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THE EFFECTS OF CHEMOTACTIC FACTORS OR COLCHICINE ON NEUTROPHIL BINDING OF PROTEIN COATED PARTICLES IN VITRO

By

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ABSTRACT

THE EFFECTS OF CHEMOTACTIC FACTORS OR COLCHICINE ON NEUTROPHIL BINDING OF PROTEIN COATED PARTICLES IN VITRO

By

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Environmental or cellular changes may alter neutrophil adherence to protein coated substrata. Since neutrophil binding to a variety of protein coated surfaces is essential for margination, motility, and phagocytosis, we investigated the effects of chemotactic factors and colchicine on neutrophil binding of bacteria, proteins, and protein coated particles. We hypothesized these agents may affect neutrophil adhesiveness by altering cellular binding site topographies or promoting the release of cellular substances that alter protein coated surfaces. Our results demonstrate the following.

Unlike isolated neutrophils, which were spherical, chemotactic factor or colchicine pretreated cells possessed distinct front or tail ends. The polarized cells bound complement, immunoglobulin, and albumin coated particles only at their front or tail ends. Regardless of pretreatment, neutrophils bound lectins and bacteria randomly on their surfaces. Time was a critical factor since neutrophils rapidly cleared surface bound particles from their attachment sites. Some particles were ingested by neutrophils immediately after binding and swept away internally from

their attachment and ingestion sites. Ingestion of particles was often dependent upon whether neutrophils were stimulated with chemotactic factors. Other protein coated particles not ingested by neutrophils were selectively redistributed in a rearward direction across the neutrophil's exterior surface. Different particles traveled at different rates on the neutrophil surface and the stimulation(s) required for surface redistributions varied. The localized binding, selective ingestion, and surface redistributions of several particles were observed to occur simultaneously on the cell. The mechanisms for maintaining very distinct binding site distributions and control of particle removal from their attachment sites remain unknown.

We also reported that neutrophil adherence to glass was almost totally prevented by coating it with a variety of human proteins. Chemotactic factors enhanced neutrophil adherence to protein coated glass as did pretreating the protein coated surface with proteolytic enzymes. Chemotactic factors may activate proteolytic enzymes on the neutrophil surface and cause neutrophils to release other proteolytic enzymes into their environment, augmenting cell adherence to protein coated glass. Neutrophil adherence to protein coated glass was inhibitable by the addition of two different specific enzyme inhibitors.

Dedicated to my wife, Jane Caswell York, and to my daughters, Katherine and Elizabeth, whose moral support, understanding, and encouragement made this work possible.

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INTRODUCTION

Human neutrophils are phagocytic leukocytes absolutely essential for human survival. They seek out, ingest, kill, and degrade particulate matter throughout the body. The first critical step for particle ingestion is the attachment of the particle to the neutrophil. Neutrophil binding of many particles can be greatly enhanced by the presence of certain proteins on the particle surface. The proteins of most interest to clinicians and researchers alike have been complement components (i.e., C_3b), immunoglobulins, and lectins. Immunoglobulins and C_3b are produced by specific and non-specific immune mechanisms and, when bound to the particle surface, "tag" that substance for removal by human phagocytic cells. Lectins are often produced by micro-organisms and are incorporated into structural components on their surface. Phagocyte attachment of lectins appears to be a very primitive cell-surface recognition system and the presence of lectins on some strains of bacteria may determine their pathogenicity [1].

Neutrophils can recognize particles with surface bound C_3b, immunoglobulin, or some lectins because neutrophils possess selective domains on their cell membrane that

specifically bind these proteins [1, 2, 3]. Very little is known about the chemical nature of these surface receptor or binding sites or the forces that enable such selective binding to C_3b, immunoglobulin, or lectin coated particles. Assuming that these receptors and binding sites are chemically and physically distinct units embedded in the neutrophil's membrane, they should obey the principles of the mosaic model of a lipid membrane. Therefore, they should be free to diffuse throughout the cell membrane and appear randomly distributed on the cell surface. Indeed, several reports have demonstrated that immunoglobulin, C_3b, and lectin coated particles bind on the neutrophil surface in an evenly distributed manner [4, 5, 6]. Agents (i.e., colchicine or chemotactic factors) morphologically polarize neutrophils or cause them to assume a shape with a recognizable front and tail end. Recent evidence indicates that chemotactic factors (i.e., CF) may also influence neutrophil binding site topographies for immunoglobulin, lectins or albumin coated particles independent of particle attachment [4, 7, 8].

This report investigates the effects of chemotactic factors and colchicine on neutrophil attachment, surface redistributions, and ingestion of lectins, bacteria, and particle bound C_3b or immunoglobulins. Emphasis was given to determining whether colchicine and CF could functionally polarize neutrophil receptors or binding site topographies

for C_3b, immunoglobulin, or lectin coated particles. A variety of cells have been shown to perform specific functions only at one region on their surface [9]. Previous reports indicate that colchicine pretreated phagocytes ingest a variety of protein coated particles only at one small region on the cell [10]. Conditions that influence receptor or binding site mobility on human neutrophils could cause specific regions on these cells to become "endocytotic windows".

A secondary issue of this study was to investigate the conditions necessary for enhancing neutrophil adherence to protein coated substrata in vitro. Chemotactic factors are agents that influence the ability of neutrophils to release lysosomal enzymes [11], possibly activate neutrophil membrane bound enzymes [12], and alter neutrophil adherence to protein coated substrata [7, 13, 14]. Therefore, a technique was devised to evaluate the effects on neutrophil adherence to protein coated substrata and enzyme modified protein coated substrata in vitro. Particular attention was given to the effects of CF on the release and activation of neutrophil enzymes that alter a variety of protein substrata and neutrophil adherence to these surfaces.

REVIEW OF LITERATURE

There has been an explosive growth of interest and research activity regarding human neutrophil physiology. The purposes of this review are to 1) briefly introduce information enabling some understanding of the physiology and physiopathology of human neutrophils and 2) review the literature pertaining to neutrophil adherence interactions important for "normal" neutrophil function.

I. Physiology and Physiopathology of the Human Neutrophil

Human blood is basically composed of two components; a cellular fraction and plasma. The cellular fraction contains several types of cells that are highly specialized to perform certain roles within the human body. The primary role of the blood neutrophil is to seek out, ingest, and destroy obligate extracellular pathogens which cause most of the acute infections in humans. Decreased numbers or functionally impaired neutrophils are incompatible with life.

At the time of birth, neutrophils are made almost exclusively in the bone marrow and the marrow will perform this role throughout the lifespan of the adult. Within the

marrow, there exists classes of stem cells which are committed to granulocytic, monocytic, erythroid, and megakarayocytic differentation. Factors regulating stem cell input into these cell lines remain poorly understood [15]. Cells destined to become neutrophils begin a mitotic phase. Following three mitotic divisions, which last approximately five days, a single stem cell produces eight differentiating cells. Following replication (i.e., mitotic phases) the cells undergo a maturation process, lasting approximately two days [16]. There are enough maturing neutrophils in the marrow, should the marrow stop functioning, to supply adult daily neutrophil requirements for about one week [17]. Various tables and figures have been provided by several authors that summarize neutrophil development in the bone marrow, staining properties, and cell morphologies during this development [18, 19].

Release from the marrow into blood sinuses is ordinarily related to the age of the cell. This process may simply be regulated by marrow anatomy. Stem cells lie deep within marrow cords and as these cells replicate and mature, they seem to be pushed onto the sinusoid surface. Therefore, release may occur on a first in-first out basis [20]. However, blood flow [21], blood concentrations of neutrophils [21], bacterial products [22], and "colony stimulating factors" [23, 24] also influence neutrophil release from the marrow. Cell maturation and acquisition of certain cell

properties (e.g., deformability, membrane fluidity, etc.) may be additional factors that also influence neutrophil release from the marrow.

Once in the blood stream, neutrophils constitute approximately 70% of the blood leukocytes. These neutrophils have a multilobed, or segmented nucleus, neutral staining granules in their cytoplasm, and are formally identified as polymorphonuclear neutrophils or PMNs. Other common names include "polys" or "segs". Isotope dilution studies [25] reveal two pools of neutrophils. Approximately 2000-7000 neutrophils per cubic micron are found in the central blood flow while almost an equal number are found to be intermittently attached to the vascular endothelium (i.e., marginated). Factors influencing interactions between these two pools remain poorly understood. Exercise, or epinepherine injected into the vasculature, have been shown to promote release of the marginated pool of neutrophils from the endothelium [21, 26, 27] and can approximately double the numbers of circulating neutrophils.

Optimal defense against infection requires intact integument and mucous membranes. Humans are in constant contact with a variety of micro-organisms and, according to cell numbers, the human body is approximately 99% prokaryotic. Following penetration of the epithelial front lines of defense by micro-organisms, inflammatory agents or chemical signals are apparently generated. It has been postulated that these

inflammatory signals may mobilize and recruit neutrophils to inflammatory sites.

What are the inflammatory signals that account for neutrophil mobilization and accumulation at inflammatory sites? Metchnikoff theorized that products released by bacterial growth within the host attracted leukocytes [28]. Since this observation in the late 1800's, numerous chemical substances have been isolated and classified as chemotactic factors (CF). Gallin provides an extensive review [29] of diverse agents that, by various criteria, have been shown to be chemotactic. Most of them are biological products generated by bacteria, immune effector cells or by enzymatic action on a variety of protein substrates. Little is known about the mechanisms by which these chemically diverse substances interact with the neutrophil and illicit a chemotactic response(s) [29, 30, 31]. Specific membrane receptors for several CF have been demonstrated [32, 33]. Some CF analogues compete for receptor sites yet do not trigger identifiable chemotactic responses [31]. Therefore, some additional event must accompany receptor-ligand formation to trigger a chemotactic response. Chemotactic factors have been shown to alter neutrophil morphology, cytoskeletal arrangements, cytoplasmic concentrations of the cyclic nuclotides, depolarize the cell membrane, increase membrane fluidity, induce ion fluxes, enhance phagocytosis, adherence, glycolysis, superoxide generation, and lysosomal enzyme release [31]. Following surface stimulation, there appears to

be a sequential series of events activated that modulate several neutrophil responses. There may be one fundamental factor that triggers these mechanisms, but, if so, it has not been determined.

Conheimn, in the late 1800's, noted that the initial leukocyte response during inflammation was the margination of these cells along the vasculature near sites of infection [28]. The marginated leukocytes were then observed to exit from the vasculature and enter the tissue compartment. Over 30 billion neutrophils are leaving the vasculature daily [21], and while in the tissue compartment neutrophils literally eat themselves to death, forming the host's second line of defense against infection. Neutrophils have never been observed re-entering the circulation and are true end cells with extremely short half lives (e.g., minutes to hours). Surprisingly, histologic sections of most non-pathologic tissues reveal few neutrophils on the mucosae. However, tissue sections obtained from infected or inflamed areas obtained in as little as one hour following infection, reveal that neutrophils emigrate to these sites to the extent that they are often the primary cell type in the region. Such recruitment and prolonged utilization can result in an immediate marked neutropenia. This prompts immature and mature neutrophils to be released from the bone marrow into the blood stream which provides a reserve force of neutrophils if needed [19, 25, 27].

Neutrophils in the vasculature are basically round intermittently adherent cells. Isolating them using various in vitro techniques [34] does not appear to alter their morphology. Yet, within seconds, in a balanced salt solution or minutes in whole blood, neutrophils "ruffle" and become polarized [35, 36, 37] when exposed to various concentrations of CF. While polarized, a distinct front (i.e., lamellapodia) and tail (i.e., uropod) end can be observed on the CF activated cells. When neutrophils migrate on a surface, migration will occur in the direction of the lamellapodia. This morphological polarization allows researchers to evaluate not only cell responsiveness to CF but also 1) cell orientation, and 2) redistribution of substances bound to the surface of the cell's membrane.

Neutrophils exiting the vasculature crawl in an amoeboid fashion. Stossel and others [38, 39] have reported the presence of actin and myosin in human neutrophils which may account for as much as 2.5% of the cell's total protein concentrations. These proteins have been implicated in the motile mechanisms of other non-muscle cells, and neutrophil mobility appears to be generated by their activity with other cellular components which allow for contraction and relaxation of the moving parts of the cell. Agents that interfere with actin polymerization interfere with neutrophil motility. Energy is needed for cell migration and inhibitors of glycolosis or the hexose monophosphate shunt also impair neutrophil migration [38, 40].

The distances neutrophils crawl in response to various CF *in vitro* is probably the most commonly used technique to clinically evaluate the functional capabilities of the cell. Chemotaxis, or the directional migration of cells towards increasing concentrations of CF, has fascinated biologists for over a hundred years, and is apparently fundamental in the biological activities of numerous motile cells [40]. Although, as numerous researchers note [13, 28, 29, 40], chemotaxis could explain neutrophil emigration to infection sites and retention of cells at that site, no *in vivo* or *in vitro* evidence have supported this hypothesis [13, 42]. In the last 20 years, numerous *in vitro* techniques have demonstrated that the rate at which neutrophils migrate is stimulated by CF and neutrophils orient and crawl up a gradient of CF *in vitro* [29, 41, 42]. Evidence for the establishment of a chemotactic gradient or neutrophil chemotaxis *in vivo* has not yet been reported.

The mechanisms by which CF influence neutrophil mobility and how neutrophils orient towards a chemotactic gradient remain unknown. It may be the nature of the adhesion between the cell and the substrata that facilitates both of these responses. Neutrophils have been shown to migrate up an adherence gradient (i.e., haptotaxis) *in vitro* [43, 44]. The effects of CF on neutrophil adherence will be discussed separately, but it should be emphasized that mechanisms controlling cell adherence influence margination, neutrophil mobility and phagocytosis.

Sites of infection have often been compared to a battlefield. Pathogens employ exotic mechanisms to immobilize host defenses and, like battles, infections are complex, involving much cell death, and destruction. Once at the inflammatory site, neutrophils ingest micro-organisms, damaged tissue, cell debris, and foreign substances. Attachment and recognition of these substances occurs at the neutrophil surface and cell-substrata interactions are dependent on the cell's surface binding sites, attachment surface, and the environment.

Following attachment, the particles may or may not be ingested. Phagocytosis is also an energy requiring process involving the flow of a hyaline pseudopod around a particle. Almost certainly, contractile proteins are important in mediating the cytoplasmic movement and pseudopod formation [29, 40, 45]. Surface modifications or opsonization by various serum proteins may be required to make particles more palatable. Attachment apparently triggers a signal for ingestion of the attached particles. Localized areas of the plasma membrane in contact with the particle invaginate, forming a pouch. This pouch, containing the particle, seals and separates from the plasma membrane forming a closed vesicle (i.e., phagosome). Two types of lysosome granules are primarily found in the neutrophil (i.e., primary and secondary granules) and within these granules, various catabolic enzymes have been isolated and characterized [46]. These lysosomes fuse with the phagosome forming a

phagolysosome where the enzymes assist in the killing and degradation of ingested matter. Neutrophil killing of microorganisms may also be mediated by plasma membrane bound ectoenzymes, superoxide radicals, lysosomal enzymes, anions, pH, or combinations of any or all these mechanisms. The success of degradation is determined largely by the properties of the ingested particles. Failure to degrade phagocytized material may be to the host's benefit, since undigested material may be needed to maintain immunologic awareness [47].

Whereas neutrophil phagocytosis is clearly essential for normal microbiocidal activity, events occurring during phagocytosis may also promote host tissue damage. Following, or coincident with plasma membrane closure during phagocytosis, constituents of lysosomal granules have been found extracellularly. External degranulation may also occur when neutrophils are adherent to non-phagocytizable substrates (i.e., reverse or frustrated phagocytosis), or following exposure to various soluble stimuli (e.g., cytochalasin B, CF, phorbol acetate, calcium ionophores, and lectins) [47]. Phagocytosis also promotes marked changes in neutrophil oxidative metabolism. Neutrophils consume large amounts of O_2, generating hydrogen peroxide, superoxide radicals, and hydroxy free radicals [46], which can also be released into the cell's environment. Lysosomal enzymes and products of O_2 metabolism released into tissue compartments can promote tissue injury. Chronic neutrophil accumulation and release

of lysosomal enzymes or oxidation products into the host environment may actually lead to secondary clinical problems such as autoimmune vasculidities, arthridities, and pneumonidities. Therefore, intricate controls must exist that normally keep this response in check. Neutrophils remove CF by endocytosis or by enzymatic degradation [12]. Several CF inactivations have also been identified in human plasma [29]. Several enzyme inhibitors which, due to capillary permeability changes during inflammation, are also in high concentrations at inflammatory sites. These enzyme inhibitors (i.e., alpha-1-antitrypsin, alpha-2-macroglobulin, and C1 inhibitor) inhibit CF generation [48], neutrophil chemotaxis [49], and lysosomal enzyme function [47]. The actions of various anti-inflammatory drugs [46] (e.g., corticosteroids, colchicine, and CF analogues) on neutrophil function is also providing insight to man's ability to prevent these clinical manifestations.

In 1954, Janeway et al described the first group of patients with recurrent infections later identified as having a phagocytic cell disorder [50]. Numerous disease processes have since been reported where this situation exists and the importance of neutrophil function is aptly illustrated by the high rates of infection and dismal survival statistics of patients with these diseases. A description of these diseases can be found elsewhere [29, 51]. Studies involving congenital or acquired neutrophil deficiencies has greatly attributed to increased understanding of the molecular events involved with several neutrophil functions. However, several

important limitations become apparent while reviewing this literature. First, the complexity of the neutrophil's interaction with other host defense mechanisms makes it difficult to evaluate neutrophil function as an independent process. Our lack of understanding of these interactions makes predictions of factors causing the abnormality(ies) almost impossible. Second, evaluations of neutrophil function (i.e., chemotaxis, phagocytosis, microbiocidal killing, adherence, etc.) only confirm diagnosis. These assays are not standardized from lab to lab and seldom contribute to understanding the mechanisms causing the abnormality. Last, ignorance of cell mechanisms that control important cell functions is amplified by our inability to treat these patients. Treatment consists of blood transfusions and preventative measures against infection (e.g., antibiotic therapy). Patients with congenital disorders of neutrophil function (i.e., chronic granulatomes disease) often suffer prolonged infections in spite of antibiotic therapy. Prognosis is almost always uncertain, unless the disease is severe and then the patient almost assuredly dies before age twenty.

II. Neutrophil-Surface Interactions: Neutrophil Adherence

A. Evaluation of Neutrophil Adherence

Neutrophil adherence to other cells, various substrata, or particulate matter appears essential for margination,

motility, and phagocytosis, all pre-requisites for neutrophil involvement in host defense. Since blood vessels are lined by endothelium, transitions from blood to tissue compartments require neutrophil-endothelial adhesions. Although the forces influencing this interaction are poorly understood, it appears to be the initial neutrophil response to inflammatory stimuli. Other neutrophil-cell interactions appear important for selective removal of damaged cells and thrombosis; but other than isolated reports very little research information is available concerning these interactions.

Early attempts to evaluate neutrophil adhesiveness utilized techniques that evaluated neutrophil margination or phagocytosis. Most of these techniques have relied on direct in vivo observations. In the early 1800's, Dutrochet observed leukocyte margination to the vascular endothelium and these observations were later confirmed and expanded upon by Addison and Conheim [28]. Elaborate models (e.g., tadpole tail [52], rabbit ear [53]) have been developed over the years to better observe blood flow and leukocyte attachment to capillary endothelium. Grant and Epstein [28] used a laser beam to selectively destroy tissue in localized areas around or on vascular endothelium and concluded that localized margination was controlled by alterations of endothelial cell adhesiveness. Advances in tissue culture techniques enabling neutrophil [34] and endothelial cell isolation [54] and culture provided researchers a means to study this cell-cell interaction in vitro. Direct observations of neutrophil

attachment to endothelial monolayers have provided data for the establishment of two separate hypotheses. Several investigators [53, 55] contend that inflammatory agents influence endothelial adherence, while others [53, 56] suggest that inflammatory and anti-inflammatory agents influence this interaction only to the extent of affecting neutrophil adherence. A third consideration, recognized by both groups is that local alterations of blood flow decreases the shearing forces between these cells facilitating leukocyte-endothelial adhesion [57]. Although conflicting results, these in vitro techniques appear instrumental in contributing to our understanding of this cell-cell interaction.

Neutrophils bind to and ingest microbes, damaged cells, and a variety of opsonized or non-opsonized particulate matter. Often, the collision rate between the attachment particle and neutrophil will determine the phagocytic rate; however, attachment does not guarantee recognition and ingestion. Suggested mechanisms of recognition by phagocytotic cells have been extensively reviewed elsewhere [9]. Although early observations of phagocytosis were primarily concerned with evaluations of the ability of neutrophils to ingest and kill microbes, these experiments broadened researchers' understanding of the diversity of surfaces neutrophils bind to. In addition, by examining the early events of phagocytosis (i.e., attachment), information was obtained to evaluate various mechanisms of attachment. Binding affinities, scatchard plots, and calculations of

association and dissociation constants were calculated. Comparisons were made to enzyme kinetics and adsorbtion isotherms to evaluate these interactions [58]. Preliminary data was obtained by direct observations of attachment in vitro and later fluorescent and radio-labeled tags accelerated and improved these calculations. Characterization of numerous types of neutrophil-surface interactions, mechanisms that control attachment and recognition, and evaluation of agents influencing these mechanisms have resulted from these studies. Research involving several of these interactions will be reviewed later. In vivo studies (i.e., "clearance assays") have proven less useful and unreproducible.

Neutrophils not only attach to endothelium, but have been observed migrating across and between cytoplasmic processes of these cells [27]. In addition, neutrophils must crawl on various substrata through extravascular spaces to sites of infection. Translocation across a surface implies that neutrophils must not only possess mechanisms for attachment but also detachment. Adhesion appears to provide the frictional forces required for movement. If hyperadhesive, cell locomotion would be minimal. Altering motile cell adherence to a substrata has been shown to influence the rate and direction of cell locomotion [44]. Electron micrographs of motile cells reveal localized plaques where the cell membrane comes in close contact (e.g., within 30 nm) with the substratum. Within these plaques are electron

dense zones of oblique and longitudinal tracts of microfibrils [59]. Little is known about the chemical nature of the membrane components that are closely associated with the attachment surface.

Most of the data used to evaluate neutrophil motility have been obtained using *in vitro* assays. Likewise, the resurgence of interest in neutrophil surface interactions has also been stimulated by the development of numerous *in vitro* assays to evaluate this parameter and its effect on motility, phagocytosis, margination, etc. Yet, *in vivo* techniques are difficult to quantitate, evaluate factors influencing the cell response, and often impossible to use on humans. In addition, cell manipulation and exposure to isolated factors are often impossible *in vivo*. There are also distinct problems involved with evaluation and interpretations of *in vitro* assessments of cell surface interactions. With the exception of evaluating neutrophil interactions with microbes or other cells, neutrophil adherence assays, like *in vitro* assessments of neutrophil motility and phagocytosis, utilize non-biological surfaces which are foreign to the cell. Glass bead columns [60], capillary tubes [61], coverslips [35], plastics [62], chambers [63], and latex [7] are but a few of the surfaces used to evaluate neutrophil adherence. Comparison of results between adherence assays are difficult not only because of different attachment surfaces, but other reasons as well. Different medias are used to sustain the cells (e.g., serum, buffered

salt solutions plus proteins, etc.) which may have different effects on the attachment surfaces of the cells. Different detachment forces are used (e.g., gravity [35], centrifugation [64], rotation [65], etc.) which confuse issues; for example, is one measuring attachment or detachment? Some techniques use whole blood, which perhaps better reflects in vivo events, but complicates interpretations with a multitude of factors influencing the cell or substrata (e.g., platelets, serum proteins, etc.). Complications also arise using isolated systems such as removing cells from their natural environment and harsh treatments required for cell isolation.

B. Neutrophil Membrane Attachment Sites

Two concepts are generally used to explain cell adhesion. First, adhesion is mediated by macromolecules that can be solubilized or secreted at the cell surface which acts as a glue or cement. Second is a recognitive system where complementary molecules on the cell and surface form "lock and key" attachments. The only differences in the theories are the number of substances involved in the interactions; the glue hypothesis involves a single substance whereas the complementary theory involves several recognition substances (i.e., receptors or binding sites). The cell membrane is a fluid lipid bilayer interspaced with protein, glycoprotein, and glycolipid molecules which appear to provide binding or receptor sites for various substances. Selective attachment of appropriate ligands to these sites thereby provides the

"radar shield" by which the cell communicates with its environment through recognition of selected substances. Electrostatic zeta potential, chemical bonding, hydrophobic and hydrophillic forces contribute to the attachment and repulsion forces between the ligand and cell [66]. Selective attachment is determined by the molecular structure of the attachment site and the ligand. The neutrophil membrane appears to contain certain domains for the selective attachment of certain foreign or protein coated surfaces. Although the chemical nature, turnover, and modulation of these domains are poorly understood, they certainly are important for host defense. Before elaborating on these attachment sites, a clear understanding of the terms "binding sites" and "receptor sites" must be developed. Following attachment of a ligand to a cell surface, the cell may or may not recognize the attachment process. High affinity and saturable attachment sites for selective ligands describes a membrane binding site. If such an attachment invokes a cell response, this membrane domain is referred to as a membrane receptor [9]. Ingestion, cell shape change, submembrane structural alterations, metabolic changes, membrane fusion, lysosomal enzyme synthesis and release, and stimulation of microbiocidal systems are examples of neutrophil responses used to evaluate cell recognition of a ligand.

Initial suggestions of a complementary-molecular theory of adherence promoted the concept of glycoproteins on cell surfaces interacting with complementary glycoproteins on

other cell surfaces as the governing force for cell adhesion. Isolated glycoproteins from different cell types do agglutinate when mixed. Proteins derived from certain plants (e.g., lectins), are able to bind with high affinity to carbohydrate or carbohydrate containing substances. They appear to be the recognition mechanisms for attachment between plant and nitrogen fixing bacteria [67]. Plant lectins also bind to the surfaces of eukarotic cells, including neutrophils, probably through interactions with various terminal sugar residues on glycoproteins, glycolipids, and glycosaminoglycans on the eukarotic cell surface. Pilated strains of gram negative bacteria attach to neutrophils in the absence of serum which appears to be dependent on the presence of lectins [1, 68] located on the bacteria's pili [69, 70]. Although other lectin based recognition mechanisms have not yet been identified for neutrophils, it may explain their affinity for substances like zymosan. Neutrophil affinities for bacterial lectins appears relatively non-specific and can be reversed by the addition of the appropriate monosaccharide. Such interactions, as pointed out by McKeever and Spicer [70], may contribute to understanding of the ability of neutrophils to interact with so many "non-specific" surfaces.

The presence or absence of serum proteins on particle surface is widely recognized as the most important factor governing neutrophil attachment to that surface. Perhaps most notable are complement components (C_3b) and antibodies.

These proteins are referred to as opsonins (i.e., to prepare food) since coating non-phagocytizable substances with them enhances their palatability.

Antibody opsonization, discovered in 1903, reconciled the controversy between Metchnikoff's followers and those that advocated the importance of antibodies in host defense against infection [71]. Antibody attachment to an antigen greatly increases the affinity (i.e., 4000X) [72] of immunoglobulin for phagocytic cells. Phagocytic cells appear to possess a selective domain on their unit membranes for binding of the Fc segment of several IgG and IgA immunoglobulins subclasses [72, 73]. Attachment of the antibody to an antigen appears to alter the Fc segment on the immunoglobulin allowing for phagocyte recognition [74]. The membrane receptors on neutrophils for antibody are probably similar to those described for macrophages, although little has been described to confirm this hypothesis. Antibody attachment to macrophages is very tight (i.e., 10-40 nm), and the bound immunoglobulin may sit in a "receptor pocket" on the membrane surface [75]. Phagocytic cells appear to have an abundance of Fc receptors (i.e., 10^5-10^6/cell) [70] and depletion of these receptors following phagocytosis does not totally exhaust their number, suggesting a marginal pool of receptors. Regeneration, requiring protein synthesis, of these receptors has been documented [76]. The chemical nature of the antibody receptor is unknown. Protease treatment of neutrophils has been reported to enhance their

antigen-antibody binding capabilities [77]. Attachment does not appear to be energy dependent and aldehyde fixation appears to destroy the Fc receptors [4].

Several techniques have been used to evaluate the Fc receptor. Various cells (e.g., RBC, bacteria, etc.) or particles (e.g., latex beads) may be coated with immunoglobulin and neutrophil binding of these cells or particles have been evaluated. Neutrophil binding of antigen-antibody complexes, in particular ferritin-antiferritin or peroxidase labeled antibody complexes, have been extremely useful for observing Fc mediated attachments using electron microscopy.

A group of eleven heat labile serum proteins (i.e., complement) comprising 10% of all serum proteins, functions in effecting immune and non-immune destruction of foreign substances [78]. These proteins appear to circulate in whole blood in unactivated forms. When activated, by either of two mechanisms, a cascade of enzyme-substrate reactions occurs, generating proteolytic fragments that influence several host defense mechanisms. Illustrations of this enzyme cascade have been presented in several reviews [78, 79]. Activation of this system may occur when either C1 reacts with an antibody-antigen complex (i.e., classical complement pathway) or when an ill-defined protein called "initiating factor" interacts with various particulate surfaces (i.e., alternative complement pathway). Regardless of the modes of activation, both systems merge at a common point, the generation of C_3 convertase. Following

the formation of this serine esterase trypsin-like enzyme, the pathways are identical. Although highly specific, trypsin-like enzymes generated in the blood clotting, fibrinolysis, and kallikrein systems also act on various complement components [80]. Several products generated in the complement cascade have a pronounced effect on inflammation (e.g., C_3a, C_5a) leukocyte responsiveness (e.g., C_3a, C_3b, C_5a, C_{567} complex) and cytolysis (e.g., C_{789}) [81]. Having already briefly discussed the role of complement components as CF, the following is a review of the literature pertaining to the complement component (i.e., C_3b) that contributes toward immune adherence.

Several recurrent infections have been observed in patients with depressed levels of C_3 [82]. When C_3 interacts with C_3 convertase, trypsin [83] or trypsin-like enzymes [80, 84], a small fragment (MW=7000) is cleaved from the amino terminal of the alpha chain and this fragment is called C_3a. The large fragment (MW=170,000), identified as C_3b, has two recognized binding sites. The first enables C_3b attachment to RBC membranes, bacteria, and other particles. These binding sites are labile and if attachment does not occur, they spontaneously decay [81]. An additional binding site is generated on C_3 following proteolytic cleavage. This one is stable, although serum inactivators can functionally destroy it [85]. It facilitates C_3b or particle bound C_3b attachment to specific receptors on "responder cells". Responder cells include human erythrocytes, platelets, B lymphocytes, and

mononuclear and granulocytic phagocytes. Formal proof for the specificity of neutrophil C_3b receptors has not been presented; however, it avidly binds C_3b coated particles, a fact not demonstrated for other complement components, excluding C_5a [33], for which a separate receptor apparently exists. Neutrophil attachment of particle bound C_3b apparently invokes a cell response [85, 86] unlike macrophage attachment [70], suggesting a true receptor exists for C_3b on the neutrophil. Binding studies on macrophages reveal fewer C_3b receptors/cell than Fc receptors; however, better techniques are needed to confirm this observation. These receptors are trypsin sensitive, and require Mg^{++} for ligand attachment [70]. Recently, neutrophil receptors for C_3b have been isolated, characterized, and antibodies prepared that appear specific for this receptor [87, 88] which should enable clarification of numerous conflicts in the literature concerning neutrophil C_3b receptors.

Two methods of evaluating C_3b mediated attachment of phagocytic cells have been used in the past. The C_3b-opsonization of various particles was first performed using fresh serum as a source of complement [5, 89, 90]. Complement deficient serum or agents that prevented activation of classical or alternative complement cascades have been used as controls for these experiments. Since other serum proteins may have influenced results, two similar approaches, using purified complement components, have become popular techniques for studying neutrophil attachment of C_3b coated

particles. The first employs exposing antibody coated particles sequentially to purified C1, C4, C2, and C3, generating a particle-Ab-C1423b complex [2]. The major disadvantages to this approach are time and expense whereas the advantages are built in controls (e.g., particle-Ab-C142) and comparisons of binding studies with particles only coated with antibody (e.g., IgM or IgG). An alternative approach has been to incubate particles with purified C3 and C3 convertase, trypsin, or trypsin like enzymes [91]. These enzymes rapidly convert C3 to C_3b which attaches and opsonizes various surfaces. The bulk of most phagocyte C_3b receptor studies have employed C_3b opsonized erythrocytes as attachment particles. However, the relative size of the RBC to the phagocytic cell prevents interpretations of receptor numbers distribution, affinities and to some extent, phagocytosis.

Only a few subclasses of antibodies are recognized by human neutrophils. However, IgM and two subclasses of IgG when bound to an antigen, activate the complement cascade. Since complement is found in the blood and extravascular compartments, it would not be unreasonable to assume most antibody-antigen complexes are also C_3b opsonized. Unlike the mononuclear phagocyte, distinct roles for complement and antibody attachment and recognition respectively have not consistently been observed for human neutrophils. Both antibody and complement opsonization have been reported to enhance particle attachment, and cell recognition (e.g., phagocytosis) [3, 88].

Neutrophils bind and ingest a wide variety of foreign substances. For lack of better terminology, the attachment of these substances has been termed "non-specific" and their membrane attachment sites as "non-specific" receptors. This appears to be a primitive recognition system, operating via mechanisms quite distinct from those mediated by the Fc and C_3b receptors, although, as previously noted, some similarity has been reported to glycoprotein recognition mechanisms. These receptors appear to have low levels of specificity and attachment may be by some non-specific force such as charge [92]. Of particular interest to researchers concerned with neutrophil surface interactions should be the complication of attachments via this process interfering with attachments wished to be studied. Numerous particles (e.g., latex beads, glutaraldehyde fixed RBCs, bacteria, etc.) may attach to neutrophils via non-specific receptors. Coating these surfaces with serum proteins, lectins, etc., may not totally prevent non-specific cell-particle interactions. In addition, neutrophil or serum enzymes may cause the erosion of serum or lectin coats from a particle's surface enabling non-specific interactions to again occur.

C. The Neutrophil Cytoskeleton: Role in Adherence

Networks of spectrum and actin are present within erythrocytes which appear to anchor membrane components. Nucleated cells appear to have a similar cytoskeleton composed of micro-tubules and micro-filaments. Although

cytoskeletal components are difficult to preserve and observe, recent electron microscopic and immunofluorescent techniques have allowed researchers to evaluate them in several cell types, including the neutrophil [6, 93].

There are two main components of the neutrophil cytoskeleton. The first are microfilaments which are approximately 60 angstroms in diameter, usually present just under the cell membrane and are most prominent at regions where the membrane has been perturbed by surface contact [6, 94]. There appears to be at least three categories of microfilaments, which Stossell [95] has shown to be comprised primarily of actin, myosin, and other poorly defined components. Microtubules, the second element of the neutrophil's cytoskeleton, are composed primarily of tubulin. This protein appears quite primitive phlyogenetically. Tubulins polymerize, *in vivo* or *in vitro*, in the presence of Ca^{++} and GTP assembling into a tube with a hollow core which may be several microns in length [96].

The bulk of the reports concerning neutrophil cytoskeletal components have used pharmocologic agents that promote assembly or dissolution of these structures. Numerous pharmacologic studies indicate that membrane dependent processes of neutrophil chemotaxis, adherence, phagocytosis, and lysosomal enzyme release are influenced by the cell's cytoskeleton.

Cytochalisns are fungal metabolites which cause structural disorganization and disruption of microfilaments perhaps by preventing gelation of actin with actin binding proteins [97]. In addition, this drug inhibits hexose and nucleoside transport [6, 90]. Numerous cell functions including phagocytosis, cell migration, and lateral movement of membrane attachment sites [11, 97, 98, 99, 100, 101] are all impaired or totally prevented following exposure to this agent. Most of these functions require active membrane movement, an apparent function of actin-myosin contractions. Although not a direct stimulus for degranulization or Ca++ influx, cytochalisns potentiate stimuli that invoke these responses (e.g., CF or Calcium ionophores [101]) and have been used extensively to study them.

Microtubule disruption by antitubulins has been shown to promote a polarization of structure and function of a variety of cells, including the neutrophil. Lectin attachment to antitubulin treated or buffer treated control neutrophils appears to be in a random, evenly distributed manner. Yet, unlike control cells, the receptor-ligand complexes "cap" or flow to the uropods of the antitubulin treated neutrophils [102, 103]. Similar observations have been reported following the attachment of C_3b or antibody opsonized particles on antitubulin treated macrophages [10, 103, 105]. Following particle redistribution, macrophage endocytosis of the particles occurred only at the uropod (i.e., polarized cell function) and at the same rate as control cells.

Microtubule depolymerization has been shown to facilitate microfilament recruitment to the uropods of antitubulin treated cells, which as Oliver suggests [106], may facilitate polarized endocytosis. Disruption of microtubules also appears to impair the chemotactic but not random migrational responsiveness of leukocytes which may be due to microtubule dependent orientation mechanisms [106, 107, 108] or the inability of these cells to release cell derived CF [42, 109].

D. Factors that Influence or Modulate Neutrophil Adherence

Adherence is an energy requiring process and the energy for it appears to be derived from glycolysis and possibly oxidative reduction [110, 111]. Adherence appears intrinsic to the cell since the cellular and plasma fractions of blood can be removed without altering the ability of neutrophils to attach to various substrata [111]. However, several proteins have been shown to modulate this adherence. It is temperature dependent [110], maximal between 25^{o} and 42^{o}C, and apparently reversible since neutrophils must be able to detach from endothelium and during migration. Anticoagulants have variable effects on neutrophil adherence, and although heparin has no direct influence, it can modify plasma induced alterations of neutrophil adherence [112]. In contrast, calcium chelators dramatically impair neutrophil adherence. Although controversial, metal cations are required for adherence [35, 110, 111]. Kaverstein [113] has shown a wide pH range (i.e., 6.3-8.8) in which no effects on leukocyte

adherence were demonstrable. Only extreme osmolality changes impair neutrophil adherence (i.e., 600 mOsm) [61]. As early as 1955, neutrophil surface charge has been a suggested mechanism of adherence [53]. Neutrophils have a negative surface charge and modification of this charge has been shown to influence adherence [92].

Several plasma proteins, in addition to antibody and complement, also influence neutrophil adherence. These substances alter either cell or substrata adhesiveness and have been extensively studied because of their obvious possible biological roles during the inflammatory response [14, 114]. Serum albumin appears to have no influence on neutrophil adhesiveness; however, it avidly binds to many surfaces inhibiting neutrophil attachment to those surfaces [14, 35], possibly by covering up attachment sites for non-specific neutrophil receptors. Baseline levels must be established to quantitate increases or decreases in neutrophil adherence *in vitro*. Researchers can control baseline levels of cell adherence to various substrata *in vitro* by varying the amounts of albumin or serum used to coat attachment surfaces [14]. Epinepherine, endotoxin, or exercise induce the production of an uncharacterized plasma protein(s) that impairs neutrophil adhesiveness *in vivo* [115]. A tremendous amount of research has focused on isolating and developing substances that reduce neutrophil adherence. These substances may provide a source for potential anti-inflammatory drugs.

Plasma proteins, other than opsonins, also augment neutrophil adherence. Of particular interest, are CF which may be generated at inflammatory sites (e.g., C_5a, bacterial filtrates, formylated peptides, etc.). Although the effects of CF on neutrophil adhesiveness in vitro is controversial [56], most researchers agree CF enhances neutrophil adherence either by influencing the cell [35, 54], or substrata [116, 117, 118] adhesiveness.

Agents shown to influence concentrations of cyclic nucleotides, calcium fluxes, microtubule organization, or charge also have been shown to modulate neutrophil adherence. Chemoattractants and other drugs or agents that promote microtubule assembly [108], decrease the net negative surface charge [92], promote calcium influxes [119], elevate intracellular concentrations of cGMP [119], also increase cell adhesiveness. In contrast, agents that decrease neutrophil adherence have been shown to increase cAMP concentrations (possible yin-yang relationship?) or inhibit calcium influx [119]. Chemoattractants enhancement of neutrophil adhesiveness appears reversible until the concentrations of CF used to pretreat the cells are in the "deactivating" ranges; then the enhanced adherence is sustained following removal of the CF [35]. Deactivation has been described as a condition where neutrophils fail to migrate in response to high concentrations of CF [120]. Several authors have suggested this apparent loss of mobility may in fact be due to the sustained enhanced adhesiveness of the cell [35, 120, 121].

Curiously enough, "priming" the cells with low concentrations of CF also dramatically impairs or alters a "normal" neutrophil response to a second higher dose of CF. Unlike deactivation, which perhaps by saturating the cell with CF overloads the cell's responding mechanisms, priming the cell with low doses of CF appears to desensitize the cell. Examples of neutrophil desensitization have been provided by several recent reports. Neutrophils primed with sub-optimal concentrations of CF fail to degranulate or aggregate [122, 123] following a second exposure to optimal doses of the CF. Neutrophil adherence also fails to be enhanced in response to CF when first primed with low concentrations of CF [35]. Indeed, their adhesiveness was reported as being reduced below levels observed for cells having never seen the CF. Therefore, this reduced adherence doesn't appear to be due to a desensitization in a classical sense. Priming appears to facilitate a response to the second CF (e.g., actually decreasing adherence and, although not yet discussed, promoting a redistribution of surface binding sites) not observed if the cells were only challenged with a single CF [7]. Desensitization must concern investigators of neutrophil adherence for two reasons. First, isolation techniques provide a harsh environment for neutrophils and this process may liberate CF which could prime control cells. This may explain why some researchers have reported that CF reduces neutrophil adherence. Second, if two CF exposures (i.e., a primary and a challenge dose) reduce neutrophil

adherence, is this reduced adherence due to the cell's failure to release enzymes or a direct effect upon the cell's adherence mechanisms (e.g., a redistribution of binding sites).

What are some of the mechanisms by which a CF could alter neutrophil adherence to a surface? Chemoattractants, by decreasing the net negative surface charge, may decrease the repulsion forces between the cell and its attachment surface [92]. This would appear to allow for more collisions, possible recognition and attachment. Chemoattractants also alter the numbers or affinity of membrane receptors for various ligands. Kay et al have reported that CF enhances the numbers of C_3b and not Fc receptors on neutrophils [124]. Likewise, CF apparently enhances the number of neutrophil binding sites for protein coated surfaces [7, 35]. The enhanced attachment of C_3b or protein coated particles did not appear to require protein synthesis. If indeed CF has increased the numbers of receptors, two conditions may explain these events. Either new membrane, with additional receptors, has been inserted into the cell or a marginal pool of "hidden" receptors were uncovered following CF stimulation. Chemoattractants have been shown to increase neutrophil volume and membrane fluidity [125]. These agents also promote degranulation and granule membranes appear to not only become incorporated into the cell membrane, but also possess surface receptors common with the cell membrane [109].

Chemoattractants also appear to polarize certain neutrophil membrane receptors and restrictions of this receptor topography would appear to influence cell function, including adherence. Walter et al [4] have demonstrated that Fc receptors, normally evenly distributed on neutrophils, redistribute to the lamellapodia of neutrophils responding to a gradient of CF. Lectin binding sites, again normally evenly distributed, also appear to occur at the lamellapodia of neutrophil orienting in a gradient of CF. The bound lectins are then observed to flow along the cell surface and "cap" on the uropods of these neutrophils [8]. Smith and Hollers have reported that a single chemotactic stimulus enhances the numbers of albumin coated particles bound by neutrophils. These particles were bound on the cell in an evenly distributed manner. However, unlike the reports on ligand capping, these binding sites, redistributed to the uropods on neutrophils exposed to sequentially increasing concentrations of CF and this redistribution was independent of ligand attachment [7].

Although the mechanism(s) by which CF promotes neutrophil membrane binding site and binding site-ligand redistributions remains speculative, it is probable that cytoskeletal alterations will eventually be linked to these processes. Chemoattractants may also alter the attachment substrata. CF may bind to various substrata, which provides a recognizable ligand on the substrata for neutrophil attachment via their chemotactic receptors. The activation

or release of neutrophil enzymes also occurs following cell stimulation by CF, which may influence cell or substrata adhesiveness. Potent inhibitors of serine esterases, some of which exist in vivo and in high concentrations at inflammatory sites, inhibit phagocytic chemotaxis, phagocytosis, enzymes released during degranulization, and super-oxide generation [8, 49, 126, 127]. Wilkinson suggested that neutrophil enzymes decrease cell attachment and mobility on protein coated surfaces possibly by cleaving attachment proteins from the substrata [118]. Enzymatic activity may also generate substances recognized by neutrophil membrane attachment sites. Neutrophil enzymes cleave fibrinogen in vitro, and apparently in vivo, in a plasmin like manner [80]. Neutrophils adhere and migrate on strands of fibrin, a property formerly used to isolate these cells. Neutrophil proteases may also influence the formation and degradation of chemoattractants. These enzymes may also loosen interstitial spaces facilitating penetration. Enzymatic modulation of neutrophil adherence may also be a result of protease activity on the cell membrane. Macrophage affinity for antibody coated particles was shown to be enhanced following pretreatment of the cells with proteases [126]. Neutrophil adhesiveness to endothelial cells was also reported to be decreased following protease pretreatment [129]. Several surface enzymes appear to be activated by CF [120] and their role in influencing cell adherence remains unknown. Chemoattractants activated

chymotrypsin like ecto-enzymes may be involved in the chemotactic activation of the cell [12].

Therefore, neutrophil ecto-enzymes or endo-enzymes, released or activated by CF or other stimuli, may influence neutrophil adherence by altering attachment surfaces, affinities of surface attachment sites, modulating concentrations of and/or responsiveness to CF, and perhaps by serving as receptors for appropriate substrates on the cell membrane.

MATERIALS AND METHODS

I. Isolation of Human Neutrophils

Human blood (5 ml) was obtained from healthy adult donors by venipuncture and immediately placed in buffered citrated vacutainer tubes (Bectin-Dickinson, Rutherford, New Jersey). The citrated blood was diluted with an equal volume of Delbecco's phosphate buffered saline (PBS) and overlaid on 3-4 ml of Ficoll-Hypaque (2.16 grams ficoll, 3.396 grams hypaque; q.s. 34 ml distilled water) (Sigma Chemical Company, St. Louis, Missouri). This solution was centrifuged (1500 rpm for 20 minutes) and the supernatant removed by suction. The cell button, consisting of granulocytes and erythrocytes, was resuspended in 9 ml of CA^{++} free PBS and erythrocytes sedimented from the suspension by dextran sedimentation as previously described [7, 14, 35]. Briefly, 1.0 ml of a 6% (W/V) solution of dextran (70,000 MW; Sigma) in PBS was added to the suspension, incubated 45 minutes at 22°C, the granulocyte rich supernatant removed and washed with an equal volume of PBS. The cells were then resuspended in PBS and counted. Rare platelets or mononuclear cells were observed in the suspension and the granulocyte-erythrocyte ratio was usually greater than 5:1. Greater than 95% of all observed granulocytes in these preparations

were neutrophils and greater than 98% of these cells remained viable hours after separation (determined by eosin exclusion). All isolated cells (10^7 cells/ml of PBS) were refrigerated (4^oC) when not used.

II. Neutrophil Pretreatment

Neutrophils (10^6 cells/0.1 ml PBS) were incubated with 0.9 ml of a specified concentration of Formyl-L-methionly-L-phenylalanine (fMP) (Sigma), colchicine (Sigma), or PBS for a selected time interval and temperature. The treated cells were then either used while in the pretreatment solutions or centrifuged (400 rpm for 5 minutes), the supernatant decanted, and tests performed on the pelleted cells. When cells were to be exposed to multiple doses of pretreatment reagents the conditions for treatment are carefully described within the results section for each experiment. All cell pretreatments were carried out in glass test tubes previously coated with 50% human serum and washed in PBS. Coating the glass with serum proteins was found to greatly reduce cell adherence to the glass during the time intervals required for treatment.

III. Neutrophil Binding of Lectins, Bacteria, and Protein Coated Spheres

Concanavalin A (ConA) and FITC labeled ConA were obtained from Sigma Chemical Company. Succynylated concanavalin A (SConA) was a generous gift from Dr. John

Wang's laboratory, Michigan State University. Lectins (10μg/ml) were incubated with pretreated neutrophils (10^6 cells/ml) for varying time intervals and the cells fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde (Sigma) for 30 minutes. The fixed cells were then washed X3 with PBS. If the lectins had not been previously labeled with fluoroscein isothrocyanate (FITC), the cell bound lectins were labeled using an immuno-histochemical "sandwich" technique. Briefly, the fixed cells with ConA attached to their surface were incubated in solutions containing rabbit anti-ConA for 1 hour at 37°C, washed in PBS, and reincubated with solutions containing FITC labeled goat anti-rabbit immunoglobulin overnight. The cells were then washed in PBS. All antisera were purchased from Behring Diagnostics, North Somerville, New Jersey. To examine for the presence of FITC labeled lectins on the cell surface, the cells were mounted on glass slides, coverslipped, and examined using a Leitz microscope (100X objective) with UV illumination.

Gram negative bacteria (a generous gift of Dr. Robert Moon's laboratory, Michigan State University) were grown (18-24 hours at 37°C in a 5% CO_2 atmosphere) in Tryptic Soy Broth (Difco Laboratories, Detroit, Michigan), heat killed (56°C for 30 minutes) and labeled with FITC as previously described [5, 89]. Briefly, 10^{10} bacteria/ml of saline were incubated 30 minutes at 37°C with FITC (1mg/ml saline adjusted to a pH of 11.0 with 1N naOH) and washed X3 in PBS.

The organisms used were Eschericia coli and piliated or non-piliated strains of Salmonella typhimurium. Neutrophils (10^6 cells) were incubated for various times at 22°C with varying concentrations of the bacteria and then fixed in 2% glutaraldehyde (10 minutes at 22°C). To remove bacteria not bound by neutrophils, the suspension was centrifuged (1500 rpm for 20 minutes) through a Ficoll-Hypaque solution (same concentration used for cell isolation). Neutrophils with attached bacteria were pelleted through the Ficoll-Hypaque suspension when centrifuged at 1500 rpm for 20 minutes, leaving the unattached bacteria in suspension. Several experiments utilized bacteria treated with human C_3 (Cordis) and fresh or heat inactivated (56°C for 30 minutes) human serum prior to incubation with neutrophils. Human serum was obtained by centrifugation of human coagulated blood. Bacterial specific immunoglobulins were absorbed from serum as previously described [5]. Briefly, 10^9 heat killed bacteria were incubated with human serum 15 minutes at 0°C, centrifuged for 10 minutes at 2000 rpm (0°C) and the serum decanted off the bacteria. The conditions for treating bacteria with human proteins and concentrations of human proteins used are clearly specified for each experiment within the Results section. Following treatment, the bacteria were washed and resuspended in the various neutrophil pretreatment solutions before incubation with neutrophils.

Microscopic examination of bacteria attached to the neutrophil surface was performed using several techniques.

The aldehyde fixed cells were washed in PBS X3, placed on a glass slide, a coverslip attached and the cells examined using the Leitz microscope (50-100X objectives) with a white light or UV illumination. The aldehyde fixed cells with attached bacteria were also prepared for scanning electron microscopic (SEM) examination as previously described [7]. Briefly, the aldehyde fixed cells were washed in distilled water and applied to poly-L-lysine coated glass coverslides (10mm diameter). The coverslip was rinsed in tap water, dehydrated in a graded series of ethanol, and critical point dried in a CO_2 liquid-gas transition (1300 psi at 42°C). The coverslips were then attached to aluminum stubs and "sputter coated" with gold (approximately 200nm in thickness). The stub was placed in a JEOL 135 C SEM and the cells examined at 3000-10,000X magnifications.

Latex spheres (Sigma) of varying diameters (0.6-1.1u) were washed X3 in PBS and treated with a variety of human serum proteins. The conditions for these treatments are carefully described for each experiment within the Results section. The reagents used include human serum, human albumin (Sigma), rabbit or goat anti-human albumin (Behring Diagnostics), and human C_3, a generous gift of Dr. Richard Patrick's laboratory (Sterling Winthroupe Research Institute, New York); and Cordis Chemical Corporation (Miami, Florida). Following all treatments, the spheres were washed in PBS and stored at 4°C. All treated spheres were resuspended in the same concentrations of solutions used to pretreat the

neutrophils. Neutrophils (10^6 cells/0.1ml of pretreatment solution) were mixed with 0.1ml of the treated spheres and incubated for various times before fixation (2% glutaraldehyde; 10 minutes at 22°C). Spheres not bound by the neutrophils were removed after aldehyde fixation using a Ficoll-Hypaque density gradient as previously described.

Several microscopic techniques were also used to evaluate neutrophil-sphere attachments after each experiment. First, aldehyde fixed cells were washed in PBS, mounted on glass slides, coverslipped and examined using a Leitz microscope (50-100X objectives) with white light or UV illumination. Neutrophils were also prepared for SEM examination as previously described. When cells were prepared for transmission electron microscopic (TEM) examination, the aldehyde fixed cells were washed in PBS and post fixed (10 minutes at 22°C) in a 0.1M phosphate buffer solution containing osmium tetroxide (1.0%). The fixed cells were then washed in PBS, incubated for 3 minutes in a graded series of ethanol (50%, 75%, and 100%), and immediately covered (2-5mm thickness) with a 50% (W/V) solution of LX 112 Resin with hardener (Polysciences, Inc., Warrington, Pa.) in absolute acetone. After a 3-hour incubation at 22°C, the resin was trimmed into 2mm squared and sectioned using an ultra microtome with a glass knife. The sections were mounted on copper grids, stained with lead citrate and uranyl acetate, and examined in a Phillip's 201 TEM at 3000-10,000X magnifications.

IV. Neutrophil Adherence to Glass or Protein Coated Glass

Multiple well tissue culture chambers (Tissue Tech) fixed onto glass microscope slides were used to evaluate neutrophil adherence to glass. Each chamber was composed of 10 individual wells. Each well held 0.4ml of fluid and provided approximately $5mm^2$ of a uniform substrata (glass) on which neutrophils could settle and attach. The glass surface could easily be treated with a variety of solutions. Solutions containing human fibrinogen (Kabi, Grade L, Stockholm, Sweden, 98% clottable; a generous gift of Dr. Douglas W. Estry, American Red Cross, Lansing, Michigan), Knox gelatin, human collagen (Sigma), human C_3 (Cordis), human serum and PBS were incubated in these chambers for various time intervals and washed with PBS. Neutrophils (10^5 cells/0.3ml) pretreated as previously described were then injected into each well, incubated (37^oC) for various times, and then the chamber inverted. The wells of the chamber were immediately detached from the glass slides. The glass slide was then immersed in methanol, and attached cells stained with Wright's stain. The slides were then air dried, and neutrophils fixed onto the glass slide were counted using an image analyzer (Optimax; Hollis, New Hampshire). The protein coated glass was, on occasion, exposed to a variety of enzymes (e.g., porcine pancreatic trypsin or chymotrypsin, Sigma) prior to use. The conditions for these treatments are specified for each experiment within the Results section.

On several occasions, specific enzyme inhibitors were also used to terminate enzyme action on these surfaces. These included L-1-tosylamide-2-phenyl-ethylchloio-methyl ketone (TPCK) and n-alpha-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma). The conditions for their use are also specified for each experiment within the Results section.

V. Presentation and Analysis of Data

The data are expressed in terms of the mean ± standard error of the mean; n represents the number of experiments. Students' test was used to assess significance. Those items compared with p values $<$ 0.005 were considered significantly different; those with p values $>$ 0.50 considered strongly related. Most often comparisons were made to a "control population" response identified within each experiment.

RESULTS

I. The Influence of Albumin or Immunoglobulin on Neutrophil Adherence of Latex Spheres

A. Sphere Pretreatment and Neutrophil Binding of Albumin or Immunoglobulin Coated Spheres

Latex spheres are uniform, available in different sizes ranging from 0.6-1.1μ in diameter, and easily observed using light microscopy, SEM, or TEM. Their ability to bind proteins enabled evaluation of neutrophil adherence of particles coated with purified human proteins.

Latex spheres were washed (X3) and reconstituted in PBS (0.1% packed sphere volume), aliquoted and used or stored at $4^{o}C$. Aliquots of the spheres were then incubated with 10% human serum albumin (HSA) in PBS for 30 minutes at $37^{o}C$. The spheres coated with HSA (albumin coated latex spheres or ACLS) were washed in PBS. Aliquots of ACLS were stored at $4^{o}C$ or reincubated with rabbit anti-human HSA (antibody coated spheres or Ab-ACLS) for 30 minutes at $37^{o}C$, washed in PBS and stored at $4^{o}C$. Isolated human neutrophils (10^{6} cells/ml PBS) were centrifuged (400 rpm for 10 minutes) into a cell pellet within serum coated 12X75 mm glass test tubes. The supernatant was discarded and the cells gently

resuspended. Washed latex spheres, ACLS or Ab-ACLS were added (0.1ml) to the neutrophil suspensions, gently mixed, and incubated for one minute at 22°C. Neutrophil adherence of the spheres was terminated by diluting the suspensions with 2ml of PBS and immediately fixing the cells with an equal volume of 2% glutaraldehyde for 10-30 minutes at 22°C. Spheres not bound by neutrophils after aldehyde fixation were removed using a Ficoll-Hypaque density gradient and the number of spheres bound by neutrophils determined using phase-contrast microscopy.

The data in Table 1 demonstrates that neutrophils avidly adhere latex spheres, and HSA coating of the spheres almost totally abolished this adherence. The HSA coated spheres were refrigerated and used over a span of several days with little variations to the results. The reduced adherence did not appear to be a direct effect of HSA on cell adherence mechanisms, since pretreating neutrophils with 10% HSA (10 minutes at 22°C) did not impair their ability to adhere (11 $\pm$ 1.0 latex spheres/neutrophil, n=2) latex spheres (0.1% packed volume). Incubation of ACLS in solutions containing rabbit anti-human albumin agglutinated ACLS, yet following the removal of the antiseras, the spheres were easily dispersed by sonification. Immunoglobulin was demonstrated as being antigenically present on the surface of the spheres days after their preparation using FITC labeled anti-rabbit immunoglobulins (n=3). The attachment of immunoglobulin to ACLS significantly ($p < 0.001$) enhanced the ability of

Table 1.

* Neutrophils (10^6 cells/0.1ml PBS) were incubated with the pretreated latex spheres (0.1% packed volume, 0.8u in diameter) for 1 minute at 22°C, diluted with 2 ml PBS and fixed with equal volumes of glutaraldehyde.

** A 1.0 volume of PBS, HSA (10% in PBS) or a variety of antiseras (1:10 titer in PBS) was used for each pretreatment. Spheres (0.05 ml of a 10% packed sphere volume) were washed in PBS after each pretreatment. After the last pretreatment the spheres were reconstituted to a 0.1% packed sphere volume with PBS.

‡ 50-100 neutrophils/test were examined using phase-contrast microscopy (100X objective), attached spheres counted and divided by the number of cells observed.

§ n = the number of individual experiments performed.

Table 1. Neutrophil Adherence of Latex Spheres, Albumin Coated Latex Spheres, and Immunoglobulin Coated Latex Spheres.*

Latex Sphere Pretreatments**			Sphere Aggregation	Average Number Spheres/Neutrophil‡	(n)§
I	II	III			
PBS (Latex Spheres)	--	--	--	8.2 ± 0.75	(3)
HSA	--	--	--	0.2 ± 0.01	(8)
HSA	Rabbit Anti-Human Albumin	--	+	7.5 ± 0.90	(8)
HSA	Rabbit Anti-Human C_3	--	--	0.1 ± 0.02	(3)
HSA	Rabbit Anti-Human Albumin	Goat Anti-Rabbit Immunoglobulin	+	0.2 ± 0.01	(4)
HSA	Rabbit Anti-Human Albumin	HSA	+	7.3 ± 0.50	(5)

neutrophils to bind the treated spheres (Table 1). This enhanced adherence persisted when the antisera was heated (56°C for 30 minutes) before use (n=3). Rabbit anti-human C_3 failed to agglutinate ACLS and spheres treated with this antisera were poorly bound by neutrophils. Although goat anti-human albumin agglutinated ACLS, it failed to enhance neutrophil adherence of the spheres. Removal of albumin from ACLS by components within the albumin specific antisera could not account for the enhanced neutrophil adherence of Ab-ACLS since re-incubation of Ab-ACLS in HSA failed to markedly diminish sphere adherence to neutrophils (Table 1). The Ab-ACLS were also used days after their preparation with little variation to the results. Sonification of ACLS did not alter neutrophil adherence of ACLS (0.2 ± 0.03 ACLS/ neutrophil, n=3).

B. Chemoattractant or Colchicine Modulation of Neutrophil Adherence of Immunoglobulin Coated Spheres

Since chemoattractants and antitubulins influence neutrophil adhesiveness to various substrata, their effects on neutrophil adherence of Ab-ACLS were studied. Neutrophils were preincubated in PBS, colchicine ($2X10^{-6}M$), or fMP for 15 minutes at 37°C. Single or multiple concentrations of fMP were used to pretreat the neutrophils. The cells were either exposed to $10^{-8}M$ or $10^{-6}M$ fMP for 10-15 minutes or exposed to $10^{-8}M$ for 5 minutes and then re-exposed to $10^{-6}M$ fMP for 10 minutes (10^{-8}-10^{-6}MfMP). All treated cells were then

centrifuged into a cell pellet, and the supernatant discarded. The cells were then gently mixed with 0.1ml of Ab-ACLS and incubated at 22°C for 1 minute. The cell mixtures were then diluted 20 fold in their respective cell pretreatment solutions, immediately fixed with an equal volume of 2% glutaraldehyde, and incubated 10-30 minutes at 37°C. Spheres not adhering to neutrophils were removed using a Ficoll-Hypaque density gradient and sphere binding evaluated using light microscopy or SEM.

The data in Table 2 demonstrates that the percentages of cells binding Ab-ACLS did not appear to be influenced by colchicine or fMP pretreatments. Chemoattractants or colchicine also did not appear to enhance neutrophil adherence of Ab-ACLS even when the concentrations of Ab-ACLS were increased 10 fold (Table 2). Decreasing Ab-ACLS concentrations to 0.01% packed volume almost totally abolished sphere adherence to neutrophils (0.55 ± 0.07 Ab-ACLS/neutrophil, n=2). The neutrophil pretreatments also did not appear to alter the fraction of cells adhering Ab-ACLS.

The topographical surface distributions of adhering Ab-ACLS were also evaluated on fMP, colchicine, or PBS treated neutrophils. The data in Table 3 demonstrates that two adherence distributions were routinely observed using light microscopy or SEM to examine the pretreated cells. Consistently greater than 90% of the cells pretreated in PBS or 10^{-8}M fMP remained spherical after pretreatment (n=11) and the majority of them bound Ab-ACLS at a single region on

Table 2.

* 10^6 neutrophils were incubated (10 minutes at 37°C) in various pretreatment solutions, centrifuged into a pellet, and the supernatant discarded.

** Ab-ACLS were reconstituted in the pretreatment solutions to a concentration of 0.1% or 1.0% packed sphere volumes. To each cell button, 0.1 ml of the spheres were added, incubated 1 minute, diluted with 2.0 ml of the pretreatment solution and immediately fixed with 2.0% glutaraldehyde. 50-100 neutrophils were examined and the number of spheres adhering to these cells counted and this number divided by the number of cells counted.

‡ 100 cells were examined for each test and the number of cells adhering spheres divided by the number of cells examined.

§ n = the number of individual experiments performed.

Table 2. The Effects of Colchicine or Chemoattractants on Neutrophil Binding of Immunoglobulin Coated Spheres (Ab-ACLS)

Cell Pretreatment *	Ab-ACLS/Neutrophil **				Fraction of Neutrophils Adhering Ab-ACLS ‡				
	0.1%	(n)	1.0%	(n)	0.1%	(n)	1.0%	(n)	§
PBS	4.5 ± 0.2	(8)	5.5 ± 0.5	(3)	0.61 ± 0.04	(5)	0.65 ± 0.05	(2)	
Colchicine ($2X10^{-6}M$)	4.5 ± 0.4	(4)	4.3 ± 0.9	(3)	0.66 ± 0.07	(5)	0.59 ± 0.05	(2)	
fMP ($10^{-8}M$)	4.9 ± 0.3	(5)	5.4 ± 0.5	(3)	0.63 ± 0.05	(3)	0.63 ± 0.03	(2)	
fMP ($10^{-6}M$)	5.1 ± 0.4	(6)	5.0 ± 0.6	(3)	0.66 ± 0.05	(5)	0.66 ± 0.05	(2)	
fMP (10^{-8}-$10^{-6}M$)	4.8 ± 0.1	(5)	4.9 ± 0.6	(3)	0.53 ± 0.06	(5)	0.67 ± 0.07	(2)	

Table 3.

* 10^6 cells were pretreated with PBS, colchicine, single or multiple doses of fMP for 10 minutes at 37°C and centrifuged into a pellet.

** Ab-ACLS (0.1%) suspended in each pretreatment solution were added (0.1 ml) to the pretreated cells, incubated 1 minute, diluted with 2 mls of the pretreatment solution and immediately fixed with an equal volume of 2.0% glutaraldehyde. Following glutaraldehyde fixation and removal of unattached spheres, the cells were examined using phase-contrast microscopy. The spheres attached on spherical cells were categorized as being random or localized. Sphere attachment on cells with distinct front and tail ends was categorized as being random, lamellapolar (front binding), or uropolar (tail end binding). For cells to be placed in a lamellapolar or uropolar category, that cell must have bound greater than 90% of all spheres exclusively at the front or tail end respectively. 100 cells were examined for each test and the percentages of cells placed within each category determined.

‡ n = the number of individual experiments performed.

Table 3. Effects of Colchicine or Chemoattractants on Neutrophil Binding Distributions for Immunoglobulin Coated Spheres (Ab-ACLS)

Cell Pretreatment *	Topographical Binding Distributions of Ab-ACLS **				
	% Random	% Localized	% Uropolar	% Lamellapolar	(n) ± ±
PBS	29 ± 1.2	71 ± 1.3	--	--	(6)
fMP (10^{-8}M)	22 ± 1.0	78 ± 2.2	--	--	(3)
Colchicine ($2X10^{-6}$M)	26 ± 1.8	--	11 ± 2.8	63 ± 3.5	(3)
fMP (10^{-6}M)	29 ± 1.2	--	--	67 ± 1.1	(3)
fMP (10^{-8}-10^{-6}M)	34 ± 1.8	--	6 ± 2.7	60 ± 3.6	(3)

their surface. Since the cells were spherical and possessed no discernable front or tail ends, this type of binding was classified as localized (Table 3). Colchicine and fMP (10^{-6}M or 10^{-8}-10^{-6}M) pretreatments caused the majority of the neutrophils (greater than 65%, n=15) to change shape, forming a polar configuration. These cells possessed a distinct front and tail end that was easily discernable using light or scanning electron microscopy. When Ab-ACLS were incubated with the polarized cells, they were observed to bind only at one pole on the cells regardless of cell pretreatment. That region was the front end or lamellapolar region of the polarized neutrophil (Table 3). When neutrophils were polarized, ruffling occurred primarily at the front of the cells and this was the principle region for attachment of Ab-ACLS (Figure 1a). Spherical neutrophils binding of Ab-ACLS at a single region was often observed to cause the cells to ruffle near the Ab-ACLS attachment sites (Figure 1b).

An attempt was made to enhance the ruffling on spherical cells and attachment of Ab-ACLS re-evaluated. Neutrophils (10^6 cells) were incubated in 10^{-6}M fMP (0.1ml) for 10 seconds and Ab-ACLS immediately added. Neutrophil adherence of the spheres was terminated 30 seconds after the addition of the spheres by aldehyde fixation. Although this short incubation period with fMP did not induce shape change, the majority of the cells (greater than 90%, n=3) were observed

Figure 1. 10^6 neutrophils were pretreated with PBS, colchicine, or various concentrations of fMP for 10 minutes, then incubated with 0.1 ml Ab-ACLS (0.1%) for 1 minute before being fixed in 2.0% glutaraldehyde. The majority of neutrophils pretreated with colchicine (2×10^{-6}M), 10^{-6}M fMP or 10^{-8}M fMP assumed a polarized morphologic configuration such that these cells possessed distinct front and tail ends. These cells were observed to bind Ab-ACLS almost exclusively on their front ends (1a). Cells pretreated with PBS or 10^{-8}M fMP remained spherical but also bound the Ab-ACLS at one restricted region on these cells. Photomicrographs of cells examined using SEM at 6000-7000 magnifications. RBCs in the background were never observed binding Ab-ACLS.

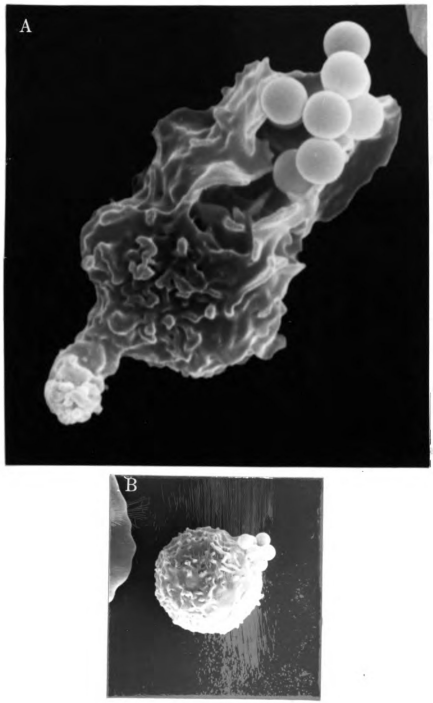


Figure 1. Neutrophil Binding of Immunoglobulin Coated Spheres (Ab-ACLS)

to ruffle extensively around their entire periphery. These cells also bound most Ab-ACLS at a single localized region (59 $\pm$ 2% of the cells examined).

C. Evaluation of the Ability of Neutrophils to Simultaneously Adhere Albumin and Immunoglobulin Coated Spheres

Neutrophils (10^6 cells/0.1ml) have been shown to adhere ACLS[7] when latex spheres were coated with 2% HSA and the sphere concentration was 5-20X greater than used in the previous experiments. Therefore, latex spheres were incubated in 2% HSA, washed in PBS, reconstituted to a 5% packed volume and neutrophil attachment re-evaluated. Neutrophils (10^6 cells) were pretreated with PBS, fMP or colchicine as previously described, the supernatant discarded, and 0.1 ml of ACLS (5% packed volume suspended in the appropriate cell pretreatment solution) added to the cell suspension. The cell-sphere mixture was gently vortexed, incubated 1 minute at 22°C, diluted 20 fold with the cell pretreatment solution, and fixed immediately with 2% glutaraldehyde.

As the data demonstrates in Table 4, fMP significantly enhanced the ability of neutrophils to bind ACLS and this enhanced adherence did not appear to be due to fMP increasing the adhesiveness of a separate population of cells. The patterns of attachment of ACLS on the pretreated neutrophils (Table 4) were also similar to those described previously [7] and very different from neutrophil attachment of Ab-ACLS

Table 4.

* 10^6 neutrophils/0.1ml were pretreated in PBS, colchicine, or fMP (0.9ml) for 10 minutes at 37°C, centrifuged into a cell pellet and the supernatant discarded. ACLS (0.1ml) were added to the cell pellet, gently mixed, incubated for 3 minutes at 22°C, and then diluted with 2 ml of 2% glutaraldehyde. Spheres not adhering to the cells were removed using a Ficoll-Hypaque density gradient.

** The numbers of ACLS adhering to 100 neutrophils/experiment were counted using light microscopy and averaged.

‡ The numbers of cells adhering more than 3 spheres were also counted and divided by total number of cells examined (100).

§ The attachment of ACLS on the spherical neutrophils was observed to be either in a random fashion or at localized regions on the cells. Polarized neutrophils (i.e., those pretreated with colchicine, 10^{-6}M fMP or 10^{-8}-10^{-6}M fMP) were observed to bind ACLS (greater than 90% of all attached spheres) on their front ends (i.e., lamellapolar adherence distributions), on their tails (i.e., uropolar), or randomly across their surface. For each experiment 100 cells were examined, the binding of ACLS on the observed cells examined, and each cell placed in one of the above categories. The ratio of the numbers of cells in each category was compared to total cells counted for each experiment and this ratio expressed as a percentage.

" n = the number of individual experiments performed.

Table 4. Neutrophil Adherence of Albumin Coated Spheres (ACLS)

Cell Pretreatment *	ACLS/Neutrophil (n=3) **	Fraction of Cells Adhering ACLS (n=2) ‡
PBS	4.4 ± 0.2	0.68 ± 0.01
Colchicine (2 X 10^{-6}M)	3.9 ± 0.4	0.70 ± 0.02
fMP (10^{-8}M)	4.6 ± 0.3	0.71 ± 0.03
fMP (10^{-7}M)	5.8 ± 0.3	0.73 ± 0.03
fMP (10^{-6}M)	8.2 ± 0.3	0.73 ± 0.02
fMP (10^{-8}-10^{-6}M)	7.6 ± 0.2	0.71 ± 0.02

Cell Pretreatment	Topographical Adherence of ACLS § (n)"				
	% Random	% Localized	% Uropolar	% Lamellapolar	(n)
PBS	93 ± 3.1	7 ± 0.04	--	--	(6)
fMP (10^{-8}M)	86 ± 4.2	14 ± 0.90	--	--	(3)
Colchicine (2 X 10^{-6}M)	35 ± 0.4	--	64 ± 1.1	--	(3)
fMP (10^{-6}M)	82 ± 2.0	--	14 ± 2.1	--	(3)
fMP (10^{-8}-10^{-6}M)	36 ± 1.5	--	60 ± 1.8	--	(3)

(Table 3). The ACLS were observed to bind in a random fashion around the entire periphery of spherical neutrophils. To the majority of polarized cells, they bound either in a random rashion (i.e., those pretreated with 10^{-6}M fMP) or almost exclusively (greater than 90% of attached spheres) on their uropolar or tail ends (i.e., those pretreated with colchicine or multiple doses of fMP, Figure 2).

Since very different binding distributions for ACLS and Ab-ACLS were observed on PBS, colchicine, and fMP pretreated neutrophils, equal aliquots of Ab-ACLS (0.2% packed sphere volume, 1.1μ in diameter) and latex spheres coated with 2% HSA (5.0% packed sphere volume, 0.8μ in diameter) were mixed in PBS, colchicine, or fMP as previously described. Consistently greater than 50% of all observed cells bound Ab-ACLS (i.e., the larger of the two populations of spheres) regardless of cell pretreatment and the adherence of ACLS (smaller spheres) appeared unrestricted by the binding of Ab-ACLS (Table 5). Neutrophil attachment distributions of Ab-ACLS and ACLS also remained unchanged on all pretreated cells. Greater than 84% of the neutrophils adhering both Ab-ACLS and ACLS bound Ab-ACLS at their lamellapodia on polarized neutrophils or at a single ruffled region on the spherical neutrophils. The adherence of ACLS on PBS or fMP (10^{-8}M or 10^{-6}M) pretreated cells also adhering Ab-ACLS, occurred in a random fashion on the majority ($78 \pm 3.4\%$, $76 \pm 5.3\%$, and $69 \pm 4.5\%$ respectively, n=3) of the cells observed. ACLS were observed attached only on the uropods on the majority of colchicine

Table 5. * 10^6 neutrophils were pretreated (10 minutes at 37°C) with various solutions, centrifuged into a pellet, and resuspended in 0.1 ml of mixtures of 0.2% Ab-ACLS (1.1μ in diameter) or 5.0% ACLS (0.8μ in diameter). Following a 1-minute incubation the cells were fixed in 1% glutaraldehyde and examined using phase-contrast microscopy.

** 100 neutrophils were examined and the number of cells adhering 1.1μ spheres counted and compared to the number of total cells examined. When examining cells adhering 1.1μ spheres, the numbers of these cells also adhering 0.8μ spheres (ACLS) were also counted and compared to the total number of cells adhering Ab-ACLS. All number ratios were expressed as a percentage.

‡ n = the number of individual experiments performed.

Table 5. Neutrophil Adherence of Immunoglobulin (Ab-ACLS) and Albumin Coated Spheres (ACLS)

Cell pretreatment *	% Neutrophils Adhering Ab-ACLS **	% Neutrophils Adhering ACLS	(n) ‡
PBS	53 ± 1.9	74 ± 4.4	(3)
fMP (10^{-8}M)	54 ± 2.8	77 ± 3.9	(3)
Colchicine (2 X 10^{-6}M)	55 ± 1.2	73 ± 5.3	(3)
fMP (10^{-6}M)	51 ± 0.7	81 ± 2.0	(3)
fMP (10^{-8}-10^{-6}M)	55 ± 3.1	76 ± 1.4	(3)

Figure 2. Neutrophils (10^6 cells) were pretreated with PBS, colchicine, and various concentrations of fMP, then incubated with 0.1 ml of ACLS (5.0%) for 1 minute and immediately fixed in glutaraldehyde. Cells pretreated in PBS (a) or 10^{-8}M fMP (b) remained spherical and most of these cells bound ACLS in a random fashion around their periphery (a,b). Neutrophils pretreated with 10^{-6}M fMP changed shape and ACLS were also observed to bind in a random fashion on the majority of these cells (c). Colchicine (d) and fMP (10^{-8}-10^{-6}M) (c) pretreated neutrophils also formed a polar configuration yet consistently appeared to bind ACLS only at their uropods (c). All photomicrographs obtained using SEM at 6000-7000 magnifications.

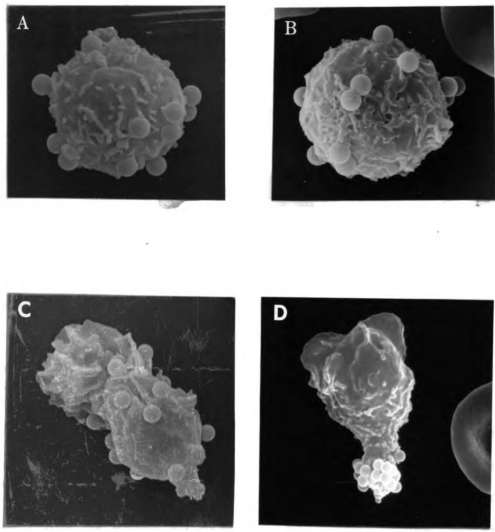


Figure 2. Neutrophil Binding of Albumin Coated Spheres (ACLS)

and fMP (10^{-8}-10^{-6}M) pretreated cells (71 $\pm$ 4.2% and 76 $\pm$ 3.4%, respectively, n=4) also adhering Ab-ACLS. Figure 3 is a collection of SEM photomicrographs which illustrate the simultaneous adherence patterns of Ab-ACLS and ACLS on various pretreated neutrophils.

D. Redistribution of Neutrophil Bound Immunoglobulin Coated Spheres

Thus far, experiments had been designed to assess neutrophil adherence and adherence topographies for protein coated spheres. Time intervals following sphere attachment to neutrophils were altered next and the effects of time on neutrophil redistribution of cell bound spheres examined. Neutrophils were incubated in PBS, colchicine or fMP, centrifuged into a cell pellet, mixed with Ab-ACLS (0.1%) or ACLS (5.0%) and incubated for 1 minute at 22°C. The neutrophil/sphere mixtures were then diluted 20 fold in their respective pretreatment solutions to restrict further attachments and incubated for 15-20 minutes prior to the addition of an equal volume of 2% glutaraldehyde. The distributions of adhering ACLS or Ab-ACLS were then evaluated as previously described using light or scanning electron microscopy. As compared to the data in Table 4, the adherence distributions of ACLS on neutrophils appeared to remain unaltered 15 minutes after their attachment (Table 6). Phase contrast and SEM examination of all pretreated cells failed to provide any evidence of endocytosis of surface bound ACLS during these time

Figure 3. Neutrophils were pretreated with PBS, colchicine, or various concentrations of fMP, then incubated with an aliquot (0.1 ml) containing equal fractions of ACLS and Ab-ACLS for 1 minute and immediately fixed with glutaraldehyde. Cells incubated with PBS or 10^{-8} fMP remained spherical and most bound ACLS (0.6μ) randomly. Ab-ACLS were bound and ingested at localized regions on the same cells (a). Cells pretreated with 10^{-6}M fMP assumed a polar configuration and most of these cells also bound ACLS randomly with Ab-ACLS binding at their front ends (b). Colchicine and fMP (10^{-8}-10^{-6}M) pretreatment apparently caused most cells to adhere ACLS at their tail ends while simultaneously binding Ab-ACLS at their front ends. All photomicrographs were taken using SEM and 6000-7000 magnifications.

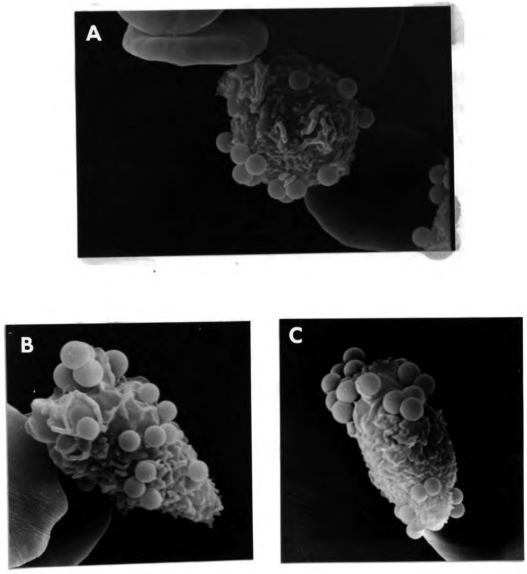


Figure 3. Simultaneous Neutrophil Adherence of Albumin (ACLS) and Immunoglobulin Coated Spheres (Ab-ACLS)

Table 6.

* Neutrophils (10^6 cells) were pretreated with PBS, colchicine or fMP at 22°C for 10 minutes, centrifuged into a pellet and the supernatant discarded. ACLS or Ab-ACLS were added (0.1 ml) to the cell pellet, gently mixed, incubated for 1 minute, diluted X20 with their respective cell pretreatment solution, and fixed with an equal volume of 2.0% glutaraldehyde after a 15-minute incubation (22°C.

** The fixed cells (100 cells) were examined using light microscopy and the numbers of cells with 80% of the spheres attached only at their front ends (L), tail ends (U), or randomly bound (R) counted. The numbers of cells placed within each category (e.g., L, U, or R) were compared to total number of cells counted and this fraction expressed as a percentage. The category with the highest percentage is given for each experiment.

‡ n = the number of individual experiments performed.

Table 6. Distributions of Albumin (ACLS) and Immunoglobulin Coated Spheres (Ab-ACLS) on Neutrophils 15 Minutes After Attachment

Cell Pretreatment *	Major Topographical Distribution **				
	ACLS	(n) ‡		Ab-ACLS	(n)
PBS	82 ± 3% R	(3)		88 ± 4% R	(3)
fMP (10^{-8}M)	78 ± 2% R	(3)		93 ± 4% R	(3)
Colchicine (2 X 10^{-6}M)	73 ± 3% U	(3)		59 ± 3% U	(8)
fMP (10^{-6}M)	74 ± 4% R	(3)		53 ± 4% U	(8)
fMP (10^{-8}-10^{-6}M)	76 ± 5% U	(3)		58 ± 3% U	(8)

intervals. When neutrophil binding of Ab-ACLS was evaluated using light microscopy, similarly pretreated cells appeared to redistribute surface bound Ab-ACLS within the same time interval (compare data in Table 3 with that in Table 6). Spherical neutrophils were observed with most Ab-ACLS distributed randomly on or inside the cell. All polarized cells were observed with Ab-ACLS on or inside their uropods. Extending the incubation period to 30 minutes prior to fixation failed to alter these results (n=5, included with data in Table 6). Only rare surface bound Ab-ACLS were observed on any of the pretreated cells when examined using SEM. This observation suggests that pretreated neutrophils were ingesting Ab-ACLS.

The time intervals needed to ingest and redistribute Ab-ACLS were then examined. The incubation period for attachment was reduced (1-10 seconds), the sphere concentration increased (1.0% packed sphere volume), and neutrophil neutrophil binding of Ab-ACLS re-evaluated. The numbers of Ab-ACLS adhering to neutrophils fixed 3-10 seconds after sphere addition were not unlike those presented in Tables 1 and 2 (n-10). It was routinely observed that, within this time frame, 5-20 spheres could be bound by all treated cells. Shortening the time interval also did not alter the binding distributions of Ab-ACLS. Greater than 56 $\pm$ 3.3% (n=8) of the spherical cells (i.e., those pretreated with PBS or 10^{-8}M fMP) were observed binding most (greater than 90%) spheres at a single localized region. More than

70 $\pm$ 3.0% (n=8) of the polarized neutrophils (i.e., those pretreated with colchicine, 10^{-6}M fMP or 10^{-8}-10^{-6}M fMP) were also observed binding Ab-ACLS almost exclusively at their lamellapodia. Pretreated neutrophils were then incubated with the spheres (3-10 seconds), diluted 20 fold with the pretreatment solutions to prevent further attachments, and glutaraldehyde fixed at various time intervals. It was not uncommon to observe, using light microscopy or SEM, Ab-ACLS endocytosis occurring at various stages on all pretreated neutrophils 10 seconds after Ab-ACLS attachment to the neutrophil surface. Within 1-3 minutes, the majority of Ab-ACLS appeared to be internalized. Following ingestion, the Ab-ACLS were observed to be randomly distributed within spherical cells and at various stages of a rearward redistribution process within polarized cells. If the spheres were 0.8μ or less, within 15 minutes spheres were observed passing through polarized neutrophils' uropolar constriction and into their uropods (Table 6). Figures 4 and 5 are a collection of light microscopic and SEM photomicrographs illustrating the events just described for Ab-ACLS redistributions on spherical and polarized neutrophils, respectively.

Two techniques were used to confirm and quantitate the ingestion of Ab-ACLS. First, spherical and polarized cells were incubated with Ab-ACLS for 5 seconds, diluted, incubated for 10 minutes and fixed in glutaraldehyde. They were then prepared for TEM examination, examined using TEM, and shown to possess the majority of cell bound Ab-ACLS within their

Figure 4. Neutrophils were pretreated with PBS or 10^{-8}M fMP for 10 minutes, then incubated with 0.1 ml of Ab-ACLS (1.0%) for 10 seconds, diluted X20 with PBS or 10^{-8}M fMP respectively and immediately or after a specified time interval, fixed with glutaraldehyde. Within 10 seconds most cells were observed with Ab-ACLS bound to a single localized region on most PBS or fMP (10^{-8}M) pretreated cells (a). Within 1 minute extensive ruffling was observed at this region of attachment (b, c). During this time frame, SEM examination of these cells (c) indicated that these spheres were probably being ingested. By 3 minutes, most externally bound spheres were not evident on these cells (e), yet many spheres were observed within the same population of cells when examined using light microscopy (d). These spheres remained randomly distributed, apparently within the cell for up to 30 minutes after attachment. SEM photomicrographs were taken at 5000-7000 magnifications and contact printed. All light photomicrographs at 1000 magnification, and enlarged 4X.

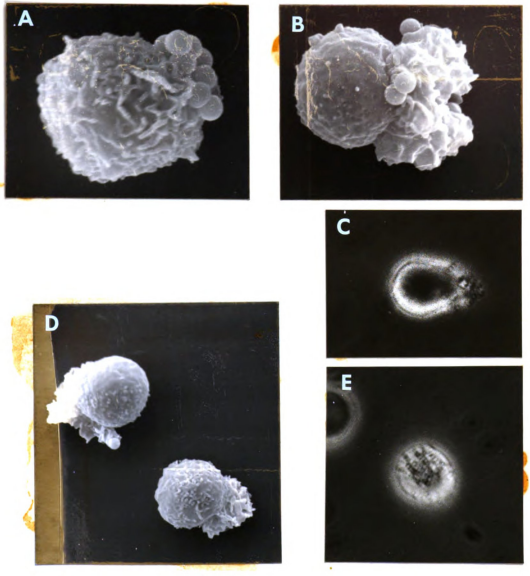


Figure 4. Redistribution of Immunoglobulin Coated Spheres (Ab-ACLS) On or Inside Spherical Neutrophils

Figure 5. Neutrophils were incubated with colchicine or fMP using conditions that cause the cells to polarize morphologically. These cells were incubated with 0.1 ml of Ab-ACLS (1.0%) for 10 seconds, diluted X20 with colchicine or fMP respectively and immediately or after a fixed interval, fixed in glutaraldehyde. The Ab-ACLS were observed to adhere on the front ends of the polarized cells (a, b) using SEM or phase contrast microscopy. Within 1 minute, SEM examination indicated that these spheres were being ingested (c). Within 3-5 minutes, spheres were absent from the majority of the cells' exterior surface (d) yet numerous spheres evident using phase contract microscopy (e) Within 10-30 minutes, the spheres were observed to redistribute rearward using phase contrast microscopy (f) and often protruding out from the cell's tail process (g). All SEM photomicrographs were taken at 6000-7000 magnifications and contact printed. All phase micrographs were taken at 1000 magnifications and enlarged at 2-5X.

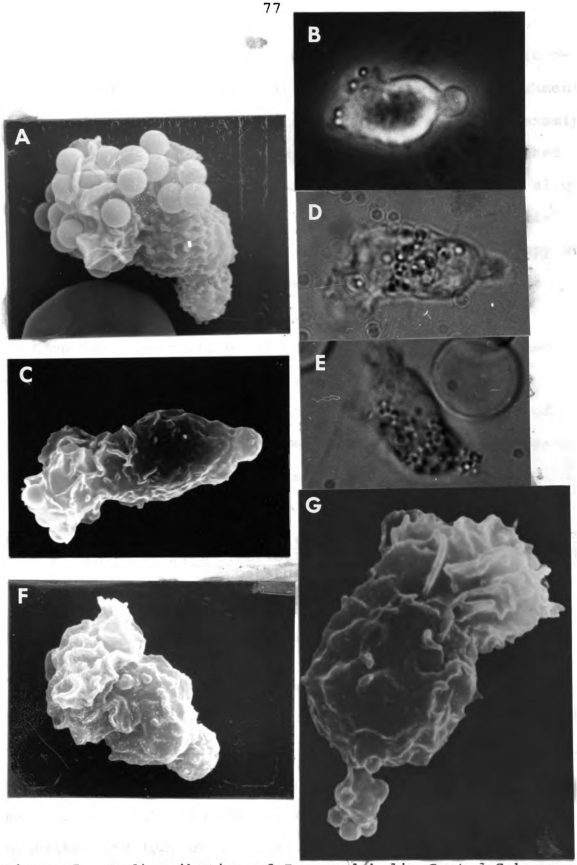


Figure 5. Redistribution of Immunoglobulin Coated Spheres (Ab-ACLS) On or Inside Polarized Neutrophils

cytoplasmic membranes (Figure 6). Next, pretreated neutrophils were aldehyde fixed 5 minutes after Ab-ACLS attachment and divided into two aliquots. The first aliquot was examined using light microscopy and the numbers of spheres attached to the pretreated cells counted and averaged. The second aliquot was prepared for SEM or incubated with FITC labeled anti-immunoglobulin and examined using SEM or light microscopy with UV irradiation (Figure 7) respectively. Visible spheres (i.e., those on the external surface of the pretreated neutrophils) using either of these microscopic techniques were also counted and averaged. The data in Table 7 summarizes these data. Consistently, greater than 85% of Ab-ACLS bound by neutrophils were not visible on the external surface of neutrophils 5 minutes after attachment.

II. Effects of Chemoattractants and Colchicine on Neutrophil Attachment and Redistribution of Lectins and Lectin-Like Substances

A. Neutrophil Binding of Lectins as Influenced by Chemoattractants and Colchicine

Neutrophils have been shown to bind Concanavalin A (ConA) and redistribute surface bound ConA rearward when pretreated with colchicine [100] or chemoattractants [8]. To extend these observations, 10^6 neutrophils were incubated (10-15 minutes at $37^{o}C$) with PBS, colchicine, or fMP as previously described and 10µg of tetravalent ConA or divalent ConA (SConA) added to the cell suspensions. These mixtures were

Figure 6. Neutrophils were pretreated with fMP (10^{-6}M) or PBS for 10 minutes, then incubated with Ab-ACLS for 10 seconds, diluted X20 with fMP or PBS and aldehyde fixed 10 minutes later. These cells were prepared for and examined using TEM. The Ab-ACLS were evident within the cytoplasmic membrane of polarized fMP pretreated cells as well as those pretreated with PBS. Spheres attached to the exterior surface of these cells' cytoplasmic membrane were rarely observed. Photomicrograph was taken at 7000 magnification. Note phagolysosome formation with spheres (arrow) and lysosomal granules.

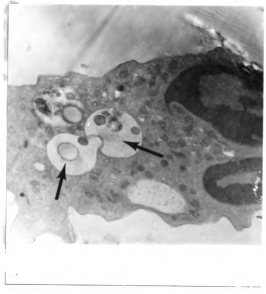


Figure 6. Examination of Neutrophils Fixed 10 Minutes After the Binding of Immunoglobulin Coated Spheres (Ab-ACLS) Using TEM

Figure 7. Following neutrophil binding of Ab-ACLS and aldehyde fixation (a) the cells were washed in PBS and reincubated with FITC labeled anti-rabbit immunoglobulin. The spheres remaining on the exterior surface of these cells were accessible for binding of the fluorescent markers and visible when exposed to a UV light source (b).

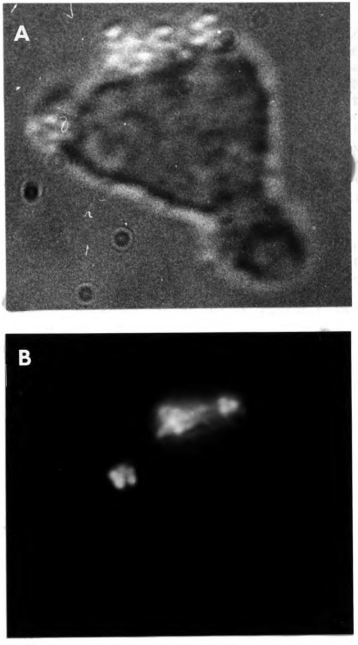


Figure 7. Neutrophil Binding of Immunoglobulin Coated Spheres (Ab-ACLS) as Observed Using Light Microscopy with White Light and UV Light Sources

Table 7.

* 10^6 neutrophils were incubated (10 minutes at 37°C) in various solutions, centrifuged into a pellet and incubated (10 seconds) with 0.1 ml of Ab-ACLS (1.0%). This mixture was diluted X20 with the various pretreatment solutions, incubated 5 minutes at 22°C and fixed in an equal volume of 2% glutaraldehyde. The cells were then divided into three aliquots, one examined using phase-contrast, another using SEM, and the third incubated overnight with FITC-anti-rabbit immunoglobulin.

** The numbers of spheres attached to 50 or 100 neutrophils were counted using light microscopy and averaged.

‡ The numbers of spheres visible using SEM (n=2) or fluorescing (n=2) using fluorescence microscopy were counted on 50 neutrophils and averaged.

§ The number of surface bound spheres minus the number of Ab-ACLS/neutrophil divided by the number of Ab-ACLS/neutrophil X 100 = % ingested.

" n = the number of individual experiments performed.

Table 7. The Effects of Time on Neutrophil Attachment and Ingestion of Immunoglobulin Coated Spheres (Ab-ACLS)

Cell Pretreatment *	Ab-ACLS/ Neutrophil **	(n)"	Surface Bound Ab-ACLS/ Neutrophil ‡	(n)	Adhering Ab-ACLS Ingested by Neutrophils §	(n)
PBS	10.7 ± 1.9	(4)	1.0 ± 0.04	(4)	91%	(4)
fMP (10^{-8}M)	11.3 ± 2.4	(4)	0.9 ± 0.06	(4)	92%	(4)
Colchicine ($2X10^{-6}$M)	10.2 ± 1.3	(4)	1.2 ± 0.06	(4)	88%	(4)
fMP (10^{-6}M)	11.0 ± 1.7	(4)	1.7 ± 0.07	(4)	85%	(4)
fMP (10^{-8}-10^{-6}M)	11.6 ± 2.0	(4)	1.4 ± 0.03	(4)	88%	(4)

then incubated for various time intervals, equal aliquots of a glutaraldehyde/paraformaldehyde mixture added, and the cell mixtures incubated for an additional 30 minutes at 22°C. The aldehyde fixed cells were then washed in PBS X3 and surface bound lectins labeled using a "sandwich" FITC conjugated antibody technique. The cells were again washed in PBS, mounted on glass slides and examined using a Leitz microscope with white light and UV illumination.

All pretreated neutrophils, when incubated with either lectin for 1-3 minutes, appeared to bind the lectins evenly across their surface (Table 8, Figure 8). The addition of 20mg alpha methyl-D-mannoside (D-mannoside) to the neutrophil lectin suspension prior to aldehyde fixation almost totally abolished lectin attachment on all pretreated cells (n=3).

Surface redistribution of neutrophil bound lectins was evaluated next. Neutrophils were pretreated as previously described and 10ug of either ConA or SConA added to the cell suspension. The mixture was then incubated 3 minutes, diluted 20 fold with the cell pretreatment solution and the cells pelleted from solution by centrifugation (400 rpm for 5 minutes). The cell pellets were resuspended in their respective pretreatment solution, incubated an additional 10 minutes, and fixed with an equal aliquot of a paraformaldehyde/ glutaraldehyde solution. Surface bound lectins were labeled using a sandwich antibody technique and the cells examined microscopically using UV and white light irradiation as previously described.

Table 8.

* 10^6 neutrophils were incubated with PBS, various concentrations of fMP, or colchicine for 15 minutes, centrifuged into a cell pellet and gently resuspended.

** A 1 ml solution containing 10µg of ConA or SConA was added to the pretreated cells and incubated with the cells for 1-3 minutes. This suspension was either immediately diluted with an equal volume of a 2% glutaraldehyde/paraformaldehyde solution or diluted X20 and incubated an additional 10-12 minutes before the addition of the aldehyde solution. Following a 3-minute incubation with the lectin, a solution containing anti-lectin immunoglobulins was added to some of the cell suspensions for 1 minute and the solution immediately diluted with an equal volume of 2% glutaraldehyde/paraformaldehyde. Neutrophil bound lectins were labeled with FITC tagged anti-lectin immunoglobulins and observed on the surface of neutrophils using a Leitz microscope with light and UV illumination. 100 cells were examined for each experiment and the distributions of bound lectins observed on these cells classified as being random or localized.

‡ n = the number of individual experiments performed.

Table 8. The Effects of Chemoattractants, Colchicine, and Time on Neutrophil Binding of Lectins and Immunoglobulin Cross-Linked Lectins

Cell Pretreatment *	ConA				SConA		Lectin/Anti-Lectin	
	1-3 min	(n)‡	15 min	(n)	1-15 min	(n)	1 min	(n)
PBS	93 ± 2	(7)	87 ± 5	(7)	91 ± 2	(6)	---	
Colchicine ($2X10^{-6}M$)	91 ± 4	(7)	46 ± 11	(7)	87 ± 5	(6)	23 ± 4	(4)
fMP ($10^{-8}M$)	93 ± 3	(7)	84 ± 5	(3)	90 ± 5	(6)	---	
fMP ($10^{-6}M$)	92 ± 3	(7)	83 ± 6	(5)	88 ± 6	(6)	21 ± 4	(4)
fMP (10^{-8}-$10^{-6}M$)	90 ± 4	(7)	39 ± 10	(7)	87 ± 5	(6)	22 ± 3	(4)

Figure 8. 10^6 neutrophils were incubated with PBS, colchicine, or various concentrations of fMP, centrifuged into a pellet, and FITC labeled ConA or Salmonella typhimurium added to the cell pellet. The topographical distributions of lectins or bacteria adhering to the neutrophils were classified as random or localized. Spherical PBS pretreated neutrophils are illustrated in Figures 8a, b, c. These cells have randomly adhering FITC labeled ConA (8b) or S. typhimurium (8a, 8c) attached randomly across their surfaces. Figures 8a and b were taken using a Leitz microscope (100 X objective) with a UV light source. Magnification approximately 1000X. Figure 8c is a photomicrograph of a PBS pretreated cell adhering S. typhimurium taken using SEM. Magnification approximately 1000X. When neutrophils were exposed to polarizing stimuli and allowed to adhere ConA or S. typhimurium they did so in a random fashion. However, a select group of cells appear to redistribute these particles rearward to their tail process in a time dependent fashion. Figures 8a, b, c illustrate polarized neutrophils with fluorescing bacteria (a) or ConA (b, c) that have redistributed to the tail processes on the cells. Magnifications approximately 1000X. The neutrophils that redistributed these bacteria rearward did not appear to ingest the bacteria as determined using SEM, for up to 30 minutes post attachment (8e, magnification 10,000X).

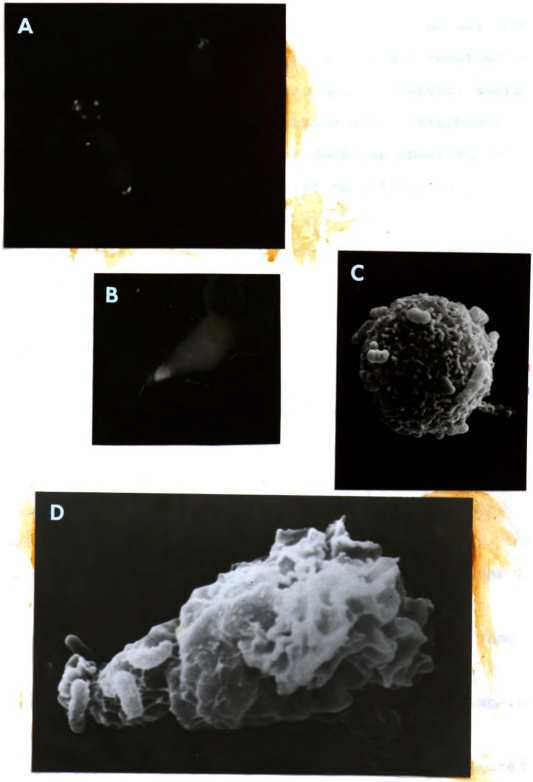


Figure 8. Neutrophil Binding of Lectins or Piliated Bacteria

The data in Table 8 demonstrates that SConA was bound on all cells in an evenly distributed manner and remained evenly distributed 15 minutes after attachment. However, surface bound ConA did appear to redistribute after attachment on a select group of neutrophils. The ConA was observed to redistribute on neutrophils exposed to colchicine or multiple increasing concentrations of fMP (10^{-8}-10^{-6}M). Greater than 50% of the polarized cells were consistently observed with surface bound ConA attached to their tail processes 15 minutes post lectin attachment (Figure 8). Unlike previous reports [8], neutrophils exposed to a single concentration of fMP (10^{-8}-10^{-6}M) failed to redistribute either lectin rearward during these time intervals (Table 8).

The effects of "crosslinking" surface bound lectins were examined next. Neutrophils were pretreated and incubated with ConA or SConA for 1 minute, centrifuged and washed as previously described. 0.1 ml of rabbit anti-ConA was then added to the cell suspension and incubated at 22°C for 1 minute. An equal aliquot of a paraformaldehyde/glutaraldehyde solution was added to the cell suspension, and the suspension incubated an additional 30 minutes at 22°C. The fixed cells were washed and surface bound lectins labeled and observed as previously described.

The surface bound lectins appeared to be aggregated or "patched" on the surfaces of cells remaining spherical after pretreatment with PBS or 10^{-8}M fMP. However, the aggregates were not observed to be localized on any single identifiable

location on the cells. Surface bound lectins on all polarized cells redistributed rearward after a brief exposure to the lectin specific antisera. Greater than 75% of all polarized cells were observed with lectins only on their uropods 1 minute after the addition of the antisera (Table 8). Redistribution of neutrophil bound lectins was not observed when non-lectin specific antisera (e.g., anti-human C_3c) were used in identical experiments (n=3).

B. The Effects of Chemoattractants and Colchicine on Neutrophil Binding of Piliated Strains of Gram Negative Bacteria

Having established a background for studying lectin attachment and redistribution on neutrophils, similar experiments were designed to evaluate neutrophil attachment and redistribution of surface bound Salmonella typhimurium, bacteria known to possess ConA like substances on their pili [1, 68]. Neutrophils (10^6 cells) were pretreated as previously described, centrifuged (400 rpm for 10 minutes) and the supernatant discarded. Bacteria with or without absorbed FITC [5] were added (0.1ml of approximately 10^{10} bacteria/ml of the various cell pretreatment solutions) to the cell button, gently mixed, and incubated at 37°C for 3-7 minutes. The cells were then fixed in 1% glutaraldehyde (10 minutes at 22°C), washed X3 in PBS, unattached bacteria removed using a Ficoll-Hypaque density gradient, and the neutrophils examined using light microscopy or prepared for and examined using SEM.

The data in Table 9 demonstrates that fMP pretreatment significantly enhanced the numbers of piliated S. typhimurium bound by neutrophils. Neutrophil binding of a non-piliated strain of S. typhimurium cultured and isolated using similar techniques, was almost non-existent (less than 0.5 bacteria/cell, n=4). Although pretreating neutrophils with 20mg/ml D-mannoside did not influence their binding of piliated bacteria (p greater than 0.90, n=3), the addition of this sugar to the bacterial suspension prior to incubation with the neutrophils (n=6) significantly (p less than 0.001) reduced the numbers of bacteria bound to neutrophils by no less than 70%. Neutrophil binding of the piliated bacteria was not significantly altered (p greater than 0.80) by the addition of 20mg/ml of glucose (n=4) to the bacterial suspension.

The binding of bacteria to the pretreated neutrophils was observed to be a random occurring event on the surface of all pretreated cells (Table 9). Therefore, the incubation period following attachment and prior to aldehyde fixation was extended to 15 minutes, and the positions of attached bacteria re-evaluated. As observed for ConA, surface bound piliated strains of S. typhimurium were apparently redistributed rearward on the surface of neutrophils pretreated with colchicine or increasing concentrations (10^{-8}-10^{-6}M) of fMP (Table 9, Figure 8). Neutrophil bound bacteria were almost exclusively observed attached to the tail ends of these polarized cells (greater than 90% of all attached bacteria)

Table 9.

* 10^6 neutrophils were incubated in 1ml of the specified solutions for 10 minutes at 37°C, centrifuged at 400 rpm for 10 minutes, and the supernatant discarded.

** FITC labeled S. typhimurium (0.1ml of a stock mixture: approximately 10^{10} bacteria/ml) suspended in the various pretreatment solutions were mixed with the neutrophil pellet, gently vortexed, and incubated for 5 minutes at 37°C. The cells were either diluted X20 in their respective cell pretreatment solutions and reincubated 10 additional minutes or immediately fixed in 1% glutaraldehyde. Unattached bacteria were removed using a Ficoll-Hypaque density gradient. The numbers of bacteria attached to 50-100 neutrophils were counted and averaged using light microscopy with UV illumination.

‡ 100 cells were examined and the distribution of attached piliated S. typhimurium classified as either being evenly distributed or localized. Those classified as localized had more than 3 bacteria attached and all attached to one area on the cell.

§ Significantly greater numbers of bacteria bound by neutrophils ($p < 0.05$) as compared to control cells (i.e., pretreated in PBS).

" n = the number of individual experiments performed.

Table 9. Conditions that Influence Neutrophil Attachment and Redistribution of Cell Bound Salmonella typhimurium

Cell Pretreatment *	Neutrophil Attachment of ** Piliated S. typhimurium (n)"		% Neutrophils that Bound ‡ S. typhimurium Randomly on Their Exterior Surface (n)		
			5 min	15 min	
PBS	4.6 ± 1.1	(10)	93 ± 4	91 ± 3	(8)
Colchicine (2 X 10^{6}M)	3.9 ± 0.8	(8)	96 ± 7	41 ± 7	(8)
fMP (10^{-6}M)	8.0 ± 1.9	(8)§	90 ± 5	86 ± 5	(3)
fMP (10^{-8}-10^{-6}M)	7.3 ± 1.2	(8)	91 ± 5	50 ± 5	(8)

15 minutes post attachment. Examination of surface bound S. typhimurium on PBS and 10^{-6}M fMP pretreated cells using light microscopy indicated that the bacteria remained randomly bound by the cells 15 minutes post attachment. Examination of the same cells using SEM revealed an almost complete absence of bacteria on the exterior surface of these cells, indicating the bacteria were apparently ingested during this time frame, yet colchicine and 10^{-8}-10^{-6}M fMP pretreated cells retained bacteria on their exterior surface for up to 30 minutes (n=6) (Figure 8).

The apparent redistribution of S. typhimurium across the neutrophil surface to its tail process may have influenced binding site topography for Ab-ACLS. Therefore, neutrophils were pretreated with colchicine or multiple increasing concentrations of fMP and incubated with S. typhimurium using conditions previously shown to redistribute the attached bacteria. The cells were then centrifuged into a cell pellet, the supernatant removed, and 0.1 ml of Ab-ACLS (0.1% packed volume) suspended in the final cell pretreatment solution added to the cell pellet. Following a 1-minute incubation (22^{o}C) the cells were fixed, washed and examined using light microscopy or SEM. A large percentage of the colchicine or fMP stimulated neutrophils (79 $\pm$ 5% and 84 $\pm$ 7% respectively, n=5) that redistributed surface bound S. typhimurium rearward also bound Ab-ACLS almost exclusively at their lamellapodia (Figure 9).

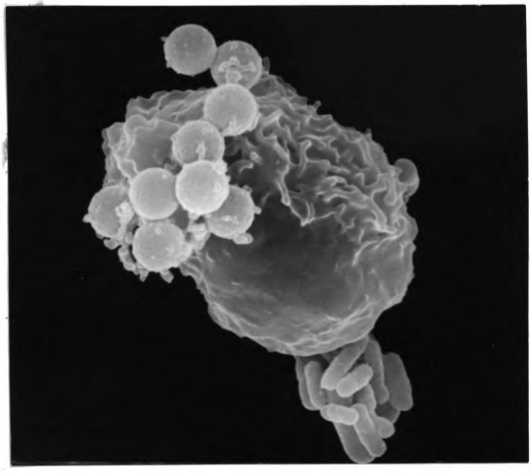
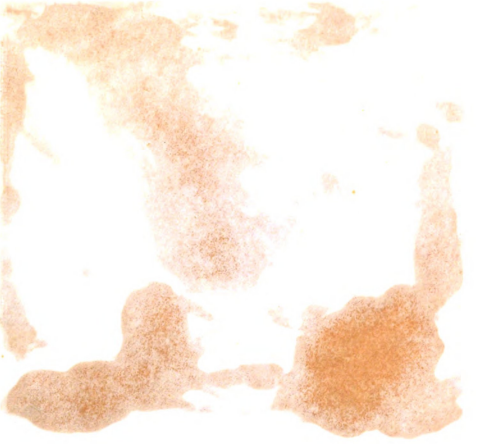


Figure 9. Neutrophil Binding of Bacteria and Immunoglobulin Coated Spheres (Ab-ACLS)

This is a SEM photomicrograph of a colchicine pretreated neutrophil magnified 12,000X. This cell was incubated with S. typhimurium for 15 minutes, Ab-ACLS added for 1 minute, and immediately fixed with 2% glutaraldehyde. The attached bacteria were observed to be adhering to the uropods on most of similarly pretreated cells while the spheres were bound almost exclusively to the fronts of the same cells.



III. The Effects of Chemoattractants and Colchicine on Neutrophil Binding of Serum and Complement Coated Particles

A. Particle Preparation

Several techniques were used to fix C_3b to the surfaces of a variety of particulate matter. First, FITC labeled gram negative bacteria (approximately 10^{10} S. typhimurium or 10^{10} E. coli/ml of PBS) were incubated (30 minutes at 37°C) in an equal volume of fresh or heat inactivated (56°C for 45 minutes) human serum. All human sera used for these experiments were preabsorbed of bacterial specific immunoglobulins [89] before use. The serum treated bacteria were then washed X3 in PBS, resuspended in PBS (10^9 bacteria/ml), and refrigerated (4°C) until used. Second, latex spheres (1.0% packed sphere volume) were washed in PBS, also incubated (30 minutes at 37°C) in fresh or heat inactivated human serum, washed X3 in PBS, and resuspended in PBS (0.1% packed sphere volume). The serum treated latex spheres were also refrigerated (4°C) until used. The final technique utilized purified human complement as a source of C_3b. Latex spheres were again washed X3 in PBS, and then resuspended in 1000 U/ml of human C_3 or trypsinized human C_3. The C_3 trypsinization procedure utilized soluble and insoluble trypsin (100 U) which had been previously shown to almost totally convert 1000 U of human C_3 to C_3b [5, 91]. Briefly, the trypsinization procedure involved adding the trypsin (100 U) to the C_3 solution and, after a 1-minute incubation

at 37°C, neutralizing the trypsin activity by adding soy bean trypsin inhibitor (100 U) or removing the insoluble trypsin by centrifugation. Following latex sphere incubation with the human C_3 or trypsinized human C_3, the spheres were washed X3 in PBS, resuspended in PBS (0.5% packed sphere volume) and also refrigerated until used.

B. Verification of the Presence of C_3b on the Surface of the Bacteria and Latex Spheres and its Effects on Neutrophil Binding of the Particles

Aliquots containing latex spheres or bacteria previously pretreated with fresh or heat inactivated serum were incubated (30 minutes at 37°C) with solutions containing rabbit or goat anti-human C_3c. Latex spheres pretreated with trypsinized human C_3 were treated in a similar fashion. Within 30 minutes, the bacteria or spheres pretreated with fresh human serum or trypsinized C_3 were agglutinated from suspension (n=4). No visible agglutination of the spheres or bacteria pretreated with heat inactivated serum occurred using the same conditions (n=4). All test particles were then washed X3 in PBS and resuspended in PBS. Solutions containing FITC labeled anti-rabbit or goat immunoglobulin were then mixed with these particles and incubated overnight at 22°C. The particles were again washed X3 and then observed microscopically using UV irradiation. The bacteria and spheres pretreated with heat inactivated serum did not fluoresce when exposed to UV irradiation conditions (n=3). Yet the bacteria or spheres pretreated with fresh serum or trypsinized human

C_3 fluoresced brilliantly when exposed to the same UV irradiation (n=3). Since C_3b was shown to be antigenically present on the particles pretreated with fresh serum or trypsinized human C_3, they will henceforth be called C_3b coated particles.

The next experiments were designed to evaluate whether the particle bound C_3b molecules would influence neutrophil binding of the particles. Neutrophils (10^6 cells) suspended in PBS were centrifuged into a pellet and the supernatant discarded. Bacteria (10^9 bacteria/0.1 ml) pretreated with serum or latex spheres (0.1% packed sphere volume) pretreated with serum, human C_3, or trypsinized human C_3 were then added (0.1ml) to the cell pellet. The neutrophil-particle suspensions were then gently mixed and incubated for 3 minutes at 22°C before 2 ml of glutaraldehyde (1.0%) were added. This suspension was then incubated (22°C) for 30 minutes, the glutaraldehyde fixed cells washed in PBS, and particles not bound by the neutrophils removed from the suspension using a Ficoll-Hypaque density gradient. The cells were then examined microscopically and the numbers of particles bound by 100 neutrophils counted and averaged.

The data in Table 10 demonstrates that neutrophil binding of the C_3b coated particles was 10-50 fold greater than neutrophil binding of particles pretreated with heat inactivated serum or purified human C_3. As previously observed when pretreating latex spheres with albumin, the treatment of latex spheres or bacteria with heat inactivated serum or purified human C_3 significantly ($p < 0.001$)

Table 10. * 10^6 neutrophils/0.1 ml of PBS were incubated (3 minutes at 22°C) with a variety of test particles and then fixed with 2 ml of glutaraldehyde. All particles not attached to the neutrophils were removed by centrifugation through a Ficoll-Hypaque density gradient.

** Heat killed bacteria (S. typhimurium) were washed X5 in PBS, labeled with FITC, washed in PBS, pretreated with serum and resuspended to a concentration of 10^9 bacteria/ml of PBS. Latex spheres were washed in PBS, pretreated with human C_3 or serum, washed in PBS (X3) and resuspended to a concentration of 0.1% packed sphere volume. All particles (1 ml) were incubated with the pretreatment solutions (10 ml) for 30 minutes at 37°C and washed in PBS before use.

‡ Following the removal of the majority of unbound particles, the glutaraldehyde fixed cells were mounted on glass slides and examined microscopically. The fluorescing bacteria or latex spheres bound to neutrophils were then counted and averaged (100X objective).

§ n = the number of individual experiments performed.

Table 10. Neutrophil Binding of Complement and Serum Coated Particles *

Particle	Particle Pretreatment **	Particles Bound/Neutrophil ‡	(n) §
Bacteria	Heat Inactivated Serum	0.4 ± 0.05	(5)
	Fresh Absorbed Serum	5.4 ± 0.72	(14)
Latex Spheres	Heat Inactivated Serum	0.2 ± 0.06	(8)
	Fresh Serum	8.9 ± 1.96	(8)
	Human C_3 (1000 U)	0.6 ± 0.15	(5)
	Trypsinized Human C_3 (1000 U)	10.3 ± 1.10	(8)

impaired neutrophil binding of the spheres or bacteria respectively (p value obtained by comparing data in Table 10 to that in Tables 1 and 9).

The C_3b coated particles were next reincubated (30 minutes at 22°C) with PBS, heat inactivated serum, goat anti-C_3c or goat anti-human immunoglobulin. Following this incubation, the particles were washed X3 in PBS and resuspended in PBS to their original concentrations. The particles (0.1 ml) were then incubated (22°C) with neutrophils (10^6 cells/0.1 ml of PBS) for 3 minutes, fixed in glutaraldehyde and the numbers of particles bound to neutrophils determined as previously described.

The data in Table 11 demonstrates that reincubating particles pretreated with fresh serum or solutions containing the trypsinized C_3 in goat anti-immunoglobulin or heat inactivated serum only minimally reduced the ability of neutrophils to bind to the particles. However, the reincubation of similarly pretreated spheres with goat anti-C_3c almost totally prevented their binding to neutrophils.

C. The Effects of Chemoattractants and Colchicine on Neutrophil Binding of C_3b Coated Particles

It has previously been reported [124], that CF may enhance the numbers of binding sites for C_3b on the surface of leukocytes. Therefore, neutrophils (10^6 cells) were incubated in PBS, various concentrations of fMP, or colchicine for 10 minutes at 37°C. The pretreated cells were

Table 11.

* 10^6 neutrophils were incubated with spheres (0.1 ml of a 0.1% packed sphere volume) pretreated as described for 3 minutes at 22°C, fixed in 2% glutaraldehyde, and unattached spheres removed by centrifuging the cell-sphere mixture through a Ficoll-Hypaque gradient.

** The spheres were incubated (30 minutes at 37°C) with fresh human serum or trypsinized human C_3 (1000 U) for 30 minutes at 37°C, washed in PBS X3, and then re-exposed to PBS, heat inactivated serum, goat anti-human immunoglobulin (1:10 titer), or goat anti-human C_3c (1:10 titer) for 30 minutes at 22°C. The spheres were then washed X3 in PBS and reconstituted in PBS to a concentration of 0.1% packed sphere volume.

‡ The fixed cells were mounted on glass slides and the numbers of spheres bound by 100 neutrophils using a Leitz microscope (100X objective) were counted and averaged.

§ n = the number of individual experiments performed.

Table 11. Blocking Neutrophil Binding of C_3b Coated Spheres with C_3c Specific Antisera *

SPHERE PREPARATION **		
First Pretreatment	Second Pretreatment	Spheres/neutrophil ‡ (n=5) §
Trypsinized Human C_3	PBS	9.3 ± 1.1
Fresh Human Serum	PBS	9.0 ± 1.2
Trypsinized Human C_3	Heat Inactivated Serum	9.2 ± 1.0
Fresh Human Serum	Heat Inactivated Serum	8.8 ± 1.3
Trypsinized Human C_3	Anti-Human Immunoglobulin	7.9 ± 1.8
Fresh Human Serum	Anti-Human Immunoglobulin	7.4 ± 1.3
Trypsinized Human C_3	Anti-Human C_3c	0.3 ± 0.1
Fresh Human Serum	Anti-Human C_3c	0.2 ± 0.1

then centrifuged into a pellet and the supernatants discarded. The C_3b coated spheres or bacteria (i.e., those pretreated with fresh serum or trypsinized C_3) (0.1 ml) were then added to the neutrophil button, the suspension gently vortexed, and incubated for 3 minutes at 22°C. Neutrophil binding of particles was terminated by the addition of 2 ml of 1% glutaraldehyde and unbound spheres or bacteria removed from the suspension by centrifuging the cell-particle mixture through a Ficoll-Hypaque gradient. The numbers of particles bound by the neutrophils were then counted and averaged (Table 12).

The data in Table 12 demonstrates that colchicine pretreated neutrophils bound roughly the same numbers of the C_3b coated particles as did the control cells (i.e., those preincubated in PBS). The data in this table also demonstrates that low concentrations of fMP (10^{-8} or 10^{-7}M) only slightly enhanced the binding of the C_3b coated particles. Yet, pretreating neutrophils with 10^{-6}M or 10^{-8}-10^{-6}M fMP significantly enhanced ($p < 0.001$) the numbers of particles bound by the neutrophils as compared to the binding of particles by the control cells. Similar percentages of cells were observed binding particles in all experiments (Table 12).

Table 12.

* 10^6 neutrophils were incubated (10 minutes at 37°C) with PBS, colchicine or fMP, centrifuged into a cell pellet and the supernatant discarded. 0.1 ml of the various C_3b coated particles were added to the cell pellet and incubated 3 minutes at 22°C. Neutrophil binding of the particles was terminated by the addition of 1% glutaraldehyde.

** Latex spheres were incubated with fresh serum (n=8) or trypsinized human C_3 (n=8) for 30 minutes at 37°C, washed in PBS and resuspended (0.1% packed sphere volume) in the appropriate neutrophil pretreatment solution. 10^9 FITC labeled S. typhimurium (n=5) were likewise incubated in fresh serum, washed, and also resuspended in PBS, colchicine, or various concentrations of fMP. Following neutrophil binding of these particles, and the addition of glutaraldehyde, unattached particles were removed and the numbers of spheres or bacteria attached to the neutrophils (100 cells examined/experiment) counted. All data for neutrophil binding of C_3b coated spheres or bacteria were combined and averaged for each experiment.

‡ 100 neutrophils were examined/experiment and the numbers of neutrophils binding 1 or more C_3b coated particles were counted, and expressed as a percentage.

§ n = the number of individual experiments performed.

Table 12. The Effects of fMP or Colchicine on the Numbers of C_3b Coated Particles Bound by Neutrophils

Cell Pretreatment *	C_3b Coated Particles/Neutrophil (n=15) ** §	% Neutrophils Adhering C_3b Coated Particles (n=9) ‡
PBS	8.6 ± 1.8	74 ± 4%
Colchicine ($2X10^{-6}M$)	7.4 ± 1.7	77 ± 3%
fMP ($10^{-8}M$)	9.9 ± 2.1	77 ± 3%
fMP ($10^{-7}M$)	10.2 ± 2.2	78 ± 3%
fMP ($10^{-6}M$)	17.6 ± 2.9	79 ± 4%
fMP (10^{-8}-$10^{-6}M$)	16.3 ± 2.3	78 ± 4%

D. The Effects of Colchicine and Time on Neutrophil Binding of C_3b Coated Particles

Since the binding affinities of colchicine and PBS pretreated neutrophils for C_3b coated particles were similar, the adhesion patterns for C_3b coated particles on these neutrophils were next examined. Neutrophils (10^6 cells) were incubated in PBS or colchicine as previously described, centrifuged (400 rpm for 5 minutes) into a cell pellet and the supernatant discarded. To this pellet, 0.1 ml of the C_3b coated bacteria (10^9 FITC labeled bacteria/0.1 ml) or C_3b coated spheres (0.1% packed sphere volume) were added, gently vortexed, incubated for 3 minutes at 22°C, and the suspension diluted 20 fold with PBS or colchicine. The neutrophil-particle suspensions were then immediately diluted with an equal volume of 2.0% glutaraldehyde. Unbound particles were removed using a Ficoll-Hypaque gradient and the neutrophils examined using a Leitz microscope with white light or UV irradiation, or by SEM as previously described.

Virtually all (98 $\pm$ 2%, n=9) of the PBS pretreated neutrophils remained spherical after treatment. Of these cells, most (92 $\pm$ 3%, n=9) bound the C_3b coated bacteria or spheres in a random fashion on their exterior surface (Figure 10). The majority of the neutrophils formed a motile configuration when exposed to colchicine (71 $\pm$ 6%, n=15). Of these neutrophils, most (89 $\pm$ 4%, n=15) were observed with C_3b coated particles bound only at one localized region, their uropods (Figure 10). It was hypothesized that either

Figure 10. 10^6 neutrophils were pretreated with PBS or colchicine ($2X10^{-6}M$) for 15 minutes at 37^oC and incubated with C_3b coated bacteria or latex spheres for 3 minutes before being fixed in glutaraldehyde. Figure 10 illustrates a typical spherical (PBS pretreated) neutrophil binding C_3b coated spheres in a random fashion. Colchicine pretreated neutrophils are illustrated in Figures b and c with C_3b coated bacteria (FITC labeled) and spheres attached only at their uropods, respectively. All micrographs were obtained using a Leitz microscope (100X objective) with light (10a) and UV illumination (10b) or by SEM (10c).

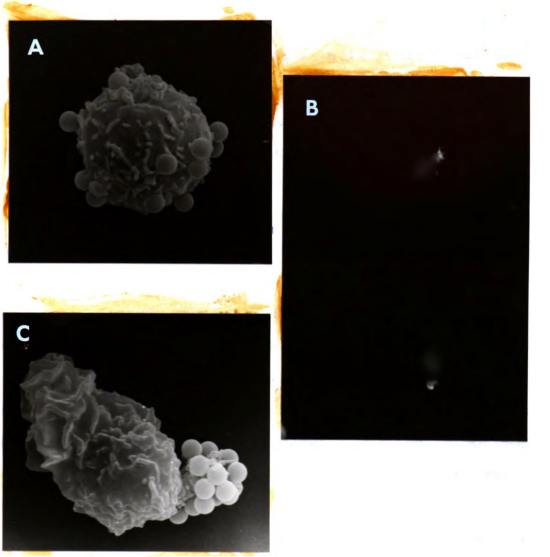


Figure 10. The Binding Topographies for C_3b Coated Particles on PBS and Colchicine Pretreated Neutrophils

colchicine pretreatment may have modified the adhesion site topography for the C_3b coated particles or that the colchicine pretreatment may have prompted a rapid redistribution of surface bound C_3b coated particles across the neutrophil surface. Therefore, three parameters of this experiment were altered and the experiment repeated. First, the concentration of C_3b coated spheres added to the pretreated neutrophils was increased (1.0% packed sphere volume). This alteration was expected to enhance the numbers of sphere-neutrophil collisions and adhesions that could occur in a short period of time. Second, the incubation time the spheres were in contact with the cells was shortened to 3-5 seconds. Last, after the 3-5 second incubation with the spheres, the neutrophil suspension was diluted 50 fold with PBS or colchicine to minimize further attachments and the suspension was either immediately or after a specified time interval fixed with an equal volume of 2.0% glutaraldehyde.

The data in Table 13 demonstrates that the PBS pretreated neutrophils were consistently observed binding the C_3b coated spheres in a random fashion (Figure 11) regardless of the time interval allowed for attachment and redistribution. Yet, the C_3b coated spheres were observed to be initially bound almost exclusively at the front ends of colchicine pretreated cells. However, 15 seconds after attachment, most of the neutrophil bound spheres were observed to be attached on the colchicine pretreated neutrophils in a random fashion. Within 60-90 seconds after attachment, the majority of these

Table 13.

* 10^6 neutrophils were incubated with PBS or colchicine for 15 minutes at 37°C, centrifuged into a cell pellet, and the supernatant discarded. C_3b coated spheres (0.1ml) were added to the cell pellet, incubated for 3-5 seconds and diluted X50 with PBS or colchicine.

** The neutrophils were either fixed with 2% glutaraldehyde immediately after their 3-5 second incubation with the spheres, or at various time intervals after being diluted with PBS or colchicine.

‡ The fixed cells were then examined using a Leitz microscope and the surface distribution of adhering spheres evaluated. Cells binding distributions were classified as random, or when greater than 80% of the cell bound spheres were attached only at the front or tail end of the cells, as lamellapolar or uropolar distributions, respectively.

§ n = the number of individual experiments performed.

Table 13. Topographical Binding Distributions for C_3b Coated Particles on PBS or Colchicine Pretreated Neutrophils as Influenced by Time

Neutrophil Pretreatment *	Time **	Topographical Binding Distribution‡		(n) §
PBS	3-5 sec	98 ± 3%	Random	(3)
	15 sec	92 ± 2%	Random	(3)
	30 sec	94 ± 3%	Random	(3)
	60 sec	90 ± 5%	Random	(3)
	180 sec	94 ± 1%	Random	(3)
	1800 sec	90 ± 4%	Random	(3)
Colchicine ($2X10^{-6}M$)	3-5 sec	80 ± 6%	Lamellapolar	(5)
	15 sec	90 ± 3%	Random	(3)
	30 sec	33 ± 4%	Uropolar	(3)
	60 sec	62 ± 4%	Uropolar	(3)
	90 sec	89 ± 5%	Uropolar	(3)
	180 sec	92 ± 5%	Uropolar	(3)
	1800 sec	94 ± 3%	Uropolar	(15)

Figure 11. 10^6 neutrophils in PBS were incubated with C_3b coated spheres for 5 seconds, diluted X50 with PBS and immediately or after a 30-minute incubation, fixed with a 2% glutaraldehyde solution. The photomicrographs (mag. X6600) illustrate the binding of C_3b coated spheres on the PBS pretreated cells 5 seconds after the addition of these spheres occurred predominantly in a random fashion (11a). The spheres appeared to be retained on the surface of the PBS pretreated cells in the same random manner 30 minutes post attachment (11b).

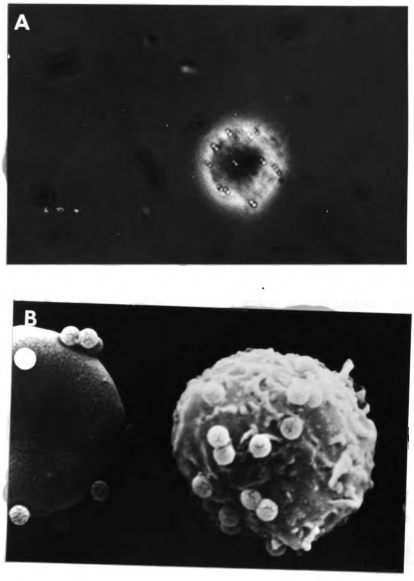


Figure 11. Random Binding of C_3b Coated Spheres on PBS Pretreated Neutrophils.

Figure 12. 10^6 neutrophils were incubated with colchicine ($2X10^{-6}M$) for 15 minutes at 37^oC, mixed with C_3b coated spheres and incubated for 5 seconds. The cell/sphere suspension was then diluted and immediately or after a specified time interval, fixed in 2% glutaraldehyde. These cells were examined using light and scanning electron microscopy. The neutrophils were most commonly observed binding the C_3b coated spheres at their lamellapodia 5 seconds after the addition of the spheres (12a, b). Within 15 seconds after binding, the most prevalent binding pattern observed was a random pattern (12c). Within 90 seconds, most cells had spheres attached only to their uropods (12d). These spheres remained attached thusly for up to 30 minutes (12e). All photomicrographs were obtained using a Leitz microscope (100X objective) with white light illumination or by SEM (12b and e).

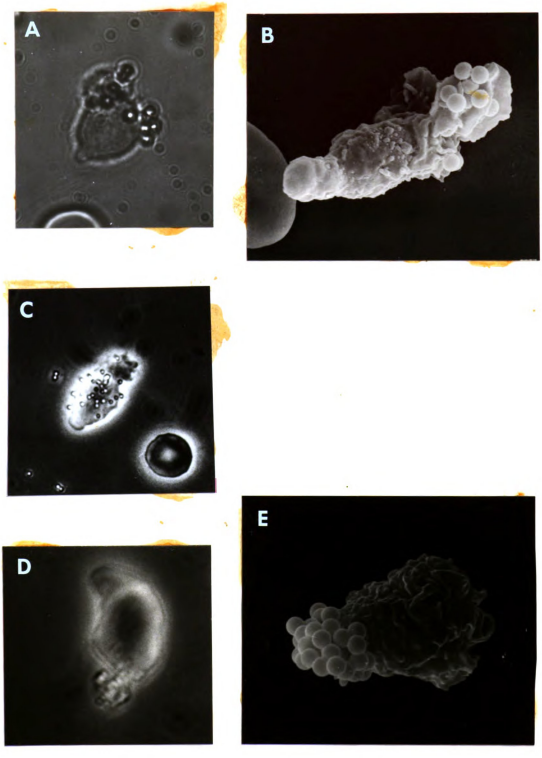


Figure 12. The Effects of Colchicine and Time on Neutrophil Binding Distributions for C_3b Coated Spheres.

same cells were observed with only C_3b coated spheres bound at their uropods (Table 13, Figure 12). The numbers of spheres bound by the PBS and colchicine pretreated cells were also counted (n=3) and found to be relatively consistent (13 ± 5 spheres/neutrophil) from experiment to experiment which suggested that binding of spheres after dilution was unlikely. Examination of neutrophils from all experiments using light microscopy and SEM failed to indicate any evidence of sphere internalization by either PBS or colchicine pretreated cells. Examination of the PBS or colchicine pretreated neutrophils using SEM also indicated that the C_3b coated spheres remained on the cells' exterior surface 30 minutes post attachment (Figures 11 and 12).

The next series of experiments were designed to evaluate if colchicine pretreated neutrophils would 1) simultaneously bind antibody and C_3b coated spheres at their lamellapodia, and 2) ingest antibody coated spheres at or near their attachment sites while selectively redistributing the C_3b coated spheres rearward across their exterior surface. Neutrophils (10^6 neutrophils) were pretreated with colchicine as previously described, centrifuged into a pellet, and the supernatant discarded. A mixture containing an equal volume of C_3b and antibody coated spheres (2.0% packed sphere volumes, respectively) was added (0.1ml) to the cell pellet and gently mixed. Two different sized spheres were used in these studies to aid in identification (e.g., 0.6μ in diameter spheres were C_3b coated and 1.1μ diameter spheres

were antibody coated). In addition, as further evidence for the selective binding of C_3b, the antibody coated spheres were prepared differently than previously described in Results Section I. The two populations of latex spheres (0.6μ and 1.1μ diameter) were first incubated separately in fresh serum. The 0.6μ C_3b coated spheres were washed X3 in PBS and set aside. The 1.1μ C_3b coated spheres were also washed X3 in PBS, then reincubated with rabbit anti-C_3c (30 minutes at 37°C), and washed again X3 in PBS. Equal aliquots of both populations of the pretreated spheres (0.6u C_3b coated and 1.1μ antibody coated) were then mixed. Following the addition of the C_3b and antibody coated spheres to the cell pellet, the cell/sphere suspension was incubated for 5 seconds at 22°C and diluted 50 fold with PBS. The neutrophils were then either immediately or after a specified time interval, fixed with 2% glutaraldehyde and the fixed cells prepared and examined as previously described.

Consistently, almost two thirds (65 $\pm$ 5%) of the cells examined (n=7, 100 cells examined per experiment) bound both the 0.6μ and 1.1μ diameter spheres 5 seconds after the addition of the spheres. Greater than 90% of these cells (94 $\pm$ 7%, n=4) were observed with spheres bound only on their lamellapodia during this time period. When the incubation period prior to fixation was extended to 15 seconds, greater than 80% of these cells (82 $\pm$ 5%) were observed with 1.1μ spheres still bound at their lamellapodia (n=5). However, most of these cells (76 $\pm$ 8%, n=5) were observed with 0.6μ

spheres now bound randomly on their exterior surface. Within 90 seconds, 74 $\pm$ 5% of the same cells were observed with 0.6μ spheres bound only on their uropods (n=5). The uropolar bound C_3b coated spheres appeared to be retained on most (63 $\pm$ 5%, n=3) cells long after (20 minutes) the antibody coated spheres were ingested. Figure 13 illustrates the binding and ingestion or redistribution of antibody and C_3b coated spheres respectively on colchicine pretreated neutrophils.

E. The Binding Distributions for C_3b Coated Particles on fMP Stimulated Neutrophils

We had previously shown that fMP enhanced neutrophil binding of C_3b coated particles. Neutrophils were again pretreated with 10^{-8}M, 10^{-6}M and 10^{-8}-10^{-6}M fMP as previously described and 0.1ml of the C_3b coated latex spheres suspension (1.0% packed sphere volume) added to the cell pellet. This mixture was gently vortexed, incubated for 3-5 seconds at 22^{o}C, and diluted 50 fold with the same concentration of fMP used previously to pretreat the cells. An aliquot of the cell suspension was then immediately withdrawn and fixed in 1% glutaraldehyde. Additional aliquots were then removed at specified time intervals and also fixed in 1.0% glutaraldehyde. Unbound spheres were removed as previously described, the cells examined using light and scanning electron microscopy, and the distributions of adhering spheres evaluated.

The majority of neutrophils (90 $\pm$ 5%, n=16) pretreated with 10^{-8}M fMP remained spherical through all experiments.

Figure 13. Neutrophils were pretreated with colchicine and incubated (5 seconds) with C_3b coated latex spheres (0.6μ in diameter) and immunoglobulin coated spheres (1.1μ in diameter) then fixed with 2% glutaraldehyde at various time intervals. Within 5 seconds of binding, the immunoglobulin and C_3b coated spheres were consistently observed to be bound in a localized fashion on these cells' lamellapodia (13a). Within 10 seconds the C_3b coated spheres were observed binding randomly on the same cells. Within 90 seconds, many of the immunoglobulin coated spheres were apparently ingested by the colchicine pretreated cells. Yet, the C_3b coated spheres were exteriorly bound on these cells' uropods (13c). All photomicrographs were obtained using SEM.

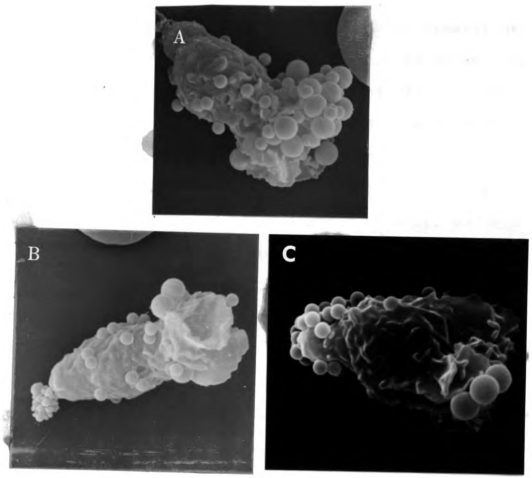


Figure 13. The Binding, Ingestion, and Redistribution of C_3b and Immunoglobulin Coated Spheres on Colchicine Pretreated Neutrophils

The data in Table 14 demonstrates that these cells also bound the C_3b coated particles randomly across their surfaces, much like PBS pretreated neutrophils. This attachment distribution was apparently unaltered 30 minutes after particle attachment. However, unlike PBS pretreated neutrophils, the fMP pretreated cells did appear to ingest the C_3b coated spheres. To quantitate this event, fMP pretreated cells were incubated with the C_3b coated spheres for 5 seconds, diluted 50 fold with fMP and immediately or after a 3 or 30-minute incubation, fixed with glutaraldehyde. The cells were then examined using light microscopy and the numbers of bound spheres counted. This number was allowed to represent the total number of externally and internally bound spheres. Two aliquots of the same cell suspension were also either incubated overnight with FITC labeled anti-C_3c, washed, and re-examined microscopically using UV illumination or prepared for and examined using SEM. The numbers of spheres fluorescing or visible on the external surface of the cells examined using SEM was then counted and allowed to represent the number of externally bound spheres. The number of spheres initially counted using light microscopy was compared to the number of externally bound spheres and this ratio indirectly proportional to the numbers of spheres ingested by the cells. The data in Table 14 demonstrates that within 30 minutes greater than 90% of all spheres were ingested by the fMP pretreated cells. Figure 14 is a collection of photomicrographs that best illustrate these events.

Table 14. The Effects of Time on the Binding and Ingestion of C_3b Coated Spheres by Neutrophils Pretreated with fMP (10^{-8}M) *

Time **	% Random Binding Distributions ‡	% Externally Bound Spheres §	(n)"
5 sec	97 ± 7	103 ± 5%	(4)
3 min	95 ± 6	84 ± 11%	(4)
30 min	94 ± 6	10 ± 3%	(4)

* 10^6 cells were incubated with 10^{-8}M fMP for 10 minutes, centrifuged into a pellet, and the supernatant discarded. Spheres (0.1% packed volume) coated with C_3b were added to the cell button, gently vortexed and incubated for 5 seconds.

** Following a 5 second incubation with the spheres, the cells were diluted X50 with 10^{-8}M fMP and immediately, 3 minutes, or 30 minutes later mixed with an equal volume of 2% glutaraldehyde.

‡ 100 cells were examined using light microscopy and the binding of the C_3b coated spheres on these cells categorized as random or "other". Random distribution refers to cells with spheres bound in an evenly distributed manner.

§ The numbers of spheres attached to 100 neutrophils were counted using light microscopy and averaged. This number was allowed to represent the total number of spheres bound/cell. Aliquots of these cells were also examined by SEM or incubated with FITC labeled anti-C_3b and examined using UV light. The numbers of spheres visible on 50 cells using SEM were counted and averaged. The number of spheres on 50 neutrophils that fluoresced after exposure to UV light were also counted and averaged. The sum of these numbers was allowed to represent the number of externally bound spheres. % externally bound spheres was obtained by comparing the ratio of the externally bound spheres to total spheres bound X 100.

" n = number of experiments performed.

Figure 14. Neutrophils (10^6 cells) pretreated with fMP (10^{-8}M) were incubated with C_3b coated spheres for 5 seconds, diluted X50 with 10^{-8}M fMP and immediately or after a specified time interval, fixed with glutaraldehyde. Neutrophil binding of C_3b coated spheres was observed to occur in a random fashion 6 seconds after the addition of the spheres using light or SEM (14a and c). These spheres were apparent on the external surface of thecells 3 minutes post attachment when observed using SEM or when the external spheres were labeled (14b) using a fluorescent label (FITC labeled anti-C_3c). However, within 10 minutes, most of the cell bound spheres failed to fluoresce when incubated with FITC labeled anti-C_3c and externally bound spheres were rarely visible on the same cell population when observed using SEM (14d).

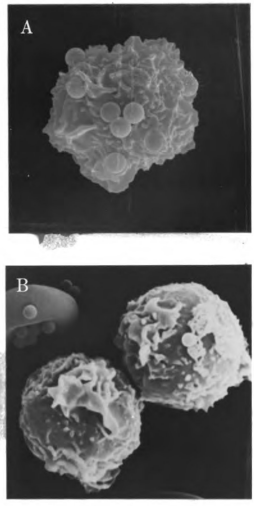
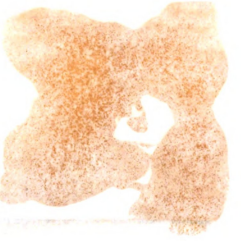


Figure 14. Neutrophil Binding of $^{-8}C_3b$ Coated Particles after Pretreatment with 10^{-8}M fMP



Neutrophils were next pretreated with shape change concentrations of fMP (i.e., 10^{-6}M or 10^{-8}-10^{-6}M fMP) and incubated with C_3b coated spheres using the same experimental conditions. The majority of these cells (76 $\pm$ 4%, n=9) formed a bipolar configuration after fMP pretreatment and like colchicine pretreated cells, possessed distinct front and tail ends. When these cells were examined using light or scanning electron microscopy, sphere binding to these cells was observed to be predominantly at their front ends 5 seconds after the addition of the spheres (Table 15). Also like colchicine pretreatment, the attached spheres were observed to be bound randomly on the fMP pretreated cells within 10 seconds (Table 15). However, when the cells were fixed 3 minutes after the addition of the spheres, a difference was observed between the fMP and colchicine pretreated cell binding of the C_3b coated spheres. Observing this difference was dependent on the technique used to examine the cells. When the cells were examined using SEM, the majority of all visible (i.e., surface bound) spheres were attached on these cells' uropods. This binding distribution was again classified as uropolar. Yet, when aliquots of the same cell population were examined using light microscopy, almost half (48 $\pm$ 4%, n=4) of the spheres were observed to be bound on or within these cells' bodies. This binding distribution, with roughly half of the observed spheres bound on a cell's tail process and the remaining half on or inside its cell body, was categorized as "mixed" (Table 15). Figure 15 is a

Figure 15. Neutrophils were pretreated with 10^{-6}M or 10^{-8}-10^{-6}M fMP and incubated 5 seconds with C_3b coated spheres. The cell/sphere suspension was diluted X50 with 10^{-6}M fMP immediately or after a specified time interval fixed with glutaraldehyde and examined using light microscopy and SEM. Initially, the spheres appeared to be bound by these polarized cells at their front ends (15a, b). Yet, 10 seconds after attachment, all spheres were bound in a random fashion (15c, d). Within minutes of attachment, externally visible spheres (visualized using SEM) were predominantly located at the cells' uropods (15e). However, when the cells were observed using light microscopy, many spheres were also observed on or inside these cells' bodies.

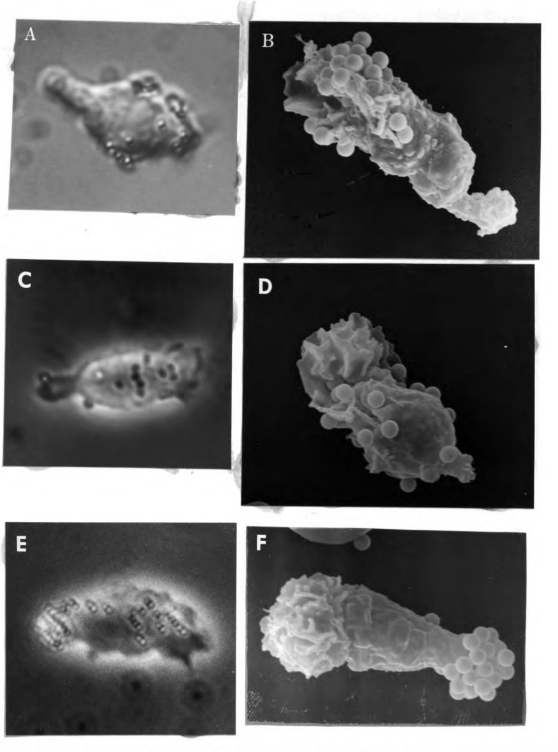


Figure 15. The Effects of Polarizing Doses of fMP and Time on Neutrophil Binding Distributions for C_3b Coated Spheres

Table 15. * 10^6 neutrophils were pretreated with 10^{-6}M or 10^{-8}-10^{-6}M fMP and incubated with C_3b coated spheres for 5 seconds. The cells were then immediately diluted X50 with 10^{-6}M fMP and immediately or after a specified time interval fixed with 2% glutaraldehyde.

** Unattached spheres were removed from the cell suspension and the cells were examined using light microscopy or SEM. Sphere attachment patterns on the cells (100/experiment) were classified as random, mixed, lamellapolar, or uropolar when the spheres were bound with no apparent pattern, in equal distributions at more than one site, or with greater than 80% of all attached spheres bound at the front or tail ends, respectively.

‡ n = the number of individual experiments performed.

Table 15. The Effects of Time on the Binding Distributions for C_3b Coated Particles on fMP Polarized Neutrophils

Cell Pretreatment *	Time	Binding Distributions **				
		Light Microscopy	(n)	SEM	(n)	‡
10^{-6}M fMP	5 sec	89 ± 5% lamellapolar	(5)	84 ± 4% lamellapolar	(3)	
	15 sec	77 ± 6% random	(5)	80 ± 6% random	(3)	
	180 sec	72 ± 4% mixed	(5)	76 ± 5% uropolar	(5)	
10^{-8}-10^{-6}M fMP	5 sec	88 ± 4% lamellapolar	(5)	88 ± 6% lamellapolar	(3)	
	15 sec	79 ± 8% random	(5)	76 ± 6% random	(3)	
	180 sec	92 ± 5% mixed	(5)	79 ± 5% uropolar	(5)	

collection of light and SEM photomicrographs that best illustrates the binding patterns for the C_3b coated spheres observed on the fMP pretreated cells as a function of time. Similar binding patterns for C_3b coated spheres were observed on neutrophils when pretreated with a single (10^{-6}M) or multiple increasing concentrations (10^{-8}-10^{-6}M) of fMP. Comparisons of the numbers of spheres, attached to neutrophils when observed using both SEM and light microscopy indicated that only approximately one half (46 $\pm$ 3%, n=4) of the spheres remained bound to these cells' exterior surface 1 minute post attachment. Similar data were obtained when the externally bound spheres were labeled with FITC labeled anti-C_3c (44 $\pm$ 7%, n=4). The externally bound spheres (i.e., those labeled with FITC labeled anti-C_3c or visible using SEM) were almost always bound on these cells' tail process. Large numbers of the externally bound spheres remained exteriorly bound 30 minutes post attachment.

The simultaneous attachment and relocation of surface bound antibody and C_3b coated spheres on fMP pretreated cells were next evaluated. The spheres were prepared as previously described in Results Section IIIE. Neutrophils were pretreated with 10^{-6}M or 10^{-8}-10^{-6}M fMP and incubated with the spheres for 5 seconds. The cell mixture was then diluted 50 fold with 10^{-6}M fMP and immediately or after a 10-60 second incubation fixed with an equal volume of 2.0% glutaraldehyde.

The cells initially bound both populations of spheres as previously described for colchicine pretreated cells. Consistently more than half of all fMP pretreated cells ($60 \pm 6\%$, n=7) bound both types of spheres. The larger (1.1μ diameter) antibody coated spheres were observed bound to the lamellapodia on $81 \pm 3\%$ (n=4) of these polarized cells, regardless of the fMP pretreatment conditions or the time interval before fixation. Most of the cells ($56 \pm 5\%$, n=8) were also observed with the smaller (0.6μ diameter) C_3b coated spheres also bound to the lamellapodia when fixed 5 seconds after the addition of the spheres. However, when the fMP pretreated cells were fixed 10 seconds after sphere attachment, $86 \pm 4\%$, (n=8) of them were observed with the C_3b coated spheres bound in a random fashion on the cell. Within 1 minute $82 \pm 5\%$, n=6 of the fMP pretreated cells were observed with exteriorly bound C_3b coated spheres only on their uropods.

Examination of the fMP pretreated cells 20 minutes post attachment using SEM indicated that the uropod bound C_3b coated spheres were the only spheres retained on the exterior surface of these cells. Figure 16 illustrates the characteristic binding of C_3b and antibody coated spheres on neutrophils when fixed 5 seconds, 10 seconds and 60 seconds after sphere attachment to the cells.

Figure 16. Neutrophils, pretreated with 10^{-6}M or 10^{-8}-10^{-6}M fMP, were incubated with antibody (1.1μ diameter) and C_3b (0.6μ diameter) coated spheres for 5 seconds. The cells were then diluted X50 with 10^{-6}M fMP and immediately or after a specified time interval, fixed with glutaraldehyde. Both populations of spheres were observed to bind at the lamellapodia (16a) on most cells. Ten seconds later, the smaller C_3b coated spheres appeared to be redistributed rearward (16b) and by 1 minute, were almost exclusively on the cells' uropods (16c). Some C_3b coated spheres were apparently being ingested (16b) en route to the uropod. Within one minute, most antibody (1.1μ diameter) coated spheres were ingested (16c).

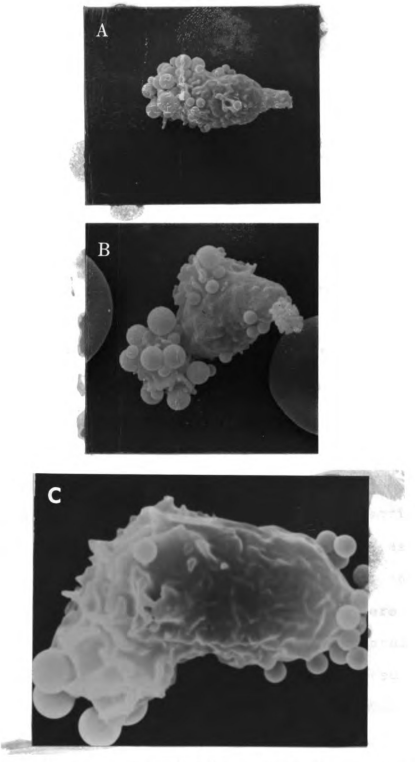


Figure 16. Simultaneous Adhesion, Ingestion, and Redistribution of Immunoglobulin and C_3b Coated Spheres on fMP Stimulated Neutrophils

IV. The Effects of Enzymes and Chemoattractants on Neutrophil Adherende to Protein Coated Substrata

A. The Effects of Chemoattractants on Neutrophil Binding of Albumin, Serum, and C_3 Coated Spheres

Others have shown that neutrophils adhere to a variety of albumin (1-2%) and serum (10%) coated substrata [7, 14, 35, 121]. These authors have also shown that CF increase neutrophil adhesiveness to these protein coated surfaces. Yet, we have shown that neutrophils rarely bound latex spheres coated with human albumin (5%), heated serum (50%), and C_3 (1000 U/ml). To determine if CF would enhance neutrophil binding of these protein coated spheres, spheres were coated with 5% HSA, 50% human heat inactivated (56°C for 30 minutes) serum and human C_3 (1000 U/ml) as previously described. The treated spheres were then washed with PBS X3 and reconstituted to a sphere concentration of 1.0% packed sphere volume. Neutrophils (10^6 cells) were incubated in PBS, or various concentrations of fMP as previously described and centrifuged into a pellet. The pretreated spheres (0.1ml) were added to the cell pellet, gently vortexed, and incubated for 3 minutes at 22°C. The cell/sphere mixture was then mixed with an equal volume of 2% glutaraldehyde and the numbers of spheres bound to the cells counted and averaged using light microscopy. The data in Table 16 demonstrates that even relatively high concentrations of fMP (10^{-6}M) fail to enhance neutrophil binding of these protein coated spheres.

Table 16. * Latex spheres were incubated with 2 ml of 5% HSA, 50% heat inactivated (56°C for 30 minutes) serum or human C_3 (1000 U/ml). The spheres were then washed X3 in PBS and reconstituted to a 1% packed sphere volume in PBS, 10^{-8}M fMP or 10^{-6}M fMP.

** Neutrophils (10^6 cells) were incubated in PBS, or fMP for 10 minutes at 22°C, centrifuged into a pellet and the supernatant discarded.

‡ The protein coated spheres (0.1ml) were added to the cell pellet, gently mixed and incubated for 3 minutes. The cell/sphere mixture was then diluted with PBS or fMP and immediately fixed with an equal volume of 2% glutaraldehyde. Spheres not bound by the cells were removed using a Ficoll-Hypaque density gradient and the numbers of spheres attached on 100 neutrophils counted and averaged using light microscopy (100X objective).

§ n = the number of individual experiments performed.

Table 16. The Effects of Chemoattractants on Neutrophil Binding of Latex Spheres Coated with Human C_3, Albumin, or Heat Inactivated Serum

Sphere Pretreatment *	Cell Pretreatment **	# Spheres/cell ‡	(n) §
5% HSA	PBS	1.4 ± 0.3	(3)
	fMP (10^{-8}M)	1.3 ± 0.4	(3)
	fMP (10^{-6}M)	1.6 ± 0.2	(3)
Heat Inactivated Serum	PBS	0.9 ± 0.1	(3)
	fMP (10^{-8}M)	1.0 ± 0.2	(3)
	fMP (10^{-6}M)	0.8 ± 0.2	(3)
Human C_3	PBS	0.3 ± 0.1	(8)
	fMP (10^{-8}M)	0.3 ± 0.1	(8)
	fMP (10^{-6}M)	0.4 ± 0.1	(8)

B. The Effects of Chemoattractants on Neutrophil Adherence to Protein Coated Glass

Neutrophils are secretory cells and CF can cause them to release lysosomal enzymes [80]. It was hypothesized that these enzymes could act upon protein coated substrata and enzyme induced alterations of the substrata might also allow neutrophils to adhere to the substrata. To evaluate this hypothesis, a flat surface was required upon which cells could settle and be allowed to act upon the substrata. Multiple well tissue chambers were selected to evaluate this process. These chambers are composed of 10 individual wells that are fixed onto a single glass slide (Figure 17).

Solutions were prepared that contained heat inactivated serum (50% in PBS, human C_3 (1000 U/ml), gelatin (1 mg/ml of PBS), purified human collagen (1 mg/ml of PBS) and purified human fibrinogen (5 mg/ml of PBS). Human serum albumin was not used in these experiments due to our inability to get this protein to consistently adhere to glass. A small volume (0.3 ml) of PBS, gelatin, collagen, human C_3, or human fibrinogen was then injected into individual isolated wells within the tissue chamber and the chamber incubated for 10 minutes at 37°C. The protein containing solutions were then removed by suction and PBS injected into all wells. This solution was also removed by suction and the rinsing process repeated X2. Neutrophils (2×10^5 cells/0.3 ml PBS) were then injected into each of the pretreated wells. The chamber was incubated (10 minutes at 37°C) and then inverted.

The individual wells were then gently removed from the glass slide and the slide rinsed in a Petri dish containing PBS. The slide was air dried, immersed in absolute methanol and attached cells stained with Wright's stain. The number of cells adhering to the glass slide circumscribed by each well was then counted using an image analyzer (10 fields, 6X objective).

Consistently between 2000 and 4000 cells were counted adhering to the area of glass pretreated only with PBS (Table 16). This count was allowed to represent 100% neutrophil adherence to the glass. Under the same conditions, neutrophil adherence to glass pretreated with serum, C_3, gelatin, collagen or fibrinogen was dramatically reduced (Table 17). Neutrophils were then incubated with various concentrations of fMP for 10 minutes prior to injection into the protein pretreated wells, and the experiment repeated as previously described. Two very different dose response curves were obtained (Figure 18), depending upon the substrata used. The effects of fMP on neutrophil adherence to C_3, gelatin, and collagen coated glass were very similar. Significant changes in adherence ($p < 0.005$) were not observed until the cells were exposed to relatively high concentrations of fMP (10^{-6}M). Yet, significant ($p < 0.050$) changes in neutrophil adherence to fibrinogen and serum coated glass resulted after neutrophil exposure to 10^{-7}M fMP (Figure 18). Maximal adhesiveness to all protein coated substrata occurred when the concentrations of fMP were near 5×10^{-6}M.

Figure 17. This diagram illustrates a tissue culture chamber with 10 isolated wells affixed on top of a single glass microscope slide. Approximately 0.3 ml of solutions containing protein or isolated neutrophils could be injected into each of the isolated compartments. The chambers were washed by removing the fluids from each well by suction. Cells injected into the chambers settled onto the glass slide by gravity and unattached cells removed by inverting the chamber and removing the compartments. The compartments were easily detached yet fixed well enough to the glass to withstand the rigors of most experiments.

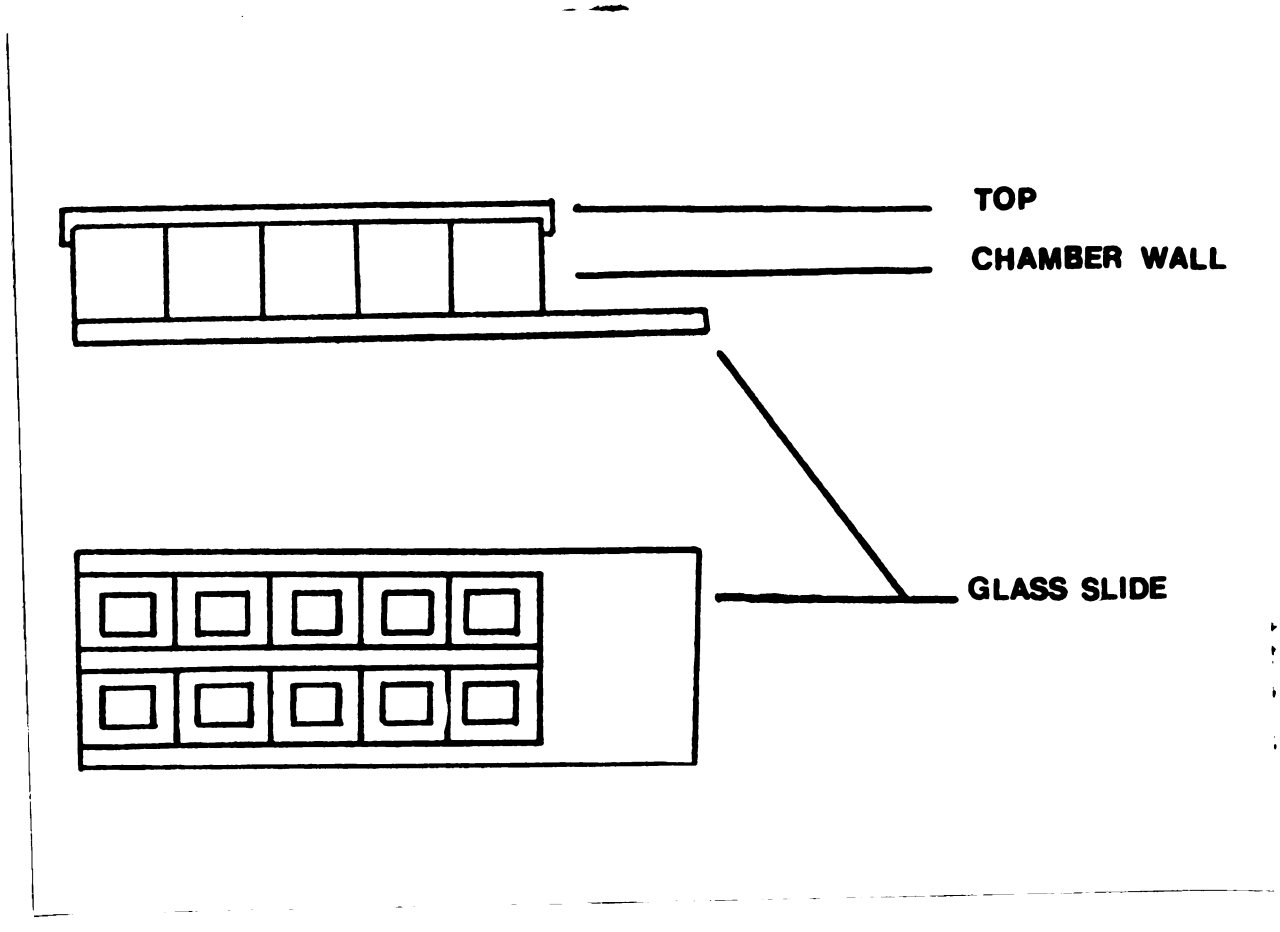


Figure 17. Diagram of a Tissue Culture Chamber

Table 17. Neutrophil Adherence to Protein Coated Glass *

Glass Pretreatment **	% Adherence	(n) ‡
PBS	100%	(7)
Human C_3 (1000 U/ml)	5 ± 2%	(7)
Gelatin (1 mg/ml)	4 ± 1%	(4)
Human Collagen (1 mg/ml)	6 ± 1%	(4)
Heat Inactivated Serum (50%)	3 ± 1%	(3)
Human Fibrinogen (5 mg/ml)	6 ± 2%	(4)

* Neutrophils (2 X 10^5 cells/0.3 ml of PBS) were injected into the tissue culture wells and incubated for 10 minutes at 37°C. Within 2 minutes all cells were previously shown to have settled onto the bottoms of these wells (i.e., glass slide). Following a 10-minute incubation, the chamber was inverted to allow unattached cells to fall off the glass slide. The tissue culture wells were then removed and the slide rinsed. The cells attached to the glass slide were fixed, stained and counted. The numbers of cells attached to the PBS pretreated glass was allowed to represent the maximum number of cells able to adhere under the conditions of the experiment (i.e., 100%). The numbers of cells adhering to the protein coated glass surface were compared to the numbers of cells adhering to PBS and expressed as a %.

** 0.3 ml of PBS or a variety of human and non-human proteins suspended in PBS were injected into individual wells of a tissue culture chamber and incubated for 10 minutes at 37°C. The solutions were then removed by suction and the wells washed X3 with PBS.

‡ n = the number of experiments.

Figure 18. Neutrophils (2 x 10^5 cells) were pretreated in PBS or various concentrations of fMP for 10 minutes at 22°C. The cells (0.3 ml) were injected into tissue culture wells and incubated for 10 minutes at 37°C. These wells were coated with human C_3, gelatin, human collagen, human fibrinogen, or heat inactivated human serum prior to the experiments. The numbers of cells adhering to the PBS or protein coated surfaces were then counted. The number of cells adhering to a PBS pretreated surface was allowed to represent the maximum number of cells able to adhere (i.e., 100% adherence). The number of cells adhering to the other surfaces were compared to the number adhering to the PBS pretreated surface and expressed as a percentage. The brackets indicate the value of the SEM. All experiments were performed with n=4.

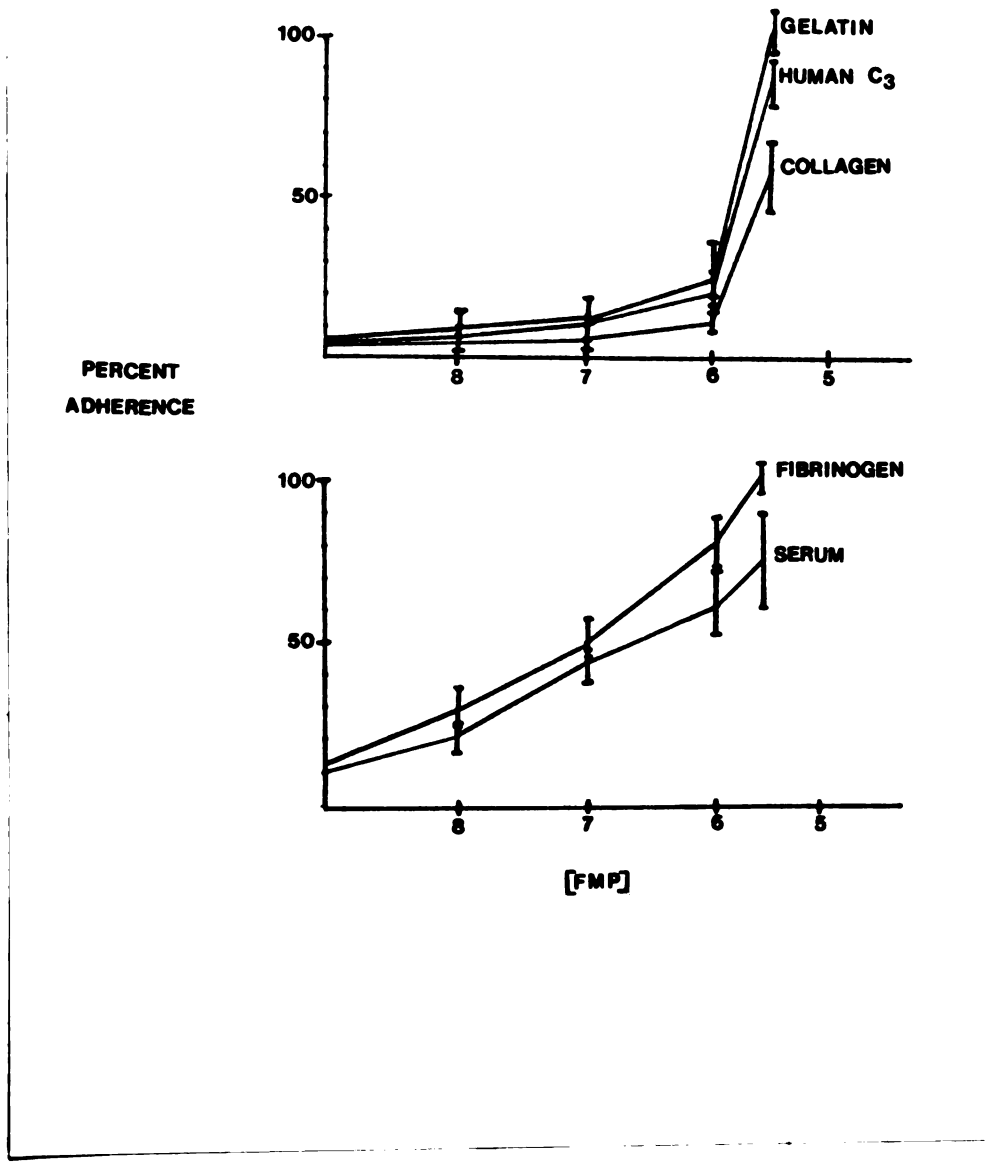


Figure 18. The Effects of Chemoattractants on Neutrophil Adherence to Protein Coated Glass

Neutrophils were next pretreated (10 minutes at 22°C) with a concentration of fMP previously shown to cause a thirty percent or greater increase in neutrophil adhesiveness to C_3, gelatin, collagen, fibrinogen or serum coated glass. These cells were then split into two aliquots. One aliquot was centrifuged, the supernatant removed, and the cells washed in PBS. The washed and unwashed cells were then injected into wells pretreated as previously described and the adhering cells counted. In addition, a second chamber was also pretreated with human C_3, collagen, serum, fibrinogen or gelatin, and washed. The supernatant from the fMP pretreated cells was then injected into the wells, incubated for 10 minutes at 37°C, the fluids removed, and each well washed in PBS. Neutrophils pretreated in PBS were then injected into these wells and adhering cells also counted. The data in Table 18 demonstrates again that PBS pretreated cells adhered very poorly to protein coated glass and fMP enhanced this adherence. This data also demonstrates that some factor within the fMP pretreated cell supernatant was apparently augmenting neutrophil adherence to C_3, gelatin, and collagen coated glass. Washing the cells dramatically lowered the numbers of cells adhering to these surfaces. Chemoattractant induced neutrophil adherence to fibrinogen and serum coated glass did not require such a factor since the washed cells adhered well. Pretreating the C_3, gelatin, and collagen (but not the fibrinogen and serum coated glass) with the fMP cell supernatants also greatly enhanced neutrophil adherence to these surfaces (Table 18).

Table 18. * Neutrophils (5 X 10^5 cells/0.3ml) were incubated in PBS or various concentrations of fMP for 10 minutes at 22°C. The fMP pretreated cells were split into two aliquots. Both aliquots were centrifuged and the supernatants removed from one and saved. This aliquot was diluted X50 with PBS, washed again, and reconstituted to its original concentration with PBS. The remaining aliquot was left suspended in its original supernatant.

** Individual wells within a tissue culture chamber were injected with PBS or various protein solutions (0.3ml) and incubated for 10 minutes at 37°C. These solutions were then removed by suction and washed with PBS. After washing, several chambers were reinjected with 0.3ml of various supernatants, incubated for an additional 10 minutes at 37°C, and washed in PBS before use.

‡ 5 X 10^5 neutrophils were injected into the pretreated wells of a tissue culture chamber and incubated for 10 minutes at 37°C. The chamber was then inverted, the wells removed and the glass slide rinsed in PBS. The slide was then immersed in absolute ethanol, attached cells stained and counted using an image analyzer (10 fields, 6X objective). A percent adherence was obtained by comparing the number of cells adhering to those adhering to untreated glass using the same experimental conditions.

§ n = the number of experiments.

Table 18. The Effects of the Cell Supernatant or fMP Pretreatment on Neutrophil Adherence to Protein Coated Glass

Neutrophil Pretreatment *	Substrata Pretreatment **	% Adherence ‡	(n) §
PBS	Human C_3 (1000 U/ml)	8 ± 3%	(7)
5 X 10^{-6}M fMP (unwashed)	Human C_3 (1000 U/ml)	84 ± 5%	(4)
5 X 10^{-6}M fMP (washed)	Human C_3 (1000 U/ml)	14 ± 6%	(4)
PBS	Human C_3 (1000 U/ml) and fMP (5 X 10^{-6}M) cell supernatant	68 ± 3%	(4)
PBS	Gelatin (1mg/ml)	4 ± 1%	(6)
5 X 10^{-6}M fMP (unwashed)	Gelatin (1mg/ml)	88 ± 8%	(6)
5 X 10^{-6}M fMP (washed)	Gelatin (1mg/ml)	12 ± 4%	(3)
PBS	Gelatin (1mg/ml) and fMP (5 X 10^{-6}M) cell supernatant	65 ± 3%	(3)
PBS	Collagen (1mg/ml)	3 ± 1%	(3)
5 X 10^{-6}M fMP (unwashed)	Collagen (1mg/ml)	62 ± 7%	(3)
5 X 10^{-6}M fMP (washed)	Collagen (1mg/ml)	9 ± 4%	(3)
PBS	Collagen (1mg/ml) and fMP (5 X 10^{-6}M) cell supernatant	49 ± 3%	(3)

Table 18 (cont'd.)

Neutrophil Pretreatment *	Substrata Pretreatment **	% Adherence ‡	(n) §
PBS	Human Fibrinogen (5mg/ml)	11 ± 3%	(4)
10^{-7}M fMP (unwashed	Human Fibrinogen (5mg/ml)	43 ± 4%	(4)
10^{-7}M fMP (washed)	Human Fibrinogen (5mg/ml)	46 ± 4%	(5)
PBS	Human Fibrinogen (5mg/ml) and fMP (10^{-7}M) cell supernatant	14 ± 3%	(3)
PBS	Heat Inactivated Serum (50%)	10 ± 2%	(7)
10^{-7}M fMP (unwashed)	Heat Inactivated Serum (50%)	40 ± 4%	(4)
10^{-7}M fMP (washed)	Heat Inactivated Serum (50%)	38 ± 5%	(4)
PBS	Heat Inactivated Serum (50%) and fMP (10^{-7}M) cell supernatant	11 ± 3%	(3)

Several authors have shown that fMP binds to protein coated glass [115, 116]. However, solutions containing fMP (10^{-5}M) were incubated in wells pretreated with all of the proteins used in the above experiments and this pretreatment failed to significantly enhance neutrophil attachment to these surfaces ($p > 0.90$, n=3).

Neutrophils contain a number of trypsin and chymotrypsin-like enzymes within their lysosomal granules [80]. Wells within tissue culture chambers were pretreated with human C_3, collagen, fibrinogen, heat inactivated serum and gelatin as previously described. To these wells, 0.3 ml of PBS, trypsin (100 U/ml of PBS) or chymotrypsin (100 U/ml of PBS) were injected and incubated for 1 minute at 37°C. The solutions were then removed using suction and the wells washed X3 in PBS. Neutrophils (2×10^5 cells, 0.3ml of PBS) were injected into each well and incubated for 10 minutes at 37°C. The chamber was then inverted, the wells removed, and the attached cells fixed, stained and counted as previously described. The data in Table 19 demonstrates that exposing the protein coated glass to either of the enzyme solutions enhanced neutrophil adherence to those surfaces. Yet, the surfaces coated with human C_3, collagen, and gelatin were most affected by trypsin and those coated with human fibrinogen or serum by chymotrypsin (Table 19).

Neutrophils were then preincubated with fMP (5×10^{-6}M) for 10 minutes at 22°C. A specific trypsin inhibitor (TLCK, 5×10^{-4}M) was then added to one aliquot of the

Table 19. * Individual wells were injected with 0.3 ml of a variety of protein solutions. The solutions were incubated for 10 minutes at 37°C and washed with PBS.

** Solutions containing either trypsin or chymotrypsin were then added to the wells and incubated for 5 minutes at 37°C. These solutions were removed by suction and washed in PBS X3.

‡ Neutrophils (2×10^5 cells/0.3ml PBS) were injected into the wells and incubated for 10 minutes at 37°C. The chamber was then inverted, the wells removed, and the adherent cells counted using an image analyzer (10 fields, 6X objective). A percentage was derived by comparing the number of adherent cells for each experiment to the number of cells adhering to untreated glass and multiplying by 100.

§ n = the number of experiments.

Table 19. The Effects of Enzymes on Neutrophil Adherence to Protein Coated Glass

Substrate Pretreatment *	Enzyme Treatment **	% Adherence ‡	(n) §
Human C_3 (1000 U/ml)	Trypsin (100 U/ml)	95 ± 7%	(7)
Collagen (1mg/ml)		98 ± 5%	(3)
Gelatin (1mg/ml)		63 ± 5%	(4)
Human Fibrinogen (5 mg/ml)		31 ± 3%	(4)
Heat Inactivated Serum (50%)		20 ± 4%	(3)
Human C_3 (1000 U/ml)	Chymotrypsin (100 U/ml)	27 ± 4%	(3)
Collagen (1mg/ml)		29 ± 5%	(3)
Gelatin (1mg/ml)		21 ± 3%	(3)
Human Fibrinogen (5mg/ml)		80 ± 7%	(4)
Heat Inactivated Serum (50%)		86 ± 8%	(3)

pretreated cell suspension. Individual wells were then pretreated with human C_3, collagen, or gelatin and washed as previously described. Neutrophils suspended in fMP with and without TLCK were then injected into the wells, incubated for 10 minutes at $37^{o}C$ and neutrophil adherence evaluated as previously described. That data in Table 20 demonstrates that TLCK effectively impaired the ability of fMP pretreated cells to adhere to C_3, collagen, and gelatin coated glass.

Neutrophils were next pretreated with $10^{-7}M$ fMP for 10 minutes at $22^{o}C$ and PBS, TLCK ($5 \times 10^{-4}M$), or TPCK (a chymotrypsin inhibitor, $10^{-6}M$) added to two aliquots of the pretreated cells. Individual wells of a tissue culture chamber were then pretreated with human fibrinogen or heat inactivated serum as previously described. The pretreated cells, with or without TPCK or TLCK, were then injected into the wells, incubated, and neutrophil adherence evaluated as previously described. The data in Table 21 demonstrates that TLCK did not markedly alter fMP pretreated neutrophil adhesiveness to fibrinogen or serum coated glass. This adherence was, however, markedly depressed by TPCK. The chymotrypsin inhibitor was not killing the cells as determined by eosin exclusion (95% viability, n=2). This inhibitor also did not appear to be damaging neutrophil adhesion mechanisms since the majority of the cells ($85 \pm 8\%$, n=3) would adhere to fibrinogen coated glass that had been pretreated with chymotrypsin as previously described.

Table 20. * Individual wells of a tissue culture chamber were injected with a variety of protein containing solutions. The solutions were incubated within these wells for 10 minutes at 37°C, then removed by suction and washed X3 with PBS.

** Neutrophils were pretreated with fMP for 10 minutes at 22°C. A trypsin inhibitor (TLCK) was then added to one aliquot of the pretreated cells.

‡ Neutrophils (2 X 10^5 cells/0.3ml PBS) were injected into the pretreated wells of a tissue culture chamber and incubated for 10 minutes at 37°C. The chamber was then inverted, the wells removed and the glass slide washed in PBS. Cells adhering to the slide were then counted using an image analyzer (10 fields, 6X objective). A percentage was obtained by comparing the numbers of cells adhering to the protein coated glass to those adhering to untreated glass.

§ n = the number of experiments performed.

Table 20. The Inhibition of a Chemoattractant Induced Neutrophil Adherence to Protein Coated Glass by a Trypsin Inhibitor

Substrata Pretreatment *	Neutrophil Pretreatment **	% Adherence ± (n=4) §
Human C_3 (1000 U/ml)	fMP (5×10^{-6}M)	88 ± 6%
	fMP (5×10^{-6}M) and TLCK (5×10^{-4}M)	31 ± 5%
Human Collagen (1mg/ml)	fMP (5×10^{-6}M)	59 ± 6%
	fMP (5×10^{-6}M) and TLCK (5×10^{-4}M)	14 ± 2%
Gelatin (1mg/ml)	fMP (5×10^{-6}M)	89 ± 6%
	fMP (5×10^{-6}M) and TLCK (5×10^{-4}M)	34 ± 4%

Table 21. * Neutrophils were incubated with fMP for 10 minutes at 22°C. Either TLCK or TPCK were added to aliquots of the cells.

** Individual wells of a tissue culture chamber were filled with solutions containing human fibrinogen or serum and incubated for 10 minutes at 37°C. The solutions were removed by suction and the wells washed X3 in PBS.

‡ Neutrophils were injected (2 X 10^5 cells) into the tissue culture chamber wells and incubated for 10 minutes at 37°C. The chamber was inverted, the wells removed, and the glass slide rinsed in PBS. Cells adhering to the glass slide were fixed, stained and counted using an image analyzer (10 fields, 6X objective). A % of adherence was calculated by comparing the numbers of cells adhering to the protein coated glass to those adhering to untreated glass, and multiplying by 100.

§ n = the number of experiments.

Table 21. The Inhibition of Chemoattractant Induced Neutrophil Adherence to Protein Coated Glass by Trypsin and Chymotrypsin Inhibitors

Neutrophil Pretreatment *	Substrata Pretreatment **	% Adherence ‡ (n=4) §
fMP (10^{-7}M)	Human Fibrinogen (5mg/ml)	44 ± 5%
fMP (10^{-7}M) and TLCK (5 X 10^{-4}M)		47 ± 3%
fMP (10^{-7}M) and TPCK (10^{-6}M)		7 ± 4%
fMP (10^{-7}M)	Heat Inactivated Human Serum (50%)	34 ± 5%
fMP (10^{-7}M) and TLCK (5 X 10^{-4}M)		36 ± 3%
fMP (10^{-7}M) and TPCK (10^{-6}M)		8 ± 4%

DISCUSSION

I. Neutrophil Binding of Protein Coated Particles

Neutrophil binding to protein coated substances is important for its locomotion and phagocytosis. A variety of neutrophil functions have been shown to be altered by the drug colchicine and various chemotactic factors. Of most importance to this report are the following; 1) these agents alter neutrophil adhesiveness to a number of protein coated surfaces [7, 35, 56] and, 2) neutrophils form a bipolar configuration with distinct front and tail ends when exposed to these agents, and 3) chemotactic factors promote the activation or release of neutrophil enzymes into their environment [91, 97, 105]. The first goal of this report was to determine whether colchicine or chemotactic factors would influence neutrophil binding of protein coated particles and bacteria. Particular emphasis was given to the effects of these agents on the topographical arrangements of neutrophil binding sites for the particles. The protein coated particles chosen for these studies were selected because of the considerable information that has accumulated about neutrophil binding of these particles.

A. Neutrophil Binding of Immunoglobulin and/or Albumin Coated Spheres

The coating of latex spheres with 5% HSA almost totally prevented neutrophil binding of the spheres. Similarly pretreated spheres were agglutinated by immunoglobulin specific for human albumin and immunoglobulin could be detected antigenically on their surfaces weeks after preparation. The immunoglobulin coated spheres were uniform, easily observed microscopically and preservable for weeks at $4^{o}C$. Unlike neutrophil attachment of albumin coated spheres, immunoglobulin coated spheres were avidly bound by human neutrophils. It was assumed that this binding was a result of the spheres' interactions with the neutrophil Fc surface receptors. As previously reported [91], human neutrophils failed to bind spheres pretreated with goat immunoglobulins, apparently because of a difference between the Fc component of goat immunoglobulins and other animal immunoglobulins.

Neither chemotactic factors nor colchicine enhanced or impaired neutrophil binding of immunoglobulin coated spheres. The spheres did adhere to neutrophils in an asymetic manner. Neutrophils isolated from whole blood and suspended in PBS or pretreated with $10^{-8}M$ fMP were spherical. Immunoglobulin coated spheres were observed binding to the majority of these spherical neutrophils at one or two localized regions. The regions were usually ruffled in appearance, and often quite extensive. The probability of additional contacts within this region was perhaps greater than the remaining smaller spherical portion of the cell. Since the cells were

spherical, it was impossible to determine if sphere binding occurred at a region common to all cells. Walter, et al have reported that immunoglobulin coated erythrocytes adhere to PBS pretreated neutrophils in a "more or less random fashion" [10]. Perhaps until additional data is obtained, this is the best description for spherical neutrophil binding of immunoglobulin coated spheres.

The majority of the isolated neutrophils were observed to form a bipolar configuration when exposed to colchicine or polarizing concentrations of fMP (e g., 10^{-6}M). Walter, et al have reported that neutrophils bind immunoglobulin coated erythrocytes only at their front ends when exposed to a gradient of chemotactic factors [10]. We also report that neutrophils bind immunoglobulin coated spheres almost exclusively at their front ends when exposed to multiple concentrations of fMP (10^{-8}-10^{-6}M). However, neutrophils pretreated with a single polarizing concentration of fMP or colchicine also bound spheres at their front ends. Therefore, the frontal binding of immunoglobulin coated spheres does not appear to be a preferential response to a gradient of chemotactic factors. The only common fact known about these cells is that they all have formed a "motile configuration". The front end of the cells was ruffled in appearance before binding of the spheres occurred. It is unlikely these cells bind spheres at any region other than their front ends, since regardless of the sphere concentration or incubation time with the spheres, sphere binding at sites other than

their front ends was rarely observed. In agreement with others, the binding of immunoglobulin coated particles on polarized neutrophils seems to occur at the most logical site [10]. The cells crawl in the direction their front end points. Collisions with particles would seem to occur most frequently at their front or "business" end.

Neutrophils rapidly ingested immunoglobulin coated spheres and did effectively remove them from their surface enabling further attachments to occur. The ingestion of spheres appeared to occur at or near the attachment site. When the cells were spherical, spheres were ingested within 1-3 minutes and they tended to fill all available internal spaces with no observable pattern. When polarized, the spheres were ingested at about the same rate, but were observed being swept away internally towards the tail end of the cell. This appeared to be a tremendously efficient method of removing particles enabling additional attachments and unencumbered ingestion to occur.

The mechanisms by which polarized neutrophils selectively bind and ingest individual particles at different sites remain unknown. The means by which internalized spheres are swept rearward also are unknown. The presence or absence of microtubules appear unimportant for these processes since chemotactic factors have been reported to promote microtubule polymerization [103] within neutrophils while colchicine causes microtubule depolymerization [93].

In the beginning of this investigation, we also verified earlier reports by Smith and Hollers [7]. Neutrophils did bind spheres coated with only 1.0% human albumin. Pretreating neutrophils with 10^{-8}M fMP enhanced their ability to bind spheres, but did not cause shape change. Increasing the concentrations of fMP used to pretreat the cells further enhanced sphere binding and caused the cells to form a bipolar configuration. Pretreating neutrophils with colchicine somewhat diminished their ability to bind the spheres yet caused shape change. Of particular interest were the binding patterns for albumin coated spheres on bipolar neutrophils pretreated with colchicine, single or multiple concentrations of fMP (i.e., 10^{-6}M, and 10^{-8}-10^{-6}M, respectively). As reported [7], neutrophils bound albumin coated spheres in a random fashion when pretreated with PBS, 10^{-8}M, or 10^{-6}M fMP. Yet, spheres were only observed bound to the tail process of cells pretreated with colchicine or multiple doses of fMP. Smith and Hollers offered this and other evidence that pretreating neutrophils with colchicine and multiple concentrations of fMP may have altered their binding site topography.

In agreement with two independent laboratories, human neutrophils appear to selectively bind two different particles (i.e., albumin and immunoglobulin coated particles) at two different locations [7, 10]. We proved that this can occur simultaneously on neutrophils. Neutrophils exposed to colchicine or multiple increasing concentrations of fMP were shown to bind immunoglobulin coated particles at their front

ends while also selectively binding albumin coated spheres at their tail ends. Spherical neutrophils or cells only exposed to a single chemotactic stimulus also bound immunoglobulin coated spheres in a localized fashion and at the same time bound albumin coated spheres randomly.

B. Neutrophil Adhesion and Surface Rearrangements of Membrane Bound Lectins and Bacteria

Concanavalin A binding to leukocytes and its subsequent surface redistribution on colchicine pretreated cells have been extensively documented in the past [6, 8, 103]. We did not quantitate the binding of ConA to neutrophils but did evaluate the topographical rearrangements of binding sites for ConA on neutrophils pretreated with chemotactic factors or colchicine. Chemotactic factors have been reported to promote the redistribution of tetravalent ConA adhesion sites to the front ends of neutrophils [8]. We were unable to document this localized adhesion pattern of fMP pretreated cells in suspension. Like others [4, 100], we observed that ConA was bound randomly on all pretreated cells. However, we do report a selective redistribution of surface bound ConA on fMP stimulated cells. The distribution of ConA on the surface of neutrophils exposed to a single dose of fMP (10^{-8}M or 10^{-6}M) did not appear to be altered with time. Yet, when cells were pretreated with multiple doses of fMP (10^{-8}-10^{-6}M) a rearrangement of surface bound ConA was observed. Most of the surface bound lectin was observed to flow to the tail

process on each of the cells or "cap". This capping process was indistinguishable from that previously reported on colchicine pretreated cells. We also observed ConA redistributing to the tail ends of colchicine pretreated cells. The redistribution of ConA on colchicine or chemotactic factor pretreated cells apparently required lectin crosslinking of receptors since bivalent ConA did not redistribute rearward on similarly pretreated cells. The redistribution of surface bound ConA to the tail ends of colchicine and chemotactic factor pretreated cells required 10 minutes at $37^{o}C$ for completion. The addition of ConA specific immunoglobulin to polarized cells with ConA previously attached promoted a much faster redistribution of surface bound ConA. This rapid redistribution (within 1 minute) occurred on all pretreated cells (e.g., 10^{-6}M fMP, 10^{-8}-10^{-6}M fMP or colchicine). The addition of immunoglobulin did more than accelerate the redistribution of ConA, since there were no restraints on the redistribution of ConA on cells pretreated with a single polarizing dose of fMP.

Salmonella typhimurium have been reported to possess ConA-like lectins on their surface pili [1, 68]. As previously reported by others [1, 68], human neutrophils failed to bind the non-piliated strain of this bacteria but did form mannose sensitive attachments with the piliated strains. Chemotactic factors enhanced neutrophil binding of the piliated bacteria and colchicine pretreatment somewhat impaired the ability of the neutrophil to bind piliated

bacteria. Neutrophil adhesion and redistributions of surface bound S. typhimurium were indistinguishable from those described for ConA. If the bacteria extensively cross-linked neutrophil binding sites, then this process did not accelerate their redistribution on the neutrophil's surface or release the restraints generated by a single chemotactic stimulus.

Several other piliated micro-organisms have been reported to be bound by neutrophils independently of serum opsonins. Some of them are apparently ingested after attachment, others are not. Bacteria bound but not ingested by neutrophils have been observed to redistribute to the tail processes of motile neutrophils after attachment. These bacteria were not internalized and remained viable [128]. The conditions affecting the selective internalization of these bacteria are unknown. S. typhimurium bound by neutrophils were also observed to redistribute rearward across the neutrophil's surface, but only after the cells were pretreated with colchicine or multiple doses of chemotactic factors. Bacteria that accumulated at the tail processes of these cells were not ingested 30 minutes after attachment. Yet, externally bound S. typhimurium were rarely observed on the surface of neutrophils exposed to a single dose of fMP. The ability of neutrophils to remove surface bound piliated bacteria by ingestion or redistribution may reside in the number of chemotactic stimuli received by the cell.

We also report that redistribution of surface bound particles does not interfere with the localized adhesion of

latex bound immunoglobulin on polarized neutrophils. S. typhimurium were bound by neutrophils pretreated with colchicine or multiple doses of fMP and, in 10 minutes, the cells redistributed the bacteria to their tail ends. Following this surface redistribution, neutrophils were still able to bind and ingest immunoglobulin coated spheres at their front ends. The redistribution of the binding sites with attached bacteria apparently did not sweep the immunoglobulin binding sites from the front end of the cell.

C. Preparation and Neutrophil Binding of C_3b Coated Latex Spheres

Two techniques were used to attach C_3 to latex spheres. Bacteria or spheres were incubated with solutions containing serum or purified human C_3. Whether fresh serum or solutions containing C_3b were used for sphere pretreatment, sphere attachments were enhanced by 20-50X as compared to neutrophil binding of spheres pretreated with heat inactivated serum or human C_3. The following evidence indicates this enhanced binding was a result of C_3b absorption to the spheres. First, the conditions used have been used in the past to fix C_3b to a variety of particles [79, 84, 91]. Second, C_3b was demonstrated to be antigenically present on the treated spheres. Third, immunoglobulins specific for C_3b (e.g., goat or rabbit anti-human C_3c) blocked (i.e., goat anti-C_3c) or modified (e.g., goat or rabbit anti-human C_3c) neutrophil binding of the treated spheres. Last, physical (i.e., sonification) or

enzymatic (i.e., trypsin) removal or latex absorbed proteins to the sphere surface were not influencing our results. The assumption was made that neutrophil binding of these particles was a result of particle bound C_3b and the neutrophil C_3b receptor interactions. Although bacteria and erythrocyte bound C_3b have been used in the past [5, 84, 97], the advantages for using C_3b coated spheres were as previously described for immunoglobulin coated spheres.

Several groups have reported that macrophages bind C_3b coated particles randomly across their surfaces [5, 91]. Bianco, et al have also shown that "activated" macrophages ingest C_3b coated particles [129]. We also observed spherical PBS pretreated neutrophils binding latex bound C_3b randomly on their surfaces. Exposing neutrophils to 10^{-8}M fMP did not morphologically alter the cells or attachment patterns for latex bound C_3b. Yet, unlike the control cells, (i.e., PBS pretreated) neutrophils exposed to even low doses of fMP ingested C_3b coated spheres. Unlike previous reports [10], colchicine pretreated neutrophils did not initially bind latex bound C_3b randomly on their surface. The initial particle attachments occurred almost exclusively at the front ends of these polarized cells. Seconds (5 seconds) later, the attached spheres traveled rearward and appeared randomly distributed. By 90 seconds, the attached spheres were almost exclusively at the cell's tail end. This was an extremely rapid clearance as compared to the surface redistribution of ConA on neutrophils. All observations of this redistribution

implied that the rearrangements were occurring on the cell surface. As previously reported for colchicine pretreated macrophages [10], we also report that neutrophil ingestion by colchicine pretreated cells was undetectable 30 minutes after the sphere-neutrophil attachment.

Adhesion of latex bound C_3b also occurred at the front ends of neutrophils pretreated with single or multiple polarizing doses of fMP. Following attachments, two neutrophil mechanisms were, however, activated for removing C_3b coated particles from the front ends of these polarized cells, and the mechanisms inseparable when the cells were examined using light microscopy. First, fMP polarized neutrophils appeared to ingest approximately half of the frontally attached spheres within 60 seconds of attachment. Following internalization, spheres traveled relatively slowly to the rear of the cells much like those spheres coated with immunoglobulin. Spheres not ingested by the fMP polarized neutrophils were redistributed rearward by a second, much faster, mechanism which occurred on the cell surface. Surface bound spheres traveled from front to tail on neutrophils in approximately 60 seconds. The different rates of rearward flow for internalized and externally bound spheres often created the impression, using light microscopy, that the spheres were randomly distributed 1-10 minutes after attachment. Each redistribution event had to be studied using light, electron, and immunochemical microscopic techniques to separate externally bound spheres from ingested spheres. As observed

on colchicine pretreated neutrophils, spheres observed attached on the tail process of fMP stimulated cells remained exteriorly bound for 30 minutes.

Neutrophils avidly bound C_3b coated spheres or bacteria. As previously reported [124], polarizing concentrations of fMP almost doubled the binding of C_3b coated spheres. Low concentrations of fMP (i.e., 10^{-8}M) did not significantly enhance sphere binding and ingestion of the spheres was relatively slow (10-20 minutes). The inability of the cells to clear spheres from the cell surface rapidly may have accounted for their lack of enhanced sphere binding. However, spheres were cleared rapidly from their attachment sites on colchicine pretreated cells, and these cells failed to bind more spheres than control cells. Only polarized cells ingesting spheres and redistributing spheres rearward on their surfaces bound significantly more spheres than the control cells. Their rate of sphere ingestion was much quicker than cells pretreated with 10^{-8}M fMP. Ingestion occurred at about the same rate as for immunoglobulin coated spheres (1-3 minutes). The rapid sphere ingestion by the cells may have exposed hidden or new binding sites for C_3b coated particles on the neutrophil surface [109].

Neutrophil binding, ingestion and redistribution of surface bound particles was again shown to be highly selective. Although neutrophils bound C_3b and immunoglobulin coated spheres simultaneously at their front ends, their fates after binding were as predicted. The immunoglobulin

coated spheres were rapidly ingested at or near their attachment sites. The C_3b coated particles were swept rearward on the same cell (e.g., colchicine pretreated) or ingested and swept rearward on the cell surface (e.g., 10^{-6}M or 10^{-8}-10^{-6}M fMP pretreated).

II. The Effects of Chemoattractants on Substrate Adhesiveness

There were two conceivable means of altering the ability of a cell to adhere to a surface. First, alter cellular adhesiveness and, second, alter the adhesiveness of the attachment surface. Our research demonstrated an example of each. Chemotactic factors were shown to enhance neutrophil binding of bacteria and spheres coated with 2% HSA or C_3b. The CF appears to have altered the neutrophil's adhesiveness for these particles. The second example was neutrophil binding of spheres coated with human C_3 before and after trypsinization. Enzymatic alteration of this protein dramatically altered particle adhesiveness.

Chemotactic factors have also been shown to cause neutrophils to release trypsin and chymotrypsin-like enzymes into their environment, and may also activate similar enzymes on the neutrophil membrane [12, 73, 99]. The second goal of this report was to determine whether enzymes could influence the adhesiveness of protein coated surfaces. To perform such experiments, another adherence assay technique

was needed that provided a surface which could be coated with proteins yet afforded a surface onto which neutrophils could settle and act on. The assay technique chosen (i.e., the tissue culture chamber) proved an accurate, reliable, simple and inexpensive technique for evaluating the ability of neutrophils to attach to a surface over an extended period of time. This technique could also be modified for utilizing automated methods of counting surface bound cells improving its accuracy and precision.

A. Neutrophil Adherence to Protein Coated Glass

As observed for similar protein coated particles, neutrophil adherence to glass coated with human C_3 or 50% heat inactivated serum was extremely poor. Neutrophils also adhered very poorly to glass coated with human collagen, fibrinogen and gelatin. Unlike neutrophil binding of particles, exposing neutrophils to CF greatly enhanced their adherence to glass coated with each of these proteins. The dose response curves reported for fMP stimulated adherence to glass coated with C_3, gelatin or collagen were similar. Neutrophil adherence was not altered until the cells were exposed to relatively high concentrations of fMP. The CF enhanced adherence to these surfaces was dramatically impaired by the addition of a trypsin specific inhibitor to the cell suspension or by washing the fMP pretreated cells prior to their addition to the adherence chamber. We demonstrated that chemotactic factor binding to the protein coated

surfaces could not explain these results. Exposing human C_3, collagen, or gelatin coated glass briefly to trypsin also greatly enhanced neutrophil adherence to the protein coated glass. These results imply 1) trypsin like enzymes released from CF stimulated neutrophils may enhance neutrophil adhesiveness to surfaces coated with trypsin sensitive substrates, and 2) these enzymes may not be released until the cell is exposed to relative high concentrations of fMP.

A different fMP dose response curve was observed when evaluating neutrophil adherence of glass coated with human fibrinogen or heat inactivated serum. Significant increases in neutrophil adherence were observed on these substrates after the cells were exposed to lower concentrations of fMP and neutrophil adherence increased proportionally with each subsequent increase of the fMP concentration. This adherence could not be diminished by washing the cells and was not inhibitable by trypsin inhibitors. Neutrophil adherence to these surfaces was greatly enhanced following a brief (1 minute) exposure of these surfaces to chymotrypsin. The addition of a chymotrypsin specific inhibitor to the CF stimulated cells greatly reduced their ability to adhere to these surfaces. It was concluded that 1) CF pretreatment may activate or release chymotrypsin like enzymes that cannot be removed or inactivated by washing the cells, 2) these enzymes can alter neutrophil adhesiveness on chymotrypsin sensitive substrates, and 3) relatively low doses of fMP can stimulate this action. Several authors have documented that

a chymotrypsin like enzyme is necessary for neutrophil locomotion on protein coated surfaces and that CF activated a surface bound chymotrypsin like enzyme on neutrophils [12, 121].

SUMMARY

We report that chemotactic factors and colchicine influence the ability of neutrophils to bind bacteria, and a variety of protein coated particles. A summary of their effects on neutrophil binding and redistribution of albumin coated spheres, lectins and bacteria, immunoglobulin coated spheres and C_3b coated particles are illustrated in Figures 19 and 20. Only the binding of lectins or piliated bacteria occurred in a random fashion on all pretreated cells. The binding of immunoglobulin or C_3b coated particles was restricted to the front ends of polarized neutrophils. The binding of albumin coated spheres was restricted to the tail process of neutrophils pretreated with colchicine or multiple doses of chemotactic factors and occurred randomly on spherical cells or those exposed to a single CF stimulus. Two methods were observed for removing particles from sites of attachment on the neutrophil surface. First, neutrophils ingested particles. All neutrophils ingested immunoglobulin coated spheres and only CF stimulated cells ingested bacteria or C_3b coated spheres. Ingested particles were swept rearward but only within polarized cells. Second, particles were swept rearward on the exterior surface of neutrophils. C_3b coated particles were redistributed rapidly in this

manner on all polarized neutrophils. Similar, but much slower redistributions of lectins or bacteria occurred only on cells exposed to colchicine or multiple concentrations of CF. Neutrophils pretreated with colchicine or CF were shown to selectively bind particles simultaneously at different regions on the same cell.

We also report that neutrophils adhere poorly to glass coated with a variety of proteins and CF enhanced neutrophil adherence to these surfaces. Brief enzymatic action on surfaces coated with protein substrates also enhanced neutrophil adherence. The adherence of CF stimulated cells to protein coated glass was impaired by the addition of enzyme inhibitors. Our results suggested trypsin and chymotrypsin like enzymes were involved in augmenting neutrophil adherence to protein coated surfaces after neutrophil exposure to a chemotactic factor.

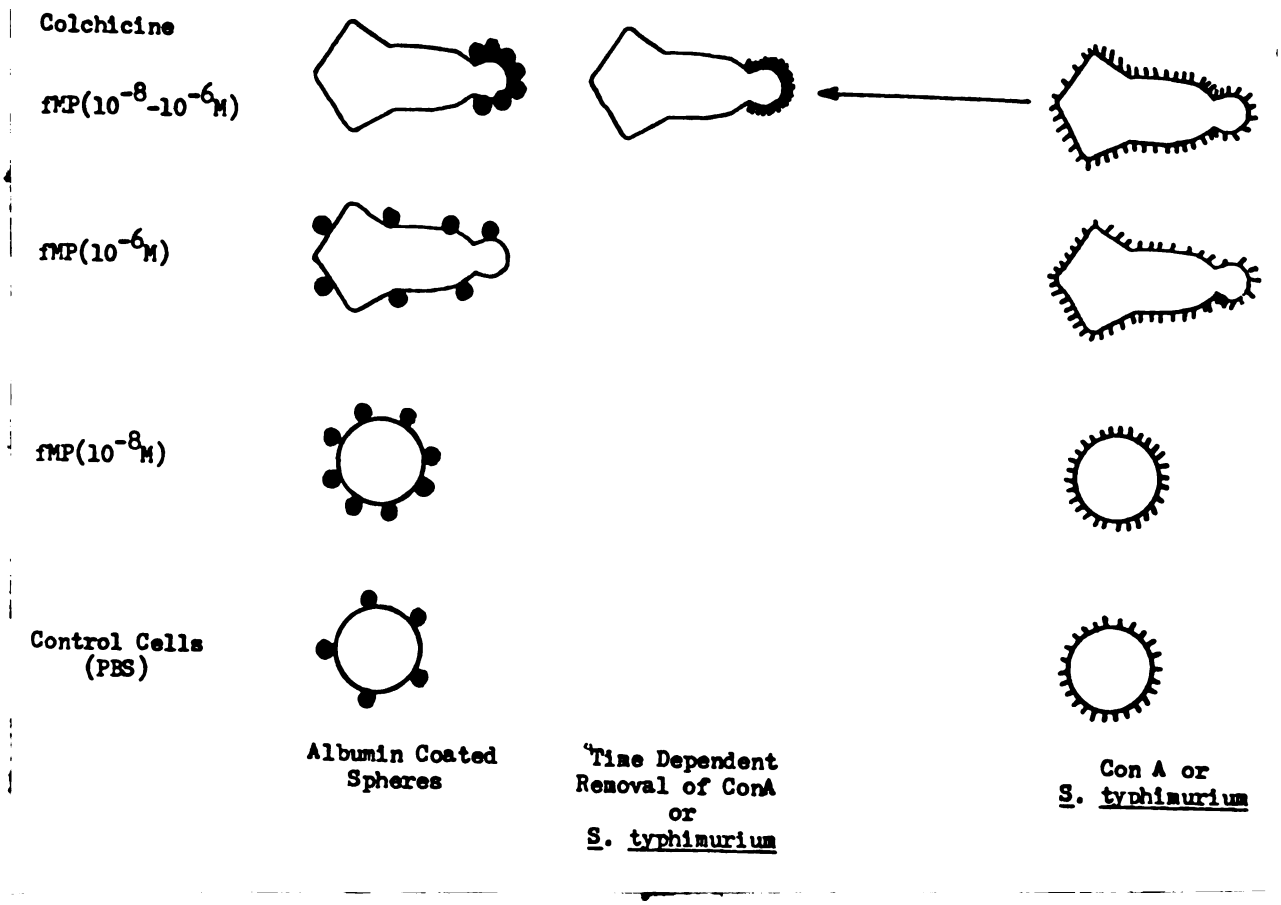


Figure 19. Summary of Binding Patterns for Albumin and Lectins or Piliated Bacteria and their Subsequent Redistribution on Control, CF, or Colchicine Pretreated Cells.

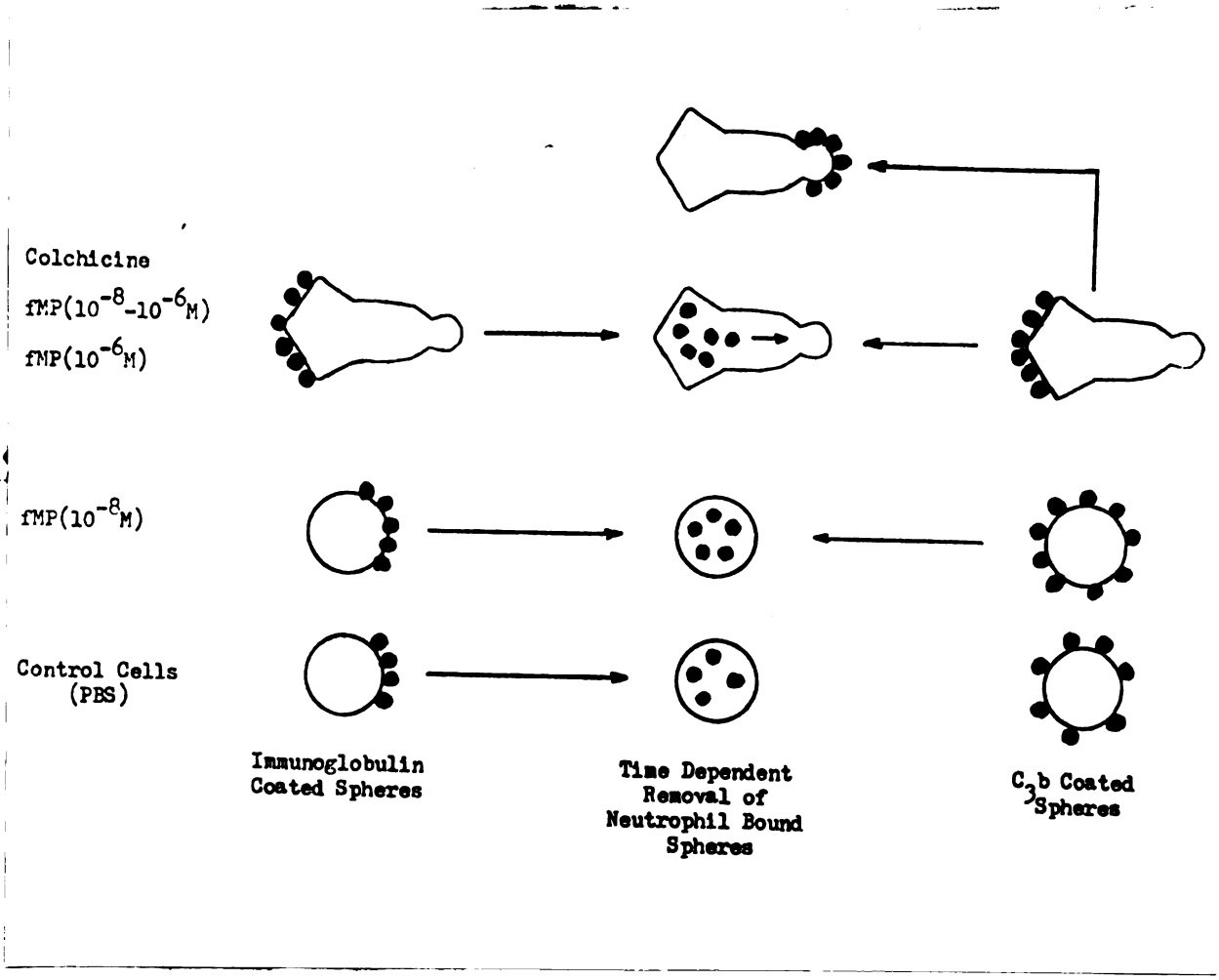


Figure 20. Summary of Binding Patterns for C_3b or Immunoglobulin Coated Particles and their Subsequent Redistribution on Control, CF, or Colchicine Pretreated Cells

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