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The City University of New York, Ph.D., 1976
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PHAGOCYTIC COMPETENCE AND C3-MEDIATED CYTOADHERENCE: DISTINCTIONS BETWEEN ADHERENT AND SUSPENSION HUMAN NEUTROPHIL POPULATIONS

by

LINDA PHAIRE WASHINGTON

A dissertation submitted to the Graduate Faculty in Biomedical Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy, the City University of New York.

1975

This manuscript has been read and accepted by the Graduate Faculty in Microbiology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Oct 8, 1975
date

Chairman of Examining Committee

Oct 8, 1975
date

Executive Officer

Supervisory Committee

The City University of New York

Abstract

PHAGOCYTIC COMPETENCE AND C3-MEDIATED CYTOADHERENCE:
DISTINCTIONS BETWEEN ADHERENT AND SUSPENSION
HUMAN NEUTROPHIL POPULATIONS

by

Linda Phaire Washington

Adviser: J. Michael Kehoe

Human neutrophils were shown to possess distinct differences in their phagocytic capacities for Staphylococcus aureus, depending upon whether they were maintained in suspension or monolayered on glass surfaces during phagocytosis. In the absence of specific opsonin suspension neutrophils demonstrated negligible levels of ingestion. Suspension cells phagocytosed the bacteria to a significant extent only in the presence of anti-staphylococcal opsonic antibody. This augmented phagocytosis or opsonization was inhibitable by preincubation of the neutrophils with a structurally homogeneous IgG3 myeloma protein or its Fc component. This implied that in suspension neutrophils, membrane bound Fc receptor sites were accessible for myeloma IgG3 to successfully compete with anti-staphylococcal IgG opsonins for Fc receptor binding.

In contrast, monolayered neutrophil populations actively phagocytosed various pathogenic S. aureus strains in the absence of staphylococcal opsonins. In the presence of anti-staphylococcal opsinins a substantial increment of the ingestion efficiency occurred, but this opsonic effect could not be blocked by preincubation of the neutrophils with IgG3 or its Fc fragment. The failure of myeloma IgG3 to effectively compete

for Fc binding sites was evident with both unaggregated and heat aggregated myeloma IgG3. This finding led to the postulate that during neutrophil adherence to glass surfaces in vitro (or to inflammed tissue surfaces in vivo?) a dynamic reorientation and topographical redistribution of Fc receptor sites occur such that they become operationally inaccessible for inhibition. This redistribution of membrane components could result in the masking of existing receptors (unlikely), the exposure of new receptors, or the local microaggregation (clustering) of existing and/or newly exposed receptor sites. Although proof of this hypothesis could best be substantiated by electron microscopic studies analyzing the distribution and the extent of binding of ferritin-labelled antigen-antibody-complement complexes on adherent and suspension neutrophils, additional findings reported in this thesis lend it further support.

Additional distinctions were found in studies concerning the C3 opsonic receptor. Both populations were challenged with C3 opsonized sheep red blood cells. Only neutrophil monolayers (adherent) demonstrated an appreciable ability to bind and ingest these EAC3 immune complexes. This specific ligand-receptor binding was inhibited by preincubation of the EAC complexes with anti-C3 antisera before presentation to neutrophils. In contrast, in suspension populations the binding of C3 opsonized erythrocytes was negligible. The activation of neutrophil monolayers is postulated to occur through perturbations and alterations of the cell membrane during the adherence phenomenon to account for the observed differences in phagocytic competence and C3 cytoadherence between adherent and suspension neutrophil populations.

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TABLE OF CONTENTS

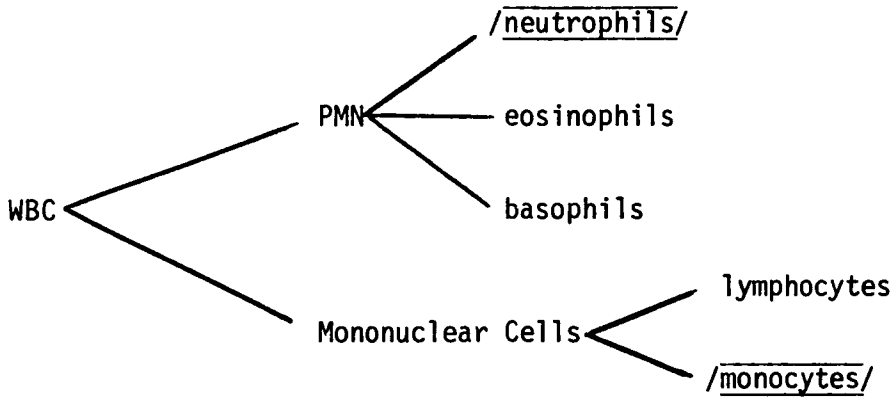
ABBREVIATIONS

FCS	Fetal calf serum.
Med 199	Tissue culture medium 199 + Hanks salts.
$1^{14}C$	The symbol for radioisotopic carbon. In glucose-$1^{14}C$ the number 1C was preferentially labelled.
EA	Sheep erythrocytes immunologically coated with IgG antibody.
EAC	Sheep erythrocytes immunologically coated with antibody (IgG + IgM) and human complement.
PMN	Polymorphonuclear leukocytes consisting of neutrophils, eosinophils, and basophils.
RBC	Sheep red blood cells.
CFU	Colony forming units.
HMS	Hexose monophosphate shunt.
PBS	Phosphate buffered saline.
MF	Microfilaments
MT	Microtubules
C'	The symbol for serum complement proteins.
Igs	Immunoglobulins
MPO	Myeloperoxidase

LIST OF ILLUSTRATIONS

I. GENERAL INTRODUCTION: THE PHAGOCYTIC DEFENSE SYSTEM

A. The Phagocytic Cells: On the basis of gross nuclear morphology circulating WBC are generally divided into polymorphonuclear and mononuclear leukocytes. Polymorphonuclear leukocytes (PMN) are composed of neutrophils, eosinophils, and basophils, whereas mononuclear cells are composed of monocytes and lymphocytes as schematically illustrated below:



Monocytes, which have the potential to differentiate into tissue macrophages (1), and neutrophils are the two major cell populations whose primary function is endocytosis, an elaborate defense mechanism whereby invading microbes and other deleterious molecules are ingested, digested, and removed from the body. Endocytosis is the general term which includes phagocytosis (ingestion of particles 1 millimicrons or larger in size), macropinocytosis (uptake of fluid or particles about .1 to 1 millimicrons in size), and micropinocytosis (uptake of material .05 millimicrons or less in diameter) (2, 3).

Two major properties of these phagocytes explain their ultimate roles as microbial killers: their arsenal of lysosomal enzymes and other anti-microbial constituents which are packaged into 1^{o} and 2^{o} membrane bound granules; and their membrane structure which is extremely

plastic and deformable (4, 5, 6). Characteristically their membranes are well suited for the active pseudopod formation and extensive membrane evaginations which are integral aspects of particle engulfment.

1. Neutrophils: Ontogeny and Maturation: Neutrophils originate in the bone marrow from pleuripotential stem cells. Although differentiation, maturation, and the kinetics of granulopoiesis are poorly understood, it appears that regulation is governed by or dependent upon hormonal influences (granulopoietins) and microenvironmental factors (7, 8).

One aspect of the kinetics of granulopoiesis is its enormous and rapid turnover which has been estimated to be in the range of 1.6×10^9 cells/kg/day (9). In the bone marrow full maturation from committed myeloblast to a mature segmented PMN is completed within 10-12 days; during this prolonged period neutrophils acquire a full armamentarium of lysosomal enzymes and anti-bacterial granule constituents, characteristic membrane deformability and phagocytic competence, all of which are crucial in supporting the neutrophil's defense function. Beginning with the committed stem cell, successive stages in differentiation are schematically illustrated as follows: myeloblast → promyelocyte → myelocyte → metamyelocyte → band form → mature multilobed neutrophil.

In the bone marrow various pools can be distinguished: a stem cell pool, a pool of proliferating cells, a maturation pool, and a storage pool. The proliferating (mitotic pool) consists of three types of cells arising successively: myeloblasts, promyelocytes, and myelocytes. In this compartment cellular division and maturation occur and, under normal conditions, the transit time from stem cell to myelocyte is 5-6 days. After the last division, following passage through the mitotic pool, neutrophils enter a non-proliferating maturation storage

compartment, which consists of cells, morphologically recognizable as metamyelocytes, band forms, and mature multilobed neutrophils. This non-proliferative maturation (no further mitosis) from metamyelocyte to mature neutrophil takes 5-6 days. Consequently, 10-12 days are required before the descendents of a committed stem cell emerge in circulation as mature segmented neutrophils (3, 10, 11).

Various other, less well understood, biochemical and structural alterations occur during maturation to account for acquired phagocytic competence. For example, neutrophil precursors relative to mature blood neutrophils are rigid, immobile, and unable to ingest particles (12, 13). Even morphologically mature band and segmented neutrophils, that constitute reserve storage pools in the marrow, ingest particles less efficiently than blood neutrophils (13, 14). Release of mature granulocytic elements into the peripheral blood appears to be dependent upon cell stickiness and deformability and upon a granulocyte releasing factor which may act upon bone marrow sinusoids (11).

Neutrophils have a relatively short life span in circulation and leave the blood in random fashion with mean half time of 6-7 hours. They then migrate into tissues where survival is variable up to 4-5 days (3, 10, 11).

(a) Origin of granule constituents: During differentiation in the marrow, two types of cytoplasmic granules are synthesized and packaged, the primary or azurophil granules and the secondary or specific granules. These two distinct types of granules differ in size, density, anti-microbial chemical constituents (15-20), and the time and mode of origin during differentiation (21).

According to the developmental sequence, the myeloblast is a relatively undifferentiated embryonic cell with a large oval nucleus and a

cytoplasm lacking granules but containing abundant ribosomes, numerous mitochondria, and a small underdeveloped Golgi complex. The promyelocyte and myelocyte are stages of intense protein synthetic and secretory activity associated with the formation of azurophil and specific granules, respectively. The large (800 millimicrons) dense azurophil granules appear early in development during the promyelocyte stage and are formed by condensation of secretory material along the proximal face of the well developed Golgi of the promyelocyte (22-24). Constituents of azurophil granules or primary lysosomes include a number of acid hydrolases, myeloperoxidases, lysozyme, hydrogen peroxide and bacterocidal cationic proteins (18, 19). The promyelocyte has azurophils exclusively. Smaller (500 millimicrons), less dense, specific granules are formed by a similar condensation process occurring along the distal face of the Golgi complex of the myelocyte (21-24). Various constituents of specific granules include alkaline phosphatase, lysozyme (2/3 total cell content), lactoferrin, and other antimicrobial agents (18, 19). The myelocyte has both specific and azurophil granules, but in varying proportions. In contrast, the band and segmented forms are non-secretory stages during which there is a progressive decrease in cell size, associated with changes in the shape of the nucleus and a gradual diminution of most cytoplasmic organelles. Band and segmental forms contain 75-90% specific granules and 10-25% azurophil granules (21). Consequently, the final product of the maturation process, the mature circulating neutrophil (11-14 microns) with a characteristic multilobed segmented nucleus, represents an expendable short-lived end cell produced in huge numbers (60-70% of the total circulating WBC) and containing the full complement of antimicrobial granules constituents, few mitochondria, but large depots of glycogen (25). Finally, it is released into circulation as a motile and

competent phagocytic cell.

2. Monocytes-Macrophages: Ontogeny and Maturation: Monocytes (and indirectly macrophages) also originate in the bone marrow; however the pattern of cell proliferation and maturation in mononuclear phagocytes is different from that in neutrophils and can be schematically illustrated as follows: monoblast → promonocyte → monocyte → macrophage. After the committed stem cell, this line has only two successive proliferating cells: the monoblast and the promonocyte. Monocytes arise from proliferating promonocytes and are released into circulation. Under normal conditions monocytes and macrophages are non-proliferative cells. Nonetheless they contain their full complement of azurophil and specific granules. During chronic inflammation, however, they can proliferate giving rise to new cells. In contrast to neutrophils, monocytes do not pass through a total maturation phase in the marrow after the last cell division, nor is there a large reserve or storage marrow pool of mature monocytes (1, 3, 26-28). Also, in contrast to neutrophils, monocytes remain in circulation 1-3 days (according to the species). They then emigrate in random fashion to various tissues where, under local influences, they differentiate and mature into larger, actively more phagocytic macrophages with increased amounts of lysosomal enzymes. Furthermore, macrophages are capable of maturation and modulation, depending on local circumstances and can form new secondary lysosomes, thereby meeting the need for new enzymes arising during their long life span in tissues (on the order of months to years, depending on the particular tissue) (2). In contrast to the relatively low numbers of circulating blood monocytes (3-7% total WBC), the macrophages are numerous and widely scattered through connective tissues (histiocytes) and the endothelial

lining of small blood vessels. Larger and more concentrated cells are present in liver sinusoids as Kupffer cells, in the lung as alveolar macrophages, and in the spleen and lymph nodes as dendritic macrophages. Consequently, monocytes do not leave the bone marrow fully matured as do neutrophils. Rather their maturation is initiated in the marrow but completed in the tissues. During their relatively long life span in the tissues, they have the capacity to synthesize new lysosomal enzymes (whereas neutrophils cannot) in response to repeated phagocytic stimulation (3, 13).

B. Stages of Phagocytosis: The phagocytic process may be conveniently divided into five discrete but interrelated stages: chemotaxis, opsonization, ingestion, degranulation, and microbial inactivation (killing). For the sake of clarity, each stage will be discussed separately.

1. Chemotaxis: Any injury to tissue, such as that following the establishment and multiplication of microbes, stimulates a sequence of defense oriented events generally referred to as the "inflammatory response." During the acute stages of inflammation (within 15-30 minutes after the initial irritant) neutrophils leave the circulation via diapedesis and accumulate at the extravascular sites of tissue injury with defined kinetic patterns (29). Later (about 4 hours), mononuclear phagocytes infiltrate the inflammed lesion and are the predominate phagocytic cells found in the late and chronic stages of inflammation. The reasons for this sequential migration of the two cell types are not entirely clear but is thought to be partially attributed to a number of factors: (a) the production of cell specific chemotactic factors, e.g., leukoegression is a powerful chemotactic stimulus specific for the mobilization of neutrophils; and C5a is specific for eosinophils and neutrophils

(30); (b) the greater motility of neutrophils is thought to explain their prompt responsiveness to chemotactic molecules and consequently their early influx into inflammed foci (13).

This vectorial, motile response or directed migration of phagocytes into inflammatory lesions is often attributed to chemotactically active molecules. The principal mechanism by which microorganisms generate soluble chemotactic molecules is through complement activation. The molecules C5a and C3a represent the most powerful stimuli to chemotaxis. There are various ways in which C3a and C5a can be generated. Antibody can react with the microbial surface antigens and this Ag-Ab complex initiates the sequential activation of C1, C4, and C2 to form the activated $\overline{C142}$ complex. This Ag-Ab $\overline{C142}$ and $\overline{C1423}$ attacks native C3 and C5, respectively, in serum to yield the low molecular weight peptides C3a and C5a. Additionally, a trimolecular complex of $\overline{C567}$ acting later in the classical complement sequence assembles and also has chemotactic effects. C3a and C5a can also be generated through the alternate pathway of C' activation, thereby bypassing the requirement for antibody and C1, C4, and C2. This is possible because C5 and especially C3 are extremely sensitive to proteolysis and serve as substrates for a wide variety of enzymes, i.e., plasmin, trypsin, $\overline{C142}$ (C3 convertase), tissue proteases, and lysosomal proteases (31, 32). Consequently, studies have shown that nonspecific proteases released from bacteria and/or damaged tissue can cleave C3 and C5 directly to yield chemotactically active C3a and C5a (33, 44). Additional chemotactic stimuli may be released from actively phagocytosing neutrophils that will attract other macrophages and neutrophils, in the absence of serum (34). Thus, there exist various <u>in vitro</u> methods to generate chemotactic stimuli with consequent mobilization of phagocytes. Whether all mechanisms operate concomi-

tantly in vivo or, whether one mechanism may be favored over the other depending upon the type of organism, the stage of infection, or the absence of specific antibody remain to be determined. Whatever the actual situation in vivo, one can see the obvious advantages in having a variety of mechanisms by which phagocytic cells are attracted to inflammed foci. Once phagocytes have been attracted and migrate into a lesion, other types of chemical substances are produced which cancel their unidirectional movement and maintain them in that general infected area. Candidates for such a phenomenon are various lymphokines which are produced by stimulated lymphocytes (13), i.e., leukocyte immobilizing factor, migration inhibitory factor, and neutrophil immobilizing factor (LIF, MIF, NIF).

It is now generally accepted that the activation of a proesterase to an esterase occurring at the cell membrane (35) is associated with the contractor mechanism to mobilize leukocytes (30). The mechanism of chemotactic mobilization has structural and molecular similarities to actin-myosin dynamics occurring in muscle contraction. Myosins with structural and enzymatic similarities to muscle myosin and with ATP hydrolyzing activity has been isolated from PMN and macrophages (13).

Genetic defects in phagocyte mobility have been described. The "lazy WBC" syndrome is characterized by recurrent infections, and peripheral neutropenia but normal bone marrow stores. These neutrophils show normal phagocytosis and bacteriocidal activities; however, they are grossly deficient in random mobility and chemotactic responses (11, 30).

2. Opsonization: Over 70 years ago it was realized that particles (microbes) that are coated with specific antibody or that have interacted with fresh serum were more rapidly and efficiently phagocytosed than un-

coated particles (36). This coating of particles with serum proteins (opsonins) resulting in accelerated endocytosis has been termed opsonization and comes from Greek meaning "to prepare for dining." Moreover, in the absence of opsonin, many virulent and/or encapsulated organisms resist or escape phagocytosis and this may account, in part, for their pathogenicity.

Quite often phagocytosis plays a decisive role in the delicate balance between host and pathogenic bacteria. Effective phagocytosis early in the course of bacterial invasion may limit the spread of bacteria and prevent ongoing infection. Alternatively, ineffective phagocytosis may lead to uncontrolled bacterial multiplication and overwhelming infection. In this respect, opsonic proteins play a critical role in ensuring that phagocytosis (ingestion) is efficient and rapid because they mediate and expedite the adherence to and subsequent interiorization of microorganisms by phagocytic cells. The capacities of opsonins to concomitantly bind microbe and phagocyte afford them the potential of bringing these two entities together and triggering engulfment. Conceptually, they act as 'liaisons' or 'bridges' between invading microbe and phagocyte. However, this is only a concept and must be interpreted with caution. The function of opsonin is more than merely mediating membrane contact of microbe and phagocyte. Although attachment is a prerequisite for engulfment, attachment does not always lead to engulfment. For example, mycoplasmas adhere spontaneously and quite avidly to macrophage membranes, but they are not ingested unless specific antimycoplasma antibodies are present (37). This is an important finding because it suggests that as a consequence of specific ligand-receptor binding, signals are transmitted to the interior of the cell which trigger the engulfment

phase. These "signals" are specific and initiated at the cell surface and determine if ingestion is to follow. Consequently, adherence is not invariably associated with ingestion unless it is mediated through complementary ligand-receptor interaction.

A variety of serum proteins with opsonic activity have been reported, i.e., tuftsin (38), C-reactive protein (39), and C5 (40). However, the two most abundant and well characterized opsonins are C3 (heat labile) and immune IgG (heat stable). Opsonization mediated by C3 and/or IgG proteins may occur by three different mechanisms: (1) Specific antibody (IgM and IgG) may act as opsonin in concert with complement by activating C3 through the classical pathway of C' activation. (2) C3 may be activated through the alternate pathway of C' activation; it can therefore function as opsonin without the requirements for antibody and/or C1, C4, and C2. (3) Specifically immune IgG alone may act as opsonin.

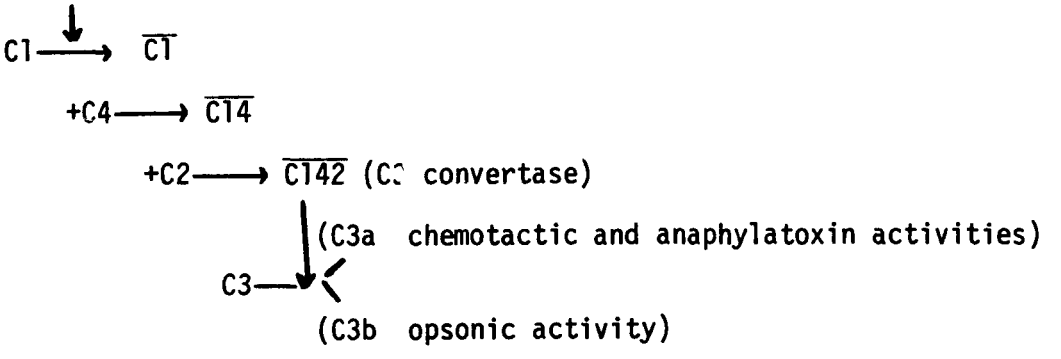
(a) C3b opsonins: Generally the protein components of the complement system (C1 through C9) exist in serum in inactive form and may undergo sequential activation. Typically, the product of one reaction is the catalyst of the next, producing a characteristic enzymatic cascade.

Neutrophils, monocytes, and macrophages possess membrane bound receptors for C3 (41, 42) and specificity for C3b has been demonstrated (43). However, since C3b opsonins do not exist in serum in active form, they must be enzymatically generated. This is effected by two major pathways: (a) via C3 convertase ($\overline{C142}$) which involves the classical pathway of C' activation and (b) via C3 activator enzyme which bypasses the requirement for C1, C4, and C2 and involves the alternate pathway of C' activation. The manner in which C3b is generated is discussed in the hope of shedding light on the various pathways in which C3 and/or anti-

body mediated opsonization can occur.

C3b generation via C3 convertase: C3, a glycoprotein with a molecular weight around 200,000, is the most abundant component of the complement system with a serum concentration of 1.2 mg/ml (44). Its generation via C3 convertase ($\overline{C142}$) requires the participation of antibody (specifically IgM, IgG1, IgG2, or IgG3), C1, C4, and C2. The triggering event here is the binding of antigen (microbe) to specific antibody (through its Fab region). This Ag-Ab binding results in a configurational change and exposes sites on the Fc region in the CH_2 domain (45, 46), capable of binding to C1q and activating the $\overline{C1}$ complex (44, 47). The activated complex, C1, activates the fourth (C4) and second (C2) components which function as a molecular complex attached to the cell surface. This activated complex $\overline{C142}$ is also termed C3 convertase because its natural substrate is C3. This newly generated enzyme cleaves C3 into a minor 7000 molecular weight fragment, C3a, which has chemotactic and anaphylatoxin activities, and a major fragment, C3b, which is able to bind to specific receptors on phagocytic cells and lymphocytes (41, 47, 48). The generation of C3b through this pathway is illustrated below (47):

Ag + specific IgG1, IgG2, IgG3, or IgM



In this manner particulate matter (microbe) is opsonized through the combined action of specific antibody and complement. Clearly one can visualize the "bridged" manner in which microbe and phagocyte are brought together with consequent membrane activation, i.e., the opsonized Ag-Ab $C\overline{1423}b$ complex can be deposited on the phagocyte membrane through specific ligand-receptor binding.

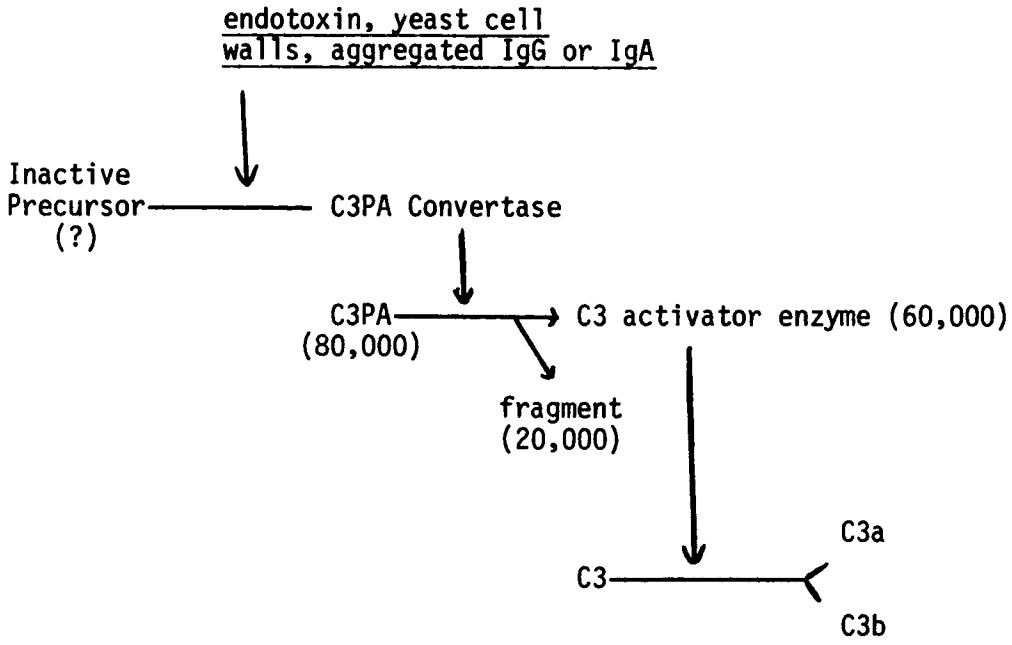
Moreover, this pathway affords efficient amplification because studies utilizing RBC, anti-RBC antibodies, C1, C4, and C3 (EAC complexes) have demonstrated that after a single immune antibody binds to a RBC and interacts with C1, C4, and C2, this $EAC\overline{142}$ complex catalyzes the binding of several hundred C3 molecules to the RBC membrane (50, 51).

C3b generation via C3 activator system: C3 is extremely sensitive to proteolysis and its initial products (C3a + C3b) appear to be the same whether cleavage is brought about by C3 convertase, lysosomal enzymes, trypsin, plasmin, or C3 activator enzyme (44, 48, 49).

In the second mechanism of opsonization, C3 may act as opsonin alone because C3b generation via C3 activator enzyme utilizes the alternate pathway of C' activation and bypasses the requirements for immune antibody, C1, C4, and C2.

The C3 activator system has a number of components and is generally initiated upon contact of serum with certain polysaccaride antigens such as endotoxins, yeast cell walls, inulin, or aggregated IgG1, IgG2, IgG3, and IgA immunoglobulins. This alternative mechanism of C3 activation is initiated when C3 proactivator enzyme (C3PA), an inactive 80,000 molecular weight glycoprotein, is enzymatically cleaved by C3PA convertase. This enzymatic cleavage of C3PA produces an enzymatically active 60,000 molecular weight component termed the C3 activator enzyme, C3A, which

has the potential to cleave C3 into C3a and C3b components, apparently with the same specificities as C3 convertase (44, 47, 49). This activation scheme is not as well characterized as the classical pathway, but a tentative scheme is illustrated below (47):



The binding of C3b to phagocytic cells is firm and hydrophobic in nature (13). In addition to its capacity to bind to phagocytes, C3 proteins have been reported to directly bind bacterial cells in the absence of immune antibody; however, the exact mechanism is unknown at present (13, 44). Nevertheless, these findings demonstrate that C3 may act alone as opsonin without the participation of antibody, C1, C4, and C2.

(b) Immune IgG as opsonin: In addition to its role as initiator in the classical pathway of C' activation, specifically immune IgG (but not IgM or IgA) can opsonize and mediate microbe-phagocyte binding and membrane activation with consequent engulfment without the par-

ticipation of C3b opsonins (53). Specific receptors for the Fc region of IgG1 and IgG3 subclasses have been demonstrated on phagocyte membranes (42, 55).

In IgG mediated opsonization, the entire molecule must be intact for expression of opsonic activity (53, 54). The $F(ab')_2$ region of the immunoglobulin molecule binds specifically to antigenic determinants on the microbial surface, whereas the Fc region attaches to the phagocyte membrane by specific ligand-receptor binding. Preliminary studies suggest that the CH_3 domain of IgG-Fc is responsible for this cytophilic binding property(56).

Three mechanisms of opsonization mediated by C3 and/or IgG have been discussed and are primarily based on analyses of in vitro studies. What is actually occurring in vivo is still speculative. Possibly C3 and IgG opsonins may have different functional roles, may act synergistically and/or as "backup" systems if one or more systems are defective. Moreover, one mechanism may be favored over the other, depending on the type of organism (encapsulated or unencapsulated), the stage of infection, and the presence or absence of specific antibody.

(c) Physiochemical distinctions between IgG and C3b opsonic receptors: The IgG and C3b receptors are distinct structural moieties as shown by differing physiochemical requirements and properties. These are tabulated below:

C3b Receptor (41)	IgG Receptor
1. Mg^{++} dependent, inhibited EDTA	1. Ca^{++} dependent
2. Inhibited by anti-C3 antisera	2. Inhibited by IgG1 and IgG3 myeloma proteins (54)
3. Not inhibited by serum IgG	3. Inhibited by anti-IgG antibodies directed against Fc determinants, i.e., rheumatoid factors. Thus, Fc not available for receptor binding (53)

4. Trypsin sensitive	4. Binding enhanced with small amounts of trypsin (57)
	5. 2×10^6 IgG sites/macrophage (57, 58)

In addition to these differing physiochemical properties, in vitro studies suggest differing functional roles for these receptors during phagocytosis. It has been postulated that the C3b receptor is primarily involved in the attachment phase, whereas participation of the IgG receptor is necessary for inducing ingestion in macrophages (59) and neutrophils (60).

3. Ingestion: Once the opsonized particle is attached to the phagocyte surface membrane and the proper intracellular signals have been transmitted and translated as a result of specific ligand-receptor interaction, the ingestion phase is promptly stimulated. Upon particle contact, the membrane deforms and the hyaline ectoplasm extends to form pseudopodia. Pseudopodia contain dense meshworks of microtubules (MT) and microfilaments (MF) and some glycogen particles - but are devoid of 1^o and 2^o granules and other cytoplasmic organelles (13). The spreading movement of the pseudopodia is similar to the spreading of phagocytes on surfaces (6, 13, 61, 62).

The pseudopodia fuse at the distal side of the particle, the consequence being the encasement of the particle within a phagocytic vessicle or phagosome, the lining of the phagosome being an inverted plasma membrane. Subsequently, the phagosome pinches off from the cell periphery and migrates centripetally away from the hyaline ectoplasm into the cytoplasm (13).

(a) Metabolism during ingestion: Engulfment in an energy

dependent episode and activates ATP generating phenomena, specifically glycolysis and glycogenolysis (64). During ingestion a substantial portion of the plasma membrane is internalized (65). The extensive membrane movements and deformations associated with ingestion depend on the provision of metabolic energy. In neutrophils and peritoneal macrophages, most of this energy is derived from glycolysis, which depends either on exogeneous glucose or on the large stores of glycogen in the neutrophil cytoplasm. In contrast, in alveolar macrophages, most of the energy comes from oxidative phosphorylation and respiration (64, 66).

In neutrophils and mononuclear phagocytes some of the metabolic concomitants of phagocytosis may be sequels of the ingestion stage, while others may be required for the act itself. Experimentally it is difficult to discern a clear cut distinction between these two alternatives. These metabolic concomitants are:

1. respiratory burst in O_2 consumption (64).
2. increased glucose utilization and lactate production (64).
3. the amount of glucose metabolized through the hexose monophosphate shunt (HMS) increases from 1% in resting to 10% in phagocytosing cells (64).
4. H_2O_2, and activated oxygen species, i.e., singlet oxygen and superoxide anion ($O\overline{2}$) are produced during the respiratory burst (67, 68).
5. increased synthesis of membrane phospholipids (69).

4. Degranulation: Selective degranulation is a complex process whereby specific and azurophil granules discharge their contents into the phagosome (phagocytic vacuole and ingested particle) forming a phagolysosome, without subjecting the cytoplasm of the phagocyte to their potentially injurious effects. Degranulation occurs in concert with ingestion, suggesting similar triggering mechanisms. In an experimental system where

engulfment was operationally eliminated (i.e., cells attached to non-phagocytosable surfaces), it was shown that aggregated IgG and C3 induced enzyme release (70, 71, 76). Thus, the interaction of ligand with IgG and/or C3 membrane receptors effects two closely integrated events: engulfment and subsequent degranulation.

Ultrastructural analyses and cinematography have revealed that once the particle is taken up into the cell (phagosome), the 1^{o} and 2^{o} granules fuse with the membrane of the phagosome and discharge their granule constituents. Only those granules situated near the vacuole degranulate into it (16, 17, 72). Histochemical data have demonstrated that specific granule fusion with the phagocytic vacuole precedes azurophil granule fusion (73). The conclusion is that selective degranulation is sequential with specific granules preceding azurophil granules. An important consequence of sequential degranulation is that the content and pH of the phagolysosome changes with time.

Two mechanisms have been described whereby neutrophils selectively release lysosomal, but not cytoplasmic enzymes from the cell interior to the external environment (70, 71, 74-76). The first model examines situations whereby neutrophils encounter immune complexes on phagocytosable surfaces, i.e., in suspension. In this model termed "regurgitation during feeding" (76) or "exocytosis" (70, 71), phagocytic vacuoles formed to enclose particles (latex, zymosan, immune complexes) remain open to the external medium while lysosomes are still in the process of merging with the innermost face of the cavity. Consequently, during degranulation into the phagocytic vacuole, regurgitation channels are formed through which lysosomal enzymes are extruded without the concomitant escape of non-lysosomal enzymes of the cytosol (70, 71, 76). In support

of the hypothesis, the release of enzyme parallels the uptake, not of the first, but of subsequent immune complexes. A corollary of this hypothesis would be that the larger the particle the greater the release of enzymes, because larger particles would be more likely to be taken into the vacuole before the cell had closed behind as compared to particles of smaller size (70, 71).

The second model examines situations in which neutrophils encounter phagocytic stimuli on non-phagocytosable surfaces, e.g., Millipore filters, and has been termed "frustrated phagocytosis" (70, 71) or "reverse endocytosis" (76). It was found that lysosomal hydrolases, but not cytoplasmic enzymes were secreted onto solid surfaces (Millipore filters) previously coated with Ag-Ab complexes or aggregated IgA and IgG, but not aggregated IgD or IgM (70, 71). Ultrastructural analyses (70, 71, 76) demonstrated that extrusion of granules occurs along the plasma membrane which is in contact with surface bound immune complexes and never on the side which was non-adherent (71). In other words, lysosomes fused directly with that section of the plasma membrane that is in contact with the phagocytic stimuli and literally extruded granule enzymes into what would normally be a phagocytic vacuole, but which, in this case, was the exterior of the cell. Phagocytic stimuli employed were aggregated IgA and IgG immunoglobulins. Aggregated IgD and IgM were ineffective, indicating that the Fc regions of IgA and IgG classes are critical for the signal transmission, probably due to interaction with specific membrane receptors (71).

The in vivo correlate of enzyme release on non-phagocytosable surfaces could be when neutrophils react with anti-tissue antibodies or immune complexes which have been deposited along a surface such as glo-

merular and vascular basement membranes, which they cannot phagotose. Nevertheless they are still capable of inducing tissue injury and evidence of enzyme release under these circumstances has been reported (77).

Defects in degranulation results in syndromes whereby microbes are resident, viable, and capable of multiplying within the phagocyte because defective degranulation contributes to impaired intracellular killing. Chediak-Higashi (CH) disease is a recessively transmitted syndrome characterized by partial albinism, photophobia and increased susceptibility to pyogenic infections. CH-neutrophils have multiple functional defects one of which is defective degranulation (78). More precisely, inefficient fusion of lysosomes with the phagosome is secondary to defective microtubule function and assembly in CH-neutrophils (79).

5. Antimicrobial Mechanisms in Neutrophils: Within 30-60 minutes after engulfment, most microbes are inactivated as shown by their failure to grow on artificial media. Killing occurs within the phagolysomes as a consequence of exposure of ingested microbes to various metabolic products and cytoplasmic granule constituents. The antimicrobial agents of the phagocyte can be divided into two major categories, O_2 dependent and O_2 independent as listed below (67):

O_2 Dependent	O_2 Independent
1. MPO-H_2O_2 - halide system	1. acid
2. MPO - independent	2. lysosomal acid hydrolases
a. O_2^- (superoxide anion)	3. lysozyme
b. H_2O_2	4. lactoferrin
c. OH radicals	5. cationic proteins
d. singlet oxygen	

a. MPO-H_2O_2-Halide system (Klebanoff system): Although engulfment in neutrophils is unaffected by anaerobiosis (64, 80), those subsequent events which ultimately lead to the death of microorganisms are, in part, dependent on the characteristic burst in O_2 consumption and oxidative metabolism that accompanies phagocytosis.

One of the best characterized and most potent microbicidal mechanisms in the phagocyte is the MPO-H_2O_2-halide system (67) which is dependent on O_2 and functions best at slightly acid pH. The components of this system, myeloperoxide, hydrogen peroxide and an oxidizable cofactor combine to form one of the most potent microbicidal systems effective against bacteria, viruses, fungi and mycoplasmas (67). The origin of these components will be briefly considered.

Myeloperoxidase, the peroxidase of neutrophils, is localized in high concentration (2-5% dry weight of cells) in primary or azurophil granules in resting neutrophils. In neutrophils, this MPO was synthesized during maturation in the bone marrow. During phagocytosis, MPO is released into the phagosome during degranulation as shown by EM cytochemical studies (21).

H_2O_2 may come from neutrophil metabolism as follows: H_2O_2 is formed during the respiratory burst and increased O_2 consumption during phagocytosis. Part of the O_2 consumed is completely reduced via a flavoprotein oxidase (NADH or NADPH ?) to H_2O_2. Another source of H_2O_2 may be from ingested bacteria. Generally lactic acid bacteria, i.e., pneumococci, streptococci, reduce O_2 to H_2O_2 and lack a catalase that converts H_2O_2 to $H_2 + O_2$. Consequently hydrogen peroxide accumulates in these organisms. Organisms which do possess catalase, i.e., staphylococci, H_2O_2 does not accumulate during metabolism (52, 63, 67).

The H_2O_2 formed by microorganisms may contribute significantly to the microbicidal activity of the phagocyte, particularly when the H_2O_2 generating system is defective, as in some cases of chronic granulomatous disease (CGD). Thus, H_2O_2 generating bacteria are readily killed by the CGD neutrophil and this is, in part, due to the replacement of a defective phagocyte peroxide generating system with one of microbial origin. Halide ions serve as the oxidizable cofactors and presumably enter the phagosome by diffusion (63).

Klebanoff (81) was the first to demonstrate the potent microbicidal activity of this system. Presumably, H_2O_2 reacts with MPO to form enzyme-substrate complexes with strong oxidative capacity. The halides (I^-, Br^-, Cl^-) are oxidized by this complex (halogenation) resulting in the formation of antimicrobial agents whose mechanism varies with the halide employed. For example when iodide is the halide employed, Klebanoff et al. showed that the iodide anion becomes covalently fixed to protein of ingested particles (67, 81) with consequent iodination (halogenation) of essential microbial components (67).

Other manifestations of the MPO-mediated bacteriocidal system have been shown to be of importance. Recently Sbarra et al. (82) have shown that the MPO-H_2O_2-Cl system can also function by decarboxylation and deamination of fatty and amino acids to yield highly reactive aldehydes. Consequently, these aldehydes cleave peptide bonds, and this process may be critical in the damage not only of microorganisms, but also of tissues.

b. MPO-independent O_2dependent systems: The MPO-H_2O_2-halide system, as first described by Klebanoff, has been gaining emphasis in the past few years. However, MPO-independent antimicrobial mechanisms which are dependent on the O_2 respiratory burst, also exist in the cell. For example, genetic MPO-deficient leukocytes retain some microbicidal acti-

vity (78, 83) although the kinetics of killing is much slower.

During the respiratory burst and increased O_2 consumption that occurs concurrently with ingestion, part of the O_2 consumed is reduced via a cyanide insensitive flavoprotein oxidase to O_2^-. The O_2^- may be further reduced to H_2O_2 or may react with H_2O_2 already formed to yield highly reactive OH radicals (63, 67). These products of O_2 metabolism (H_2O_2, O_2^-, $OH^\cdot$) in the absence of MPO have antimicrobial activities on their own and will be discussed briefly.

H_2O_2: At relatively high concentration, in the absence of MPO, H_2O_2 has considerable bacteriocidal activity. However, all microorganisms are not equally susceptible to H_2O_2 action, e.g., those which produce catalase can degrade H_2O_2 and this affords them some protection against the H_2O_2-dependent antimicrobial systems (67). H_2O_2 microbicidal activity is also increased in the presence of ascorbic acid (84) and has been referred to as the ascorbate-H_2O_2 system. Although H_2O_2 has microbicidal activity *per se*, its antiviral, antifungal and antibacterial activities are markedly potentiated by MPO in the presence of halide ions (67).

Superoxide Anion (O_2^-): The superoxide anion, O_2^-, is formed by the univalent reduction of oxygen by a cyanide insensitive oxidase. For example: $HO_2 \longrightarrow H^+ + O_2^-$

The production of this highly reactive radical is increased during phagocytosis (68, 85) and has properties of both oxidant and reductant. When it functions as a reductant, O_2^- is oxidized to O_2; when it functions as oxidant, O_2^- is reduced to H_2O_2. Two molecules interact spontaneously as a dismutation reaction with the formation of $O_2 + H_2O_2$, schematically illustrated as follows (67):

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Whether the O_2^- anion, an activated oxygen species, has direct toxic effects was initially proposed based on the presence and distribution of superoxide dismutase in various microbial species (67), which makes them resistant to the O_2^-. For example, superoxide dismutase removes O_2^- by increasing its dismutation into H_2O_2. Consequently, a lot of work has centered on these activated oxygen species, i.e., O_2^-, singlet oxygen and OH and their possible roles as direct microbicidal elements. However, the evidence concerning O_2^- is somewhat contradictory and confusing. Some studies have demonstrated that phagocyte bacteriocidal activity was prevented by the presence of this dismutase, particularly when attached to latex particles (86).

However, other studies demonstrated that the catalase negative bacteria killed by neutrophils in CGD all have superoxide dismutase (63). This suggests that the MPO-H_2O_2-halide system and not superoxide itself is the major bacteriocidal peroxidative mechanism in the phagosomes or, that it represents a backup system.

Thus the direct toxicity of O_2^- remains to be elucidated. What is known, however, is that O_2^- production is increased during phagocytosis and serves as an intermediate in the synthesis of H_2O_2, which is a proven bacteriocidal agent. Furthermore, the microbicidal activity of O_2^- appears to be weak when compared to H_2O_2 formed from it when combined with other components of the MPO-halide antimicrobial system (67). The point is that the evidence for O_2^- being directly toxic is not conclusive. However, it is known that much greater bacteriocidal components are formed when O_2^- serves as an intermediate metabolite in H_2O_2 synthesis. Consequently, H_2O_2 becomes one of the major components in the MPO-

mediated antimicrobial system.

Hydroxyl radicals: Hydroxyl radicals ($OH^{\cdot}$) are formed by the interaction of H_2O_2 and O_2^-.

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\cdot}$$

The role of $OH^{\cdot}$ as a microbicidal agent has been suggested, but killing is more rapid when components of MPO system are added to the reaction mixture (67).

c. O_2-independent antimicrobial systems: Exposure of intact neutrophils to an atmosphere of nitrogen does not totally abolish antimicrobial activities, suggesting that other "backup" mechanisms are operative which are not dependent upon O_2 (67). These include acid, lysozyme, lactoferrin, and granular cationic proteins.

Acidity: During phagocytosis, the acid in the phagocytic vacuole (phagolysosome) vary in the range of pH 3-6.5 (21, 87). Among the candidates for the source of this acid pH is lactic acid which is produced during ingestion. This acidity has a number of functions: (1) the acid medium has selective toxicity for certain organisms such as pneumococci whereas others like lactobacilli grow well at acid pH; (2) the acid pH creates favorable conditions for the MPO-mediated antimicrobial system; (3) acid facilitates digestion of killed intracellular organisms by the lysosomal acid hydrolases (21).

Lysozyme: This basic protein, of molecular weight 14,500 daltons, is present in both azurophil and specific granules. This enzyme is muralytic and hydrolyzes glycosidic bonds between N-acetymuramic acid and acetyl glucosamine. Consequently, it has the potential to solubilize bacterial cell wall peptidoglycans. Quite expectedly, bacteria vary

in their susceptibilities to lysozyme attack based on differences in cell wall structure. Micrococcus lysodeikticus is very susceptible whereas S. aureus organisms are resistant to lysozyme, unless specific antibody + C' are present (67). The lysis of gram (-) organisms through the synergistic effects of ascorbate, H_2O_2 and lysozyme has also been reported (84). Due to these variable susceptibilities, the importance of lysozyme primarily as a microbicidal agent, has been re-evaluated. It has been suggested that lysozyme serves more of a digestive rather than microbicidal function (67).

Lactoferrin: This is a bacteriostatic agent that is present in specific granules (18-20). Its bacteriostatic property is primarily related to its iron chelating capacity. It inhibits growth by binding the iron required as an essential microbial nutrient.

Cationic proteins: The specific granules of human neutrophils contain heat stable, acid resistant, cationic proteins which can be separated into seven bands by electrophoretic techniques, and each has different antimicrobial specificities (88). These proteins are released into the phagocytic vacuole whereby they localize at the surface of the ingested organism (67, 89, 90).

The precise mechanism of bacteriocidal activity is yet to be defined. However, it is felt that these strongly cationic proteins bind to acid groups on the organisms and interfere with viability and growth, possibly by damaging the permeability barriers of both gram (+) and gram (-) organisms (91).

Antimicrobial Mechanisms in Mononuclear Phagocytes: In mononuclear phagocytes the antimicrobial armamentarium is similar to neutrophils, but differs in a number of respects. Although lysozyme,

H_2O_2 and acid are important antimicrobial agents, mononuclear phagocytes lack lactoferrin and cationic proteins. Also, although alveolar macrophages lack MPO (3, 63), the MPO-halide-H_2O_2 may still be operative because they contain catalase. Catalase can substitute for MPO and catalyze oxidation of substrates in the presence of H_2O_2, and has similar antimicrobial activity at acid pH in the presence of halide ions and H_2O_2 (63, 67).

Lipid peroxidation, occurring in human monocytes and alveolar macrophages, serves as another mechanism by which macrophages potentiate the action of H_2O_2. For example, malonyldialdehyde, a catabolite of lipid peroxides has been shown to have antibacterial activity (63).

It has become increasingly clear that a number of antimicrobial mechanisms exist in phagocytic cells. At present the MPO mediated H_2O_2-MPO-halide seems to be of primary importance among the various mechanisms. Which antimicrobial mechanism predominates in vivo remains to be proven. However, this "overkill" capacity is extremely advantageous, particularly in situations where one system may be defective or absent. Consequently, in MPO-deficiency, organisms may be handled less efficiently but adequately by the "backup" O_2 independent and/or dependent systems (67).

C. Membrane Dynamics and Regulation in Phagocytic Cells

1. Introduction: The fluid mosaic model of membrane organization (92) has provided an impetus for many studies and speculations geared at gaining information concerning the molecular and regulatory mechanisms involved during those cellular events where active membrane movement and phenomena are of critical importance. For example, endocytosis, lysosomal secretion, chemotaxis, loss of contact inhibition

and neoplastic proliferation, cell spreading, cell attachment to a surface, Ag-binding and lymphocyte transformation all involve membrane participation.

Phagocytosis first involves attachment of a particle to cell surface receptors. When the proper signals are transmitted, engulfment proceeds through processes involving membrane invagination, pseudopodial spreading, and pseudopodial fusion at the distal side of the particle. The final outcome is the encasement of the particle in a phagocytic vacuole, the lining of this structure being an inverted plasma membrane. Werb and Cohn's (65) observations on cholesterol exchange and plasma membrane 5' nucleotidase markers have shown that a substantial portion of the plasma membrane is internalized during ingestion. Other very recent observations are equally pertinent and shed light on other membrane dynamics that occur during phagocytosis, chemotaxis, lysosomal degranulation, and cellular spreading. These studies may provide insight on fundamental questions of importance to all specialties of cellular physiology and biology. For example:

a. What signals are transmitted and translated to the interior of the cell as a result of surface receptor-ligand binding to bring about such effector mechanisms as pseudopod formation, membrane fusion, and directed cell movement?

b. How is surface topography altered, i.e., how is receptor distribution and motility regulated during active events of selected membrane internalization?

c. What is the nature of the cytoplasmic organelles which regulate/modulate/direct these processes? Microtubular (MT) and microfilamentous (MF) structures would appear to be good candidates.

Microfilaments (fine filament 6nm in diameter) are prominent in the peripheral cytoplasm of macrophages, particularly at points of cellular attachment to surfaces (13, 62). Such filaments have been identified in many cells as actin polymers in equilibrium with actin monomers (63). MT differ from actin filaments in being hollow fibers, with a larger diameter (24nm) that are composed of tubulin subunits (63). They are primarily localized to the endoplasm of phagocytes and also appear to insert into the region of actin filaments at the cell periphery, particularly at points of cellular attachment to a surface (62, 63). More is known about the behavior of MT during phagocytosis-oriented cellular events because of its pharmacologic specificities.

Colchicine and the vinca alkaloids, vincristine and vinblastine, have played a key role as tools in the investigation of microtubule (MT) function because of their binding to tubulin, the subunit protein of which MT are formed (93). It has been postulated that the colchicine binding site is one of the protein interaction sites of tubulin. Consequently, a tubulin-colchicine complex is incapable of associating with a second tubulin molecule. The ability of colchicine to disrupt assembled MT would therefore depend upon the stability of the MT, or the degree of dynamic equilibrium between tubulin monomer-polymer subunits. Vinca alkaloids also disrupt MT polymerization (93). It has been hypothesized that colchicine, vincristine, and vinblastine disrupt MT assembly by preventing assembly reactions rather than by directly disrupting preformed MT. These association-dissociation equilibrium properties of MT subunit assembly play key roles in the function of these organelles (93). The state of subunit assembly is strongly temperature dependent and modulated by cyclic nucleotides. Cyclic AMP (cAMP) presumably promotes dis-

assembly whereas cGMP stimulates polymerization, thereby modulating the association-dissociation equilibrum (93, 94). Based on these data concerning the sensitivity of microtubular organization and function on physiological temperatures, cyclic nucleotides, colchicine, vinblastine, and vincristine and its insensitivity to luminocolchicine (a colchicine derivative which lacks the ability to bind to tubulin (93), many investigators have studied the effects of these drugs and experimental conditions on other cellular processes such as surface reorganization, phagocytosis, chemotaxis, lysosomal, secretion, and spreading. The effects of these drugs have been studied primarily to gain insight on the role of MT in these various effector mechanisms.

2. Modulation of Membrane Topography During Ingestion and MT Involvement: Recent studies have shown that characteristic changes in membrane organization are induced after exposure of cells to phagocytic stimuli. In turn, these surface changes are regulated by structures involved in contractile processes inside the cell. It has been demonstrated that during neutrophil phagocytosis, transport carriers for purine nucleosides, purine bases, and amino acids are selectively preserved on the outer surface of the cell. While the glycoprotein receptors for two plant lectins, concanavalin A (Con A) and Ricinus communis agglutinin (RCA) are selectively removed from the plasma membrane during neutrophil phagocytosis (96-98). Moreover, the population of lectin receptors that remain on the cell surface after phagocytosis is different from the population that is internalized. This indicates that there is clearly a change in the distribution and nature of lectin receptors after phagocytosis (96, 97).

Knowledge concerning distance of these Con A binding sites from phagocytic receptors, IgG and C3b would be enlightening particularly because Con A has been shown to inhibit phagocytosis (98). Nevertheless, further investigations showed that intracellular MT were particularly important in regulating this segregative movement of membrane proteins during ingestion. Colchicine was found to abolish selective preservation of transport sites on the cell surface and the removal of lectin receptors during phagocytosis. The postulate is that phagocytosis alters surface topography by inducing the selective movement of binding sites into membrane undergoing internalization and that MT are required for this directed movement and topographical reorganization. Disassembly of MT with colchicine releases these subcellular constraints on movement so that proteins show random internalization during ingestion (79, 95, 97).

Other studies concerned with selective membrane mobilization during ingestion have demonstrated that the membrane response to a phagocytic stimulus is segmental. In other words, the phagocytic stimulus is confined to that segment of the plasma membrane immediately adjacent to the particle (99). Ingestion of one particle does not trigger generalized membrane internalization. It would be interesting to study the effect of colchicine, vinblastine, and cyclic nucleoties on this segmental membrane response. Light might thus be shed on the roles of MT during this process.

3. MT Membrane Dynamics During Degranulation, Chemotaxis, Spreading and Attachment to Glass: Neutrophils selectively release lysosomal enzyme constituents to the external environment when exposed to non-phagocytosable stimuli such as immune complexes on non-phagocytosable Millipore filter surfaces (70, 71, 76). This selective secretory pro-

cess, not involving cell death, offers a good system to study the underlying mechanisms regulating lysosomal release, primarily because one has operationally dissociated engulfment from degranulation. Investigations concerning the mechanism of this release demonstrated reciprocal effects of cGMP and cAMP. Cyclic AMP, which favors MT disassembly, inhibits lysosomal enzyme release whereas cGMP, which favors assembly, enhances release (74-76, 100). Moreover, electron microscopic analysis (76) demonstrated that cells challenged with a phagocytic stimulus in the presence of cAMP contained fewer MT than in the absence of cAMP. cGMP, on the other hand, enhances MT assembly and is associated with a very noticeable alignment of lysosomes with tubule proteins. These findings led to the postulate that cyclic nucleotides regulate lysosomal traffic and movement to the cell periphery and fusion of these granules with the plasma membrane by a direct or indirect effect on MT assembly. Consequently, MT exert a positive influence on lysosomal release when in the aggregated state (76). Other investigations have provided ultrastructural evidence of less lysosomal mobilization and degranulation in the presence of colchicine (100). The conclusion is that MT facilitate the "getting together" of lysosome and phagosome and the effects of colchicine may, in part, be dependent upon the inhibition of this process and the associated extracellular release of granule enzymes (100).

Additional studies have shown that aggregated IgG and IgA can directly stimulate selective neutrophil degranulation (101). However, for effective secretion, the IgG and IgA molecules had to be particulate or bound to a surface. Soluble aggregates in suspension were ineffective. This indicates that activation occurs at the cell surface during specific receptor-ligand binding and points out the importance of surfaces

in that activation. This may result from increased binding affinity of IgG with membrane receptors if IgG is concentrated on the surface and/or from cell membrane alterations induced by cell attachment to the surface (35, 101). This specific secretion required Ca^{++} and stimulation was inhibited by DFP (diisopropyl phosphofluoridate), an irreversible serine esterase inhibitor (35).

Electron microscopic analysis of MT organization during cell attachment to glass also emphasizes the importance of surfaces in activating cellular effector processes. Axline, et al. found a high density of MT and microfilaments adjacent to part of the membranes of macrophages that were in contact with a glass surface. This MT organization mimicked that seen after challenge with a phagocytic stimulus (62). This is consistent with the hypothesis that cell attachment and spreading on surfaces may represent the cell's attempt to phagocytose a particle of infinite size, i.e., "frustrated phagocytosis" (61, 71).

Chemotaxis (63) and macrophage spreading (6, 103) on glass surfaces are also inhibited by colchicine and vinblastine and requires ATP (102).

From these studies it has become increasingly clear that the neutrophil membrane bound receptor system and the system involved in membrane mobility, motility and alteration of cell shape form a dynamic communication with each other via cytoplasmic MT and microfilaments. Cyclic nucleotides cAMP and cGMP reciprocally modulate this communication, directly or indirectly. This necessitates the existence of mechanisms which couple ectoenzymes (serine esterases, ATPases) and surface receptors on the outer membrane with guanyl cyclase on the inner membrane (104) with resultant signal transmission. These closely integrated

mechanisms (chemotaxis, lysosomal secretion, and phagocytosis), as demonstrated by inhibition with DFP, a serine esterase inhibitor, seem to require the participation of "activable esterases" or proenzymes within the granulocyte which are converted to active form when chemotactic, phagocytic, or secretory responses are initiated (35, 105). Furthermore, it points up similarities in terms of cell activation, MT mobilization, and the dynamic regulation of membrane movement and cell motility among closely integrated events of the phagocytic defense system which includes chemotaxis, cell attachment and spreading on surfaces, ingestion and lysosomal secretion.

D. Objectives of the Present Study

The present study was concerned with the processes by which phagocytosable materials are taken into the human neutrophil. The major goals and objectives were to evaluate the importance of neutrophil surface adherence and its effects upon phagocytosis in the presence of opsonin, be it IgG or complement mediated. The results indicated that, when compared with neutrophils in suspension, adherent cell populations are far more efficient during phagocytosis.

An examination was also made of the differential display of certain membrane receptors by human neutrophils under conditions of suspension culture as compared with adherent cells. These receptors were analyzed with special reference to the interaction of human neutrophils with IgG immunoglobulin and a component of the complement system.

The findings will be related to probable in vivo correlates of the neutrophil phagocytic function.

II. DIFFERENTIAL PHAGOCYTIC CAPACITIES OF ADHERENT AND SUSPENSION HUMAN NEUTROPHIL POPULATIONS.

A. Introduction

The phagocytic capacities of neutrophils, monocytes, and macrophages are known to be modulated by a number of factors, among which humoral antibodies and complement components (opsonins) are of primary importance. The presence of membrane receptors for IgG and C3 on these phagocytic cells has been established (41-43, 53-55, 106). Although these studies have shown specificities for IgG1 and IgG3 subclasses, the participation of other immunoglobulin subclasses remains controversial (101).

Nonetheless, the requirement for IgG and/or C3 opsonic proteins for efficient phagocytosis is generally accepted, and is based on studies which show that in the absence of specific opsonin, most pathogenic bacteria escape phagocytosis.

These phagocytic cells have the capacity to adhere to glass surfaces *in vitro*. Subsequent spreading of the plasma membrane can be induced under certain conditions (5, 6, 103, 107). The extent to which adherence alters or influences function is not yet clear, although some data suggest that significant biochemical and morphological changes (108), in addition to changes in phagocytic capacities (109), may occur with adherent macrophages.

In the present study, marked differences were observed in the phagocytic capacities of adherent and suspension human neutrophils toward various coagulase positive pathogenic strains of *S. aureus*. In the absence of specific opsonin, adherent neutrophils represented a more efficient phagocytic population. Moreover, clear differences in opsonic

uptake were evident between the two populations in their response to the phagocytosis inhibiting effect of an IgG3 immunoglobulin molecule or its Fc fragment.

B. Materials and Methods

1. Isolation of Neutrophils: Human neutrophils were isolated from peripheral whole blood as described by Henson (76), with minor modifications.

Venous blood was collected from healthy donors into 1/7 volume of sterile acid citrate dextrose (ACD), as anticoagulant. ACD was prepared as described by Rapoport (110). Na citrate-$2H_2O$ (1.330 grams), citric acid-H_2O (.470 grams), and anhydrous glucose (3.00 grams) were mixed together and made up to a volume of 100 ml with deionized distilled water and autoclaved for 30 minutes at 30 pounds of pressure. The tubes containing the anticoagulated blood were centrifuged at 2000 rpm for 30 minutes at room temperature. The platelet-rich plasma was removed and a curved bent tip pipette was employed to remove the "buffy coat" which contained predominantly mononuclear cells. The tubes were then filled with an equal volume of 6% dextran (type 200C, Sigma Co.) in pyrogen free .15M NaCl. The red blood cells (RBC) were then allowed to sediment for 60-90 minutes at 37°C. The supernatant containing the polymorphonuclear leukocytes (PMN) was then removed and pelleted at 1300 rpm for 10 minutes at room temperature. The pellet was resuspended in 5-10 ml of .87% NH_4Cl in water to lyse residual RBC. After 30 minutes at room temperature, the tubes were filled with tissue culture medium 199 + Hank's salts containing 10^4 units of penicillin-streptomycin (Grand Island Biological Co.) and 10% fetal calf serum (FCS) (Microbiological Assoc.). In all studies the FCS had been previously heat inactivated at

56°C for 30 minutes to inactivate serum complement components. The PMN population was sedimented at 1300 rpm for 10 minutes at 4°C and subsequently washed 2-3 times with large volumes of medium 199 + 10% FCS and resuspended in this medium to a concentration of 2 x 10^6 cells/ml. Subsequently these cells were utilized in adherent and/or suspension cell systems.

This technique regularly yielded 80-90% pure neutrophils, as shown by hemocytometer analysis. In all studies, sterile tissue culture techniques were rigorously employed.

2. Bacterial Preparations: S. aureus organisms of phage types 80/81, 52, 52A, and the Cowan strain (kindly provided by Dr. Schaefler, New York Dept of Health) were employed in most of the studies. All four strains were mannitol positive and coagulase positive.

The encapsulated organisms used were Klebsiella pneumoniae and Streptococcus pneumoniae, type III (kindly provided by Dr. E. Bottone, Mt. Sinai Hospital).

All bacterial types were grown in glucose broth for 4-5 hours at 37°C in a shaking water bath. Streptococcus pneumoniae required 10% calf serum in glucose broth for proper growth.

After 4-5 hours of growth the bacterial cultures were washed two times with 50 ml of phosphate buffered saline, pH 7.0 (PBS), pelleted at 3500 rpm for 10 minutes and resuspended to a concentration of 2 x 10^8/.5 ml in PBS (0.5M Na_2HPO_4 in .15M NaCl titrated to pH 7.0 with 1M Na H_2PO_4).

Quantitative determinations of the bacteria were based on prepared standard curves of colony forming units/ml (CFU/ml) versus Klett values.

a. Quantitative determination of bacteria: Standard curves of bacteria were constructed by relating the turbidity of the bacterial culture or its Klett value to the number of colony forming units/ml (CFU/ml). Klett values were obtained from an analytical apparatus which employs a photoelectric colorimeter to measure the turbidity of the bacterial culture. (Klett-Sumerson photoelectric colorimeter, model 800-3; Klett Mfg Co., Inc.). With this standard curve of Klett value versus CFU/ml, one can quantitate the number of bacteria in the preparation by determining the Klett value and extrapolating the CFU/ml.

Log phase bacteria were always employed; bacteria were grown up for 4-6 hours in glucose broth and the Klett value of this culture was determined. The number of viable bacteria corresponding to the Klett value was quantitated by preparing serial dilutions (10^4, 10^5, 10^6,.... ....10^{10}) of this log phase culture and streaking the diluted culture on blood agar plates; after 18-24 hours of incubation at 31°C, the number of bacteria was determined by counting the number of colony forming units and multiplying this figure by the dilution factor to obtain a final value of CFU/ml.

Standard curves of Klett value versus CFU/ml were constructed for the following bacteria: S. aureus, type 52 (Figure 1), S. aureus - Cowan strain (Figure 2), Klebsiella pneumoniae (Figure 3), and Streptococcus pneumoniae, type III (Figure 4). The growth curve for S. aureus, type 52 was also utilized for the quantitation of S. aureus types 80/81 and 52A.

Staphylococcus aureus, type 52

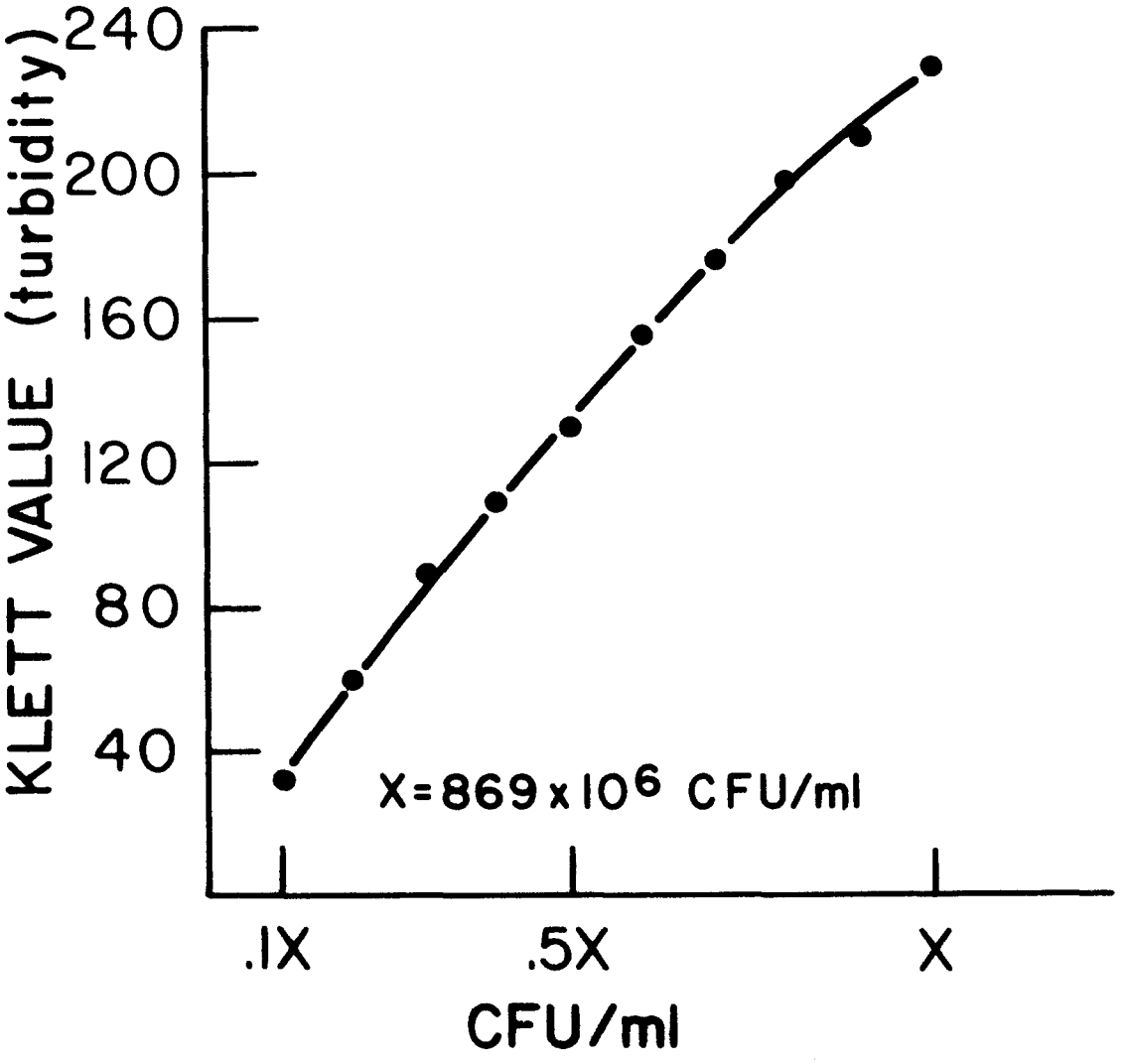


Figure 1: Staphylococcal aureus, type 52: Relationship of turbidity to the number of viable colonies.

This standard curve was constructed for S. aureus, type 52 and illustrates the proportional relationship between the turbidity (Klett value) of a log phase staphylococcal culture and the number of colony forming units/ml (CFU/ml). Utilizing this standard curve, once the Klett value of a log phase staphylococcal culture was determined on a Klett-Sumerson photoelectric colorimeter, the number of CFU/ml was determined by extrapolation.

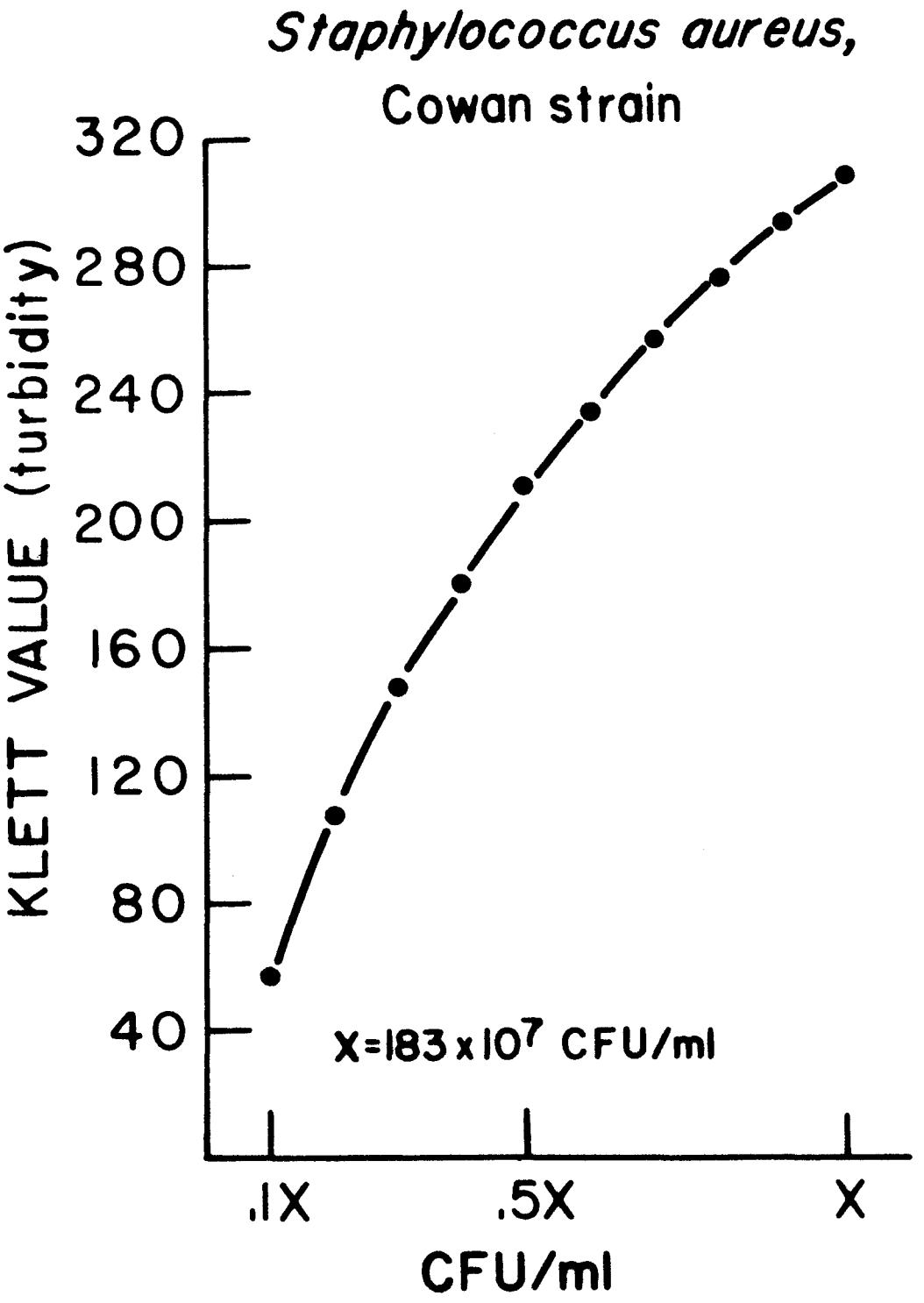

Staphylococcus aureus,
Cowan strain
320
280
240
200
160
120
80
40
KLETT VALUE (turbidity)
X=183 x 10⁷ CFU/ml
.1X
.5X
X
CFU/ml

Figure 2: Staphylococcus aureus, Cowan strain: Relationship of turbidity to the number of viable colonies.

This standard curve was constructed for S. aureus, Cowan strain, and illustrates the proportional relationship between the Klett value (turbidity) of a log phase culture and the number of colony forming units/ml (CFU/ml). Utilizing this standard curve, once the Klett value of a log phase culture was determined on a Klett-Summerson photoelectric colorimeter, the number of CFU/ml was determined by extrapolation.

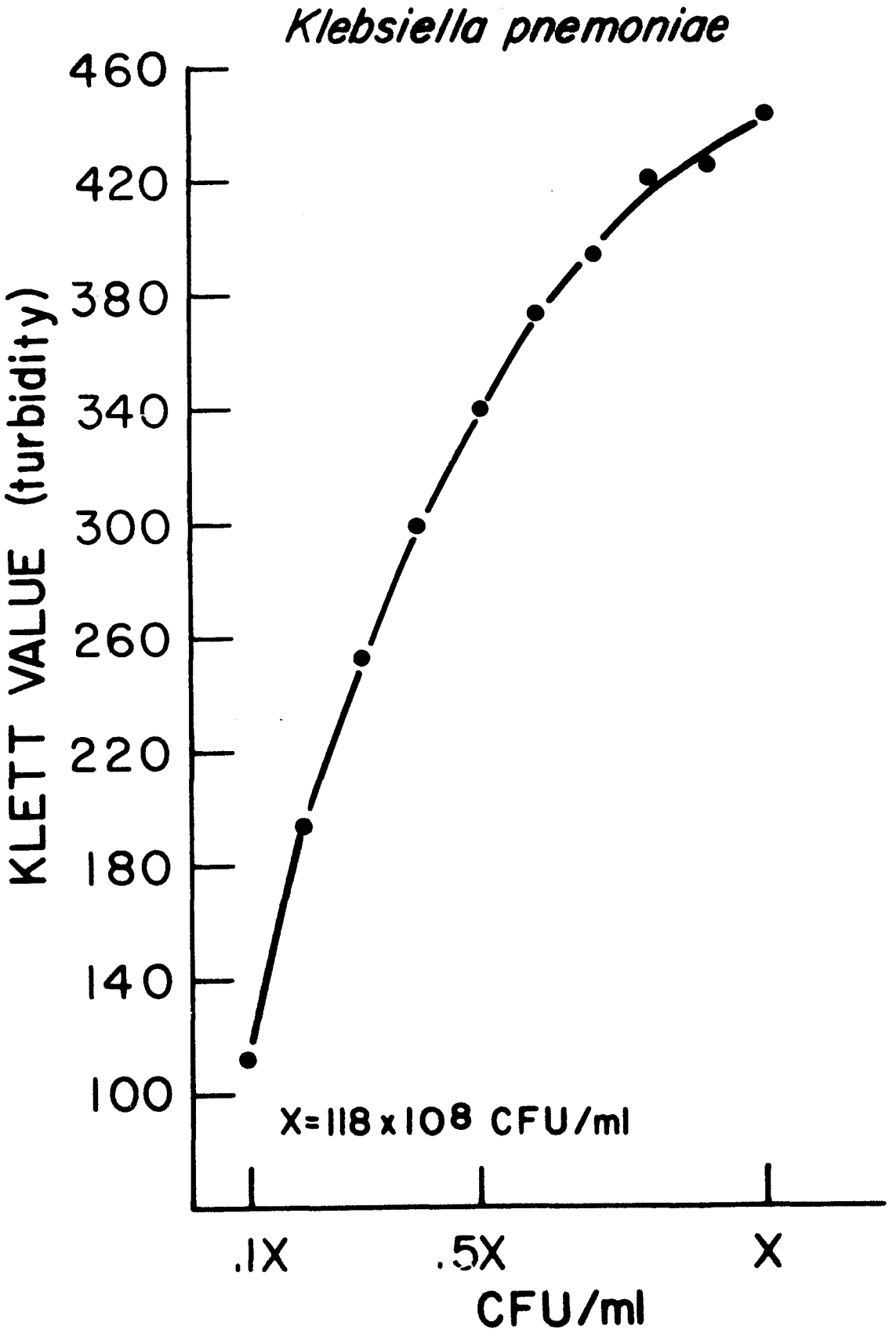

Klebsiella pnemoniae
460
420
380
340
300
260
220
180
140
100
KLETT VALUE (turbidity)
X=118 x 10⁸ CFU/ml
.1X
.5X
X
CFU/ml

Figure 3: Klebsiella pneumoniae: Relationship of turbidity to the number of viable colonies.

This standard curve was constructed for encapsulated K. pneumoniae and illustrates the proportional relationship between the turbidity (Klett value) of a log phase Klebsiella culture and the number of colony forming units/ml (CFU/ml). Utilizing this standard curve, once the Klett value of a log phase Klebsiella culture was determined on a Klett-Summerson photoelectric colorimeter, the number of CFU/ml was determined by extrapolation.

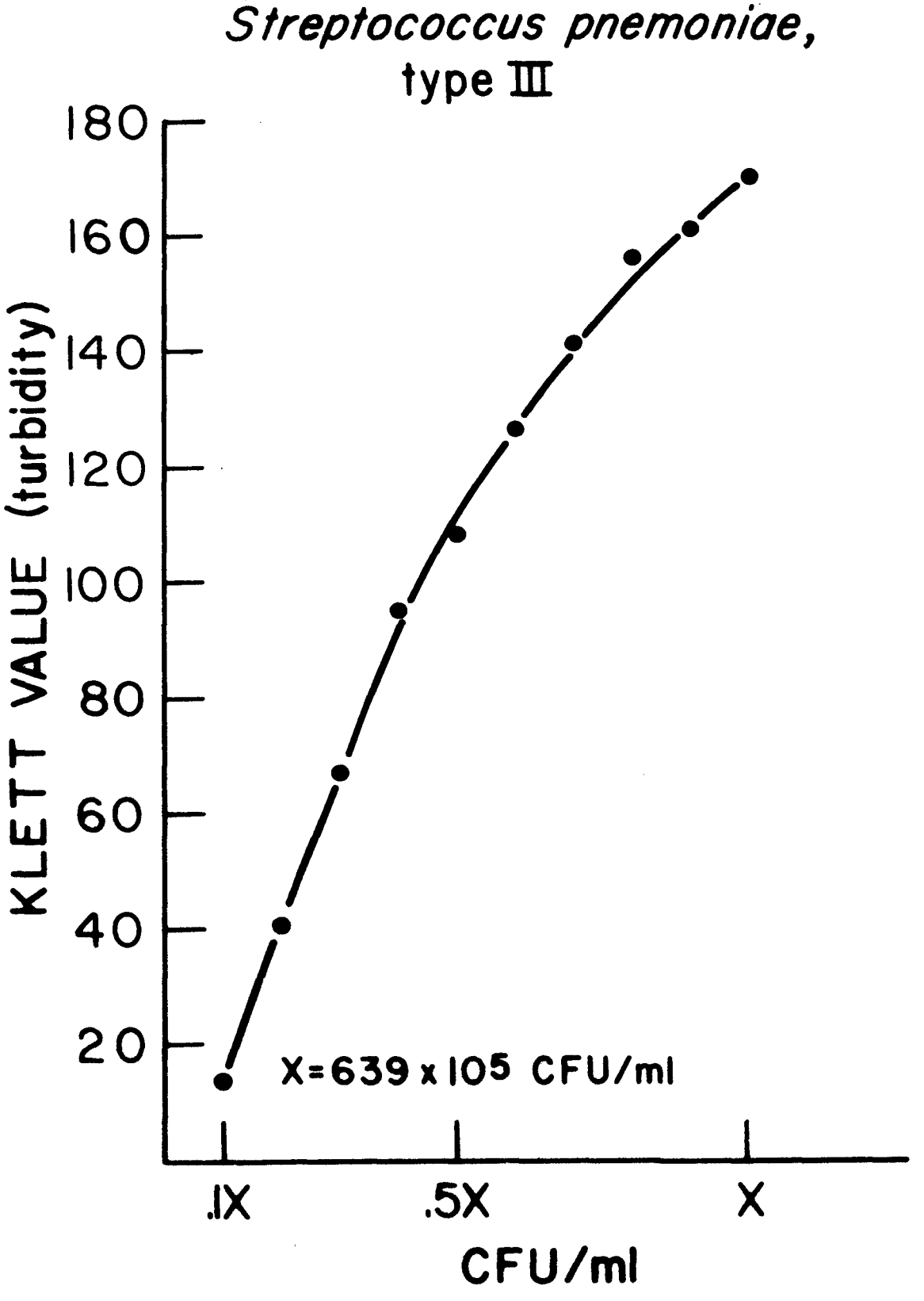

Streptococcus pnemoniae,
type III
180
160
140
120
100
80
60
40
20
KLETT VALUE (turbidity)
X=639 x 10⁵ CFU/ml
.1X
.5X
X
CFU/ml

Figure 4: Streptococcus pneumoniae: Relationship of turbidity to the number of viable colonies.

This standard curve was constructed for encapsulated Streptococcus pneumoniae, type III and illustrates the proportional relationship between the turbidity (Klett value) of a log phase streptococcal culture and the number of colony forming units/ml (CFU/ml). Utilizing this standard curve, once the Klett value of a log phase streptococcal culture was determined on a Klett-Summerson photoelectric colorimeter, the number of CFU/ml was determined by extrapolation.

b. Determination of coagulase production: Nearly all strains of S. aureus which are pathogenic for man produce coagulase, an enzyme which clots plasma. This property and DNAse production have been cited as the best correlates for pathogenecity (111).

S. aureus organisms were grown for 16-24 hours in glucose broth. 0.5 ml of this staphylococcal culture was mixed with .5 ml of citrated rabbit plasma (Bacto-coagulase plasma, Difco Laboratories) and incubated together for 1-4 hours at 37°C. Two control tubes, one containing a known coagulase positive culture and the other containing uninnoculated plasma, were included. Clot formation was determined by visual examination and a positive culture clotted the plasma usually within a 1-4 hour period. All strains of S. aureus utilized (80/81, 52, 52A, and Cowan strain) were coagulase positive.

3. Opsonization Procedures: All sera used for opsonization of S. aureus organisms were heat inactivated for 30 minutes at 56°C and diluted to a titre of 1:16 with PBS, pH 7.0.

The anti-staphylococcal opsonic serum (kindly provided by Dr. J. D Capra) was obtained from a human patient wich chronic staphylococcal osteomyelitis.

All S. aureus types, at a concentration of 2×10^8CFU/.5 ml, were opsonized with an equal volume of anti-staphylococcal opsonic serum (OS), normal human serum (NHS), or mock opsonized with an equal volume of PBS at 37°C for 30-60 minutes in a gently shaking water bath.

After that time, 100 microliters of the mixture, which contained 2×10^7CFU, were used as a source of opsonized or mock opsonized S. aureus in the phagocytic assay.

K. pneumoniae and S. pneumoniae were mock opsonized with an equal

volume of PBS, pH 7.0, as a control of these organisms in the absence of their immune antibodies.

The ratio of bacteria:neutrophils was 10:1.

4a. Phagocytic assay with adherent neutrophils: Neutrophils were resuspended to a concentration of 2 x 10^6 cells/ml in medium 199 + 10% FCS. One ml aliquots were dispensed into Leighton tubes with inserted coverslips (Bellco Plastics). The cells were allowed to adhere to coverslips (10mm x 35mm) for 30 minutes at 37°C (Figure 5A). After that time the coverslips were washed 2-3 times with 1 ml of medium 199 without FCS to remove nonadherent cells. Finally 1 ml of fresh medium 199 without FCS was added and cell monolayers challenged with 2 x 10^7 opsonized or mock opsonized bacteria (.1 ml).

When the IgG3 myeloma protein and its Fc fragment were used as inhibitors (aggregated or unaggregated) they were preincubated with adherent neutrophils at a concentration of 100 micrograms/2 x 10^6 neutrophils for 20-30 minutes at 37°C before challenge with opsonized or mock opsonized bacteria.

Phagocytosis proceeded for 30 minutes at 37°C with the ratio of bacteria to neutrophils 10:1. At the end of 30 minutes, phagocytosis was stopped in all tubes by the addition of one drop of 10% sodium azide. The coverslips were removed from the Leighton tubes and vigorously dipped (with subsequent drainage on paper towels) into five different tubes containing 50 ml of medium 199. Washed cells were then fixed in methanol for at least 10 minutes and stained with Wright's and Giemsa dyes.

The extent of phagocytosis was quantitated by light microscopic examination under oil immersion (1000 x magnification) of stained cells. Neutrophils were scored as positive if they contained one or more

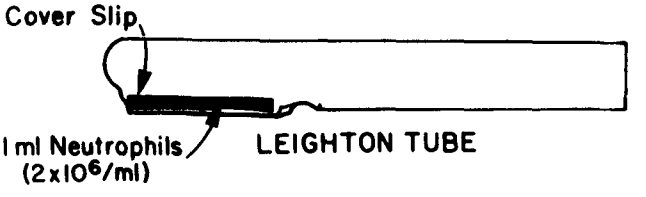
organisms per neutrophil. The degree or quality of ingestion was also determined by scoring the distribution frequency of 1-4, 5-10, or greater than 10 organisms per 100 neutrophils. At least one hundred neutrophils were counted per coverslip, and all studies were performed in duplicate or triplicate.

4b. Phagocytic assay with suspension neutrophils: The method employed was modified from Quie et al. (53). Highly enriched neutrophil preparations were resuspended in medium 199 + 10% FCS for 30 minutes at 37°C. After 30 minutes, the cells were pelleted at 1000 rpm for 10 minutes and the pellet washed two times with medium 199 without FCS. (This 30 minute incubation in medium 199 + 10% FCS with subsequent washes in 199 without FCS was performed in order to mimic conditions in the adherent cell system during monolayering protocol, thereby minimizing any artifactual differences in the two cell systems.) Neutrophils were resuspended in medium 199 at a concentration of 2×10^6/ml. One ml aliquots were dispensed into 13mm x 125mm 5 ml plastic tubes with screw caps (Falcon Plastics), and challenged with 2×10^7 opsonized or mock opsonized bacteria. The final volume of the phagocytic mixture was 1.1 ml. The phagocytic assay was carried out in tubes gently tumbled end-over-end in a Roto Rack (Fisher Scientific) for 30 minutes (Figure 5B).

When inhibitors were utilized, neutrophils were preincubated with appropriate inhibitors (IgG3 or Fc) at a concentration of 100 micrograms/2×10^6 neutrophils for 20-30 minutes at 37°C tumbling end-over-end in the Roto Rack, before challenge with opsonized or mock opsonized bacteria.

At the end of 30 minutes, phagocytosis was stopped in all suspen-

(A) PHAGOCYTIC ASSAY WITH "ADHERENT" NEUTROPHILS:



(B) PHAGOCYTIC ASSAY WITH "SUSPENSION" NEUTROPHILS:

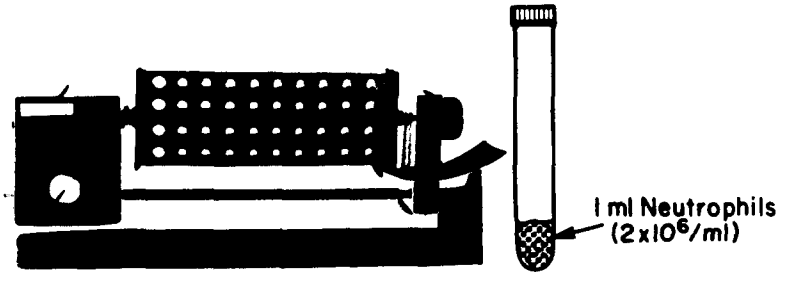


Figure 5: Equipment utilized for the phagocytic assays with suspension and adherent neutrophils.

This illustration shows the two types of equipment which were utilized to assay phagocytosis with adherent and suspension neutrophils.

A. The Leighton tube with its inserted coverslip (10mm x 35mm) were used for the phagocytic assay with adherent neutrophil populations; 2×10^6 neutrophils (2×10^6/ml) were allowed to monolayer onto coverslips for 30 minutes at 37°C. Subsequently, the monolayers were washed with medium 199 to remove nonadherent cells. Then 1 ml of fresh medium 199 was added and cell monolayers were challenged with 2×10^7 (.1 ml) of opsonized or mock opsonized bacteria.

B. The Roto Rack was used for the phagocytic assay with suspension neutrophils under comparable conditions as that used with adherent neutrophils. 2×10^6 neutrophils (2×10^6/ml) were dispensed into 13mm x 125mm 5 ml plastic tubes with screw caps; subsequently they were challenged with 2×10^7 (.1 ml) opsonized or mock opsonized bacteria. The tubes were placed in the Roto Rack and gentle revolving, allowing the tubes to tumble end-over-end, maintained the cells in suspension during the entire assay. The final volume of the phagocytic mixture in both systems was 1.1 ml.

pension tubes by the addition of one drop of 10% sodium azide. The 1.1 ml content of each tube was dispensed into Leighton tubes with inserted coverslips; cells were allowed to adhere for 5-15 minutes at room temperature in the presence of azide (this step was necessary in order to get an even monolayer of suspension neutrophils for proper microscopic examination; allowing cells to monolayer in the presence of azide inhibited further phagocytosis). After 15 minutes, the cells were washed by vigorously dipping coverslips into 5 tubes containing 50 ml of medium 199. Coverslips were drained on paper towels, cells fixed in methanol for at least 10 minutes, and then stained with Wright's and Giemsa dyes.

The extent of phagocytosis was quantitated by light microscopic analysis, under oil immersion of stained cells, exactly as described for adherent neutrophils.

5. Isolation of Homogeneous IgG3: Plasma from a patient (Jo) with multiple myeloma (kindly provided by Dr. J. D. Capra) containing homogeneous immunoglobulin of IgG3 subclass served as the source of IgG, its Fab and Fc fragments.

IgG was isolated from plasma by ion-exchange chromatography on DEAE-A50 Sephadex (Pharmacia), equilibrated in .05M PO_4 buffer, pH 7.85 (1M NaH_2PO_4 was tritiated to pH 7.85 with 1M Na_2HPO_4 and this stock solution was diluted 1:20 with water). At this particular ionic strength and pH, IgG does not adsorb to the ion-exchange resin; therefore it eluted with the equilibrating buffer while all other serum proteins adsorbed to the resin. The IgG eluant was collected for 18-24 hours and concentrated in an Amicon Ultrafiltration Unit (Amicon Corporation, Lexington, Mass.) containing a UM-50 membrane. Its purity was confirmed by Ouchterlony double diffusion analysis against the follow-

ing antisera: rabbit anti-human IgG and rabbit anti-normal human serum (Behring Diagnostics).

6. Preparation of Fc and Fab from IgG3: For the preparation of Fc and Fab fragments, homogeneous IgG3 was digested with trypsin as described by Edelman et al. with modifications (112).

IgG3 was dialyzed overnight in NH_4HCO_3 buffer, pH 8.0 and digested with trypsin (TPCK-trypsin) at an enzyme to substrate ratio of 1:50 for 1 hour at 56°C. After 1 hour, digestion was stopped by the addition of soybean trypsin inhibitor at an enzyme to inhibitor ratio of 1:2.

Trypsin digests were fractionated by Pevikon block electrophoresis (113). The Pevikon (Connaught Laboratories) was washed and equilibrated with Barbital buffer (.04M sodium diethyl barbiturate) pH 8.6 and zonal electrophoresis carried out in that buffer for 18-20 hours at 450 volts.

The paper strips for determining protein migration were stained with Brom-phenol-Blue (1% dissolved in 95% ethanol) and destained with a 3:1 ratio of H_2O:glacial acetic acid.

Fc and Fab fragments were eluted separately from the Pevikon matrix with barbital buffer and concentrated with an Amicon Ultrafiltration Unit containing a PM-10 membrane.

The purity of each fragment was determined by double diffusion analysis. The suspected Fc component formed a precipitin arc of identity with whole IgG3 against anti-Fc specific antisera (Figure 6); the suspected Fab formed a precipitin arc of identity with whole IgG3 against anti-Fab specific antisera (Figure 7). Both antisera were commercially prepared (Behring Diagnostics).

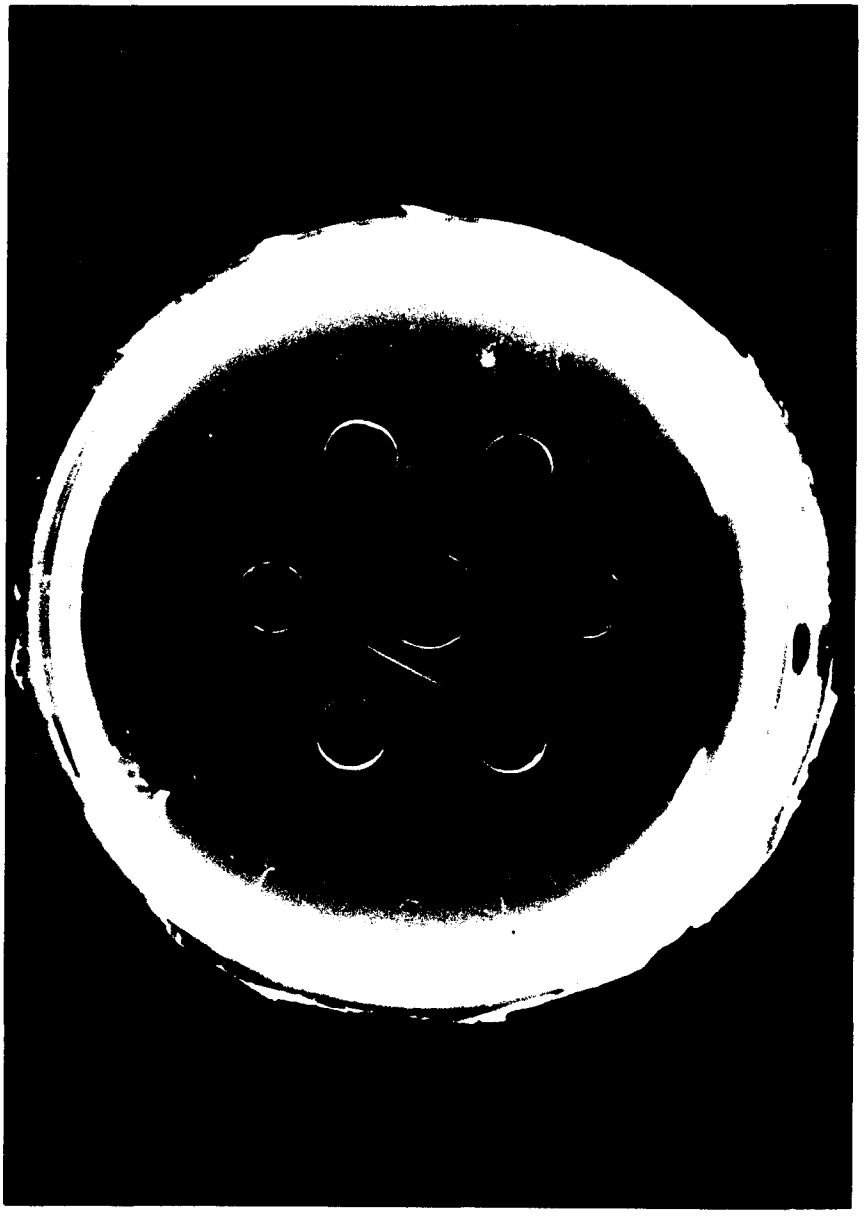


Figure 6: Ouchterlony double diffusion analysis: Purity of Fc isolated from homogeneous IgG3.

This photographic illustration demonstrates the antigenic relationships among whole IgG3 and its suspected Fab and Fc fragments. The center well contains anti-Fc specific antisera (Behring Diagnostics). The Fc component formed a precipitin arc of identity with whole IgG3 against anti-Fc antisera; whereas the suspected Fab component shows no Fc contamination as demonstrated by the absence of a Ag-Ab reaction with anti-Fc specific antisera.

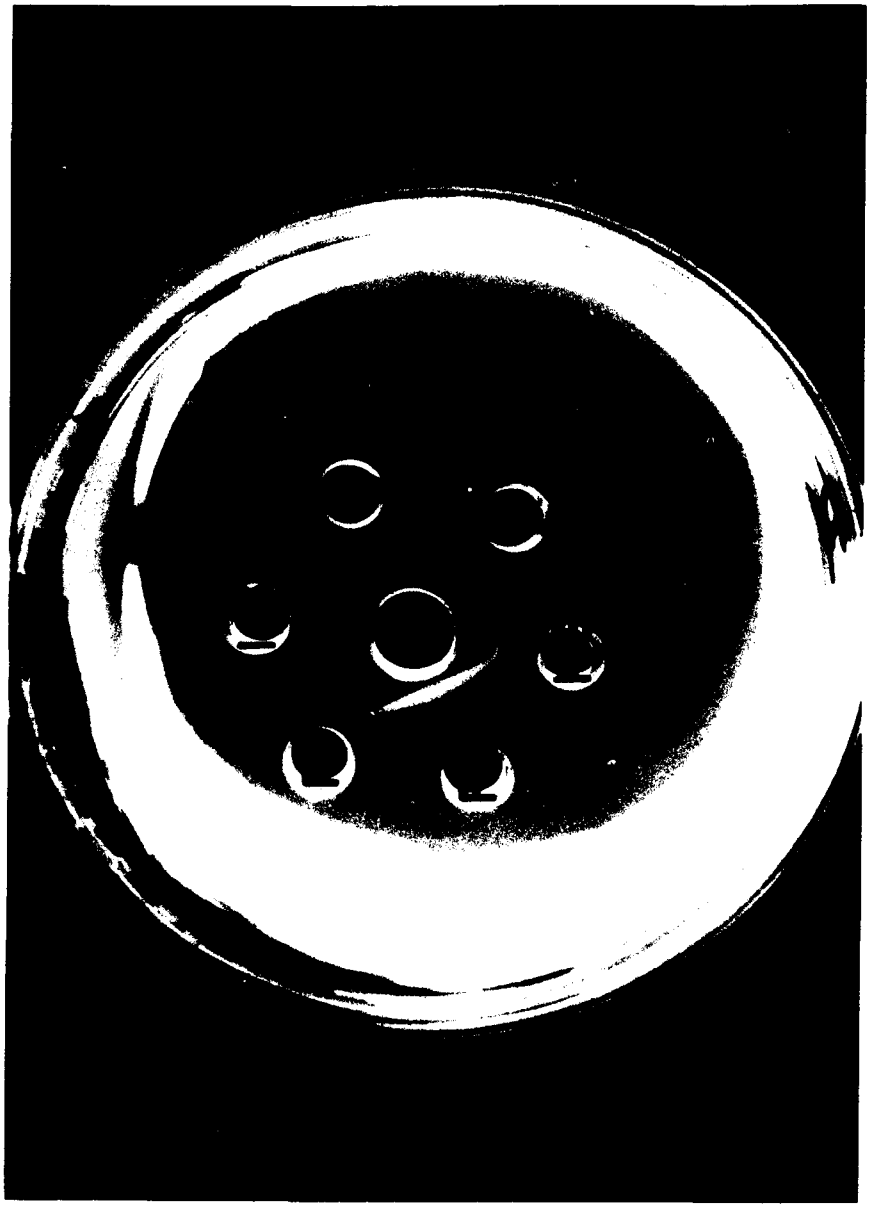


Figure 7: Ouchterlony double diffusion analysis: Purity of Fab isolated from homogeneous IgG3.

This illustration demonstrates the antigenic relationships among whole IgG3 and its suspected Fc and Fab fragments. The center well contains anti-Fab specific antisera (Behring Diagnostics). The Fab component formed a precipitin arc of identity with IgG3 against anti-Fab specific antisera; whereas the Fc component shows no Ag-Ab reaction with anti-Fab antisera.

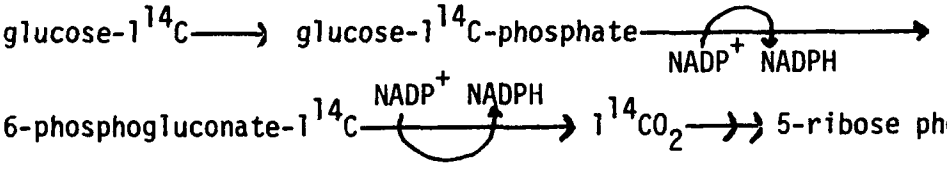
IgG3 and its Fc and Fab fragments were dialyzed into PBS, pH 7.0, and resuspended at a concentration of 1 mg/ml; .1 ml or .2 ml (100-200 micrograms) of each suspension was used in inhibition studies.

7. Fixation and Staining: After phagocytosis, washed coverslips (containing neutrophils) were dispensed into 15mm x 125mm glass tubes and submerged in 5 ml of methanol (Fisher Scientific); fixation in methanol proceeded for at least 10 minutes at room temperature. Afterwards, the methanol was decanted and 5 ml of Wright's staining solution (Fisher Scientific) was added to tubes. After 8-10 minutes, the Wright's staining solution was poured off and the coverslips were rinsed twice with tap water. Subsequently, 5 ml of Giemsa stain (Fisher Scientific), diluted 1:20 with tap water, was added to the tubes; after 10-15 minutes, the coverslips were rinsed twice with deionized distilled water and gently drained (5 seconds) on paper towels.

Coverslips were mounted, cell side down, onto glass microscope slides with Permount (Fisher Scientific), a commonly used histological adhesive.

8. Assay of Hexose Monophosphate Shunt (HMS) Activity: The hexose monophosphate shunt (also called phosphogluconate pathway or pentose phosphage pathway) is an alternate pathway of glucose degradation. Its activity is increased 4-10 times during phagocytosis and has been used as an indicator of phagocytic function (64, 66).

HMS activity was indirectly assayed by quantitating the amount of $1^{14}CO_2$ released from the oxidation of glucose-$1^{14}C$. For example,



The flasks used to assay $1^{14}CO_2$ production were 25 ml Warburg flasks housing an immovable center well in which a removable center cup was placed.

In the HMS assay with suspension neutrophils .2 ml of neutrophils (2×10^6) were added to the bottom of the Warburg flasks containing .2 ml of opsonized or unopsonized heat killed bacteria (2×10^8) + 25 lambda of glucose-$1^{14}C$ (10 microcuries/ml). The bacteria were heat killed by boiling for 30 minutes; they were washed two times with 50 ml of PBS, pH 7.0 before opsonization with an equal volume of anti-staphylococcal opsonic serum. The ratio of bacteria:neutrophils was 100:1 and the total volume, at this stage in the assay, was 1.25 ml. Blank controls consisted of 1 ml of medium 199 + 25 lambda of glucose-$1^{14}C$ and resting controls contained .2 ml of neutrophils (2×10^6) + .8 ml of medium 199 + 25 lambda of glucose-$1^{14}C$.

In the assay with adherent neutrophils, the protocol was comparable except that neutrophils were allowed to monolayer onto the bottom of the Warburg flasks before they were challenged with bacteria. The total volume of 1.25 ml was maintained by adding .8 ml of medium 199 to washed monolayers, .2 ml of opsonized or unopsonized bacteria + 25 lambda of glucose-$1^{14}C$.

All flasks were then covered air tight with a rubber stopper and incubated for 30 minutes at 37°C, rocking gently. After 30 minutes, with the aid of a 1 ml syringe and needle, .4 ml of hyamine hydroxide was injected onto the center cup (to capture released $1^{14}CO_2$) and 1 ml of 2N HCl was added to the cells located on the bottom of the flask. The flasks were reincubated for another 30 minutes, rocking gently at 37°C. After 30 minutes, the center cup containing the released $1^{14}CO_2$, was re-

moved, gently wiped on the outside, and placed in 10 ml of toluene, a standard scintillation cocktail. The amount of $1^{14}C$ radioactivity was determined on a liquid scintillation counter.

9. Double Diffusion Analyses: Double diffusion analyses, originally described by Ouchterlony (114) were carried out in circular dishes (Petri dishes) containing 1%-1.5% ionagar in Veranol buffer, pH 8.6, with .002% sodium azide.

10. Aggregation of IgG: The method of heat aggregation was adopted from MacLennan et al. (115, 116). IgG or its Fab or Fc fragments, at a concentration of 1 mg/ml, were aggregated by heat at $63^{o}C$ for 20-30 minutes.

11. Protein Concentration Determinations: Protein concentrations determined utilizing the Folin-Lowry procedure (117). 2 ml of a mixture containing 21 ml of Folin reagent, .21 ml of 2% sodium tartarate and .21 ml of 1% $CuSO_2$, was added to various dilutions (i.e., 5, 10, 25, or 50 microliters) of the protein in question. This mixture was allowed to stand for 20 minutes at room temperature. After 20 minutes, .2 ml of 50% phenol (1:1 dilution with distilled water) solution was added to each tube and immediately mixed with a Vortex agitator (Fisher Scientific). The tubes were allowed to stand at room temperature for an additional 30 minutes. Subsequently the optical density at 700nm (Tungsten lamp) of all tubes was recorded.

The mg/ml was estimated utilizing the linear portion of a previously prepared IgG Standard Folin Curve which related the optical density to the mg/ml.

C. Results

1. Phagocytic Efficiencies in the Absence of Specific Opsonin: The ability of suspension versus adherent neutrophil populations to phagocytose coagulase positive S. aureus organisms of phage types 52, 52A, 80/81, and the Cowan strain of S. aureus was investigated. In this study and all subsequent studies, not only was the percentage of neutrophils participating in the ingestion process recorded (bottom half of Figure 8), but I also assessed which percentage of these positive neutrophils contained 1-4, 5-10, or greater than 10 S. aureus organisms per neutrophil. These distribution frequencies (top half of Figure 8) concerning the numbers of intracellular S. aureus per positive neutrophil was always recorded.

Clear distinctions were observed in the phagocytic efficiencies of these two neutrophil populations when they were challenged with pathogenic S. aureus in the absence of antistaphylococcal immune opsonins (Figure 8). In the absence of antibody, suspension neutrophils exhibited low levels of ingestion of any of the strains tested (Figures 8, 9b); under comparable conditions, however, in adherent neutrophils, there were both a substantially greater percentage of neutrophils participating in the ingestion process and greater numbers of organisms ingested per positive neutrophil (Figures 8, 9a).

On the whole, it became evident that in the absence of immune opsonins, neutrophils which were adherent to glass surfaces during phagocytosis represented an actively more phagocytic cell population (Figures 9, 9a) as opposed to neutrophils kept in suspension during the entire period of phagocytosis (Figures 8, 9b).

PHAGOCYTIC CAPACITIES OF
"ADHERENT" vs "SUSPENSION" NEUTROPHILS CHALLENGED WITH
S. aureus IN THE ABSENCE OF IMMUNE ANTIBODIES

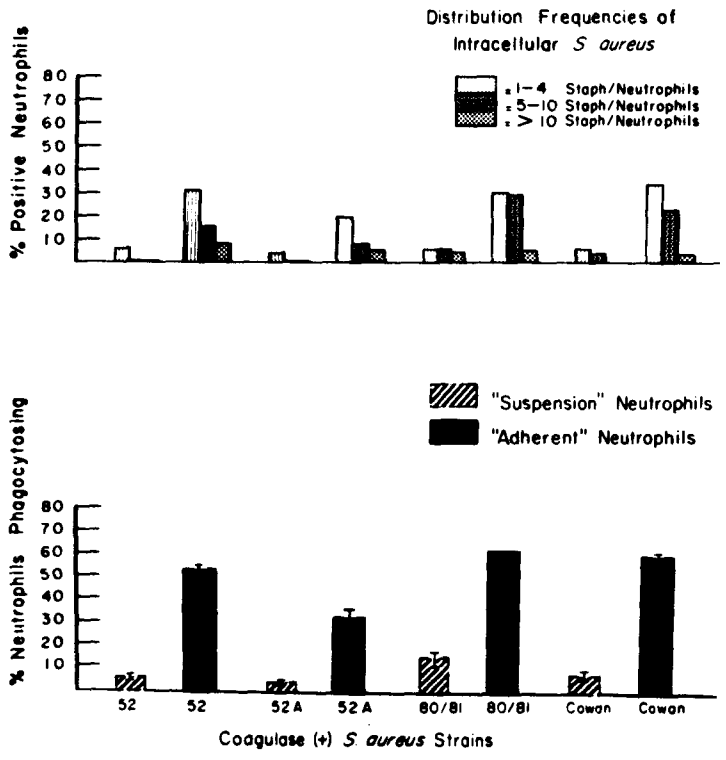


Figure 8: Phagocytic capacities of adherent and suspension neutrophils challenged with mock opsonized S. aureus.

Suspension neutrophils (diagonally stripped bars) and adherent neutrophils (solid black bars) were challenged with coagulase positive, pathogenic strains of S. aureus of phage types 52, 52A, 80/81, and the Cowan strain. The extent of phagocytosis was determined by light microscopic examination (at 1000 magnification) of stained cells. Neutrophils were scored as positive if they contained one or more bacteria per neutrophil (see bottom 1/2 of graph). The quality of ingestion was also assessed by scoring the distribution frequencies of 1-4, 5-10, or greater than 10 organisms per neutrophil on the positive cells (see top 1/2 of graph).

In the absence of antistaphylococcal opsonins, suspension neutrophils exhibited very low levels of ingestion of these organisms; in contrast, adherent neutrophils showed a significantly greater percentage of cells actively engaged in phagocytosis and greater numbers of intracellular bacteria were present per positive neutrophils. Consequently, adherent neutrophils represented an actively more phagocytic population.

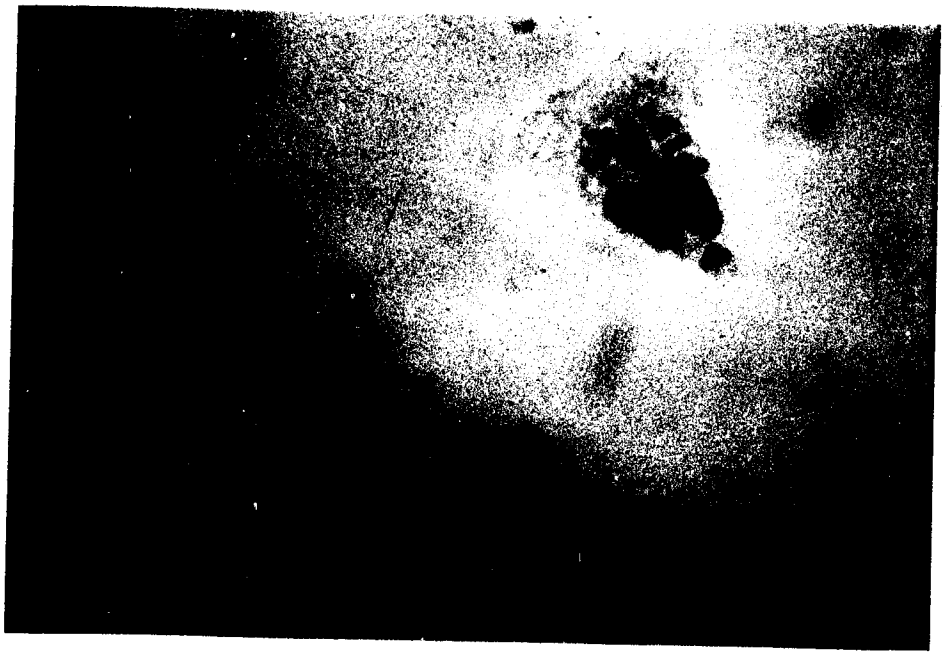


Figure 9: a. Light micrograph of adherent neutrophils challenged with mock opsonized S. aureus.

Light micrograph (1000 x magnification) demonstrating the ability of adherent neutrophils to phagocytose pathogenic S. aureus organisms even though antistaphylococcal opsonic antibodies were absent in the reaction mixture.

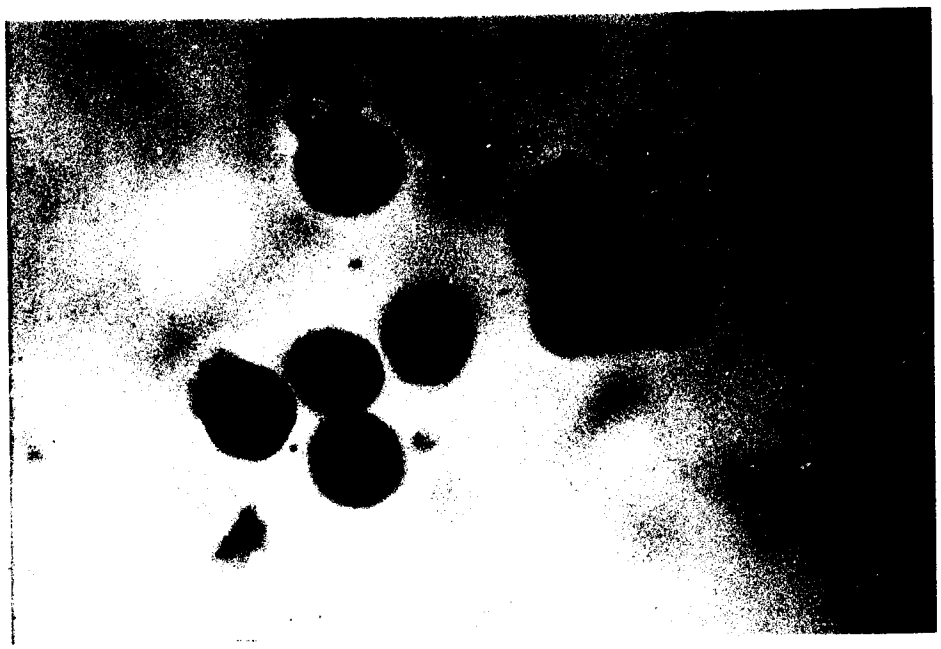


Figure 9: b. Light micrograph of suspension neutrophils challenged with mock opsonized S. aureus.

Light micrograph (1000 x magnification) demonstrating the failure of suspension neutrophils to appreciably phagocytose pathogenic S. aureus organisms in the absence of antistaphylococcal opsonic antibodies.

2. Phagocytic Efficiencies in the Presence of Antistaphylococcal Opsonin: The phagocytic capacities of suspension versus adherent neutrophils challenged staphylococcal and antistaphylococcal opsonic complexes (opsonized S. aureus) were studied (Figure 10).

In the presence of specifically immune antibodies, a marked qualitative and quantitative opsonic enhancement was consistently observed with suspension neutrophils. This opsonic increment was highest with S. aureus phage types 52 and 52A and lowest with S. aureus phage type 80/81 and the Cowan strain. I suspect that these differences in efficiencies in the suspension system may be related to slight differences in the antigenic determinants (i.e., exposed immunodominant determinants) of the various S. aureus types. The hyperimmune opsonic serum was obtained from a patient with chronic staphylococcal osteomyelitis. However, the predominant S. aureus type in that infection was never determined. In any event, in the presence of immune opsonin in the suspension system, there were substantially higher percentages of neutrophils actively engaged in phagocytosis and greater numbers of bacteria per positive neutrophil (Figures 10, 11b).

With neutrophil monolayers (adherent cells) an opsonic enhancement on ingestion was also observed. There were higher percentages of neutrophils actively engaged in phagocytosis. However, the opsonic increment, over that seen in the absence of antistaphylococcal antibodies, was particularly manifest in the greater numbers of ingested bacteria per positive neutrophil. For example, there were more neutrophils with 5-10 and greater than 10 intracellular bacteria (Figures 10, 11a).

Thus, in both neutrophil populations, I was able to demonstrate an opsonic enhancement on ingestion in the presence of antistaphylococcal opsonic antibodies.

PHAGOCYTOSIS OF
S. aureus + ANTI-STAPH IMMUNE COMPLEXES BY
"ADHERENT" vs. "SUSPENSION" NEUTROPHILS

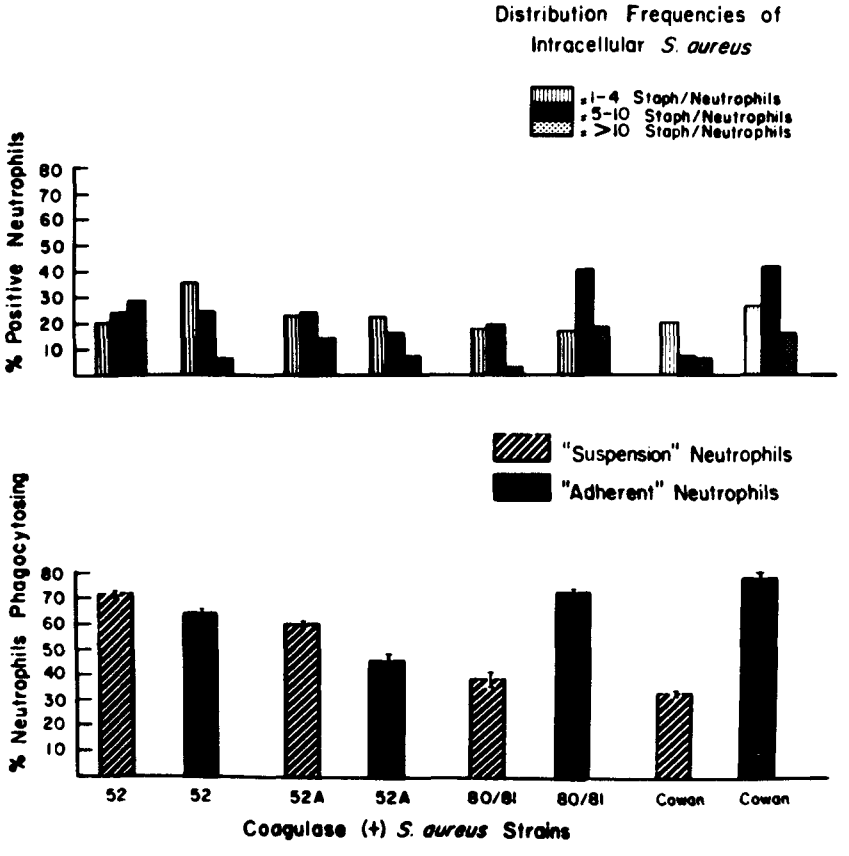


Figure 10: Phagocytosis of S. aureus - Antistaphylococcal opsonic complexes by adherent and suspension neutrophils.

Suspension neutrophils (diagonally striped bars) and adherent neutrophils (solid black bars) were challenged with various coagulase positive, pathogenic strains of S. aureus which had been opsonized with antistaphylococcal antibodies.

In both neutrophil populations, there was evidence of a pronouned opsonic enhancement on ingestion efficiencies. Suspension populations showed increased percentages of neutrophils actively phagocytosing (see bottom 1/2 of graph) and greater numbers of intracellular bacteria were present per positive neutrophil (see top 1/2 of graph). Adherent neutrophils showed higher percentage of cells engaged in ingestion; however, the opsonic increment, over that seen in the absence of opsonin, was particularly manifest in greater numbers of intracellular bacteria per positive neutrophil.

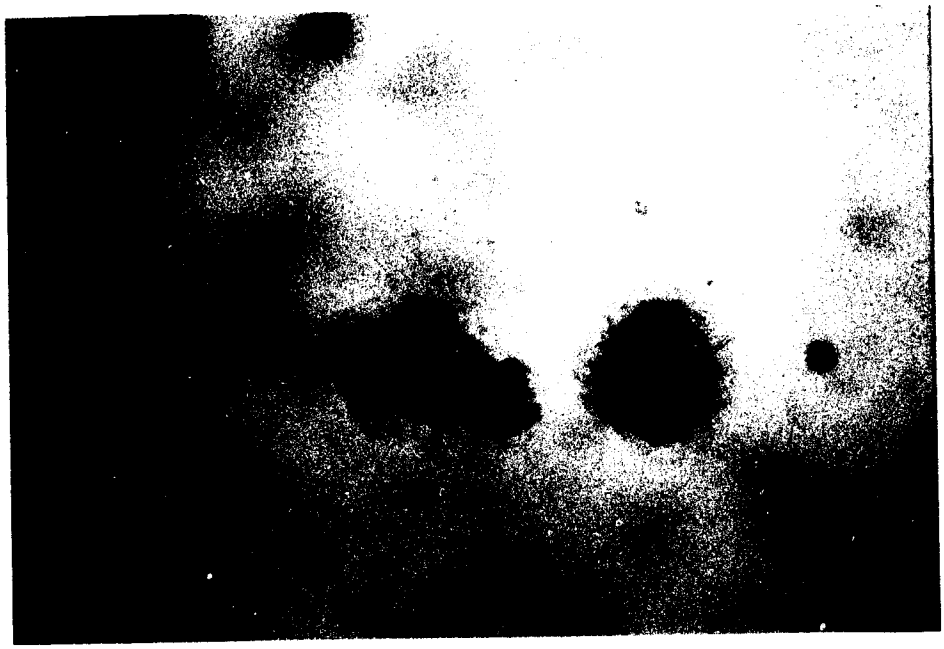


Figure 11a: Light micrograph showing opsonic enhancement by adherent neutrophils.

Light micrograph (1000 x magnification) demonstrating the increased efficiencies of adherent neutrophils to phagocytosed S. aureus which had been opsonized with antistaphylococcal antibodies.

Figure 11b: Light micrograph showing opsonic enhancement by suspension neutrophils.

Light micrograph (1000 x magnification) demonstrating the pronounced opsonic increment on ingestion efficiencies (over that seen in the absence of immune antibodies) by suspension neutrophils which had been challenged with opsonized S. aureus.

3. Ability of a Homogeneous Human IgG3 Myeloma Protein and its Fc Fragment to Block Opsonic Ingestion of S. aureus by Suspension Neutrophils: Presumably, immunoglobulin mediated opsonization is possible because neutrophils possess plasma mambrane bound receptors for the Fc region of the IgG molecule (53, 55). Specificity has been shown for the IgG3 and IgGl subclasses. Therefore, subsequent experiments were conducted to examine the ability of a structurally homogeneous IgG3 myeloma protein or its Fc fragment to inhibit the uptake of opsonized S. aureus by competing with antistaphylococcal IgG opsonins for IgG-Fc receptor sites on the neutrophil membrane.

The presentation of S. aureus + antistaph immune complexes resulted in a distinctly enhanced opsonic effect. This augmented phagocytosis was completely inhibitable by preincubation of suspension neutrophils with 100 micrograms IgG3/2 x 10^6 neutrophils, or an equivalent quantity of its Fc fragment. This efficient blockage of phagocytosis occurred with both the aggregated and unaggregated intact IgG3 molecule or its purified Fc fragment (Figure 12).

Figures 13a and 13b are light micrographs of that experiment. An opsonic enhancement is evident when suspension neutrophils were challenged with opsonized S. aureus (Figure 13a); however, this augmented uptake was effectively blocked by preincubating neutrophils with an IgG3 myeloma protein (Figure 13b).

ABILITY OF IgG3 & ITS Fc FRAGMENT TO BLOCK OPSONIC INGESTION OF *S. aureus* BY "SUSPENSION" NEUTROPHILS

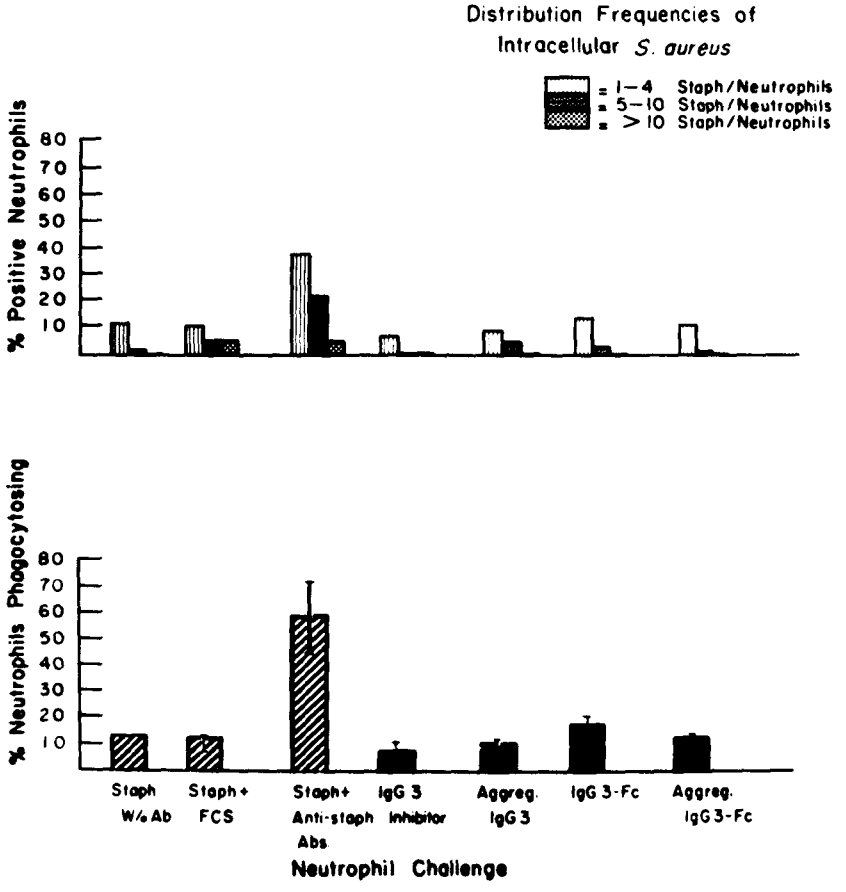
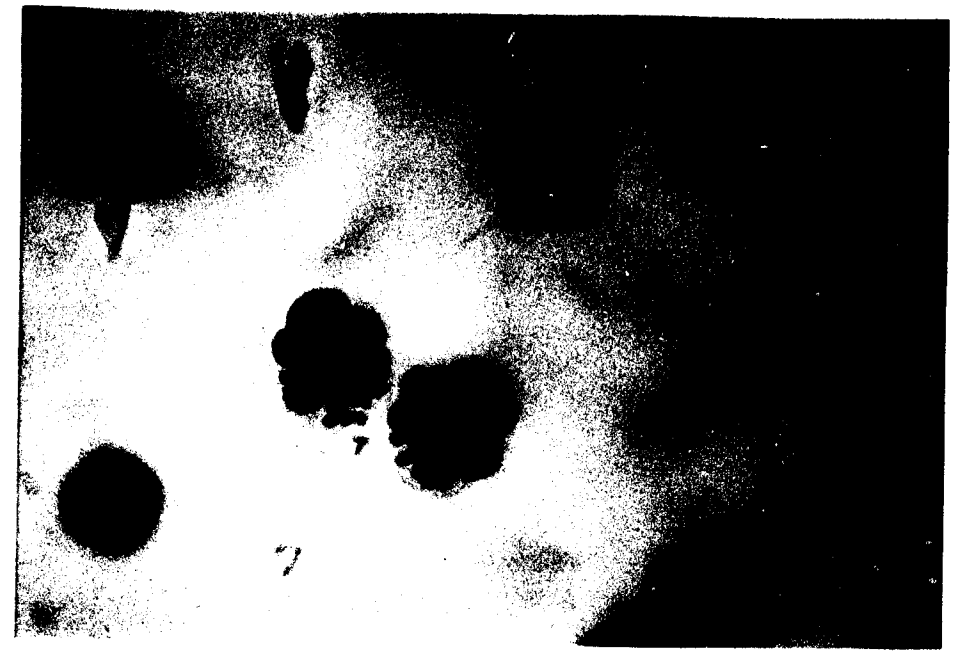


Figure 12: Ability of an IgG3 myeloma protein to inhibit ingestion of opsonized S. aureus by suspension neutrophils.

The ability of a structurally homogeneous IgG3 myeloma protein or its Fc fragment to inhibit the uptake of opsonized S. aureus, by competing with antistaphylococcal IgG opsonins for membrane bound IgG-Fc receptor sites on suspension neutrophils, was investigated. In the absence of inhibitors, neutrophils showed efficient ingestion of S. aureus + antistaphylococcal immune complexes. However, if suspension neutrophils had been preincubated with 100 micrograms of IgG3 or IgG-Fc (solid black bars), it was possible to block the subsequent uptake of opsonized S. aureus. Effective inhibition occurred with unaggregated or heat aggregated IgG3 and IgG3-Fc.



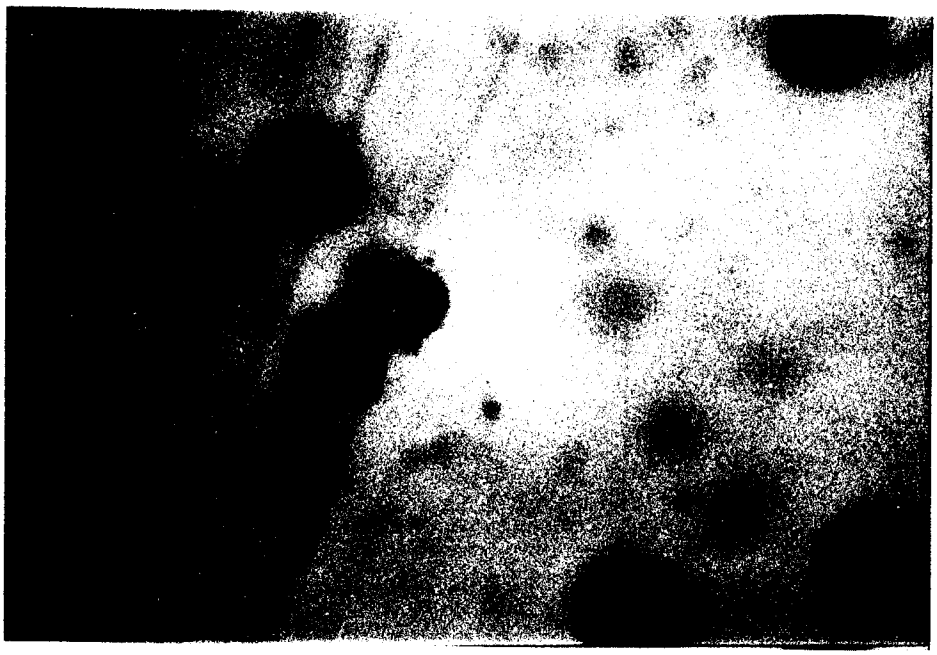


Figure 13: a. Light micrograph (1000 x magnification) showing opsonic ingestion of S. aureus by suspension neutrophils.

b. Light micrograph (1000 x magnification) showing the ability of IgG3 to block opsonic ingestion of S. aureus by suspension neutrophils.

4. Effect of a Human IgG3 Myeloma Protein and its Fc Fragment on Opsonic Ingestion by Adherent Neutrophils: In contrast to the results with comparable suspension neutrophils, I could not block the augmented uptake of staphylococcal-antistaphylococcal opsonic complexes by preincubating adherent neutrophils with IgG3 or its Fc fragment at the same concentration (Figure 14). Figure 15a is a light micrograph of a typical experiment in which adherent neutrophils were challenged with opsonized S. aureus. An opsonic enhancement is evident. However, this opsonic enhancement or augmented phagocytosis of staphylococcal-antistaphylococcal complexes was not inhibitable if neutrophils were preincubated with free intact IgG3 (Figure 15b). In addition, it was not possible to effectively inhibit this opsonization with adherent neutrophils even if the intact IgG3 myeloma protein was preincubated with neutrophils before they were allowed to adhere to glass coverslips and subsequently challenged with specifically opsonized S. aureus.

Taken at face value, this observed difference between suspension and adherent neutrophil populations suggests that in neutrophil monolayers, the proposed membrane bound IgG-Fc receptors are inaccessible for effective inhibition or blockage with free unbound IgG3. Thus, the failure of IgG3 to effectively compete with antistaphylococcal IgG opsonins for IgG-Fc receptor sites in the adherent cell system may be related to a spatial reorientation or topographical rearrangement of IgG receptors during the phenomenon of glass adherence. Considering the extensive deformability and translocation of the cell membrane that occur as cells settle on glass (4, 5), it is not unreasonable to postulate that some sort of topographical relocalization of membrane

INABILITY OF IgG OR ITS Fc FRAGMENT TO INHIBIT ENHANCED OPSONIC INGESTION OF *S. aureus* BY "ADHERENT" NEUTROPHILS

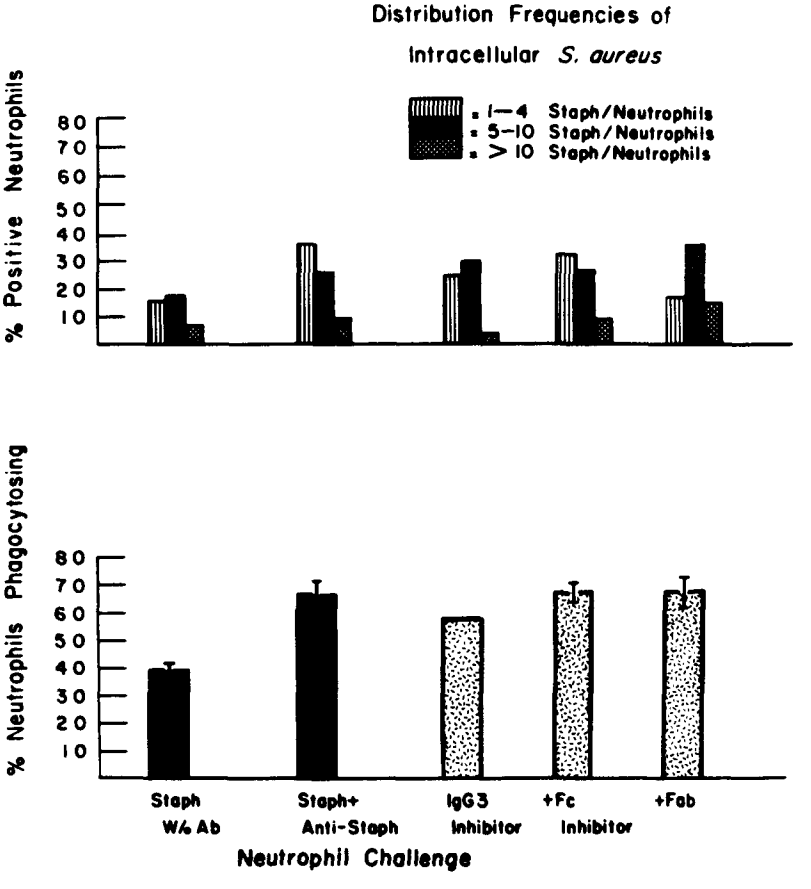


Figure 14: Failure of IgG3 to block opsonic ingestion of S. aureus by adherent neutrophils.

Although a pronounced opsonic enhancement on ingestion was also evident with adherent neutrophils challenged with S. aureus + antistaphylococcal immune complexes, this augmented uptake was not inhibitable by preincubating the cells with IgG3 or IgG3-Fc, as previously shown with suspension neutrophils. These results demonstrate another distinction between the two neutrophil populations.

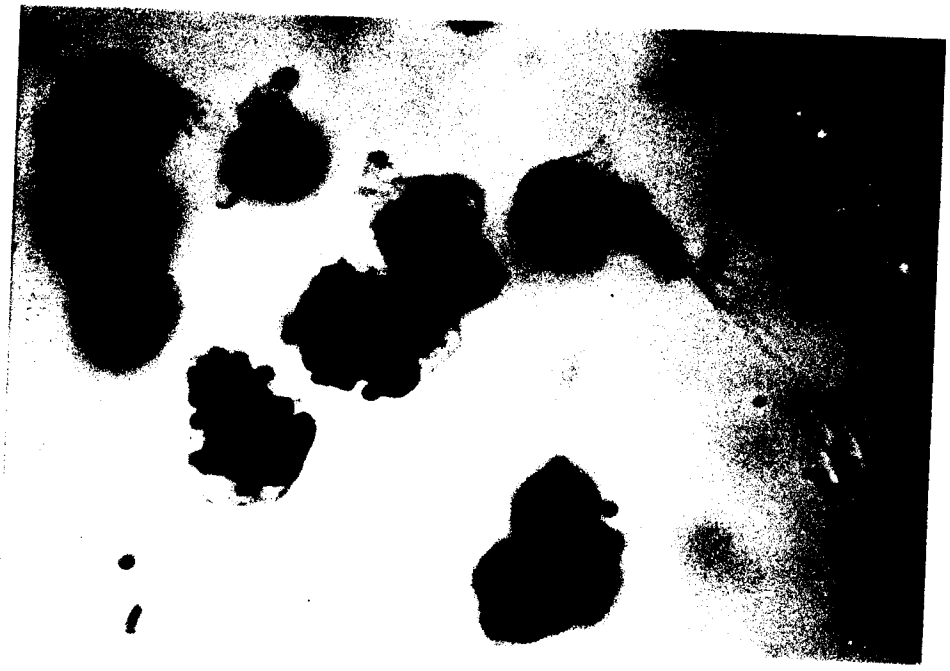


Figure 15: a. Light micrograph (1000 x magnification) demonstrating opsonic ingestion of S. aureus by adherent neutrophils.

b. Light micrograph (1000 x magnification) demonstrating the failure of IgG3 to inhibit opsonic ingestion of S. aureus adherent neutrophils.

receptor sites occurs during these extensive membrane perturbations that accompanies glass adherence. Such a relocalization of receptor sites may be manifest as (1) a clustering (microaggregation) of already exposed IgG receptor sites; (2) the exposure of additional IgG receptor sites; or (3) the masking or internalization of existing receptor sites. I tend to favor postulates (1) and (2). Perhaps adherence results in local aggregation of IgG receptor sites such that a tighter binding or higher energy of interaction could occur between neutrophils and Ag-Ab complexes than between neutrophils and free, uncomplexed IgG molecules. Possibly the binding of neutrophil and free IgG-Fc is a weak one and can be displaced by Ag-Ab complexes which interact more efficiently with aggregated or clustered receptors. In considering postulate (2), perhaps additional receptor sites are exposed during glass adherence which makes them accessible for stimulation by staphylococcal-antistaphylococcal immune complexes. Consequently, inhibition could be overcome by stimulation of newly exposed IgG receptor sites.

In any event, realizing that the major differences observed between the two neutrophil populations were in some way related to the phenomenon of glass adherence, I subsequently studied what effects, if any, monolayering neutrophils for varying time periods had on the phagocytic efficiencies of these cells.

5. Effect of Allowing Neutrophils to Monolayer for Varying Time Periods on Ingestion Efficiency. I investigated what effects the time allotted to allow neutrophils to adhere to glass surfaces had on the efficiency of phagocytosis of S. aureus in the absence of antistaphylococcal opsonic antibodies (Figure 16). Neutrophils were allowed to adhere to glass coverslips for 15, 30, 60, 90, and 120 minutes, and

EFFECTS OF MONOLAYERING NEUTROPHILS FOR VARIOUS TIME PERIODS ON THE EFFICIENCY OF INGESTION OF "MOCK OPSONIZED" *S. aureus*

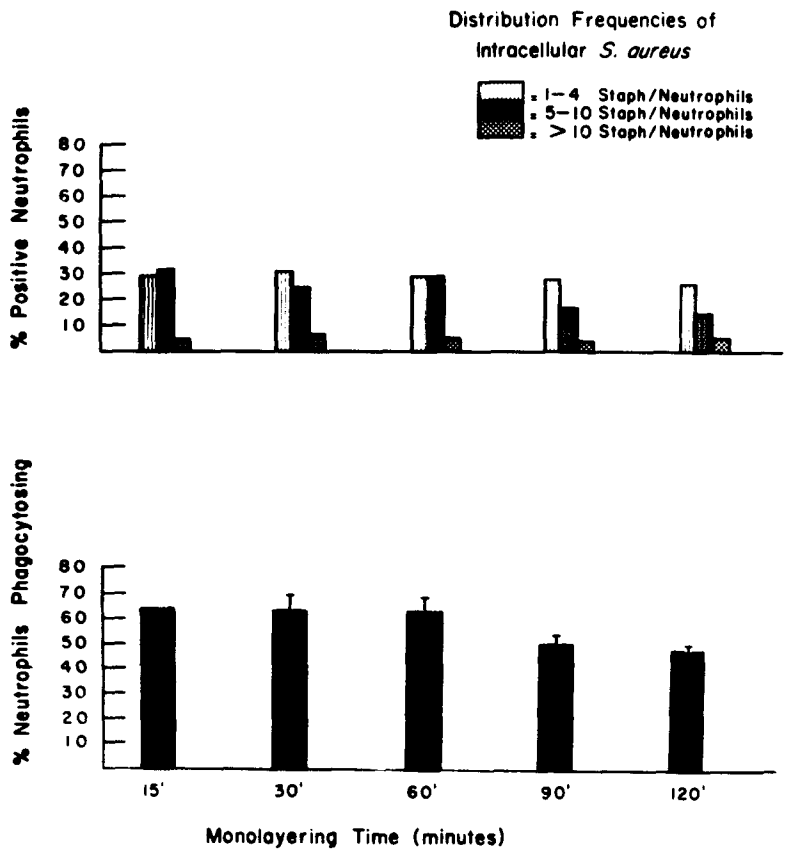


Figure 16: Effects of allowing neutrophils to monolayer for various time periods on the efficiency of ingestion.

Neutrophils were allowed to adhere to glass surfaces for 15, 30, 60, 90, and 120 minutes before they were challenged with S. aureus in the absence of specific opsonin. Ingestion efficiencies were comparable when adherence occurred over a 60 minute time period. After 90 and 120 minutes, phagocytic efficiencies diminished slightly.

subsequently the monolayers were challenged with S. aureus. Consistent with my earlier findings, neutrophil monolayers ingested S. aureus remarkably well in the absence of immune opsonin. There were no significant differences in the percentages of neutrophils phagocytosing nor in the quality of ingestion whether cells were monolayered for 15, 30, or 60 minutes. After 90 and 120 minutes the phagocytic efficiency of monolayered cells decreased slightly (Figure 16).

6. Effects of Allowing Neutrophils to Monolayer in the Absence of FCS. Neutrophils were allowed to adhere to glass surfaces in tissue culture medium 199 (med 199) containing either 10% FCS, 10% normal calf serum, 15% bovine serum albumin (BSA), or without FCS (Figure 17). All sera were heat inactivated. It became apparent that neutrophils could be monolayered in med 199 in the absence of 10% FCS. However, the potential of these adherent monolayers to substantially phagocytose S. aureus in the absence of immune opsonin was abolished (Figure 17). This was the first clue that the efficient phagocytic potential of adherent monolayers, in the absence of specific opsonin, was complex and related to at least two factors: (1) A moiety present in decomplemented FCS, and (2) the adherence phenomenon itself. This became apparent when suspension neutrophils were also cultured in med 199 + 10% FCS; suspension neutrophils still did not possess the comparable enhanced potential, as adherent cells did, to phagocytose unopsonized S. aureus. Also, if adherent neutrophils were allowed to monolayer in the absence of FCS, the nonspecific phagocytic capability was lost (Figure 17). Consequently, neutrophils must be adherent to glass surfaces for FCS to potentiate the nonspecific ingestion of mock opsonized S. aureus. This implies that the moiety in FCS

ABILITY OF FCS AND CALF SERUM TO
STIMULATE INGESTION OF *S. aureus* IN THE ABSENCE OF
IMMUNE ANTIBODIES BY "ADHERENT" NEUTROPHILS

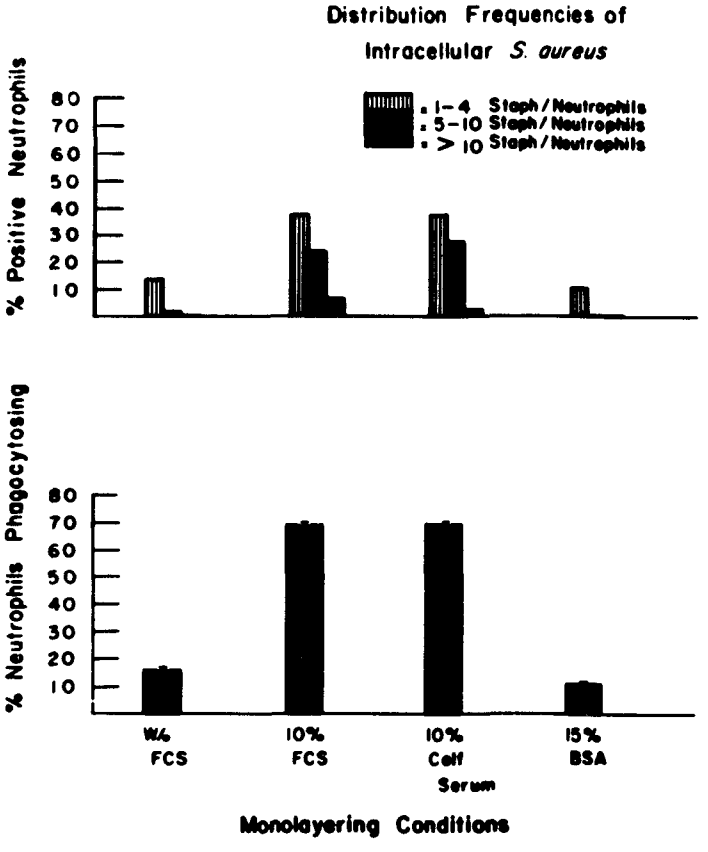


Figure 17: Effects of allowing neutrophils to monolayer in the absence of FCS.

Inspection of this figure illustrates that even though neutrophils could adhere to surfaces when cultured in medium 199 without FCS, the efficient ingestion of unopsonized S. aureus was abolished. Consequently, it became apparent that FCS and normal calf serum contained a moiety (or moieties?) capable of activating membrane components which are exposed, presumably during surface adherence, to account for the observed potential of adherent monolayers to ingest unopsonized S. aureus.

The stimulatory activity of FCS most probably was directed at phagocyte membrane components (as opposed to bacterial membrane components), because after adherence occurred the monolayers were washed 2-3 times with medium 199 without FCS before any subsequent bacterial challenge.

interacts with neutrophil (membrane?) components that are exposed only after glass adherence. The phagocytosis stimulating activity was not unique to components present in FCS since normal calf serum possessed comparable phagocytosis-stimulating activity on neutrophil monolayers. Cells were also monolayered in med 199 containing 15% BSA. This control was included to rule out the possibility that the phagocytosis-stimulating activity was not due to a nonspecific interaction of neutrophil monolayers with any protein. Monolayers cultured in med 199 + 15% BSA lacked the potential to ingest unopsonized S. aureus (Figure 17).

This finding of a phagocytosis-stimulating activity in FCS and bovine serum is not unprecedented. It has been known for some time that bovine serum (decomplemented) also contains intrinsic factors capable of stimulating pinosome formation in cultivated mouse macrophages (118). More recently, a macroglobulin component of calf serum was found to interact opsonically with motile Bacillus subtilis and Bacillus cereus organisms, mediating their adherence to mouse macrophages in culture (119).

Realizing that the nonspecific phagocytic potential of adherent neutrophils was related to a moiety (or moieties?) present in FCS (and normal calf serum), subsequent experiments were focused on (a) testing the ability of FCS to stimulate nonspecific ingestion of highly encapsulated bacteria; (b) constructing a dose-dependent curve relating the percentage of FCS used in the monolayering protocol with the efficiency of ingestion of S. aureus (opsonized and mock opsonized), and (c) isolating IgG from FCS and investigating its ability to activate the observed ingestion of unopsonized S. aureus.

7. Interaction of Adherent Neutrophils with Unopsonized Encapsulated Bacteria: The capability of unfractionated FCS to stimulate adherent neutrophils to ingest highly encapsulated K. pneumoniae and S. pneumoniae, type III, in the absence of their respective antibodies, was examined (Figure 18). Despite the previous observation that adherent neutrophils, allowed to monolayer in med 199 containing 10% FCS, could ingest S. aureus in the absence of antistaphylococcal antibodies, they could not phagocytose these highly encapsulated bacteria in the absence of their respective antibodies (Figure 18).

8. Phagocytic Capacity of Adherent Neutrophils: Dose Dependency on FCS: Adherent neutrophils were allowed to monolayer onto glass surfaces in med 199 containing 0, .01%, .1%, 1%, 5%, 10%, 15%, and 25% FCS. Subsequently the neutrophil monolayers were challenged with mock opsonized or opsonized S. aureus (Figure 19).

In the absence of antistaphylococcal opsonin I observed a dose dependent relationship between the percentage of FCS used in the monolayering protocol and the efficiency of ingestion of S. aureus by these adherent neutrophils. The peak phagocytic activity was observed when 10% FCS was utilized.

In the presence of immune opsonin, the dose dependent rise in phagocytic efficiency was still apparent, peaking again in the presence of 10% FCS. However, the dose dependent rise was more pronounced in the absence of antistaphylococcal antibodies. With higher percentages of FCS (15%, 25%), the efficiency of ingestion of opsonized and unopsonized S. aureus decreased slightly.

FAILURE OF "ADHERENT" NEUTROPHILS TO
PHAGOCYTOSE UNOPSONIZED ENCAPSULATED BACTERIA

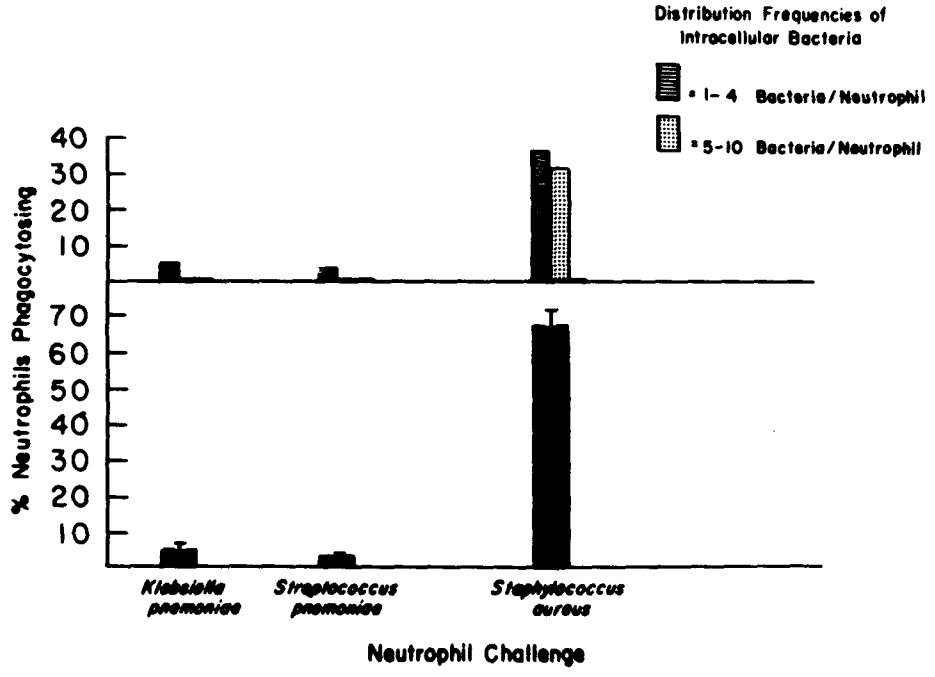


Figure 18: Inability of FCS to stimulate ingestion of unopsonized encapsulated bacteria by adherent neutrophils.

The ability of adherent monolayers (cultured in medium 199 + 10% FCS) to ingest highly encapsulated K. pneumoniae and S. pneumoniae in the absence of their respective opsonic antibodies was studied. Although phagocytosis of unopsonized S. aureus occurred, the phagocytic cells failed to ingest unopsonized encapsulated bacteria.

PHAGOCYTIC EFFICIENCIES OF
"ADHERENT" NEUTROPHILS: DOSE-DEPENDENCY OF FCS

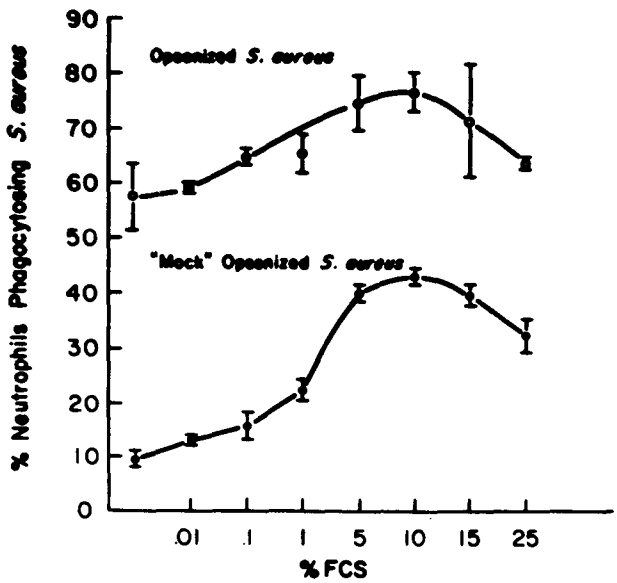


Figure 19: Phagocytic capacity of adherent neutrophils: Dose dependency on FCS.

Adherent neutrophils demonstrated a dose dependent rise in the efficiencies with which they ingested unopsonized and specifically opsonized S. aureus. The optimal activity in both cases was observed when 10% FCS was used to monolayer phagocytes.

When cells were allowed to monolayer in the absence of FCS (0% FCS), although the ability to ingest unopsonized S. aureus was abolished, the cells nevertheless remained functionally active. This was demonstrated by their continued ability to ingest opsonized S. aureus under similar experimental conditions.

9. Characterization and Isolation of IgG from Fetal Calf Serum (FCS): Ouchterlony double diffusion analysis of both FCS and normal calf serum revealed the presence of IgG immunoglobulins in these sera (Figure 20a). The precipitin arc due to the antigen-antibody reaction of bovine IgG (control) + antibovine IgG formed complete identity with that of FCS + antibovine IgG and calf serum + antibovine IgG.

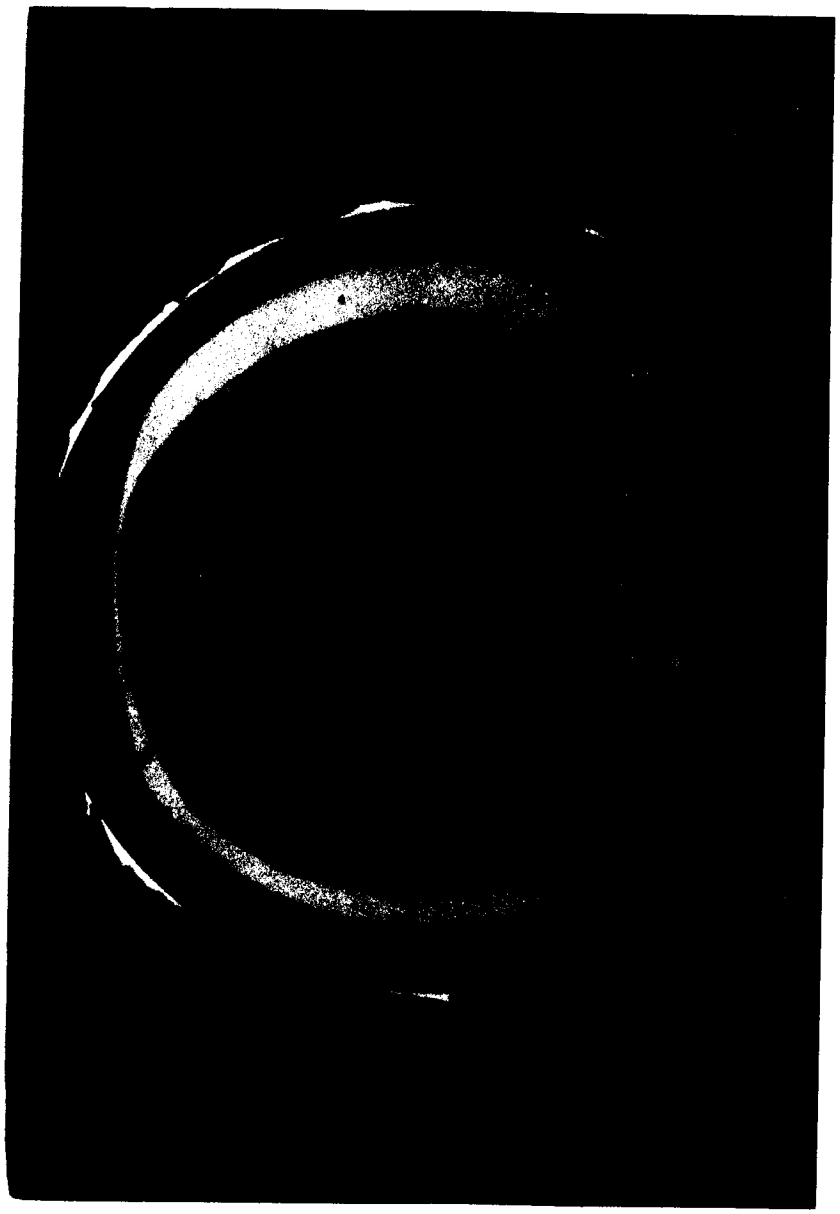
IgG was fractionated from FCS by passing FCS (50 ml) over a DEAE-A50 ion exchange column in .05M PO_4 buffer, pH 7.85 at 4^oC. Figure 20b shows this chromatographic elution profile.

Peaks I and II were cut and concentrated (Amicon concentrator) and subjected to double diffusion analyses against antiserum to normal bovine serum (Figure 20c). Peaks I and II formed a precipitin arc of identity with each other and with bovine IgG (control). This signified that peaks I + II were pure and represented immunoglobulins of the IgG class. Because the goat antibovine normal serum used was against the entire fraction of bovine serum (i.e., not subclass specific), I suspect that peaks I + II probably represented the differential elution of two bovine IgG subclasses.

The estimated concentration of IgG in the FCS was 10 mg/ml; this was a midpoint estimate based on the known concentration range of IgG in normal human serum (8-16 mg/ml).

Both peaks were separately concentrated down to 10 mg/ml, and 10 ml of each peak was added to 90 ml of med 199. Subsequently med 199 + peak I and med 199 + peak II were used to monolayer neutrophils.

10. Effect of FCS-IgG on the Ingestion of Unopsonized Bacteria: Neutrophils were allowed to adhere to glass coverslips in med 199 containing either 10% FCS, 0% FCS, or IgG isolates from FCS (peaks I + II)



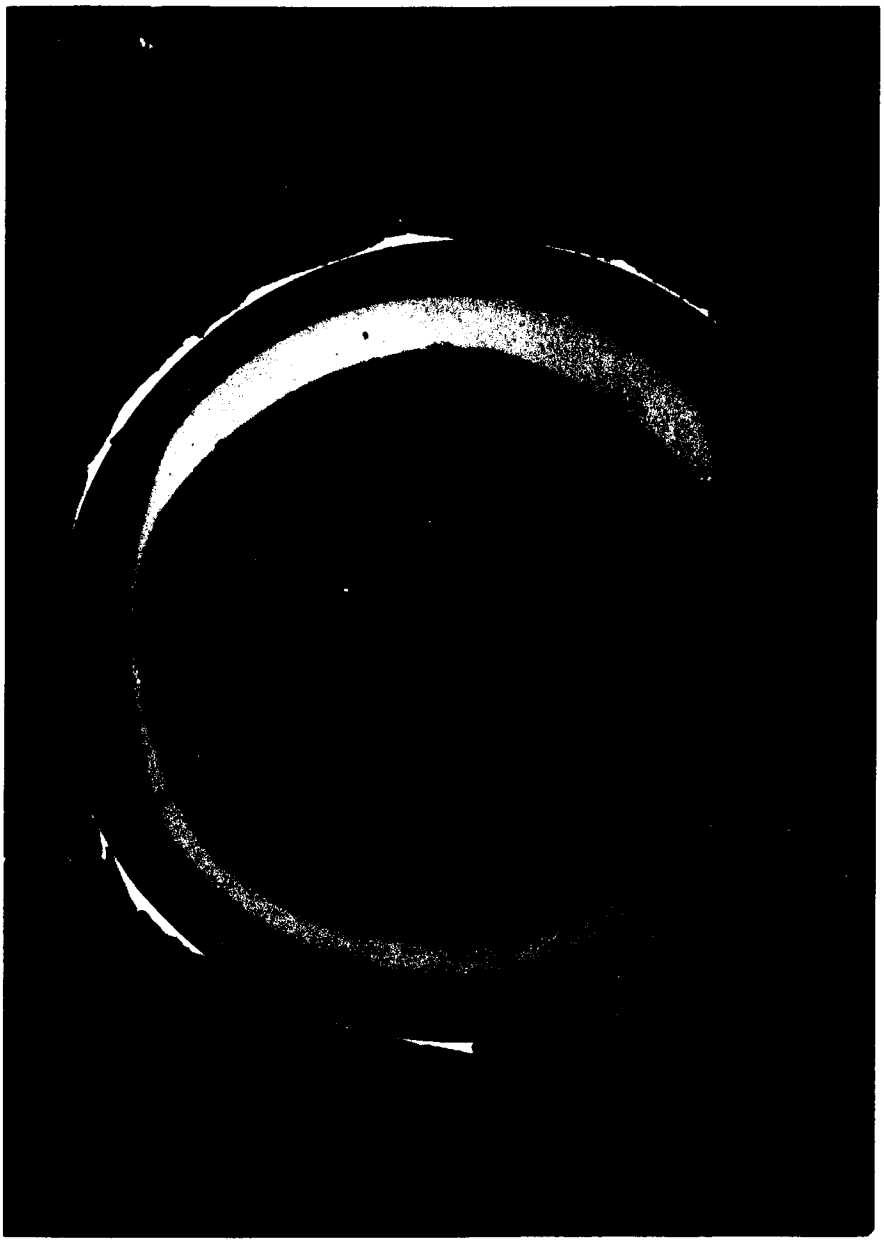


Figure 20: a. Ouchterlony double diffusion analysis demonstrating the presence of IgG in FCS.

This photograph demonstrates the antigenic relationships among bovine IgG (well #2), fetal calf serum (well #1), and adult calf serum (well #3). The center well contains antibovine IgG specific antisera. Thus the FCS used in my studies contained IgG immunoglobulins. The ability of FCS-IgG isolates to stimulate ingestion of unopsonized S. aureus was therefore tested in subsequent studies.

CHROMATOGRAPHIC ELUTION PROFILE

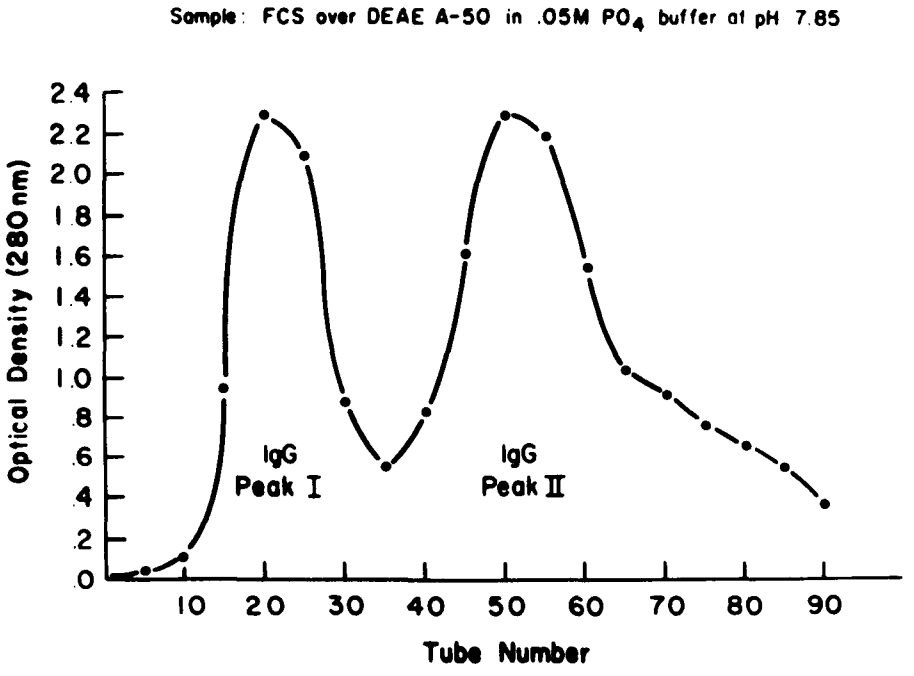


Figure 20. b. Chromatograph elution profile of fractionation of IgG from FCS.

This illustration shows the elution profile obtained when fetal calf serum (50 ml) was subjected to ion exchange chromatographic fractionation over a DEAE-A50 Sephadex column, equilibrated with .05M PO_4 buffer, pH 7.85. Each fraction contains 4 ml. Peaks I and II, the suspected IgG components, were cut, concentrated, and subjected to double diffusion analysis.

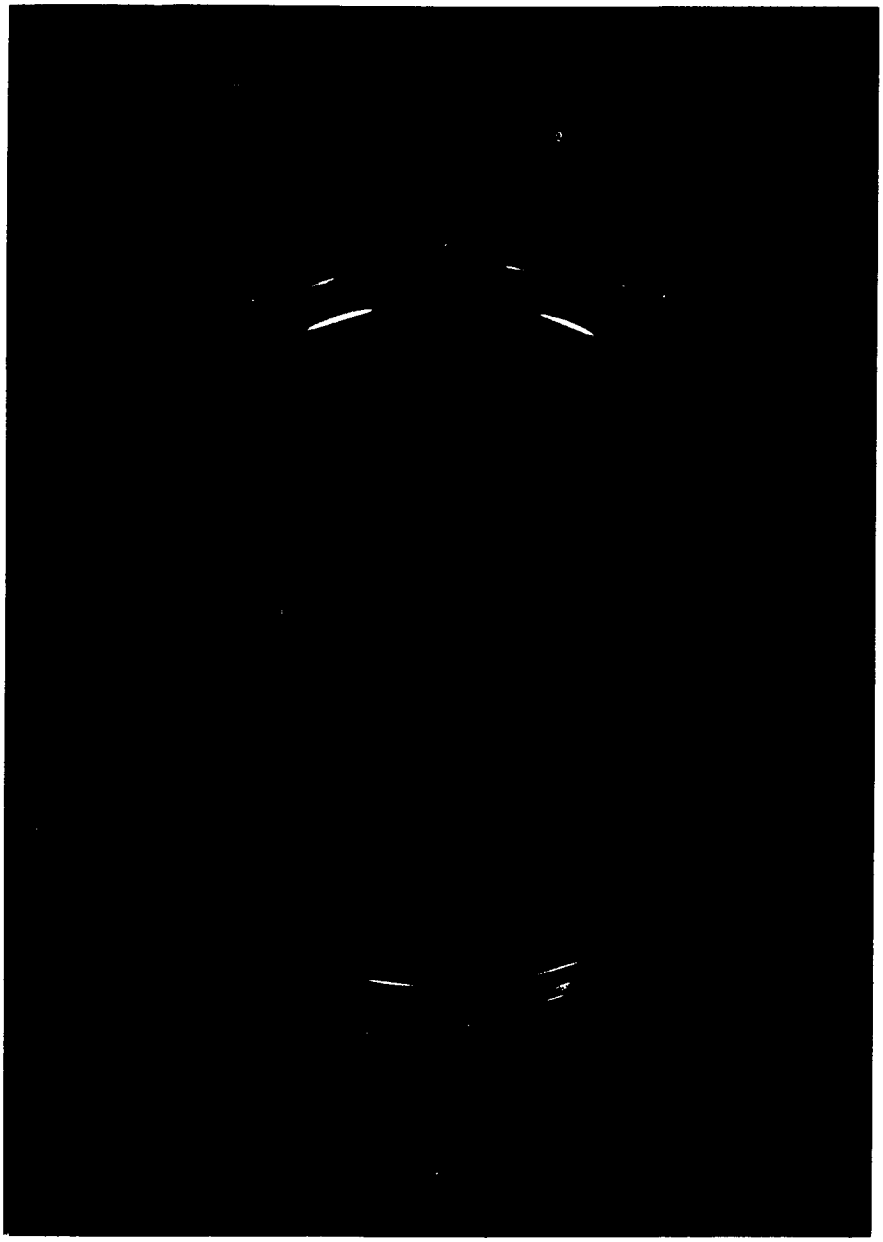
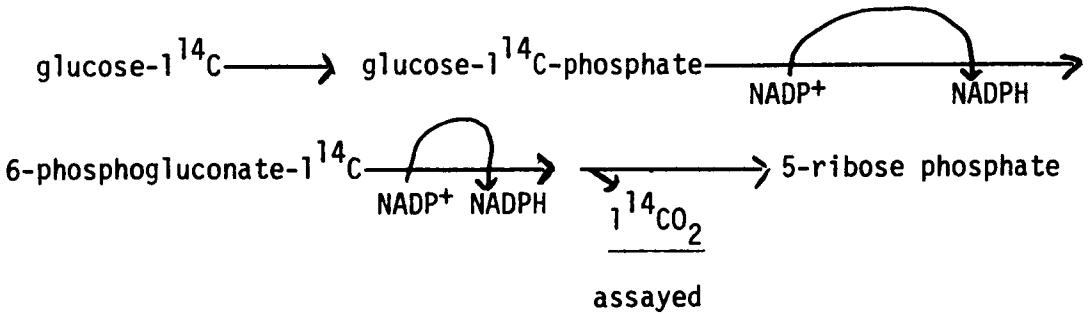


Figure 20: c. Ouchterlony double diffusion analysis demonstrating the purity of IgG isolated from FCS.

This illustration shows antigenic identities among peaks I (well #1) and II (well #2), and bovine IgG (control). The center well contains goat antibovine normal serum. Peaks I and II formed a precipitin arc of identity with each other and with control bovine IgG. Presumably peaks I and II represent the differential elution of two bovine IgG subclasses uncontaminated with any other serum proteins.

(Figure 21). Used at its estimated physiologic concentration (10 mg/ml) in 10% FCS, neither IgG peak potentiated the nonspecific ingestion of unopsonized S. aureus by adherent neutrophils when compared to that activity observed with unfractionated FCS (Figure 17). Therefore, whatever the moiety in FCS responsible for activating nonspecific phagocytosis (ingestion in the absence of immune opsonin) by adherent neutrophils, it was not related to the IgG components.

11. Comparison of Hexose Monophosphate Shunt (HMS) Activities in Both Neutrophil Populations: It has been previously demonstrated that during a phagocytic pulse, metabolic energy is required for efficient ingestion. The energy for ingestion in neutrophils comes largely from the degradation of glucose (glycolysis). In actively phagocytosing cells, some of the glucose is degraded to CO_2 via the HMS (pentose phosphate pathway). Consequently, in actively phagocytosing cells, HMS activity increases 4-10 fold above that observed in resting neutrophils (64, 66). The assay used to assess the oxidation of glucose to CO_2 via the HMS commonly measures the oxidation of radioactively labelled glucose-1^{14}C to 1$^{14}CO_2$. The following scheme represents that pathway:



Hexose monophosphate shunt activity was studied in suspension and adherent neutrophil populations under resting conditions, that is, no

FAILURE OF
FCS-IgG ISOLATE TO STIMULATE PHAGOCYTOSIS OF
"MOCK" OPSONIZED *S. aureus* BY "ADHERENT" NEUTROPHILS

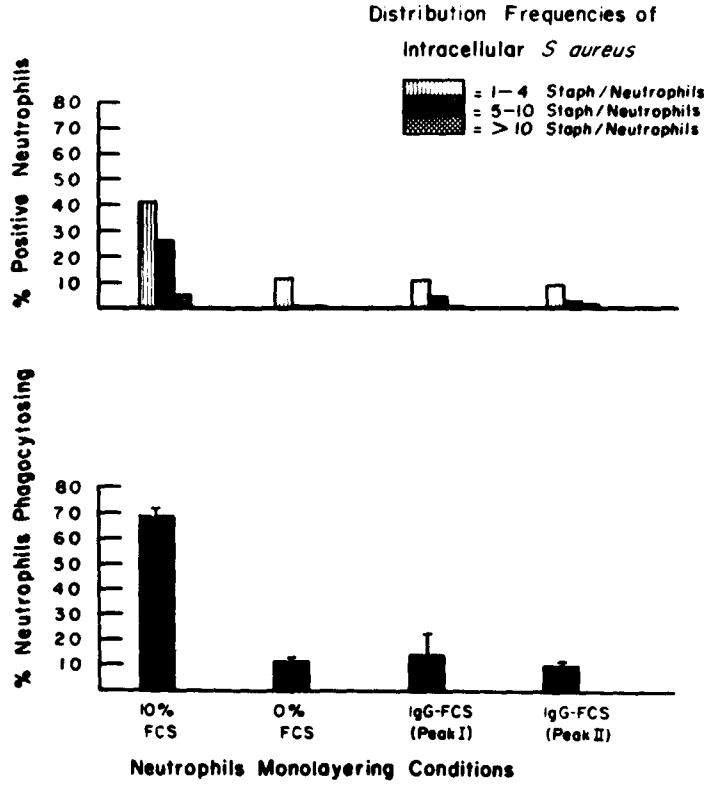


Figure 21: Failure of FCS-IgG isolates to stimulate ingestion of mock opsonized S. aureus by adherent neutrophils.

IgG isolates from FCS (peaks I and II) used at physiologic concentrations in 10% FCS failed to stimulate phagocytosis of unopsonized S. aureus as compared to activity observed with unfractionated FCS.

phagocytic challenge, and under conditions of a phagocytic challenge with opsonized or mock opsonized bacteria (Figure 22).

In terms of HMS activity, there were no apparent differences between the two neutrophil populations under resting conditions. However, it should be kept in mind that HMS activity was assayed after the cells adhered to glass surfaces. It is quite conceivable that metabolic differences in HMS activity may be observed if assayed in resting populations as the adherence phenomenon is initiated.

Under conditions of a phagocytic challenge with mock opsonized S. aureus, there were no significant differences between the two neutrophil populations. This finding was unexpected because, by microscopic analysis, adherent neutrophils were, by far, more actively phagocytic.

Both neutrophil populations showed evidence of an opsonic effect. When challenged with S. aureus-antistaphylococcal antibody complexes, there was approximately a 4-fold increment in HMS activity (Figure 22).

D. Discussion

This study has shown that physiologic differences are demonstrable between populations of human neutrophils that are adherent to glass surfaces as compared with comparable populations that are maintained in suspension during phagocytosis (Figure 5).

Specifically, the ability of suspension versus adherent neutrophil populations to phagocytose coagulase positive pathogenic strains of S. aureus of phage types 80/81, 52, 52A, and the Cowan S. aureus strain was investigated. Clear differences were observed in the phagocytic efficiencies of the two neutrophil populations. In the absence of antistaphylococcal antibodies, adherent neutrophils showed a sub-

COMPARISON OF
HEXOSE MONOPHOSPHATE ACTIVITY IN
"ADHERENT" AND "SUSPENSION" NEUTROPHILS

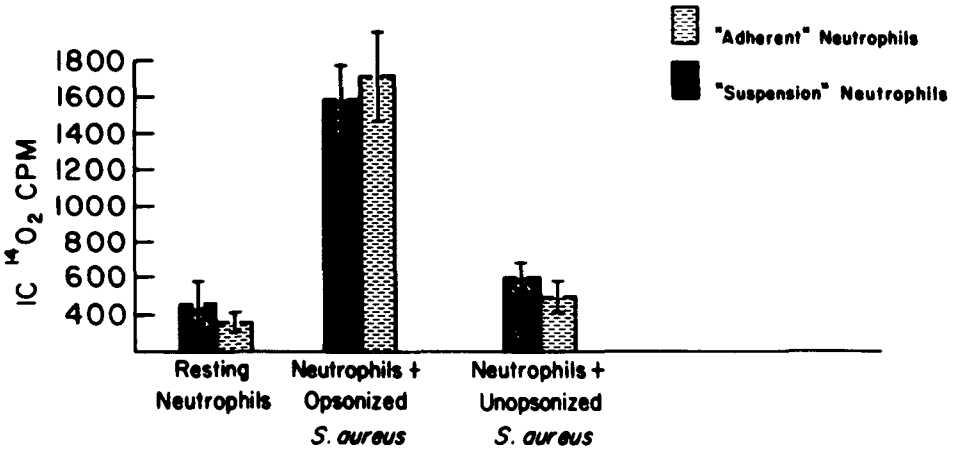


Figure 22: Comparison of hexose monophosphate shunt activities in adherent and suspension neutrophils.

Glucose degradation to CO_2 via the hexose monophosphate shunt was compared in adherent and suspension neutrophil populations under resting conditions (no phagocytic pulse), and after challenge with opsonized and unopsonized S. aureus. No significant differences in HMS activity was observed in the two cell populations under resting conditions or during active phagocytosis.

stantial qualitative and quantitative efficiency of ingestion. In contrast, suspension neutrophils, in the absence of opsonin, showed relatively low or negligible levels of ingestion of these organisms (Figures 8, 9a, 9b). I postulate that the adhesion of neutrophils to glass surfaces may, by some yet unknown mechanism, activate the phagocytic process to account for these differences. These observations could have important in vivo correlates in acute S. aureus infections such as endocarditis. For example, when neutrophils have entered extravascular sites of tissue injury where concentrations of opsonic antibodies are presumably lower than that in the bloodstream, or during the acute stages of inflammation, when antibody titers are low (i.e., preantibody phase of infection), phagocytosis of these organisms might still occur very efficiently. Phagocytosis of encapsulated bacteria in vivo, in the absence of opsonizing antibody, was first demonstrated in experimental lesions produced with a type I pneumococcus and K. penumoniae (120). In both subcutaneous and pulmonary lesions phagocytosis was evident within the first few hours of infection, although no specific antibody could be found in the blood or local tissues. Specific in vitro investigations revealed that this form of phagocytosis resulted from a trapping of the bacteria by leukocytes against tissue surfaces (120, 121). This phenomenon of "surface phagocytosis" and my observations with adherent neutrophils could be due, in part, to membrane activation during the adherence of phagocytes to the surfaces of glass or inflammed tissues.

Another distinction between the two neutrophil populations was observed in inhibition studies. When suspension neutrophils were challenged with specifically opsonized S. aureus, a pronounced opsonic enhancement of ingestion was observed. This opsonic effect could be

inhibited by preincubation of suspension neutrophils with free IgG3 or its Fc fragment (Figures 12, 13a, 13b). In contrast, when adherent neutrophils were challenged with opsonized S. aureus, phagocytosis was qualitatively and quantitatively augmented above that which occurred in the absence of specific antibodies. However, this could not be inhibited or blocked by preincubation of adherent cells with free IgG3 or its Fc fragment (Figures 14, 15a, 15b). These data imply that the postulated neutrophil membrane bound IgG-Fc receptor (53, 55) becomes inaccessible for inhibition during the adherence of these cells to glass surfaces. This may be related to a spatial reorientation or density redistribution of IgG-Fc receptor sites after neutrophils have adhered to glass (or tissue) surfaces. It is also possible that the binding of free IgG3 to adherent neutrophils is weak so the protein can easily be displaced by S. aureus-antistaphylococcal immune complexes. The latter possibility is not unprecedented because it has been shown that radiolabelled antigen-antibody complexes have tighter binding affinities to mouse macrophages than the binding conferred by antibody alone (122, 123).

Although I observed a substantially heightened ability of adherent neutrophils to phagocytize S. aureus very efficiently in the absence of antistaphylococcal antibodies, this activity could not be demonstrated with highly encapsulated K. pneumoniae or S. pneumoniae in the absence of their specific antibodies (Figure 18). A precise explanation for this specificity is not yet available but may be related to the antiphagocytic capsules surrounding these organisms.

In this study, it became apparent that a moiety in FCS and normal calf sera (decomplemented) could be correlated (when used in

the monolayer protocol) with the efficient ingestion of unopsonized S. aureus by adherent neutrophils. This activation by FCS was dose dependent. Previous investigations have shown that calf sera contain intrinsic factors capable of stimulating pinosome formation and opsonically mediating adherence of motile bacteria to mouse macrophages (118, 119).

I isolated IgG from FCS. Subsequent utilization of the IgG isolate (in the monolayer protocol) revealed that phagocytosis-stimulating activity could not be ascribed to the IgG components of FCS.

Scanning electron microscopic analysis has revealed differences in plasma membranes of glass adherent versus suspension neutrophils (124). Electron microscopic studies of macrophage attachment have shown that cells first extend finger-like projections and veil-like processes which are very similar to the morphological changes in membranes that occur during phagocytosis. Electron microscopic analyses of glass-attached and free surfaces of cultivated macrophages have shown differences in the orientation of microtubules and microfilaments in the subplasmalemmal regions. Moreover, after a phagocytic pulse with polystyrene particles, microtubular and microfilamentous components of free surfaces translocated and oriented themselves in a manner very similar to that seen in the glass attached surfaces (62).

The studies presented here have demonstrated that in the absence of immune opsonin, adherent but not suspension neutrophils represent an actively more phagocytic population. Previous investigations of enhanced phagocytosis by adherent phagocytes (125, 126) are all consistent with the postulate that cell attachment to glass (4, 5, 124), cellular spreading on glass (6, 103), and phagocytosis, are all func-

tionally related phenomena dependent on alterations, activations, and perturbations of the cell membrane.

III. INTERACTION OF ADHERENT VERSUS SUSPENSION NEUTROPHILS WITH IgG-COATED AND COMPLEMENT-COATED ERYTHROCYTES

A. Introduction: Neutrophils have membrane receptors for the Fc portion of IgG (53, 55) and for C3b, the modified third component of serum complement (41, 43). These receptors have usually been demonstrated by light microscopy of neutrophil monolayers forming rosettes with and/or ingesting erythrocytes coated with IgG antibodies (EA-7S) or coated with antibodies and complement components (EAC). It is generally believed that these membrane bound IgG-Fc and C3b receptors mediate and facilitate the attachment and subsequent interiorization of immune complexes by phagocytic cells. Previous studies have shown significant differences in the phagocytic efficiencies of suspension versus adherent neutrophils when challenged with unopsonized bacteria (126, 127). Generally, neutrophils which have adhered to glass surfaces and are subsequently challenged with unopsonized S. aureus are more efficient phagocytes than neutrophils in suspension. When challenged with specifically opsonized S. aureus, both neutrophil populations showed an opsonic enhancement of ingestion. However, opsonization by suspension neutrophils was inhibitable by preincubation of the cells with an IgG3 myeloma protein or its Fc fragment. Opsonization by adherent neutrophils was not inhibited under similar conditions (127). Since neutrophils have extremely short half-life in the bloodstream (6-7 hours) (3, 11), and reside mainly in extravascular sites and tissue surfaces, particularly during the acute stages of inflammation, I believe that the adherent cell system may more closely reflect the in vivo situation.

The objective of this aspect of the study was to investigate possible differences in C3 receptors between suspension and adherent neutrophil populations. The results indicate that adherent neutrophils possess the capacity to bind to and ingest EAC immune complexes very efficiently, while suspension neutrophils show negligible EAC binding.

B. Materials and Methods

1. Isolation of Neutrophils: Human neutrophils were isolated from peripheral whole blood by dextran sedimentation and hypotonic lysis and resuspended to a concentration of 2×10^6 ml in med 199 + 10% FCS, as described elsewhere (Chapter II).

2. Isolation of Total WBC: For studies concerning monocytes and neutrophils, total white blood cells (WBC) were isolated from peripheral blood. Venous blood was collected from healthy human donors into 1/7 volume of ACD (anticoagulant).

The tubes containing the blood were filled with an equal volume of 6% dextran in pyrogen free .15M NaCl. The RBC were allowed to sediment for 60-90 minutes at 37°C. The supernatant containing mononuclear and polymorphonuclear leukocytes and residual RBC was taken and pelleted at 1300 rpm for 10 minutes. The pellet was resuspended in 5-10 ml of .87% NH_4Cl in H_2O to lyse residual RBC. After 30 minutes at room temperature the tubes were filled with med 199 + 10% FCS and the leukocytes sedimented at 1300 rpm for 10 minutes at 4°C. Subsequently, the pellet was washed 2-3 times with large volumes of med 199 + 10% FCS and the cells resuspended to a concentration of 2×10^6/ml in this medium. This protocol was extremely similar to the neutrophil isolation protocol except that the step involving the removal of the "buffy coat" (predominantly lymphocytes and monocytes) was omitted here.

3. Preparation of EA and EAC Immune Complexes: The method utilized to sensitize sheep red blood cells (SRBC) with antibody and/or complement components was adopted from previous studies (128, 129).

10 ml of SRBC in Alsever's solution (Cordis Laboratories) were washed 4 times with PBS, pH 7.0 (0.5M Na_2HPO_2 in .15M NaCl was titrated to pH 7.0 with 1M Na_2PHO_4). After the final wash the supernatant was discarded, the RBC pellet resuspended in 25 ml PBS, and the concentration of SRBC enumerated by hemocytometer analysis. The erythrocytes were resuspended to a concentration of 2×10^9/ml and 1 ml aliquots were dispensed into 12mm x 75mm 5 ml plastic tubes (Falcon Plastics). Each tube was centrifuged at 2000 rpm for 2 minutes and resuspended in .9 ml of med 199.

Sheep erythrocytes (E), were sensitized with either subagglutinating dilutions of rabbit anti-Forssman antisera containing only the IgG fraction (EA-7S), the IgM fraction (EA-19S), or both the IgG and IgM fractions (EA-hemolysin). All anti-Forssman antisera were obtained from Cordis Laboratories and are directed against membrane bound Forssman antigens on the RBC membrane. Forssman antigens are possessed by a variety of phylogenetically unrelated species (heterogenetic antigens) and found usually in the tissues and organs. However, in sheep, the Forssman antigen is present on the red blood cells and usually absent in the tissues and organs of sheep (130). Anti-SRBC antisera (7S, 19S, or hemolysin) were diluted 1:16, 1:40, and 1:80 with PBS, pH 7.0. .1 ml of the respective dilutions was added to .9 ml of SRBC (2×10^9) to give final antibody dilutions of 1:160, 1:400, or 1:800 respectively. As a control, a source of unsensitized SRBC (E), .9 ml SRBC were mixed with .1 ml of PBS. All EA and E (control) com-

plexes were incubated at 37°C for 30 minutes in a gently shaking water bath. After 30 minutes, the sensitized RBC (EA_s) were washed 3 times with PBS and resuspended to a volume of .9 ml with PBS and used for the preparation of EAC, or the pellets were resuspended in 1 ml of med 199 (2×10^9 RBC/ml). For EA studies, .1 ml of the latter EA preparations was added to 2×10^6 neutrophils (or leukocytes). Thus the final ratio of RBC:phagocytes was 100:1. In studies concerning the binding of antibody coated RBC to neutrophils, EA-7S was always employed at final antibody dilutions of 1:160, 1:400, or 1:800.

For the preparation of EAC complexes, fresh human serum (isologous) served as a source of complement proteins. 3 ml of venous blood was collected and allowed to clot at 37°C for 1-2 hours. The clotted blood was centrifuged at 3000 rpm for 20 minutes, the serum collected and diluted 1:10 with PBS. .1 ml of this dilution was added to .9 ml of washed EA (prepared with the hemolysin fraction at a final dilution of 1:800) and incubated at 37°C for an additional 60 minutes in a gently shaking water bath. After 60 minutes, EAC complexes were washed 3 times with PBS and resuspended to a volume of 1 ml (2×10^9 RBC/ml) in med 199. .1 ml of the EAC (2×10^8 RBC/ml) mixture was added to 2×10^6 neutrophils or leukocytes. Thus, the final dilution of serum was 1:100 and that of antibody was 1:800 in all EAC preparations.

4. Inhibition Studies with Anti-C3: Preparation of EAC-Anti-C3 Complexes: For the preparation of EAC + anti-C3 complexes, .1 ml of goat anti-human C3 antiserum (anti-beta 1C-Cordis Laboratories) was added to .1 ml of a 1:10 dilution of fresh human serum, which served as a source of C3. The serum + anti-C3 mixture was incubated for 30-45 minutes at 37°C in a gently shaking water bath.

1 ml of EA, prepared with the hemolysin fraction at a dilution of 1:800 and containing 2 x 10^9 RBC/ml, was washed 3 times with PBS, pH 7.0 and resuspended to a volume of .8 ml. Subsequently .2 ml of the C′-anti-C3 mixture was added to .8 ml of the EA complexes and incubated for 60 minutes, gently shaking, in a 37°C water bath. Afterwards, this EAC-anti-C3 mixture was washed 3 times with PBS, pH 7.0 and resuspended to a volume of 1 ml (2 x 10^9 RBC/ml). .1 ml of EAC anti-C3 immune complexes was added to 2 x 10^6 neutrophils or 2 x 10^6 leukocytes, thereby maintaining the 100:1 ratio of RBC:phagocytes.

5. Phagocytic Assays: Phagocytosis was assayed with adherent and suspension neutrophil (or leukocyte) populations as described and detailed in Chapter II, with the following modifications: E, EA, EAC, and EAC + anti-C3 complexes were incubated with both neutrophil populations at a 100:1 ratio of RBC neutrophils. Phagocytosis proceeded for 1 hour at 37°C and was stopped in all tubes by the addition of one drop of 10% sodium azide.

The extent of phagocytosis was quantitated by light microscopic examination at 1000 x magnification on stained coverslips mounted on glass microscopic slides. Neutrophils (or leukocytes) were scored positive for ingestion if they contained one or more intracellular RBC/neutrophil; neutrophils were scored as a positive rosette (positive for attachment) if they were surrounded by three or more membrane-bound RBC/neutrophil. At least 100 neutrophils were enumerated per coverslip and the total percentage of positive neutrophils was recorded. The specific type of interaction (attachment and/or ingestion) was also determined by separately scoring the distribution frequency (per 100

neutrophils) of the percentage of neutrophils with RBC-rosettes only, the percentage of neutrophils with ingested RBC only, and the percentage of neutrophils showing combined resettes and ingestion. All studies were performed in duplicate or triplicate.

C. Results

1. Interaction of Adherent and Suspension Neutrophils with IgG Coated Erythrocytes (EA-7S Complexes): Sheep red blood cells were immunologically coated with the IgG fraction (7S) of rabbit anti-SRBC antisera (Cordis Laboratories). Controls consisted of uncoated erythrocytes (E). The interaction of these E and EA-7S complexes with both populations of neutrophils was investigated (Figure 23).

Neither neutrophil population interacted nonspecifically with unsensitized E. Neither neutrophil population bound to (rosette formation) nor ingested specifically coated EA complexes. This latter negative finding was not related to technical manipulations because monocytes, which often comprised a 5-10% contamination, still showed EA-7S binding and/or ingestion (Figures 24, 25).

2. Differential Capacity of Adherent and Suspension Neutrophils to Bind and Ingest Complement-Coated Erythrocytes (EAC Complexes): In this study, serum complement was used to sensitize freshly prepared EA complexes to form new complexes of EAC. The interaction of EAC complexes with both neutrophil populations was then studied (Figure 23).

Adherent neutrophils demonstrated a pronounced ability to specifically bind and ingest EAC complexes. Attachment of EAC to the neutrophil membrane (rosette formation) predominated over ingestion (Figure 23, see top half of graph). EAC rosette formation around adherent

INTERACTION OF
"ADHERENT" vs "SUSPENSION" NEUTROPHILS WITH
Ab-COATED & COMPLEMENT-COATED ERYTHROCYTES

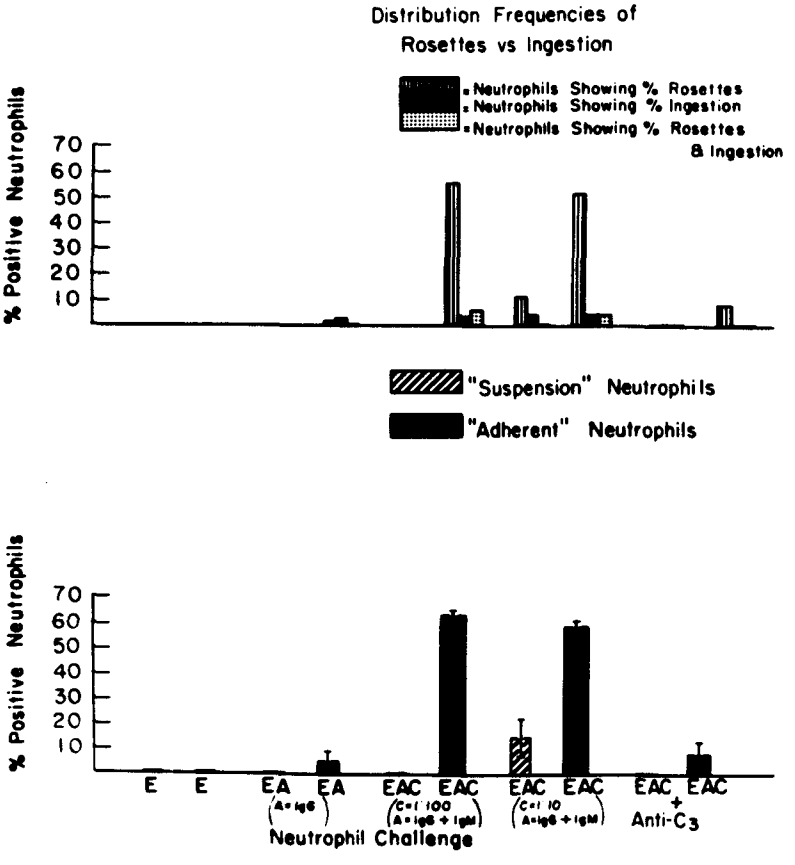


Figure 23: Interaction of adherent and suspension neutrophils with IgG-coated and complement-coated erythrocytes (EA and EAC).

The capacities of suspension and adherent neutrophil populations to bind and/or ingest erythrocytes immunologically coated with IgG antibody (EA) or antibody (IgM + IgG) and complement components (EAC) were compared. Adherent neutrophils efficiently bound to and ingested EAC complexes; attachment and rosette formation predominated over ingestion. This finding is consistent with very recent studies which show that the C3 receptor is primarily involved in the attachment phase of phagocytosis (59, 60). This EAC binding was inhibited by preincubation of C' with anti-C3 antisera. In contrast, suspension neutrophils lacked significant ability to bind to or ingest EAC complexes.

Neither neutrophil population significantly bound nor nigested EA-7S complexes.

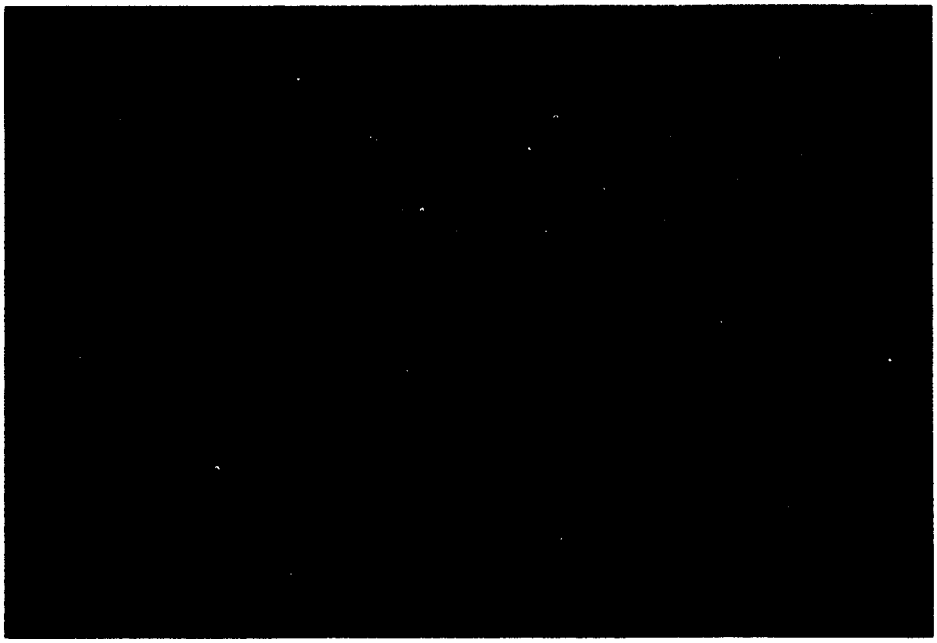
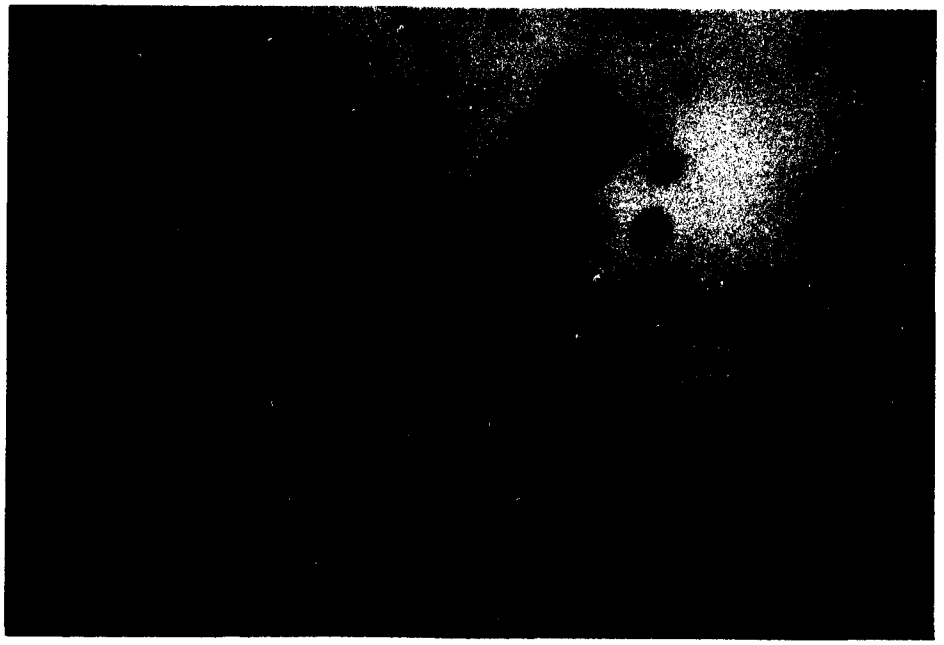


Figure 24: Light micrographs (1000 x magnification) demonstrating the failure of suspension neutrophils to bind IgG-coated erythrocytes (EA).

Suspension neutrophils failed to interact with IgG coated RBC. However, also seen in the field are contaminating monocytes which demonstrated rosette formation and ingestion of EA.



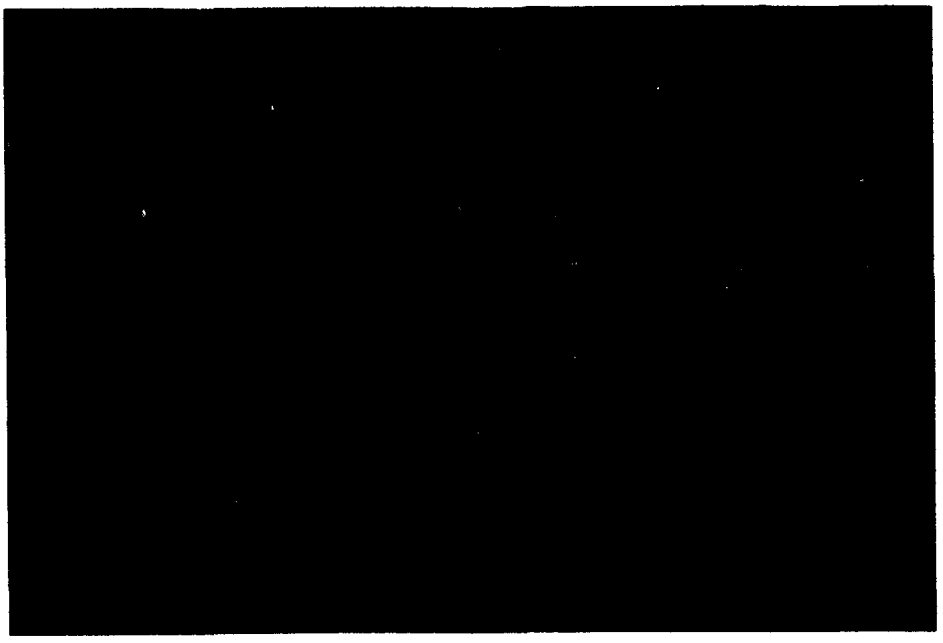


Figure 25: Light micrographs (1000 x magnification) showing the failure of adherent neutrophils to bind IgG-coated erythrocytes (EA).

Adherent neutrophils also failed to effectively interact with EA-IgG complexes. However, monocytes again contaminating the preparation (5-10%) showed efficient ingestion and rosette formation of these complexes. The reasons for this are discussed and may be related to quantitative differences in the number of IgG receptor sites on monocytes versus neutrophils.

neutrophils was evident at both dilutions (1:10 and 1:100) of serum complement (Figure 26).

By preincubating serum complement with anti-C3 antisera before interaction with EA, EAC142 complexes but not EAC1423 complexes will be formed. In this manner, one can demonstrate the binding specificity for the modified, enzymatically split, third component of complement, C3. In these experiments, I could inhibit the binding of EAC to neutrophils by preincubating complement with anti-C3 specific antiserum. This finding agrees with recent report by other investigators (41, 43). Figure 27 is a light micrograph demonstrating this inhibition of EAC binding to neutrophils by an anti-C3 specific antiserum.

In contras^{+}, however, suspension neutrophils neither bound nor ingested EAC complexes when prepared with a 1:100 dilution of complement (Figure 23), whereas monocytes contaminating the suspension system demonstrated clear EAC binding and ingestion (Figure 28). At a 1:10 dilution of complement, EAC binding (rosetting) was evident in approximately 15% of the suspension neutrophil populations and in 60% of the adherent cell populations. In both systems, this binding was inhibitable with anti-C3 antiserum.

D. Discussion

In this investigation the capacities of suspension and adherent neutrophil populations to bind to and/or ingest erythrocytes sensitized with antibody (EA) and erythrocytes sensitized with antibody and complement components (EAC) were compared. I consistently observed significant differences in the capacities of the two neutrophil populations to bind and ingest EAC complexes. Adherent neutrophils

Figure 26: Light micrographs (1000 x magnification) demonstrating EAC rosette formation around adherent neutrophils.

Adherent neutrophils demonstrated an appreciable ability to bind and ingest C'-coated RBC with rosette formation predominating over ingestion. This demonstrates another significant distinction between adherent and suspension neutrophils.

Figure 27: Light micrograph (1000 x magnification) demonstrating inhibition of EAC binding to adherent neutrophils by anti-C3 antiserum.

EAC binding to adherent monolayers could be inhibited by preincubating serum C' with anti-C3 specific antisera.

Figure 28: Light micrograph (1000 x magnification) showing the failure of suspension neutrophils to bind EAC complexes.

Suspension neutrophils failed to interact with C' coated RBC. However, a contaminating monocyte is seen in this field demonstrating competent and clear EAC rosette formation.

showed a striking ability to bind and ingest EAC (Figures 23 and 26). Attachment and rosette formation predominated over ingestion. In contrast, suspension neutrophils lacked the ability to bind or ingest EAC complexes. In all cell suspensions, contaminating monocytes (5-10% of the total cell population) did demonstrate the capacity to ingest and bind EAC complexes (Figure 28). At a 1:10 dilution of serum complement (EAC, 1:10), although some C-dependent RBC hemolysis did occur, I was able to demonstrate basal level binding of EAC to suspension neutrophils (15%). However, the rosette formation was never as extensive (quantitatively and qualitatively) as for adherent neutrophils (60%). EAC binding in both systems was inhibited by preincubation of serum C' with anti-C3 antiserum (Figures 23 and 27). These distinctions in the capacities with which suspension (0-15%) and adherent (60%) neutrophils bind C' coated red blood cells (EAC) suggest quantitative differences in the numbers of exposed and/or accessible membrane bound C3 receptor sites on the two neutrophil populations. Perhaps during adherence, additional C3 sites are exposed or locally aggregated at the cell surface, permitting more efficient EAC binding.

Neither neutrophil population significantly bound or ingested EA-7S complexes. However, the contaminating monocytes did show evidence of ingestion and rosette formation (Figures 23-25). Failure to demonstrate EA binding to neutrophils while monocytes showed efficient EA binding (54, 129) may be related to differences in the number of IgG receptor sites, or in their spatial arrangement, orientation and/or density on the membranes of monocytes and neutrophils. Previous investigations have shown that EA binding to neutrophils required the use of hyperimmune serum (55), which suggests that a greater num-

ber of IgG molecules must be present on the erythrocyte membrane for effective neutrophil interaction.

The marked differences in the efficiencies of adherent versus suspension neutrophils to bind EAC complexes implies differences in the spatial orientation and availability of C3 receptor sites on the two neutrophil populations. I am not suggesting that suspension neutrophils lack C3 receptor sites. The efficient ingestion and killing of virulent and/or encapsulated bacteria by suspension neutrophils clearly requires the participation of complement components (131-133). These observations suggest that neutrophils kept in suspension during phagocytosis do indeed possess receptor sites for C3. The inability to show EAC binding with suspension neutrophils in the present study merely suggests that not enough C3 receptor sites are present on the suspension cell surface for effective EAC interaction (i.e., C3-mediated cytoadherence).

The 'fluid mosaic model' of membrane structure (92) implies that physical or chemical perturbations of the cell membrane, such as surface adherence, may alter the orientation or distribution of membrane components. In accordance with this concept the cell membranes of phagocytic cells exhibit various kinds of mechanical behavior which are under metabolic control. Morphologic studies have shown that as relatively spherical macrophages settle and adhere to glass surfaces, their surfaces flatten and are thrown into irregular flaps and ruffles, with extensive cytoplasmic projections and microvillus formation (4). Scanning electron microscopy has shown that the primary points of attachment between EAC complexes and lymphocytes are through the tips of the microvilli, suggesting that these areas may have specialized recognition

functions (134). During the phenomenon of glass adherence, phagocyte membranes undergo extensive microvillus formation. The observation that the macrophage (membrane) can be induced to spread during glass adherence on various substrates (6, 102, 103) and that neutrophils also have this property (Douglas, S. D., unpublished observation) is further evidence for membrane mobility and deformability.

My studies have shown distinct differences in the capacities of adherent versus suspension neutrophils to bind and ingest EAC complexes. These findings may be related to differences in the localization and orientation of C3 receptor sites on the membranes of the two neutrophil populations. My tentative hypothesis is that during adherence of neutrophils to glass a rearrangement and reorganization of membrane bound C3 receptor sites occurs such that additional sites are exposed at the cell surface and become accessible for efficient EAC binding. Proof of this hypothesis could perhaps be substantiated by electron microscopic studies of the distribution and extent of binding of ferritin-labelled antigen-antibody-complement complexes on suspension and adherent neutrophil populations.

GENERAL DISCUSSION

Neutrophils, monocytes, and macrophages possess surface membrane receptors for IgG-Fc (54, 55) and the modified third component of complement (41, 42), specifically C3b (43). It is generally felt that these function as opsonic receptors and are critical for efficient opsonization.

The membranes of phagocytes also exhibit properties of extensive plasticity, motility and deformability. Clearly these properties are critical attributes for the extensive membrane movements, pseudopodial formations, and evaginations which are integral aspects of chemotaxis, particle engulfments, and lysosomal secretion. Consistently, these phagocytes have an unusual capacity to adhere to glass surfaces in vitro (4, 5, 107). Subsequent motility and spreading of the plasma membranes can be induced (6, 102, 103). However, the extent to which adherence alters function is not yet clear.

Scanning electron microscopic analyses have shown morphological and topographical differences in the plasma membranes of glass adherent versus suspension neutrophils (124). Transmission electron microscopic analyses have shown differences in the orientations of microtubules and microfilaments in the plasmalemma of glass attached and free surfaces of macrophages (62). In the mid sixties, it was shown that after in vitro cultivation of blood monocytes on glass surfaces, a sequence of biochemical and morphological changes occurred whereby these cells matured into tissue macrophages (108). Related studies also demonstrated that alveolar macrophages exhibit 30 times the phagocytic capa-

city towards particles after 3-4 weeks in culture on glass surfaces than that seen after one day (125).

The question of whether any differences exist in the phagocytic efficiencies of adherent versus suspension neutrophil populations was a major objective of my thesis investigation. This project was proposed in the hope of gaining insight on how adherence influences phagocytic function. The studies reported in this thesis show that human neutrophils possess distinct differences in their phagocytic capacities towards S. aureus organisms depending upon whether they were in suspension cultures or monolayered on glass surfaces. Suspension cells phagocytosed the bacteria to a significant extent only in the presence of opsonic antibody and this phagocytosis could be blocked by preincubation of neutrophils with a human IgG3 myeloma protein or its Fc fragment. In contrast, the monolayered cells (adherent) actively phagocytosed various pathogenic staphylococcal organisms in the absence of opsonic antibody. The activation of monolayered neutrophils is postulated to occur through perturbations of the membrane during the adherence phenomenon. In the presence of opsonin, an increment of ingestion occurred but this opsonic effect could not be blocked by the IgG3 protein or its Fc component.

Neutrophils have an extremely short half life in the bloodstream (6-7 hours). Their residence in extravascular sites and on tissue surfaces is on the order of 4-5 days (3, 11). Consequently I felt that the adherent cell system was a closer reflection of what may actually be occurring in vivo. Thus, in terms of physiological consequences, it makes more sense that specific opsonization remained uninhibitable in the presence of free unbound IgG.

Studies focused on examining differences in the C3 opsonic receptor are noteworthy. Adherent cells bound to and ingested C3 opsonized RBC. This specific ligand-receptor interaction was inhibitable with anti-C3 antisera. In contrast, suspension neutrophils did not appreciably bind to or ingest these EAC3 complexes under comparable assay conditions.

In accordance with the fluid mosaic model of membrane structure (92), my tentative hypothesis is that glass adherence physically perturbs the membrane and alters the topographical orientation and density distribution of opsonic receptor membrane components. Perhaps during adherence additional C3 sites are exposed or existing ones are locally clustered or microaggregated at the cell surface, thereby permitting more efficient EAC3 binding (cytoadherence). In the same fashion, local microaggregation of Fc receptors may also occur in the adherent state. The consequence of surface receptor aggregation could reasonably be increased binding affinity of antigen-antibody complexes with the clustered Fc receptors. This could explain why free unbound myeloma IgG failed to inhibit the adherent cell system. Possibly myeloma binding is weak and can be displaced by higher affinity Ag-Ab complexes.

Support for this hypothesis comes from recent analysis on the specific binding of EAC3 complexes to lymphoblastoid Raji cells. Reduction of free membrane movement by treating Raji cells with glutaraldehyde abolished C3 dependent cytoadherence (rosette formation). Immunofluorescent studies with soluble C3 and anti-C3 conjugated FITC (fluorescein isothiocynate) revealed that the binding of C3 induced microaggregation and a patchy distribution of membrane bound C3 receptors (135). The hypothesis is that local microaggregation of C3

receptors is a prerequisite for efficient C3 mediated cytoadherence. These findings lend support to the postulate stated above, which is based on data presented in this thesis investigation.

SUMMARY

This investigation has shown that physiologic differences are demonstrable between populations of neutrophils that are adherent to glass surfaces as compared with comparable populations that are maintained in suspension during phagocytosis. Specifically differences were observed during nonspecific ingestion, during IgG-mediated opsonization, and lastly during C3-mediated opsonization (cytoadherence).

1. Both populations of neutrophils were challenged with various coagulase (+) pathogenic strains of S. aureus (80/81, 52A, 52, and the Cowan strain). When challenged with unopsonized S. aureus, neutrophil monolayers (adherent) demonstrated adequate phagocytosis. In contrast, suspension neutrophils showed negligible levels of phagocytosis of all strains tested. Evidence indicated that in the absence of immune IgG opsonins, neutrophil monolayers represented a more efficient phagocytic population.

2. When suspension neutrophils were challenged with S. aureus opsonized with antistaphylococcal antibodies, a pronounced enhancement on ingestion was observed. This augmented ingestion was inhibitable by preincubating neutrophils with a structurally homogeneous IgG3 protein or its Fc component. Effective inhibition occurred with heat aggregated or unaggregated IgG3 and IgG3-Fc. This implied that membrane bound Fc receptor sites on suspension populations were operationally accessible for effective inhibition. Stated another way, homogeneous IgG3 successfully competed with antistaphylococcal IgG opsonins for Fc receptors on the neutrophil membrane.

3. A marked opsonic enhancement on ingestion was also evident with adherent neutrophils challenged with S. aureus + antistaphylococcal immune complexes. However, this augmented uptake was not inhibitable by preincubating neutrophil monolayers with heat aggregated or unaggregated homogeneous IgG3 or IgG3-Fc. These results demonstrated another distinction between the two neutrophil populations.

4. It was realized that the major differences between the two neutrophil populations were, in part, related to the phenomenon of glass adherence. Subsequent experiments were focused on allowing neutrophils to monolayer for varying time periods before challenge with unopsonized S. aureus. Ingestion efficiencies of unopsonized S. aureus were adequate and comparable when adherence occurred over a 15-60 minute time period. After 90 and 120 minutes, phagocytic efficiencies diminished slightly.

5. It became apparent that even though neutrophils could be monolayered with tissue culture medium, in the absence of 10% FCS, the potentialities of these adherent cells to substantially ingest unopsonized S. aureus was abolished. This was the first clue that the efficient phagocytic potential of adherent monolayers, in the absence of opsonin, were related to at least two factors: A moiety in decomplemented FCS and the adherence phenomenon itself. Presumably, both FCS and normal calf serum contained components capable of activating phagocyte membrane components which had been exposed during surface adherence.

6. It was demonstrated that the phagocytosis-stimulating activity of FCS, on adherent monolayers, was dose dependent. However, although phagocytosis of unopsonized S. aureus remained demonstrable, these monolayers failed to ingest encapsulated K. pneumoniae and S. pneumoniae, type III, in the absence of their respective antibodies.

7. Double diffusion analyses revealed the presence of IgG in fetal and normal calf sera. When IgG was fractionated from FCS by DEAE ion exchange chromatography, two peaks eluted differentially. Nevertheless, in double diffusion studies both peaks demonstrated antigenic identity with control bovine IgG.

8. At physiologic concentrations in 10% FCS, IgG isolates from FCS (I and II) were utilized in the tissue culture medium to monolayer neutrophils. Neither IgG peak potentiated ingestion of unopsonized S. aureus, as compared to the activity observed with unfractionated FCS. Consequently, phagocytosis-stimulatory activity could not be ascribed to the IgG component of FCS.

9. Comparison of HMS activities in adherent and suspension neutrophils revealed no significant differences in the two cell populations under resting conditions or during active phagocytosis.

10. Studies concerning C3 mediated opsonization (i.e., binding of EAC3 immune complexes) revealed another distinction between the two neutrophil populations. Adherent monolayers demonstrated a pronounced and efficient capacity to bind to (rosette formation) and ingest C3-opsonized erythrocytes. This binding was inhibitable with anti-C3 antisera. In contrast, suspension neutrophils failed to interact appreciably with C3 coated RBC.

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