactic neutrophil migration in Boyden assays. The reason(s) for this requirement are unknown. The following experiments were designed to determine the effects generated by the addition of albumin

Table 1. The effects of bovine or human serum albumin (BSA or HSA respectively) in a gradient on neutrophil migration

HSA concentration in the cell compartment (mg/ml)	HSA concentration in the stimulus compartment (mg/ml)			
	0	3.5	35	140
0	26*	32	42	76
3.5	33	35		
35	67		80	
140	88			90
BSA concentration in the cell compartment (mg/ml)	**BSA concentration in the stimulus compartment (mg/ml)**			
	0	10	30	50
0	41*	61	88	115
10	75	100	110	
30	87	120	119	
50	106			128

*Numbers represent the distances of migration (μm) as determined by the leading front technique with 60 or 90 minute incubations for HSA or BSA respectively.

Table 2. Effects of casein in a gradient on neutrophil migration

Casein concentration in the cell compartment (mg/ml)	Casein concentration in the stimulus compartment (mg/ml)				
	0	0.1	1	5	10
0	25*	29	56	110	120
0.1	33	32			
1	56		65		
5	70			85	
10	60				79

*Numbers represent distances of migration (mμ) as determined by the leading front technique; incubation 45 minutes.

Table 3. Alterations of neutrophil morphology when the cells were suspended in various reagents*

Solution in Cell Suspension	Round $\bar{x} \pm$ SEM	Motile $\bar{x} \pm$ SEM	n	p
Hanks' Buffer	90 ± 3	10 ± 3	3	
Human Serum Albumin (35 mg/ml)	88 ± 5	12 ± 5	3	> 0.5
Casein (1 mg/ml)	13 ± 5	87 ± 5	3	< 0.01

*The morphology of the neutrophile in suspension was determined microscopically (50X oil objective) after fixing the cells with glutaraldehyde; cells were classified as round or, when pseudopids and uropods present, as motile.

to Boyden assays.

Neutrophil adherence and the distances of neutrophil migration on filters treated with albumin were assessed by the leading front technique (Figure 3). Increasing the concentrations of albumin used to pretreat the filters enhanced migration but decreased neutrophil adherence. The distribution of cells in the treated filters was also determined (Figure 4). Increasing the concentrations of albumin to treat the filters increased the numbers of neutrophils getting into the filters.

The effects of adding albumin to the cell suspension on neutrophil migration were determined previously (Table 1). In addition, albumin was placed in the cell suspension and adherence to filters was determined. Neutrophil adherence and leading front measurements were similar when albumin was used to treat the filters or placed in solution with the cells (Figure 5).

The effects of albumin on neutrophil adherence to glass were determined using the slide chamber technique. Glass coverslips were pretreated with the same solutions used to pretreat the filters. Cell adherence was significantly decreased when albumin was used to treat the coverslips (Table 4). The addition of albumin to the cell suspension also significantly reduced adherence to untreated glass (Table 4).

Albumin has been shown to be required in the cell suspension to assess neutrophil migration in response to several chemotactic solutions, including C5a and fMetPhe.[34] Micropore filters (3 μm pore size) were soaked in solutions of albumin, albumin then fMetPhe, or HBSS and placed in blind well chambers. Cells were placed in the cell

Figure 3. The effects of pretreating micropore filters with solutions of human or bovine serum albumin (HSA or BSA respectively) on neutrophil adherence and random migration. The filters were pretreated with albumin solutions or Hanks' buffer (HBSS) for 4 minutes and washed by passing 20 ml of HBSS through the filter. Top panel presents the distance of migration determined by the leading front technique with 120 or 75 minutes incubation for filters treated with BSA or HSA respectively. Lower panel presents the adherence of neutrophils to filters pretreated with albumin relative to the adherence to filters pretreated with HBSS. The number above each bar is the number of duplicate determinations and the vertical line represents ± 1 SEM. All measurements within each of the 4 groups are significantly different ($p < 0.02$).

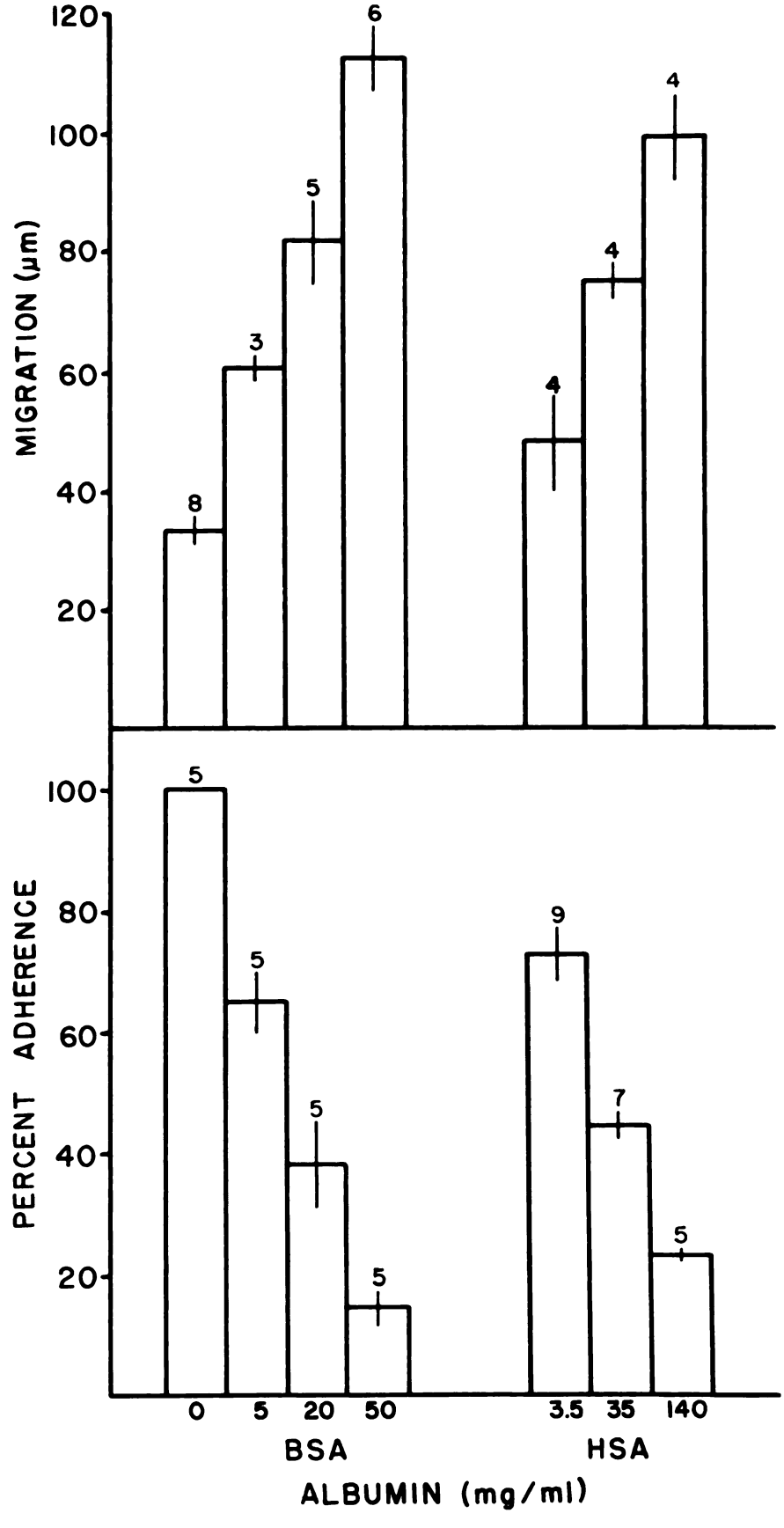
MIGRATION (μm)
120
100
80
60
40
20
8
3
5
6
4
4
4
PERCENT ADHERENCE
100
80
60
40
20
5
5
5
5
9
7
5
0
5
20
50
3.5
35
140
BSA
HSA
ALBUMIN (mg/ml)

Figure 3.

Figure 4: The effects of pretreating micropore filters with solutions of albumin (BSA) on neutrophil distributions. Neutrophil distribution was assessed after neturophils had migrated into treated filters for 120 minutes. Filters were incubated in solutions of albumin or HBSS for 4 minutes and washed by passing 20 ml of HBSS through the filters. The solid dots represent the mean number of neutrophils counted/40X microscopic field at the specified distances into the filters from the cell origin. Each dot represents the mean for 10 counts. The numbers to the left of the first count (30 μm into the filter) for each curve are the albumin concnetrations (mg/ml) used to treat the filters.

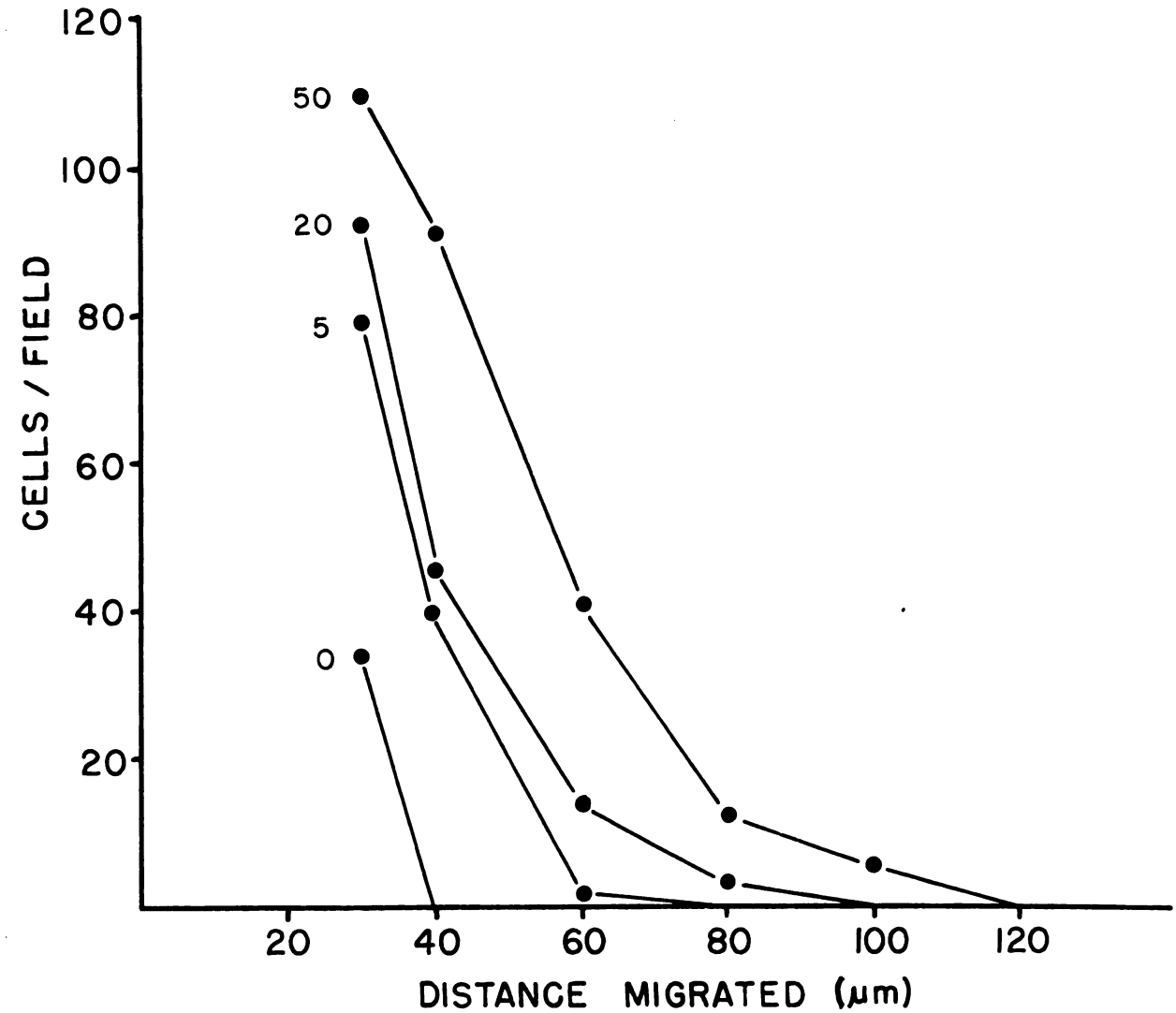
120
100
80
60
40
20
CELLS / FIELD
50
20
5
0
20
40
60
80
100
120
DISTANCE MIGRATED (μm)

Figure 4.

Figure 5: The effects of bovine serum albumin (BSA) on neutrophil adherence when used to treat micropore filters or when placed in the cell suspension. The dashed lines represent filters pretreated with albumin solutions and the solid lines are with albumin in the cell suspension. The distances neutrophils migrated (top graph) were determined by the leading front technique with incubation times of 120 minutes for experiments using filters pretreated with BSA (incubated for 4 minutes and washed by passing 20 ml of Hands' buffer through the filters) or 90 minutes when BSA was in the cell suspension. Adherence (bottom graph) to filters pretreated with BSA or with BAS in solution is relative to the adherence to filters in the absence of albumin. The solid dots are the means of measurements from duplicate experiments and the vertical lines are ±1 SEM.

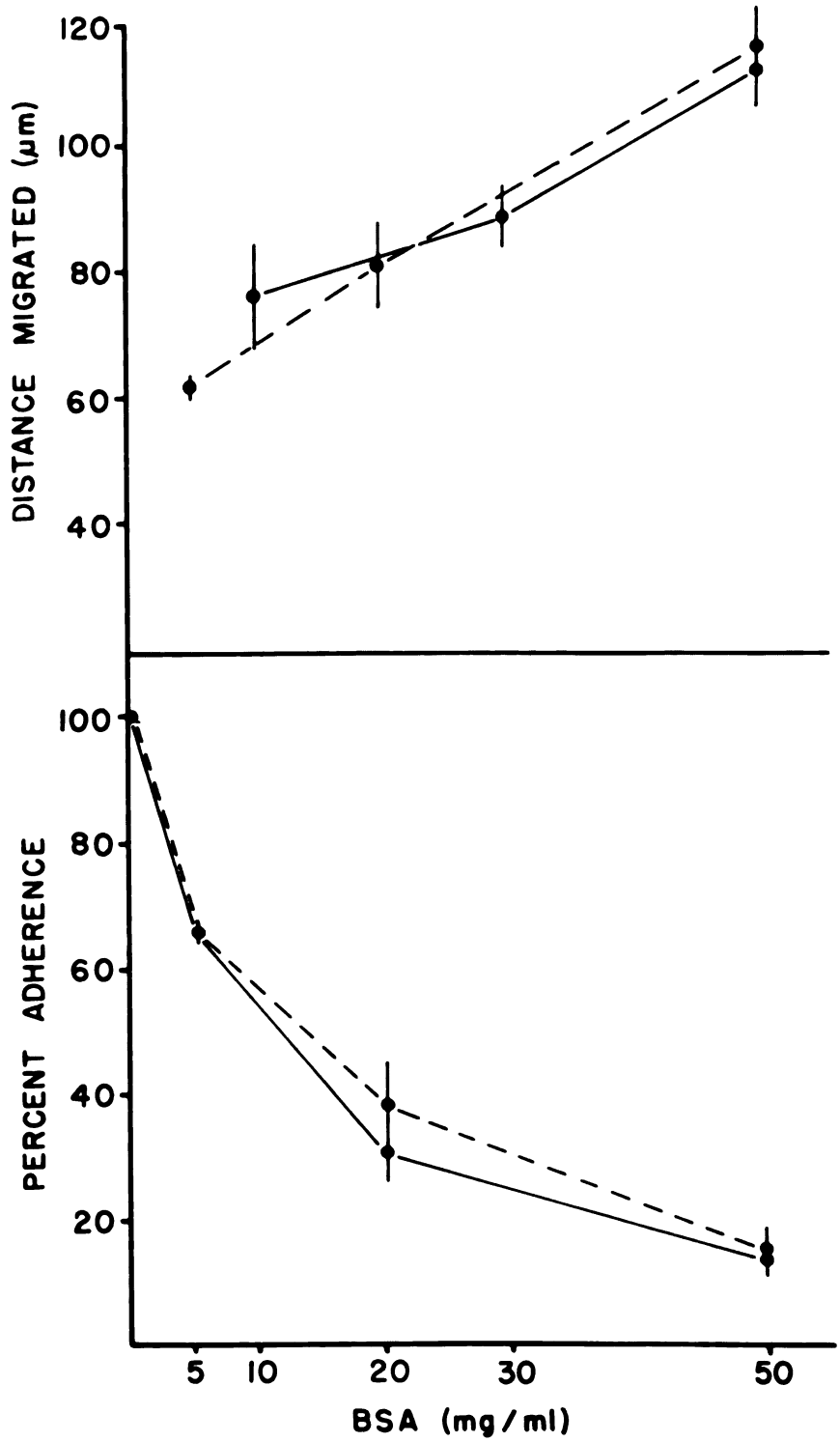
DISTANCE MIGRATED (μm)
120
100
80
60
40
PERCENT ADHERENCE
100
80
60
40
20
5
10
20
30
50
BSA (mg/ml)

Figure 5.

Table 4. The effects of bovine or human serum albumin (BSA or HSA respectively) on neutrophil adherence to glass

Glass pretreatment*	PMN suspended in	Glass adherence** % ± SEM	n
HBSS	HBSS	100	4
HBSS	BSA (20mg/ml)	79 ± 5***	3
HBSS	HSA (35mg/ml)	30 ± 9***	3
BSA (20mg/ml)	HBSS	71 ± 10***	4
HSA (35mg/ml)	HBSS	50 ± 7***	3

*Glass coverslips were incubated in the specified solutions for 2 minutes and washed X3 in Hanks' buffer (HBSS).

**Adherence was determined by the slide chamber technique.

***Significantly different from control values ($P < 0.05$).

compartment and fMetPhe or HBSS in the stimulus compartment. A chemotactic response to fMetPhe was observed only with filters pretreated with albumin and fMetPhe did not affect migration when allowed to bind to micropore filters (Table 5).

The Effects of Pretreating Neutrophils with Chemotactic Factors on Adherence and Migration

Previous experiments have shown that incubation of neutrophils with high concentrations of chemotactic factors significantly enhance cell attachment to glass coated with ablumin.[30] Cells preincubated with chemotactic solutions have also been shown to be less responsive when migration was assessed in chemotaxis chambers.[32] To expand these observations, adherence and random migration of neutrophils preincubated

Table 5: The effects of pretreating micropore filters with bovine serum albumin on the chemotactic response of neutrophils to formylated peptides (fMetPhe)

Filter pretreatment*	Stimulus compartment	Distance of neutrophil migration** (μm ± SEM)	n
HBSS	HBSS	28 ± 1	8
HBSS	fMetPhe (10^{-6}M)	27 ± 2**	3
fMetPhe (10^{-6}M)	HBSS	28 ± 2**	3
BSA (20mg/ml)	HBSS	53 ± 5	8
BSA (20mg/ml)	fMetPhe (10^{-6}M)	83 ± 4***	8
BSA (20mg/ml) then fMetPhe (10-6M)	HBSS	55 ± 7****	4

*Filters were incubated with the sepcified solutions for 4 minutes and washed by passing 20 ml of HBSS through the filters. Hanks' buffer was abbreviated as HBSS.

**Mean distances of migration determined by the leading front technique; incubations, 45 minutes.

***Not significantly different from control values ($P > 0.5$).

****Significantly different from control values ($P < 0.01$).

in chemotactic solutions were assessed in the blind well chamber using filters pretreated with solutions of albumin. Neutrophils pretreated with fMetPhe (10^{-5}M) did not migrate as far as control cells preincubated in HBSS (Figure 6, top panel), and they were more adherent (Figure 6, bottom panel). Increasing the albumin concentration used to pretreat the filters reduced adherence and enhanced migration of cells preincubated in fMetPhe or HBSS. The adherence and leading front data derived from preincubating cells in fMetPhe and exposing them to filters treated with albumin at concentrations of 50 mg/ml were not significantly different from results obtained when cells preincubated in HBSS were exposed to filters pretreated with albumin at concentrations of 20 mg/ml. Results similar to those in Figure 4, for migration and adherence were obtained when neutrophils were preincubated with 40 μg of protein/ml of C5a (43±5 μm and 68±4% respectively using filters treated with 20 mg/ml of albumin).

Since random migration of neutrophils preincubated with C5a or fMetPhe was indistinguishable, the responsiveness of the preincubated cells to a second chemotactic stimulus was assessed. The formylated peptides (10^{-6}M) were placed in the stimulus compartment and the migration of cells preincubated in fMetPhe or C5a on filters treated with albumin was assessed by the leading front technique. Unlike random migration, significant differences were noted in the chemotactic migration of preincubated neutrophils (Figure 7). Minimal mirgation in response to fMetPhe was observed for cells preincubated in fMetPhe. Though the response of cells preincubated in C5a was significantly increased, it was not as high as in control cells preincubated in HBSS. When the concentration of albumin used to pretreat

Figure 6: The effects of pretreating micropore filters with Bovine serum albumin (BSA) on the adherence and random migration of deactivated neutrophils. Neutrophils were incubated in 10^{-5}M fMetPhe (hatched bars) or Hanks' buffer (HBSS) (open bars) for 30 minutes at 37°C and washed X3 with HBSS. Micropore filters were incubated with albumin or HBSS for 4 minutes and washed by passing 20 ml of HBSS through the filter. The top panel shows the distances neutrophils migrated which were determined by the leading front technique with 90 minute incubation. The bottom panel shows the adherence measurements relative to the adherence of neutrophils incubated in HBSS to filters treated with HBSS. The numbers above the bars are the numbers of duplicate experiments and the vertical lines represent ±1 SEM. All measurements for each group of experiments (i.e. cells pretreated with HBSS or fMetPhe) for adherence or the distances of migration are significantly different.

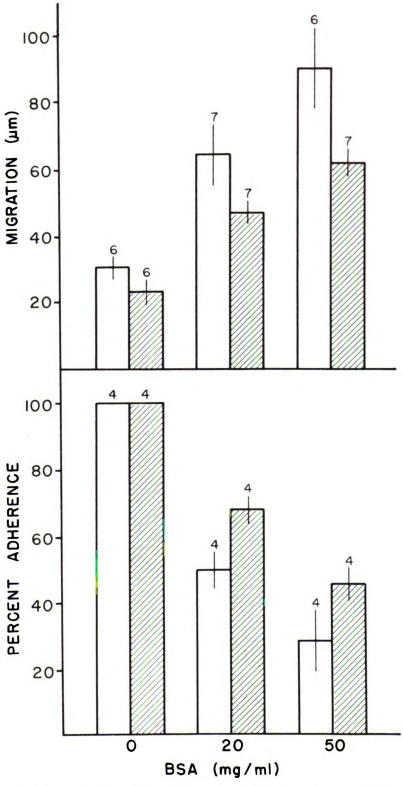
MIGRATION (µm)
100
80
60
40
20
6
6
7
7
6
7
PERCENT ADHERENCE
100
80
60
40
20
4
4
4
4
4
4
0
20
50
BSA (mg/ml)

Figure 6.

Figure 7: The effects of pretreating micropore filters with Bovine serum albumin (BSA) on the chemotactic migration of deactivated neutrophils towards fMetPhe. Neutrophils were incubated in fMetPhe (open bars, C5a (hatched bars), or Hanks' buffer (HBSS) (closed bars) at 37°C for 30 minutes and washed X2 with HBSS. The bars are the distances of migration as determined by the leading front technique with 45 minutes incubation. Albumin solutions were incubated with micropore filters for 4 minutes and then the filters were washed by passing 20 ml HBSS through the filters. The number above each bar is the number of duplicate experiments and the vertical line is ±1 SEM. All measurements for each of the 2 groups are significantly different ($P < 0.01$).

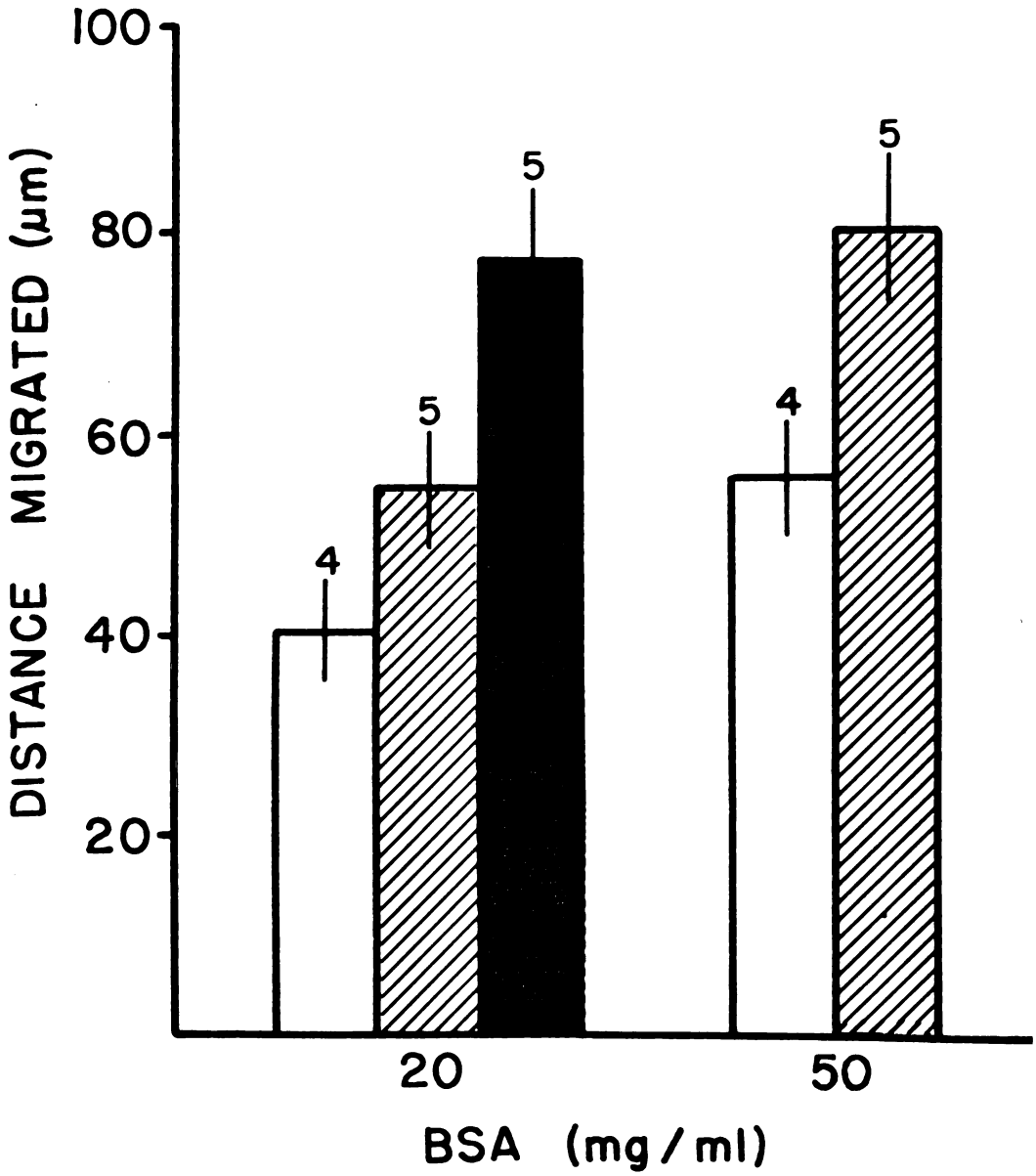

Figure 7.

the filters was increased to 50 mg/ml, the distances of chemotactic migration for cells preincubated in either chemotactic factor increased. However, only for cells preincubated in C5a and placed on filters treated with 50 mg/ml of albumin were the distances of migration comparable to control cells preincubated in HBSS on filters treated with 20 mg/ml of albumin (Figure 7).

Effects of Pretreating Micropore Filters and Glass with Chemotactic Solutions on Neutrophil Adherence and Migration

Micropore filters were pretreated with various chemotactic solutions or HBSS, washed, and placed in the blind well chamber. As shown in Table 6, filters pretreated with solutions of serum, ZAS, or casein had significantly decreased numbers of attached cells. Migration in these filters was also significantly increased. Only two chemotactic solutions (fMetPhe and C5a) when allowed to bind to the filter surface did not affect neutrophil adherence or migration. Pretreatment of the filters with serum produced essentially the same results as pretreating with human serum albumin (Figure 8). These effects remained when serum was heated at 56C or when heparin (20 U/ml) was added to the serum solution.

Adherence to glass pretreated with chemotactic solutions was determined with the slide chamber technique. The adherence of neutrophils suspended in HBSS to glass coated with solutions of serum, ZAS, and casein was significantly reduced (Table 6).

The Effects of Chemotactic Factors in Solution on Neutrophil Adherence and Migration

Pretreatment of filters or glass with chemokinetic solutions (albumin) produced similar effects on adherence and migration to

Table 6: The effects of pretreating micropore filters with chemotactic solutions on neutrophil adherence and migration

Substrate pretreatment*	Glass adherence % ± SEM**	n	Filter adherence % ± SEM**	n	Distances migrated μm ± SEM***	n	p****
HBSS	100	5	100	6	30 ± 4	8	
fMetPhe (10^{-6}M)	100 ± 2	3	97 ± 2	3	29 ± 3	3	
C5a 30 μg protein/ml)	97 ± 4	3	101 ± 3	3	27 ± 3	3	
Serum (10%)	30 ± 5	3	53 ± 8	6	66 ± 6	5	****
ZAS (10%)	26 ± 2	3	55 ± 11	4	63 ± 6	4	****
Casein (1mg/ml)	33 ± 7	3	31 ± 8	4	63 ± 7	4	****

*Glass xoverslips were incubated for 2 minutes in the specified solutions and washed X3 in HBSS; filters were incubated in these solutions for 4 minutes and washed by passing 20 ml of Hanks' buffer (HBSS) through the filters. Zymosan activated serum was abbrwviated as ZAS.

**Neutrophil adherence to glass was determined by the slide chamber technique and filter adherence by the new modified Boyden assay.

***The distances of migration were determined by the leading front technique; incubation times were 60 minutes with fMetPhe or C5a, 90 minutes for serum and ZAS, and 45 minutes for casein.

****Significantly different ($P < 0.01$) from control values (HBSS pretreatment) for all parameters measured.

Figure 8: The effects of pretreating micropore filters with solutions of human serum albumin (HSA) or serum on neutrophil adherence and migration. The filters were incubated in solutions of serum, HSA, or Hanks' buffer (HBSS) for 4 minutes and washed by passing 20 ml of HBSS through the filters. The solid lines are the distances neutrophils migrated as determined by the leading front technique with incubation times of 90 or 75 minutes for experiments using filters treated with serum or HSA respectively. The dashed lines refer to the adherence of neutrophils to filters treated with serum or HSA relative to the adherence to filters treated with HBSS. The solid dots represent measurements using filters pretreated with serum (n = 3) and the open dots are measurements with filters pretreated with HSA solutions (n = 3). The concentrations of serum used to pretreat the filter were based on estimates of albumin concentrations in normal serum.

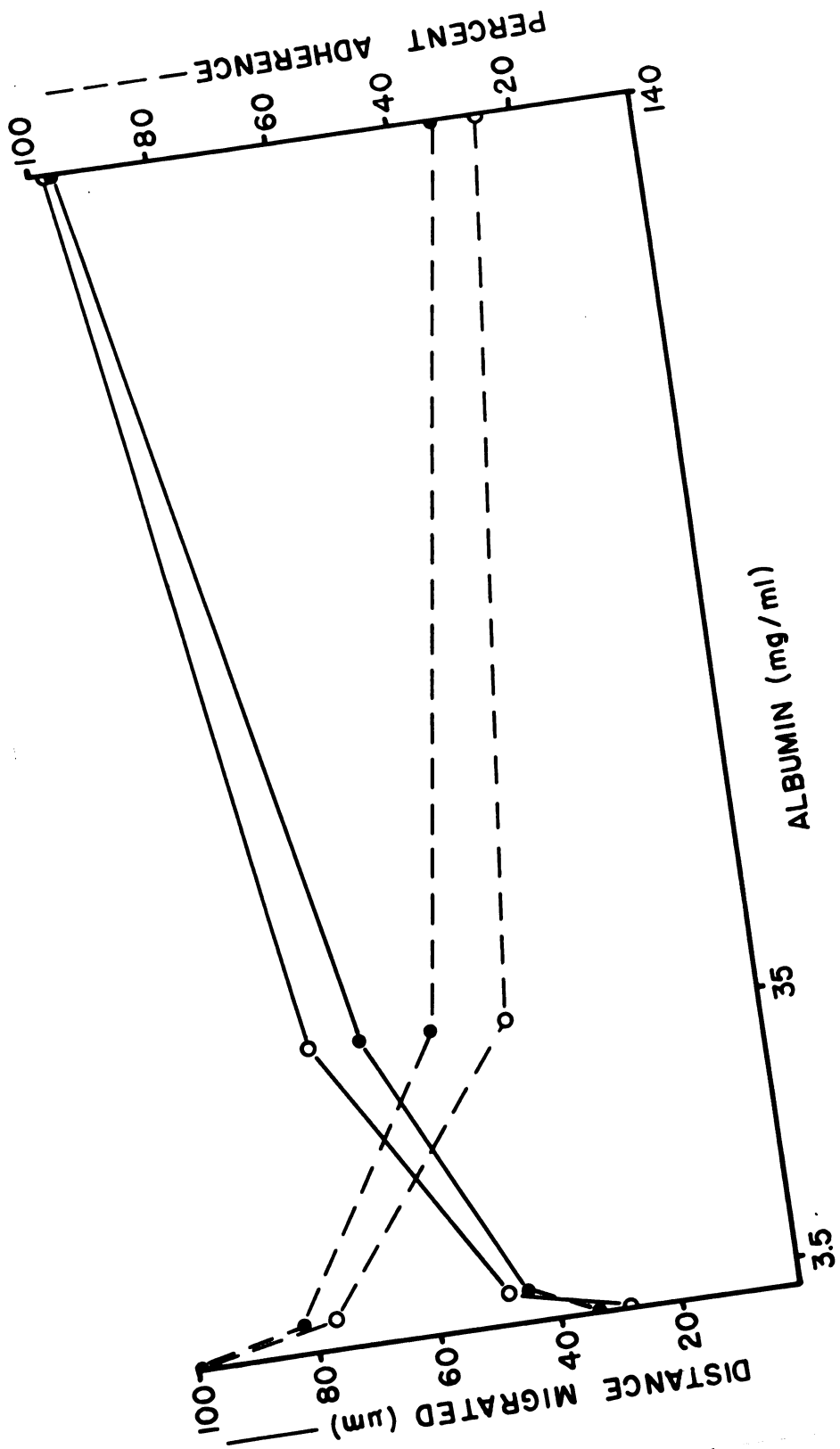
PERCENT ADHERENCE
100
80
60
40
20
140
ALBUMIN (mg/ml)
35
3.5
DISTANCE MIGRATED (μm)
100
80
60
40
20

Figure 8.

solutions of serum, ZAS, and casein which are chemotactic. Experiments were designed to assess adherence and migration with the chemotactic factors in the cell or stimulus compartments of the blind well chamber. The first chemotactic factors tested were fMetPhe and C5a because of our observations that these agents did not affect cell behavior when used to coat the filter. Micropore filters were coated with albumin since chemotactic migration in response to C5a and fMetPhe did not occur in the absence of albumin as demonstrated in earlier experiments. In either compartment, both chemotactic factors significantly increased neutrophil migration and adherence (Table 7). Adherence and migration were greater when the chemotactic factor was in the stimulus compartment. The levels of adherence were not as high as observed when chemotactic factors were preincubated with the cells (Figure 6).

The other chemotactic solutions (serum, ZAS, and casein) were tested in a similar manner with the exception that the filters were pretreated with the chemotactic factors instead of albumin. The concentrations of chemotactic factors used to pretreat the filters were determined as sufficient to prevent additional binding of the chemotactic factor to the filter. The results show that all the chemotactic factors tested significantly increased adherence and migration when in solution (Table 7).

The effects produced by serum in the stimulus compartment were particularly interesting. The migrational responses (leading front measurements) for serum as a chemotactic agent in the stimulus compartment were similar to those produced by ZAS. However, the

Table 7. The effects of chemotactic solutions in the gradient on neutrophil adherence and migration

Filter* pretreatment	Cell compartment	Stimulus compartment	Filter** adherence % ± SEM	n	Distance*** migrated µm ± SEM	n	p****
BSA (20mg/ml)	HBSS	HBSS	50 ± 7	8	58 ± 4	8	
" "	fMetPhe (10^{-6}M)	HBSS	78 ± 4	6	77 ± 4	4	****
" "	HBSS	fMetPhe (10^{-6}M)	84 ± 5	8	83 ± 8	8	****
" "	C5a (30 µg protein/ml)	HBSS	75 ± 6	3	71 ± 6	3	****
" "	HBSS	C5a (30 µg protein/ml)	84 ± 9	3	80 ± 3	3	****
Serum (10%)	HBSS	HBSS	53 ± 5	6	40 ± 3	5	
" "	Serum (10%)	HBSS	69 ± 2	3	83 ± 3	3	****
" "	HBSS	Serum (10%)	76 ± 5	3	86 ± 7	3	****
ZAS (10%)	HBSS	HBSS	55 ± 4	2	42 ± 5	2	
" "	ZAS (10%)	HBSS	82 ± 2	2	75 ± 12	2	****
" "	HBSS	ZAS (10%)	95 ± 3	4	83 ± 7	4	****
Casein (1mg/ml)	HBSS	HBSS	31 ± 8	3	53 ± 3	3	
" "	Casein (1mg/ml)	HBSS	61 ± 3	3	70 ± 8	3	****
" "	HBSS	Casein (1mg/ml)	88 ± 11	3	113 ± 12	3	****

*Filters were incubated in the specified solutions for 4 minutes and washed by passing 20ml of HBSS through the filters. Abbreviations of reagents used to treat the filter are; bovine serum albumin (BSA), zymosan activated serum (ZAS), and Hanks' buffer (HBSS).

**Adherence was determined by a new modification of the Boyden assay.

***The distances of migration were determined by the leading fron technique; incubation, 45 minutes.

****Significantly different ($P < 0.05$) compare to control volues for adherence and migration.

distribution of cells responding to ZAS was greater (Figure 9). When seurm was heated at 560 for 30 minutes or when heparin (20 U/ml) was added to the serum solution and placed in the cell suspension, adherence to glass was reduced. Heating serum or the addition of heparin to serum in the cell suspension also increased the number of neutrophils migrating through the filter (Table 8).

Table 8: The effects of heating serum or the addition of heparin to serum on neutrophil adherence and migration

Cell compartment	Stimulus compartment	Glass* adherence % ± SEM	Cell** counts $\bar{x}$ ± SEM	n	p
Serum (10%)	Serum (10%)	--	156 ± 40	4	--
Heated Serum*** (10%)	Serum (10%)	--	612 ± 85	4	0.001
Serum (10%) and Heparin (20 U/ml)	Serum (10%)	--	572 ± 71	4	0.001
Serum (10%)	--	81 ± 4	--	14	--
Heated Serum *** (10%)	--	25 ± 5	--	4	0.001
Serum (10%) and Heparin (20 U/ml)	--	24 ± 5	--	13	0.001

*Adherence determined in the slide chamber with untreated coverslips.

**Cell counts determined by counting the cells on the bottom surface of the filter in 10 microscopic fields; incubation 3 hours.

***Serum was heated at 56C for 30 minutes.

Figure 9: The distribution of neutrophils responding to serum or zymosan activated serum (ZAS) in the stimulus compartment of the blind well chambers. The filters were pretreated with serum or ZAS (10%) for 4 minutes and washed by passing 20 ml of HBSS through the filters. The numbers of cells in a 40X microscopic field were counted at 20 μm intervals through the filter. Three field cores were counted on each of duplicate filters and averaged. The open dots are the counts with serum in the stimulus compartment and serum used to treat the filter. The closed dots are with ZAS used in a similar manner. The S represents the bottom surface of the filter.

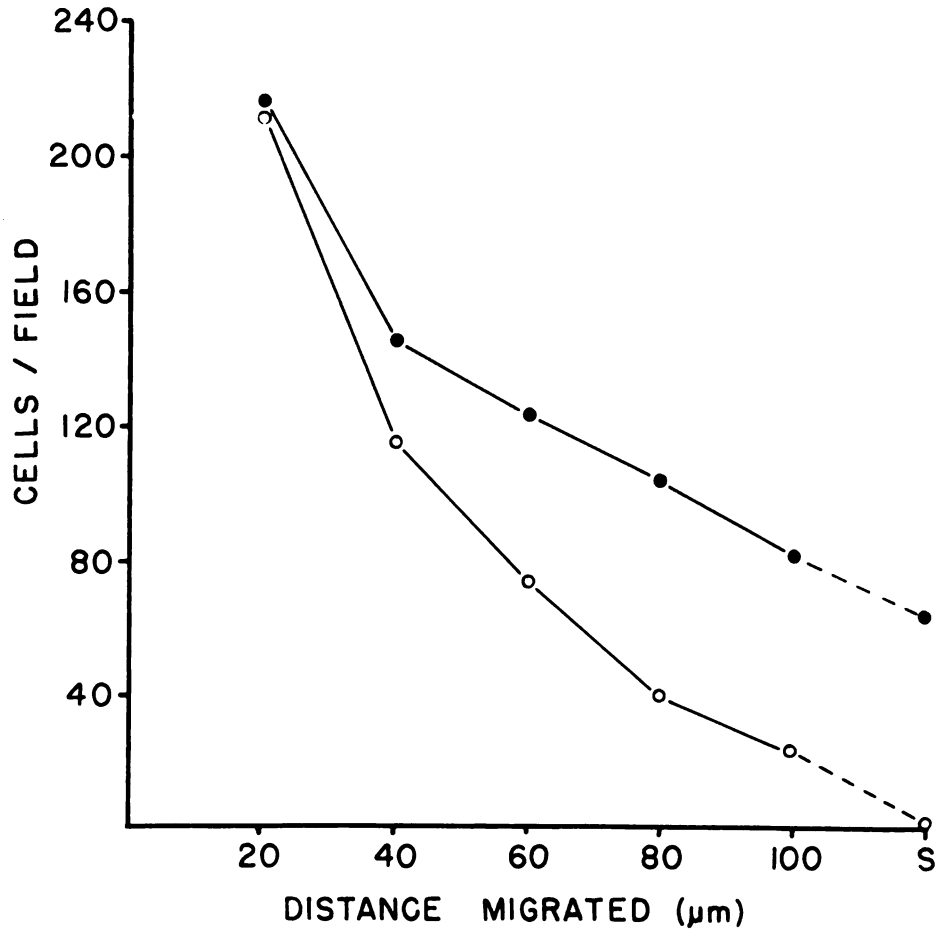
240
200
160
120
80
40
CELLS / FIELD
20
40
60
80
100
S
DISTANCE MIGRATED (μm)

Figure 9.

DISCUSSION

The demonstration of chemotaxis *in vitro* seems to require the addition of protein to the culture medium.[20] Albumin is commonly used as a protein supplement for Boyden assays. Although the specific functions of albumin are not understood, some possibilities have been suggested. Since albumin has a chemokinetic effect in the absence of chemotactic factory,[34] one possibility is that it influences the mechanisms of cell motility. Yet, several workers have shown that albumin does not alter these mechanisms,.[35] We have observed that preincubation of neutrophils with albumin did not alter adherence, migration or neutrophil morphology. Wilkinson has suggested that albumin may act as a carrier for chemotactic factors and is necessary for neutrophils recognition of some chemotactic factors, including fMet peptides.[34] Yet, neutrophils have been shown to bind and respond to fMet peptides in the absence of albumin.[1,30,35] Keller has suggested that albumin facilitates motility by reducing neutrophil adherence.[19]

The results of our experiments support Keller's hypothesis. Neutrophil adherence was reduced as much as 80% by increasing the concentrations of albumin used to coat the micropore filters or glass. In addition, treating the filters with albumin increased the distance neutrophils migrated and the numbers of neutrophils getting into the filter by 2-3 fold. Coating the filters with albumin

resulted in an obvious chemotactic response to fMetPhe, yet fMetPhe did not affect neutrophil behavior by binding to filters coated with albumin. A major role for albumin in random and directed neutrophil locomotion seems to be altering cell-substratum interactions, thereby decreasing adherence and facilitating movement. Numerous experiments in the past were designed with albumin in the stimulus compartment. The results of our experiments suggest that albumin in the stimulus compartment would decrease neutrophil adherence and may promote the loss of cells from the bottom surface of the filter. The loss of cells from the filter surface would interfere with interpretations of cell migration when cells accumulating on the bottom of the filter were counted.

Effects of Chemotactic Factors on Cellular Adhesiveness

Since modifications to the surface on which cells crawl appear to influence neutrophil locomotion, the effects of cellular alterations were studied. Incubation of neutrophils with chemotactic factors increases their adhesiveness to glass and plastic.[26,30] We incubated neutrophils with 2 chemotactic solutions using conditions that "deactivate" neutrophils.[14,32] Deactivation has been defined as the irreversible inhibition of neutrophil locomotive responsiveness after incubation with high concentrations of chemotactic factors. The cause for this decreased responsiveness is unclear. In our experiments, incubation of neutrophils in chemotactic solutions not only resulted in decreased random locomotion, but also increased adhesiveness to albumin treated filters. Smith et al. have reported that this increased adhesiveness is not spontaneously reversible within

the incubation times used in our study.[30] However, increasing the albumin concentrations used to coat the filters restored the locomotion of deactivated neutrophils to control levels. The substratum apparently could be modified to reduce adherence, thus restoring locomotion.

To expand on these observations, locomotive responsiveness of deactivated neutrophils to a second chemotactic stimulus was tested. Neutrophils exposed to high concentrations of chemotactic factors (e.g., fMetPhe) for a period of time exhibited increased metabolic activity, release of lysosomal enzymes, change in cellular shape and an increase in ability to attach to foreign surfaces.[30,36] When the chemotactic factors were removed, the cells appeared in some ways to return to a baseline level of activity. Metabolic activity returned to control levels[16] and the cells rounded up morphologically.[30] However, the cell's adhesiveness remained significantly elevated[30] and migration was minimal. Restimulation of deactivated cells with a new chemotactic factor resulted in another burst of metabolic activity,[16] reformation of a polar configuration,[30] and under these conditions where adhesiveness remained high, reduced translocation. However, the chemotactic response to fMetPhe of neutrophils preincubated in C5a was restored by treating the filters with increasing amounts of albumin. This response could not be produced for cells preincubated in fMetPhe when rechallenged with fMetPhe. These results support the views that deactivation results in increased cellular adhesiveness,[30] that deactivated neutrophils can respond to other chemotactic factors,[16,30] and that neutrophils have separate receptors for C5a and fMetPhe.[35] Our results imply that the reduced locomotion following

deactivation may be linked to increased cellular adhesiveness. Conditions that produce deactivation did not affect the cell's locomotor mechanisms or ability to sense chemotactic factors, as has been suggested by others.

Thus far, we have presented evidence that decreased neutrophil adhesiveness enhances locomotion and increased adhesiveness hinders locomotion. A paradox seems to exist in that chemotactic factor, in a gradient, significantly increase adherence and locomotion. However, the degree of adhesiveness may be dependent on the magnitude of the chemotactic stimulus. Differences in adhesiveness were observed with different concentrations of fMet Phe when used in the deactivation (10^{-5}M) and chemotaxis (10^{-6}M) experiments. Optimal levels of chemotactic factors bring about a moderate increase in adhesiveness, but the activation of the locomotive mechanism may be sufficient to overcome any tethering effects of the cell's attachment to the substratum. Moreover, if attachment and detachment are separable phenomena governed by distinct mechanisms (a concept not addressed by the techniques in this paper),[33] enhancement of attachment may be an integral part of the mechanism of directed migration.

Effects of Cehmotactic Factors on the Substratum

Dierich et al. have reported that the chemotactic factor in casein binds to micropore filters thereby enhancing neutrophil migration.[10] In our experiments, serum or ZAS solution, like casein, when used to coat filters also enhanced neutrophil migration by 2-3 fold. In addition, treatment of filters or glass with these chemotactic solutions decreased neutrophil adherence. However, these

chemotactic factors (and fMetPhe or C5a) in a gradient increased neutrophil adherence. Since chemotactic solutions are mixtures, the adherence data may indicate that different agents in these solutions affect adherence and locomotion. In support of this concept are the findings that 1) C5a did not affect neutrophil behavior by binding to mircopore filters or glass, though it is generally accepted as a chemotactic factor in serum and activated serum; and 2) the data on adherence and migration were similar when serum or HSA was used to coat filters or glass. Thus, it seems that the chemotactic solutions, serum, ZAS, and casein, generate both an albumin-like effect (i.e., decrease adherence by an effect on the substratum) and a chemotactic factor-like effect (i.e., increase the adhesiveness of neutrophils). The agent in serum that increases neutrophil adherence when in a gradient was sensitive to heat and the addition of heparin. Smith et al. have reported that serum, unlike the other chemotactic factors did not induce shape change when incubated with neutrophils.[30] The heat and heparin sensitivity of serum and failure to induce shape change suggest that the agent in serum generating these effects is not C5a. These observations may influence interpretations of data derived from the Boyden assay, in particular, clinical investigations using serum and activated serum. Unknown variations in concentrations of agents which affect the neutrophil or the filter surface hinders evaluations of factors affecting locomotion.

In conclusion, it was demonstrated that the chemokinetic properties of albumin may be due to reducing neutrophil adherence to several substrates in vitro. Reduced cellular adhesiveness to the substratum appears essential for random and directed cell migration.

In addition, chemotactic factors increase neutrophil adhesiveness to glass or micropore filters and may, in some instances, retard cell migration. Several chemotactic factors were demonstrated as affecting both the substratum and cellular adhesiveness which hinders evaluations of neutrophil migration.

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