Neutrophil plasma membrane reorganization following stimulation with N-formyl-met-leu-phe in the presence of dihydrocytochalasin B
by Goutam Mukherjee

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
Superoxide (O2) production by neutrophils stimulated with chemotactic peptide fMLF is transient, but increases in rate and duration after pretreatment of the cells with dihydrocytochalasin B (dhCB), suggesting a possible role for the membrane skeleton in the regulation of the O2 generating system. Analysis of plasma membranes from these cells by density gradient sedimentation showed no significant variations in the distribution of plasma membrane markers between control and dhCB-treated cells, while a significant redistribution of plasma membrane markers was observed in dhCB+fMLF-treated cells. Instead of being localized in 31-35% sucrose, as in former two groups, plasma membrane markers were broadly distributed over 25-50% sucrose in the dhCB+fMLF-treated cells. In addition, ~80% degranulation was reached in these cells, while little granule release (<5%) was observed in control and dhCB-treated cells. Analysis of gradient fractions for membrane skeletal components (actin and fodrin) and NADPH oxidase components (cytochrome b and p47-phox) in dhCB+fMLF-treated cells demonstrated that the distribution of fodrin, actin, cytochrome b, and p47-phox followed the broad distribution of plasma membrane markers, with an overall increase in membrane-associated actin. The distribution of O2 generating activity remained confined to a narrow peak at ~50% sucrose. These results demonstrate that a heterogenous surface membrane of higher density and a differential distribution of proteins and O2 generating activities is created after dhCB+fMLF treatment with only a subfraction of the reorganized plasma membrane containing all of the components necessary for active O2 generation. Our results support a role for plasma membrane lateral organization and participation of the membrane skeleton in the regulation of the 0'2 generating system. The distribution of Mac-1 on similarly treated neutrophils demonstrated symmetric distribution of the integrin on the plasma membrane of control cells, and dHCB treated cells, but forms aggregates and is polarized in distribution in dHCB+fMLF treated cells in suspension conditions. Under adherent condition, the distribution of Mac-1 on dHCB+fMLF treated cells was primarily restricted to the lamellipodia. These observations suggest that the chemoattractant induced redistribution of Mac-1 on the protruding ends of neutrophils might play a role in directed transmigration.

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Date 12/4/95

This thesis is dedicated to my father Sri Debi Mukherjee, who would probably be the proudest man to know that I earned my Ph.D.

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ABSTRACT

Superoxide (O_2^-) production by neutrophils stimulated with chemotactic peptide fMLF is transient, but increases in rate and duration after pretreatment of the cells with dihydrocytochalasin B (dhCB), suggesting a possible role for the membrane skeleton in the regulation of the O_2^- generating system. Analysis of plasma membranes from these cells by density gradient sedimentation showed no significant variations in the distribution of plasma membrane markers between control and dhCB-treated cells, while a significant redistribution of plasma membrane markers was observed in dhCB+fMLF-treated cells. Instead of being localized in 31-35% sucrose, as in former two groups, plasma membrane markers were broadly distributed over 25-50% sucrose in the dhCB+fMLF-treated cells. In addition, ~80% degranulation was reached in these cells, while little granule release (<5%) was observed in control and dhCB-treated cells. Analysis of gradient fractions for membrane skeletal components (actin and fodrin) and NADPH oxidase components (cytochrome b and p47-*phox*) in dhCB+fMLF-treated cells demonstrated that the distribution of fodrin, actin, cytochrome b, and p47-*phox* followed the broad distribution of plasma membrane markers, with an overall increase in membrane-associated actin. The distribution of O_2^- generating activity remained confined to a narrow peak at ~50% sucrose. These results demonstrate that a heterogenous surface membrane of higher density and a differential distribution of proteins and O_2^- generating activities is created after dhCB+fMLF treatment with only a subfraction of the reorganized plasma membrane containing all of the components necessary for active O_2^- generation. Our results support a role for plasma membrane lateral organization and participation of the membrane skeleton in the regulation of the O^-_2 generating system. The distribution of Mac-1 on similarly treated neutrophils demonstrated symmetric distribution of the integrin on the plasma membrane of control cells, and dHCB treated cells, but forms aggregates and is polarized in distribution in dHCB+fMLF treated cells in suspension conditions. Under adherent condition, the distribution of Mac-1 on dHCB+fMLF treated cells was primarily restricted to the lamellipodia. These observations suggest that the chemoattractant induced redistribution of Mac-1 on the protruding ends of neutrophils might play a role in directed transmigration.

CHAPTER 1

INTRODUCTION

General

Neutrophils are the body's primary defense against invading bacteria. They detect chemical footprints of bacterial invasion, migrate to the site and destroy them. The killing machinery of neutrophils depends upon different intracellularly stored degradative enzymes and the cascade of toxic oxygen radicals generated by the production of superoxide anions (O^-_2), known as the respiratory burst.

The primary goal of the research described in this thesis is to understand in molecular detail the changes taking place in the neutrophil plasma membrane resulting in the generation of O^-_2. Briefly, we isolated peripheral blood neutrophils and stimulated the cells with chemoattractant *N*-formyl methionyl leucyl phenylalanine (fMLF) in presence of actin filament depolymerizing agent dihydrocytochalasin B. We studied the resulting alteration in plasma membrane architecture, and the redistribution of the cytoskeletal, adhesive, secretory, and respiratory burst proteins. To help put this research in a broader perspective, it is important to understand the historical aspects of inflammatory biology and the present state of understanding of its cellular and molecular basis. Central to this understanding is the role of neutrophils in inflammation and host defense. A brief review of the literature follows.

Historic Perspective

The importance of infectious organisms, and the role of inflammation in protection against infection, is well understood and accepted by the medical and scientific community. It took more than a century of research, however, to lay the foundation for our present day understanding of the biology of inflammation. In 1860, Louis Pasteur discovered the abundance of microorganisms in our environment. In 1870, he discovered bacterial involvement in silkworm mortality and in anthrax. His work with bacteria in disease established bacteriology as a new field of study in science. In the following years, the work of great scientists such as Koch, Lister, Roser, Pasteur, Loffler, Roux, Yersin, and others, greatly enhanced the knowledge of the role bacteria play in disease (1). As more species of bacteria were isolated and characterized, their role in the pathogenesis of disease in man and animals was also better understood.

The new field of inflammatory biology stimulated great interest among physiologists as to how animals respond to infection. In 1882, Ilya Metchnikoff performed his classical experiments on transparent starfish larva. When he introduced a rose thorn into the starfish larva, motile cells accumulated around the thorn. Ingenious and intuitive reasoning led Metchnikoff to suggest that a similar event must take place "in a man who runs a splinter into his

finger." This experiment formed the basis of the study of inflammation and phagocytosis in host defense (2). Metchnikoff later extended his experiments to fungal infections in Daphnia and studied the interactions between the invading fungus and the host's phagocytes. Simultaneously, Julius Cohnheim in 1882 performed his seminal experiments on microscopic vascular events that occur in acutely inflamed frog mesentery. His observations included capillary dilation, transduction of fluid, leukocyte adhesion to the capillary walls and migration of leukocytes into the extravascular space (2).

Initial work in the field of host defense was primarily restricted to the study of cellular defense mechanisms. As knowledge of host immunity (both humoral and cellular) began to accumulate in the late 19th century, a conflicting battle ensued to establish the importance of one kind of immunity over another (3). An extensive scientific effort followed, and, by the end of 19th century, three components of blood i.e., phagocytic cells, antibodies, and the complement cascade were recognized to be important in host defense (2). Nearly another century of derivative research revealed many of the features of human and animal host defense. A brief description of the components of host defense of the human body is presented in the following sections.

The Cellular Constituents of Blood

Blood is a highly specialized connective tissue in which blood cells are suspended in a protein rich liquid plasma. Approximately 50% of blood volume is plasma, 45% is erythrocytes, and the rest platelets and leukocytes. The erythrocytes in mammals are non-nucleated biconcave disks that are rich in hemoglobin and carry out the oxygen and carbon dioxide transport function. Neutrophils are the main bactericidal constituents of the leukocytes. Leukocytes in general have a wide range of functions and can be histochemically classified, using Romanowsky type staining, into granulocytes (granulated cytoplasm) and agranulocytes (lack of granulation in the cytoplasm). These stains can also help to differentiate the granulocytes and agranulocytes into further subclasses.

On a stained blood smear granulocytes can be further divided into: a) neutrophils which are approximately 9 to 15 μm in diameter with a heterochromatic multilobed nucleus, pink staining cytoplasm, and a moderate number of azurophilic granules; b) eosinophils, 9 to 15 μm in diameter with usually a bi-lobed heterochromatic nucleus and closely packed large red/orange eosinophilic granules; and c) basophils, 10 to 16 μm in diameter with two or three lobed heterochromatic nuclei and violet colored spherical cytoplasmic granules.

Agranulocytic leukocytes consist of lymphocytes (~10 μm) and monocytes (15 to 20 μm). Lymphocytes are characterized by rounded kidney shaped nuclei with coarse chromatin and marginal agranular cytoplasm. Monocytes are the largest of the white blood cells, ranging from 14 to 20 μm in diameter. Their nuclei are horseshoe shaped and vary in size with a delicate network of chromatin and abundant gray or blue staining cytoplasm.

Platelets are the smallest cells in the blood. They are ovoid and 2 to 4 μm in diameter and are devoid of nuclei. They contain a central blue granular zone, the granulomere, with a lighter, clear periphery, the hyalomere (4).

Origin, Maturation and Microscopic Structure of Neutrophils

Neutrophils originate from stem cells in the bone marrow. They are the major white blood cell type and 55-60% of the bone marrow is dedicated to their production (5). Pluripotent stem cells in the bone marrow give rise to myeloblasts which are undifferentiated cells with large oval nuclei and few or no granules (6). Multiple cytokines have been implicated in the growth and development of neutrophils and other blood cells from progenitor stem cells. *In vitro* studies using a semisolid matrix demonstrated that granulocyte colony stimulating factor (GM-

CSF) stimulates formation of granulocyte/monocyte colonies from human bone marrow cells. Other cytokines which also give rise to neutrophil colonies are granulocyte colony stimulating factor (G-CSF), and interleukin-3 (IL-3) or multi colony stimulating factor (Multi-CSF) (7,8). Myeloblasts differentiate to promyelocytes, and, at this stage, the azurophilic granules are formed. Promyelocytes mature into myelocytes and, at this stage, secondary granules are formed. Myelocytes are the last mitotic cells in the maturation process of neutrophils. At this stage neutrophils acquire locomotor capacity in response to chemotactic factors (9).

During the next two stages of neutrophil development, the nucleus undergoes a series of morphological changes progressing from a slight indentation in metamyelocyte stage to immature 'band' form and on to the typical multilobed nucleus in mature neutrophils.

The exact nature of how different growth factors regulate neutrophil development in the bone marrow is not clear, even though the respective receptors and the signal transduction pathways have been identified. However, it has been shown that activated T-lymphocytes release GM-CSF which, in turn, increases production of neutrophils and monocytes and enhances the neutrophils' responsiveness to endogenous and exogenous stimuli (8). Similarly, administration of IL-3 in humans and monkeys increased the

total white cell count over three fold (10). Of the three growth stimulating factors mentioned, G-CSF is the only neutrophil specific CSF. Unlike GM-CSF and IL-3, which play vital roles during infection, G-CSF may play a role in day to day regulation of neutrophil maturation (11). When administered in vivo, G-CSF causes an increase in circulating neutrophil numbers in a dose dependent manner (12).

A mature neutrophil (not band cells) is identified by its multilobed nucleus and the presence of large (~500 nm) azurophilic or primary and smaller (~200 nm) specific or secondary granules (6). The name azurophilic comes from the fact that these granules have an affinity for azure stains and stain reddish purple with Wright's stain. These granules are formed at the promyelocyte stage and are visible in normal bone marrow smears. As the cells mature into the myelocytic stage, they lose this distinct staining characteristic, and it was thought that the azurophilic granules differentiate into secondary granules. It is now known that the secondary granules are formed independently from azurophilic granules at the myelocyte state, but specific granules become more numerous than azurophilic granules during the myelocyte stage because the formation of azurophilic granules ceases after the promyelocyte stage, and, as a result, the azurophil granules per cell is reduced by mitosis, whereas specific granules continue to be

produced by the daughter cells (13). Azurophilic granules contain many hydrolytic, degradative and bacteriocidal constituents including myeloperoxidase, numerous defensins, lysozyme and elastase. The secondary granules lack peroxidase but contain lactoferrin, lysozyme, vitamin B_{12} - binding protein, collagenase and other defensive and regulatory proteins. The membranes of these granules also contain stores of plasma membrane proteins including cytochrome b_{558}, adhesins, and certain receptors (14). A more complete list of the contents of each granule is shown in Table 1.

The whole process of neutrophil maturation takes approximately 11 days. The time of circulation for neutrophil in the blood is nearly 10 hours, after which time they migrate to the tissue and probably live for 1 to 2 days (15,16). Approximately $1X10^{10}$ neutrophils enter and leave the circulatory system every day (17). Some research suggests that the spleen is a possible site for destruction of neutrophils while others suggest that they migrate through the colonic epithelium into the intestine (18).

Neutrophils and Related Events in Bacterial Infection in Human

As evidenced in the late 19th century, during bacterial or mechanical insults neutrophils and macrophages are mobilized toward the wound site. This directed migration of

cells is facilitated by fragments of complement C5, and chemotactic gradients of bacterial degradation products and the interleukins. The predominant role of neutrophils once they reach their destination, is to secrete degradative enzymes, antibacterial peptides and toxic oxygen species. These agents provide an "antibiotic shield" by producing oxygen radicals and directly killing the bacteria (19). Neutrophils also play an important role in wound healing by secreting cytokines such as transforming growth factor β (TGF-β), Interleukin-8 (Il-8), and Interleukin-1 (IL-1). Neutrophil serine proteases such as elastase and cathepsin G degrade matrix components producing peptide fragments which can also act as monocyte chemoattractants (20).

In the blood, neutrophils are distributed in circulating and marginated pools (21). The number of neutrophils in circulating pools increases with increases in the rate of blood flow, exercise, and/or epinephrine levels (22-24). Most of the cells in marginating pools are sequestered in post-capillary venules (25).

To reach a site of infection or injury, circulating neutrophils have to marginate and migrate through the endothelium (diapedesis or transepithelial migration). In normal subjects the marginating neutrophils exhibit reversible rolling. The rolling is governed by low affinity attachment of neutrophils to the endothelium. Leukocyte endothelial cell adhesion molecule -1 (LECAM-1) on

neutrophils, and endothelial leukocyte adhesion molecule -1 (ELAM-1) on endothelial cells are implicated in this cellular interaction. The process of diapedesis begins with tight adhesion of neutrophils to the vascular endothelium. The capillary endothelium close to the site of inflammation becomes both the target of and the producer of cytokines. The adhesion is governed by a set of ligand-receptor interactions other than those involved in rolling (26). Various by-products of inflammation including bacterial endotoxin or lipopolysaccharide (LPS), chemoattractants, serum components, IL-1, and tumor necrosis factor (TNF) increase the expression of adhesion molecules on endothelial cells. Examples of these adhesion molecules are different selectins (with lectin domain) and integrins. All selectins have a calcium dependent lectin- or carbohydrate binding motif, epidermal growth factor like motif and complement regulatory motif (27). The selectins are classified into three different classes called L-selectin, E-selectin, and P-selectin. Depending on the activation state, both neutrophils and endothelial cells express different selectin molecules on the surface which helps in neutrophil homing. Integrins are a group of heterodimeric glycoproteins which act as adhesion-recognization receptors in regulating cellular adherence to other cells or to the extracellular matrix (28). Integrins are classified into families depending on the β subunit. Approximately 8 distinct types

of β subunits have been identified. Major neutrophil integrins are LFA-1, Mac-1, and gp150/95. All of them share the same β_2 subunit (also known as CD18), but different α subunits. LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$), and gp150/95 ($\alpha_X\beta_2$) are also known as CD11a/CD18, CD11b/CD18, and CD11c/CD18 respectively by World Health Organization nomenclature (29,30).

The state and level of expression of these molecules varies depending on the stimulatory conditions. Unstimulated neutrophils exhibit a weak binding to the endothelial cell adhesion molecule ICAM-1 via CD11a/CD18 (31). When stimulated with chemotactic factors, a stronger binding is mediated through CD11b/CD18 (MAC-1) on neutrophils and ICAM-1 on endothelial cells (32). CD11b/CD18 also mediates an adherence-dependent respiratory burst in neutrophils stimulated with chemoattractants, which is characterized by a massive release of H_2O_2 (33). Endothelial cells also demonstrate an increased adherence for neutrophils when exposed to inflammatory mediators, IL-1, TNF, or LPS (34). A higher number of ICAM-1, and ELAM-1 receptors are expressed by stimulated endothelial cells (35).

After binding to the endothelial cells, neutrophils migrate across the endothelial monolayer to reach the tissue space. Although the mechanism of this process is not completely understood, it has been shown that the trans-

migration involves CD11a/CD18, CD11b/CD18, and ICAM-1 molecules (34,36-38). Once in the tissue space neutrophils reach the site of infection by chemotaxis which is governed by various chemoattractants.

Neutrophil Response to Chemoattractants

Multiple chemoattractants play an active role in the body to direct neutrophil migration towards the site of infection. Some of the well studied chemoattractants are a complement protein fragment C5a, interleukin-8, leukotriene B_4 (LTB_4), and platelet activating factor. During bacterial infection, bacteria release N-formyl methionyl peptides at the site of infection which directs neutrophil chemotaxis. Several formylated peptides were found to be chemoattractants *in vitro* (39). It was found that conservation of N-formyl-met at position 1 and phe at position 3 was important for chemoattractant function (40). Over the years the synthetic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) has emerged as an important molecular tool to study the antibacterial responses of neutrophils *in vitro*. Numerous studies have described the effects of fMLF on neutrophils (for a review see 41). These include morphological polarization, chemotaxis in a concentration gradient, production of superoxide anion (O^-_2) and secretion of the contents of intracellular storage compartments (degranulation). The role fMLF plays in

bacterial infection *in vivo* is not clear, even though the peptide has been isolated from *E. coli* and *S. sanguis* (42-44)

There are specific receptors for fMLF (FPR) on the cells. The receptor is a heptahelical G protein coupled glycoprotein with molecular weight approximately 50-70 kD (45-47). There are approximately 50,000-100,1000 fMLF receptors (FPR) per cell (41). The receptors exist in a dynamic cycling in the cell. Initial studies with radiolabeled ligands have indicated that the ligand is internalized following binding to the receptor (48,49). Subsequent studies by Sklar et al. found that the internalized ligand-receptor complex localizes in the Golgi fraction (50), and, as a result, the number of cell surface receptors decrease, however, the number of cell surface receptors eventually increase again, possibly by recycling and replenishment from intracellular reservoirs which Jesaitis and coworkers have shown to be the specific granules (51,52).

The receptor on the plasma membrane exists in two affinity states. One is lower (K_d ~ 20 nM) (53). The lower affinity receptor is accessible to G-protein and takes part in signal transduction, but shortly after occupancy at 37^{0}C. FPR is converted to a slowly dissociating, high affinity (K_d ~ 0.3 nM) form and is associated with the cytoskeleton (54).

Chemotaxis

Chemotaxis is a special form of cellular locomotion in response to chemical signals, in which the responding cell becomes oriented, and moves up a concentration gradient of an attractant. The term chemotaxis was coined by Pfeffer in 1884 to describe directed migration of plant cells (55).

Neutrophils demonstrate a highly sensitive and relatively rapid chemotactic response. *In vitro*, when exposed to fMLF or other chemoattractants 95% of the blood neutrophils become polarized within a few minutes of stimulation with chemotactic factors such as fMLF or C5a (56). Neutrophils polarize anterioposteriorly in the direction of first contact by the ligand. Morphological polarization is followed by asymmetric distribution of receptors to the anterior region of the cell with a residual number of receptors remaining localized in the posterior portion (57). The cells then migrate directly up the concentration gradient of chemoattractants in a relatively straight path with few turns (58).

In contrast, if the chemoattractant is present in isotropic concentration, it activates cellular polarization, and enhanced motility, but in a random direction. This random locomotion of leukocytes is called chemokinesis and is characterized by a series of linear movements of constant speed, punctuated by changes of direction. This type of

locomotion has been called "random walk with persistence of direction" (59,60). Chemokinesis activates a wide range of cellular functions distinct from chemotaxis. Many of the chemoattractants, e.g., fMLF, LTB4, and C5a, also act as chemokinetic factors (61). In contrast to an anterioposterior orientation in the chemotactic gradient, the cells still polarize but orient or extend lamellopodia in random directions in an isotropic concentration of activators (56).

Neutrophil motility in a chemotactic gradient is thought to be mediated by a process of protrusion of microspikes and lamellipodia which, once in contact with the extracellular matrix, form an adherence plaque. The rest of the cell moves forward by contracting towards the adherence plaques. It has been shown that adherence plaques are the sites of interaction between the intracellular cytoskeletal network and the extracellular matrix via integrin molecules and so may be regulated by their mechanisms.

In general, chemoattractants bind to specific G-protein-coupled receptors and stimulate discrete biological responses (62,63). The cellular response is dependent upon the concentration of the chemoattractant, and at lower concentrations (~0.1-1 nM) of the stimulant, the cells respond by chemotaxis. For secretory responses or an oxidative burst, at least 20 fold higher concentrations are needed (64). This bimodal nature of the chemoattractant

response suggests a separate kinetic and biological signalling pathways in neutrophils.

Some of the well studied chemoattractants for neutrophils are bacterial formyl peptides (fMLF), products of the complement cascade (C5a), platelet activating factor (PAF), leukotrine B_4 (LTB_4), interleukin-8 (IL-8), etc. As mentioned before, all of these chemicals elicit chemotaxis at lower concentrations and a cytotoxic response at higher concentrations.

Significant effort has been focused on the molecular events following chemoattractant occupancy of the receptor. Following ligand binding, the chemoattractant receptors direct a signal to the intracellularly coupled heterotrimeric GTP-binding protein G_i which results in dissociation of the $G_{i\alpha}$ from $G_{i\beta\gamma}$ subunits. The activated Gi2-α then stimulates phospholipase C (PLC) which in turn mediates rapid hydrolysis of phosphatidyl inositol 4,5-biphosphate (PIP_2) into inositol triphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG).

$$\text{Receptor}.G_{i\alpha\beta\gamma} \text{ ------} > G_{i\alpha} \text{ ------} > \text{PLC}$$

$$PIP_2 \text{ ------} > IP_3 + \text{DAG}$$

In the case of the cytotoxic response, this is followed by calcium influx and activation of phosphatidylcholine-specific phospholipase D.

Intracellular influx of calcium following ligand binding and activation of the signal transduction pathway plays an essential role in cell activation. IP_3 induces release of calcium from the intracellular stores and raises the level of cytosolic free calcium and also form an influx of extracellular calcium. The primary intracellular calcium storage sites in neutrophils are distinct from mitochondria and the endoplasmic reticulum and have been termed "calciosomes" (65). Calcium ionophores, in the presence of extracellular calcium, can also induce a rise in cytosolic calcium and trigger neutrophil degranulation, aggregation of the cells, superoxide production and polarization (66,67), and it is postulated that calcium acts as a second messenger in the cell. Some authors suggested that fMLF induced secretion in neutrophil is mediated by rise in the Ca^{2+} through signal transduction pathway (68). In the presence of free-calcium quenchers in the cell, the ability of fMLF to produce superoxide was found to be reduced and so was the accumulation of DAG. In general, peptide chemoattractants are more potent than the lipid chemoattractants in mediating sustained elevation of Ca^{2+} levels in the cell. The higher Ca^{2+} level in the cytosol for a longer period of time leads to additional DAG production from sources other than PIP_2, most likely phosphatidylcholine (PC) (67).

DAG produced from PIP_2 and other pathways can activate protein kinase C which mediates many of the sustained

cellular responses to chemoattractants. Accumulation of DAG in the cells has a definite biphasic character. Initial kinetics are similar to the production of IP_3. Secondary sustained accumulation continues after the production of IP_3 has largely diminished, probably from a different precursor, possibly phosphatidylcholine (PC). The direct pathway of DAG production from PC can be activated by calcium ionophores, phorbol esters, or different growth factors (69,70). In an interesting experiment by Snyder et al., low doses (motility inducing) of chemoattractants induced rapid (~10 sec) IP_3 and DAG accumulation. At higher doses however, the sustained production of DAG was mediated by phospholipase D and correlated with cytotoxic activities that probably involve calcium/protein kinase C activation. However the exact relationship between calcium, protein kinase C, and phospholipase D is unknown (64).

Although significant biophysical work has been carried out in an effort to link the biochemical events with the chemotactic function, the precise mechanism by which neutrophils form pseudopods that extend in the appropriate direction is still not understood. Several mechanisms have been proposed to take part in this process, including the force generated by actin polymerization, localized change in osmotic force, and the membrane bending moment generated by various members of signal transduction pathways.

Several actin associated enzymes are known to generate contractile forces e.g., myosin, dynein, and kinesin, but whether actin polymerization itself can generate the forward moment necessary for membrane protrusion is questionable (71). It has been documented that the acrosomal process of the sperm of the sea cucumber, Thynone, can extend 90μm in 10 seconds. Tilney et al. found that these acrosomal processes are filled with actin polymers with their barbed end oriented towards the plasma membrane. From this observation, they concluded that actin polymerization is responsible for the protrusion (72). But, later it was found that the rate of actin polymerization in the acrosomal protrusion is too slow to account for this process (71). Further studies by the same group of researchers later suggested that the protrusions are formed by osmotically generated hydrostatic forces, and actin polymerization acts to form a rigid supporting structure to stabilize it (73). The same model might not explain the formation of lamellipodia and microspikes in neutrophils, which grow much slower than the acrosomal sac, and might not be limited by the rate of actin polymerization (71).

Even though actin polymerization has long been implicated in cell motility, some authors suggest that the process of polymerization itself is unable to generate the force necessary to produce pseudopods. They suggest that an imbalance of osmotic pressure equilibrium is brought forth

by actin polymerization at the membrane, and this unbalanced osmotic force drives the cellular protrusion. According to their model, at the protruding end, the actin-profilin complex is probably cleaved by PIP_2 (74), releasing G-actin and free profilin which results in a net doubling of the particle count. This net gain in molecule number creates higher osmotic flux and drives the boundary. The rate of formation of polymeric F-actin from newly formed G-actin at the tip is determined by the diffusion rate constant of the actin-profilin complex (71). How this process couples with the chemotactic peptide receptor occupancy is not clear.

Several chemical events in the signal transduction pathway have been implicated to be the mechanochemical driving factors for cell motility. PIP_2 which is generated by stimulation of the signal transduction pathway via the FPR could play a direct role in the formation of cellular processes. Also, the cleavage of PIP2 can produce DAG which has been implicated in causing the membrane bending moment and can buckle the membrane outward if the local concentration is sufficiently high (75). Cleavage of PIP2 releases IP3. IP3 itself and IP3 mediated Ca^{2+} release activates solating factors like gelsolin which severs actin strands, thus reducing its elastic modulus which helps in the protrusion process and play a role in remodelling of the cortical actin gel (73). It is possible that all these mechanisms participate in unison for active pseudopod

formation. Insights into these biomechanical processes might help us understand the implication of our work more.

Oxygen-dependent Cytotoxicity

When stimulated neutrophils reach the site of infection, they need to phagocytose and kill the invading, generally opsonized microorganism. The microbicidal and cytotoxic system of neutrophils can be grouped into two mechanistically distinct processes, one involving oxygen and the other oxygen independent.

Neutrophils undergo a marked increase in oxygen consumption when actively destroying microorganisms. Studies by Mandell and Vet et al., have shown that this increase in O_2 consumption is cyanide insensitive and is not used for generation of ATP but rather for microbicidal activity in the generation of toxic oxygen species (76,77). The importance of this respiratory burst has been demonstrated in the case of chronic granulomatous disease (CGD). Neutrophils from CGD patients do not display the characteristic respiratory burst and lack the microbicidal capability of normal cells, resulting in recurrent severe infection for the host (78,79). The initial microbicidal product of a neutrophil oxidative burst is O^-_2 which is formed by the single electron reduction of molecular oxygen (80).

$$O_2 + e^- \text{------>} O^-_2$$

By itself O^-_2 is probably not directly toxic to bacterial cells because it reacts 'sluggishly' with many biologically important molecules (81,82). It has also been suggested that in a nonpolar environment of the membrane, the toxicity of O^-_2 is increased when its reactions are not in competition with the proton requiring dismutase reaction. It also acts as a powerful base that may be highly reactive with lipid membranes (83,84).

One potential acceptor for O^-_2 could be nitric oxide ($\cdot NO$). Nitric Oxide reacts rapidly with O^-_2 to form a very strong oxidant, peroxynitrile anion ($ONOO^-$). It has been shown that activated neutrophils and macrophages can produce nitric oxide and O^-_2 at the same rate. The existence of this pathway in human neutrophils is debated. The source for nitric oxide in the human could be endothelial or other cells (85).

At low pH, O^-_2 can be protonated to form a perhydroxyl radical ($HO\cdot_2$) which is a considerably stronger oxidant than O^-_2 .

$$O^-_2 + H^+ \rightleftarrows HO\cdot_2$$

This shift could be significant in the localized environment of the phagosomes where the pH can be as low as pH=3

(83,84). Due to its relatively low reactivity and low, steady state concentration, O^-_2 can diffuse over significant distances in the cell and pass through anion channels (86,87). *In vitro*, the presence of O^-_2 is tested by its ability to reduce ferricytochrome C which leads to a change in absorbance at 550nm.

At physiologic pH, dismutation of O^-_2 can occur spontaneously but is efficiently catalyzed by superoxide dismutase (SOD) resulting in hydrogen peroxide (see reaction below) (88).

$$O^-_2 + O^-_2 + 2H^+ \text{ ----- } O_2 + H_2O_2$$

Stimulated neutrophils, therefore, produce a large amount of H_2O_2 (89-91). This well known microbicidal agent can diffuse a considerable distance through the membranes and protein rich fluids of cells. It is one of the key toxic microbicidal intermediates of the oxidative burst (89). Autologous damage of neutrophils by H_2O_2 is prevented by the presence of the enzyme catalase (92), glutathione and by components of the glutathione cycle (93).

Neutrophils can also form additional toxic derivatives from H_2O_2, hypochlorous acid (HOCl) which is also a very potent microbicidal agent, in conjunction with myeloperoxidase from azurophilic granules and halides (mainly chloride ions) (94). The MPO-H_2O_2-Cl^- system can

also generate hydroxyl radicals ($OH^{\cdot}$) (see reaction below) (95).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-$$

$$HOCl + O_2^- \dashrightarrow Cl^- + O_2 + OH.$$

HOCl can react with O^-_2 to form both $OH^{\cdot}$ and $^1O_2$ (96). $OH^{\cdot}$ is one of the most potent oxidants known and thus may be important in phagocytic toxicity. However its short diffusion distance may limit its role in the process (85). Hydroxyl radicals can also be generated from H_2O_2 by three different pathways: a) by the Fenton reaction, b) Haber-Weiss reaction, and c) autoxidation of iron (97-99).

NADPH oxidase system

The microbicidal reactive oxygen cascade begins with the generation of O_2^-. There is almost universal agreement that this electron transfer requires a low potential, b type, cytochrome in the plasma membrane or phagosome membrane. This cytochrome is called cytochrome b_{-245} based on midpoint potential of -245 mV, or cytochrome b_{558} or b_{559} which refers to the wavelength of the alpha-band of the reduced cytochrome. Cytochrome b_{558} has the lowest midpoint potential of any mammalian cytochrome b, which gives it the capability to directly reduce molecular oxygen to O_2^-. It is a heterodimeric protein consisting of a noncovalantly

associated large subunit (molecular weight 91 kD), and small subunit (molecular weight of 22 kD). Defects of either subunit results in the genetic disorder, chronic granulomatous disease (CGD) (100). Cytochrome b_{558} is found primarily in phagocytic cells like neutrophils, monocytes, macrophages and eosinophils, but a low amount of this protein is also found in B lymphocytes (101). Neutrophils have dual pools of cytochrome b located in the plasma membrane (PM) and in the specific granules. Following appropriate stimulation of the cells, some of the cytochrome b from the specific granules is translocated to the PM, apparently by a pool of subsurface vesicles or a tubular network that extends through the cytoplasm (102). Previous research from our lab has shown that in active O^-_2 generating cells, cytochrome b_{558} and superoxide generating activity co-isolates with the cytoskeleton (103).

The electrons necessary for reduction of molecular oxygen comes from NADPH and is catalyzed by an enzyme system called NADPH oxidase system. It is a multi-component system which is activated by the cells to allow transfer of electrons of cytosolic NADPH to cytochrome b, and subsequently to molecular oxygen.

Three cytosolic proteins also take part in an activated NADPH system (p47phox, p67phox, and rac-2) (104-107). Upon stimulation, these components are translocated to the

membrane bound oxidase complex to form the oxidase (108,109).

P47phox is a cytosolic protein with a calculated mass of 44.7 kD. It was discovered by its absence in the cytosol from neutrophils in autosomal recessive CGD patients in cell free reconstitution assays (CFRA) (110). The necessity of p47phox phosphorylation in forming an active NADPH oxidase was not clear for a while, but multiple studies have shown that the rate and extent of phosphorylation of the protein correlated closely with activation of respiratory burst (111,112). In later years the cDNA for this protein was cloned and the recombinant protein was shown to reconstitute the activity of cytosol from autosomal CGD patients lacking p47phox (105). The majority of p47phox in the cell is localized in the cytosol. In fact 0.5% of neutrophil cytosolic protein is p47phox (104). Upon activation with appropriate stimuli, about 10% of the cellular pool of p47phox is translocated to the plasma membrane (100). For p47phox to translocate to the membrane, the presence of cytochrome b_{558} is necessary indicating that cytochrome b_{558} may be the docking site for p47phox (113). Also, phosphorylation is essential for this translocation (114).

Several groups of scientists working with either whole or fractionated cytosol (ion exchange chromatography, ammonium sulphate precipitation) discovered the necessity of another cytosolic component other than p47phox (115,116).

It is a 67 kD cytosolic protein and constitutes 0.3% of all the neutrophil cytosolic proteins (117). The cDNA for p67phox has been isolated and recombinant protein can reconstitute oxidase activity of complementary CGD patient cytosol (106). Upon activation, about 10% of the cytosolic p67phox is translocated to the PM where it participates in the formation of active NADPH oxidase complex (100). Translocation of p67phox does not take place in the absence of p47phox suggesting p47phox could be a cofactor for p67phox or helps in docking to cytochrome b_{558} (113). Previous studies from our lab have shown that in active O^-_2 producing cells, membrane p47phox was located in the Triton X-100-insoluble fraction indicating cytoskeletal association (118). However, the exact role of cytoskeletal network in the formation and the regulation of NADPH oxidase is not yet clear.

Degranulation

One of the important cytotoxic mechanisms employed by neutrophils is degranulation. Early investigators such as Metchnikoff and Ehrlich observed that neutrophils lose cytoplasmic granularity when engaged in an inflammatory responses. The significance of this cellular event however was not understood until 1920 when Graham observed that the loss of peroxidase staining granules from the cytoplasm was correlated to phagocytosis. He noted that the granules

disappeared progressively as the number of bacterial inclusions increased (119). Robineaux and Frederick in 1955, and Hirsch and Cohn in 1960, used phase contrast microscopy to demonstrate that degranulation occurs in phagocytic neutrophils (120,121).

More detailed understanding of the connection of degranulation with phagocytosis emerged from the work of Hirsch using microcinematography, where he showed that degranulation occurs almost exclusively at the edges of phagocytic vacuoles. Electron microscopic studies by Zucker-Franklin and Hirsch later confirmed that degranulation resulted from the fusion of granule membranes with the membrane of phagocytic vacuoles followed by extrusion of granule contents into the vacuoles (122, 123).

Many of these studies were done on rabbit or chicken polymorphonuclear leukocytes because of their larger granule size but similar events were observed in human cells as well. This form of degranulation, where the discharge was in the phagosomes, was predicted by Metchnikoff who concluded that neutrophil's "digestive ferments" were normally not "thrown off by phagocyte". A second form of degranulation was observed, however, where granular contents were released to the extracellular environment. Especially in case of streptococcus and staphylococcus infection, neutrophil degranulation involving exocytosis was observed. Two bacterial exotoxins (streptolysin from streptococcus and

leukocidin from staphylococcus) were shown to be involved in this process by causing leakage in the plasma membrane (124,125). The phenomenon of extracellular degranulation appeared to be relevant to the pathogenesis of tissue destruction caused by these organisms, and is thought to be distinct from degranulation which occurs during phagocytosis. Later studies, however, indicated that intact neutrophils could release granular contents extracellularly during phagocytosis (123,126-128).

In 1974, Leffell and Spitznagel made the important observation that during phagocytosis, specific granule degranulation was directed primarily towards the outside of the cell, whereas azurophilic degranulation was confined primarily to the phagosomes (128). This preferential degranulation was explained by the observations of Brentwood and Henson who realized that specific granules degranulate first and are more likely to release their contents into phagosomes that are not completely closed (129).

A few modes of exocytotic degranulation in association with phagocytosis have been documented. Sometimes this exocytotic degranulation happens when the phagosomes are not completely enclosed (130). In other instances, neutrophils can adhere to opsonized surfaces which cannot be engulfed. Degranulation then occurs at the plasma membrane. This phenomenon has been described as frustrated phagocytosis (131,132). Exocytotic degranulation also occurs without any

association with phagocytosis. Specific granules are more prone to degranulation at the plasma membrane level with soluble stimuli, and a number of agents have been shown to induce exocytosis. These include chemoattractants, hydroxyeicotetranoic acid, calcium ionophores (e.g., A23187), PMA, and lectins such as concanavalin A (133-136). Some authors suggest that specific granules are true secretory granules of neutrophils (137). All of the above mentioned chemicals, when used alone, induce degranulation of no more than half of the specific granule markers. At higher concentrations of the stimuli, some azurophilic granule discharge is also observed (136).

Cytochalasins enhance chemoattractant mediated secretion of granular content in neutrophils (138). The exact mechanism is unknown, but different theories prevail. Some authors suggested that in presence of cytochalasins the phagosomes cannot get sealed off when in contact with particulate stimuli (139,140). Poste and Allison suggested that, in unstimulated cells, a submembranous "bundle" of cortical microfilaments limit the access of vesicle-granules to the cytoplasmic face of the plasma membrane, and cytochalasins enhance secretion by rendering the necessary contact (141). It has also been suggested that in addition to disruption of actin filaments, cytochalasins also enhance the stimulus-response coupling in general and potentiate secretion by yet unknown pathway (142). The chemoattractant

fMLF alone can induce only 50% degranulation of the total granule contents, but by keeping dHCB in all the reaction buffers through subcellular fractionation, we achieved a level of degranulation (>85%) not reported in the literature before. More will be discussed about this topic in Chapter 2. A list of some of the contents of specific granules, azurophilic granules, and the plasma membrane is listed in the table below (table 1, page 31).

Table 1
Constituents of Human Neutrophil Granules and Plasma Membrane

Azurophilic granules	Specific granules	Membrane fractions
Acid hydrolases	Lysozyme	***Alkaline phosphatase***
Acid β-glycerophosphate	***Lactoferrin***	Acid *p*-nitrophenyl2
β-glucoronidase	Vitamin B_{12}-binding	-phosphatase
N-acetyl-β-glucosaminidase	-protein	HLA Ag
β-galactosidase	Collagenase	Mg^{+}ATPase
5′-nucleotidase	Gelatinase	Receptors
Cathepsin	Cytochrome b	fMLF
Neutral Proteases	β_2 microglobulin	Mel14
Cathepsin G	Plasminogen activator	
Elastase	Histaminase	
Cationic Proteins	Receptors	
Myeloperoxidase	fMLF	
Lysozyme	CD11b/CD18	
Acid mucopolysaccharide		

markers used in our studies
Table adopted form refs. (14,143-145)

Regulation of Neutrophil Responses

Previous studies by Jesaitis and coworkers have shown that fMLF induced O^{-}_2 production by neutrophils is a transient phenomenon (146). The results of stimulation, e.g., O^{-}_2 production and degranulation, are initiated

rapidly and terminate within 2 to 5 minutes. Similarly, intracellular messengers such as IP_3, calcium, and cAMP also return to normal levels within 5 minutes following a peak at 30 seconds. The autotermination of the response affects a number of components of the signal transductional pathways. Several mechanisms have been suggested for various receptor-ligand systems, e.g., degradation of ligand, loss of receptors, uncoupling of receptor and G protein, uncoupling of receptor/phospholipase C and inhibition of calcium flux.

The transient responses initiated in neutrophils by fMLF are terminated by several possible biochemical mechanisms. The ligand might be hydrolyzed by the classical lysosomal-endosomal pathway following internalization of the ligand-receptor complex. Interestingly, while lysosomal hydrolysis could not account for the total rate of ligand hydrolysis, an external membrane bound metalloproteinase, neutral endopeptidase (NEP), was found which is an extremely efficient system for fMLF hydrolysis and removes the chemoattractant (147).

An increased level of cAMP occurring through a calcium dependent pathway, also acts as a termination mechanism in chemoattractant induced cell activation (148). Concanavalin induced superoxide production is not pertussis toxin sensitive and does not alter binding of fMLF, but is sensitive to elevated cAMP (149). This indicates that the

inhibitory action of cAMP is not due to the effects on the receptor or its associated G protein (150).

Another mechanism for control of activation is phosphorylation of the agonist occupied receptor. Homologous desensitization of the N-formyl peptide receptor involves a receptor specific kinase. Treatment of neutrophils with fMLF results in a decrease of receptor-G-protein coupling as measured subsequently in membrane preparations (151). Phosphorylation of serines and threonines near the carboxyl terminus and on the third cytoplasmic loop of some G protein coupled receptors initiates binding of arrestin, a regulatory molecule, and renders it inaccessible to G proteins. Most of the research on this type of system has been studied using beta-adrenergic and rhodopsin receptors which are homologous to the fMLF receptors in these respects (152,153).

Heterologous desensitization involving multiple ligand-receptors, rather than homologous desensitization with specific ligand-receptor involvement, has been implicated in the case of fMLF and C5a receptors. Working with co-transfected cells, Disdbury et al. have shown that C5a and fMLF receptors desensitize each other's response even though the ligands C5a and fMLF do not cross react with the other's receptor (152). Disdbury called this response "receptor-class-specific desensitization" which postulates that both receptors might compete for the same G proteins or a

receptor-class-specific-kinase. Heterologous desensitization has also been shown in beta-adrenergic receptors mediated by cAMP dependent protein kinase (152).

It has been suggested that the cytoskeleton play a role in this regulation (154), because cytochalasins, potent inhibitors of actin polymerization, potentiate such responses in magnitude and duration. Understanding the role of the cytoskeleton in the regulation may provide important clues to its relative role in termination.

In 1988 a novel mechanism of receptor specific desensitization in neutrophils was proposed by Jesaitis and coworkers that could explain the turn off of O^-_2 generation by neutrophils *in vitro* when stimulated with fMLF. Physical separation of the receptor from G-protein was proposed by lateral segregation in the membrane plane into domains enriched in actin and fodrin. Thus the accessibility of G-protein to receptor is controlled and thus signal transduction regulated (155).

Neutrophil responsiveness to chemoattractants could also be positively regulated by a variety of physiologic and pharmacologic stimuli. Potentiation of neutrophil response is observed as an increased rate and extent of chemoattractant response. This phenomenon is called priming (64). Various physiological stimuli like cytokines, including TNF-α, GM-CSF, and IFN-γ, and pharmacological agent like cytochalasins prime neutrophils and increase the

rate and duration of the secretory component and oxidative burst of the chemoattractant response. However, by themselves these chemicals produce only minor secretory responses. The mechanism of priming is not clear, although GM-CSF has been known to increase the number of fMLF receptors on the surface (156,157).

An increase in receptor number, however, is not always the mechanism for increased responsiveness. GM-CSF and TNF-α increase superoxide production by neutrophils in response to pretreatment with non-receptor mediated activating agents such as sodium fluoride (158). The priming effect of IFN-γ has been attributed to phospholipase D-mediated phosphatidylcholine hydrolysis (159). Interestingly, GM-CSF or TNF-α cannot enhance superoxide production induced by PMA, probably because PMA acts directly on protein kinase C (PKC) (158).

Many of the lipid chemoattractants are poor stimuli for superoxide production and act mainly as priming chemicals for fMLF stimulated responses (160). Priming with cytokines usually requires exposure for several minutes to several hours. The calcium ionophore, A23817, has been shown to prime neutrophils for secretory responses induced by fMLF. FMLF alone can only induce secretion by neutrophils of 50 % of the total content, whereas priming the cells with A23187 induces secretion up to 85% of maximum (161). Monoclonal antibodies against Mac-1 also

enhances adhesion dependent respiratory burst of neutrophils stimulated with chemoattractants (33).

Goals of This Dissertation

The chemoattractant-induced oxidative burst results from assembly of NADPH-oxidase from a number of soluble and membrane-bound components at the membrane. Although much is known about the identity of these proteins from studies in cell-free systems, the exact sequence of the events in the assembly process in intact cells is not known. In addition, the role of the plasma membrane in the assembly process is not clearly understood. It has been suggested that there are different structural and functional sub-domains in the plasma membrane and they might take part in the regulation of the activation process (103,162). Previous studies have also shown that the membrane skeleton plays an important role in the lateral compartmentalization. In fMLF desensitized neutrophils, the fMLF receptors are converted to high affinity, GTP -insensitive form and co-localizes with Triton X-100-insoluble membrane skeleton. Jesaitis et. al. in 1993 have shown that the possible cytoskeletal candidate for receptor anchoring in the heavy PM domain is actin (163). We are trying to understand the organizational role of neutrophil cytoskeleton and the PM in resting, primed and activated neutrophils. To address this question we have used dihydrocytochalasin B (DHCB), a dihydro

derivative from cytochalasin B which is a metabolite from the mold *Helminthosporium demantioideum*, can inhibit actin polymerization, thus disrupting the cytoskeleton. DHCB does not bind to or inhibit the hexose transport system, and shows greater selectivity than cytochalasin B in its effect on the cytoskeleton. DHCB has also been shown to potentiate O^-_2 production in fMLF- stimulated cells, probably by deregulating receptor desensitization. DHCB can also enhance degranulation and the expression of cell surface receptors, reduce desensitization; and stop or slow receptor internalization (154). Thus dHCB provides a useful tool with which to examine the organization of the PM and the role of membrane skeleton in fMLF stimulated assembly of the NADPH oxidase system (in the membrane).

The long term goal of this research is to understand the molecular basis of regulation of chemoattractant activation. The immediate goal of this dissertation is to ascertain the role of plasma membrane organization in NADPH oxidase function. To reach that goal we studied at the molecular level the effect of dHCB and fMLF on the a) organization and assembly of the NADPH oxidase system in the plasma membrane, b) alteration in the composition and structure of the plasma membrane in different stimulatory conditions, c) the association of membrane skeletal system with the active oxidase system, and d) the relationship of adhesion molecules to the process.

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CHAPTER 2

REMODELLING OF THE PLASMA MEMBRANE AFTER STIMULATION OF NEUTROPHILS WITH F-MET-LEU-PHE AND DIHYDROCYTOCHALASIN B: IDENTIFICATION OF MEMBRANE SUBDOMAINS CONTAINING NADPH OXIDASE ACTIVITY

Introduction

Neutrophils are the body's primary cellular defense against invading microorganisms (1,2). The ability of these cells to produce superoxide anion (O_2^-) is vital to this host defense function, as is exemplified by individuals with chronic granulomatous disease (CGD)(3). Neutrophils from patients with this inherited disorder are unable to make O_2^-, and this leads to an enhanced susceptibility to bacterial and fungal infections (3,4).

Activation of the O_2^- generating system in neutrophils causes a large increase in O_2 consumption resulting in the generation of a cascade of toxic oxygen species (2) into the phagolysosomes as well as into the surrounding milieu (1,5,6). Therefore, inappropriate triggering of the respiratory burst can result in diverse inflammatory tissue damage (7). The oxidative burst results from the activation of a membrane bound NADPH oxidase which is inactive in unstimulated cells (8).

The NADPH oxidase is a multicomponent electron transport system that is assembled on the plasma membrane from a number of soluble and membrane-bound components (8,9). The membrane-

associated components include a low potential b-type cytochrome (10), which is now thought to be a flavocytochrome (11,12), and possibly Rap1A, a low molecular weight GTP-binding protein that associates with the cytochrome (13). The cytosolic components involved in the NADPH oxidase are p47-*phox*, p67-*phox*, and another low molecular weight GTP-binding protein, Rac (Rac1 and/or Rac2)(14-18). All three of these proteins have been shown to translocate to the plasma membrane during oxidase activation (19,20). Although the detailed biochemical events and sequence of assembly of these components is still unknown, a significant amount of information has been derived from biochemical studies utilizing a cell-free reconstitution system composed of membranes and cytosol and an activating agent such as sodium dodecyl sulfate (SDS) or arachidonic acid (21-23). In contrast, much less is known about the specific details of how this system assembles and is regulated in the plasma membrane in intact cells. Thus, further studies are necessary to identify cellular architectural parameters important for the regulation of the assembly, activation, and regulation of the O_2^- generating system in intact cells.

Neutrophils possess a variety of signal transduction pathways that can activate the respiratory burst (24). These include receptors for different soluble and particulate stimuli, including N-formylated bacterial oligopeptides, C5a, aggregated immunoglobulin, etc. (25). Activation of these

pathways depends on a variety of factors, including adherence of the cells, surface perturbation, and integrity of the cellular actin network. All of these factors could be expected to influence the organization of the cell surface where both the activating receptor and NADPH oxidase system reside. Previously, we hypothesized that the lateral compartmentalization of receptors, effectors, and intermediate regulatory components plays a role in the regulation of O_2^- production and controls the accessibility of receptors and transducing proteins (26,27). In both cases, the membrane skeleton was implicated in playing an important role in this lateral compartmentalization. Because dihydrocytochalasin B (dHCB), a fungal-derived compound that can inhibit actin polymerization (28,29), has been shown to potentiate O_2^- production by fMLF, enhance degranulation and the expression of cell surface receptors, reduce desensitization, and stop or slow receptor internalization in neutrophils (30), we felt that dHCB would provide a useful tool with which the organization of the plasma membrane and role of the membrane skeleton in the NADPH oxidase could be examined.

In this study, we compared the composition of plasma membranes isolated from neutrophils treated with dHCB alone or stimulated with fMLF in the presence of dHCB and analyzed the composition of these membranes with respect to the relative distribution of membrane skeletal components, NADPH oxidase components, and O_2^- generating activity. In order to

prevent subtle changes in cellular and membrane morphology that occur when dHCB and fMLF are removed at stages following stimulation, dHCB and fMLF concentrations were maintained constant throughout all subsequent steps in sample preparation. Our results suggest that: 1) the neutrophil plasma membrane undergoes major remodelling following stimulation with dHCB+fMLF, and these cells are almost completely degranulated (>80%), 2) the composition of the plasma membrane changes radically under such conditions, 3) O_2^- production is localized to only a subdomain of the reorganized plasma membrane, and 4) the amount of membrane-bound actin increases significantly in stimulated cells. These results further support the involvement of the membrane skeleton and plasma membrane lateral organization in maintaining the active O_2^- generating complex.

Material and Methods

Chemicals

The chemicals used in these studies were of the highest quality commercially available. Monoclonal anti-actin antibody was purchased from Amersham (Arlington Heights, IL). Affinity purified rabbit anti-human brain fodrin antibody was a kind gift from Dr. Jon Morrow (Department of Pathology, Yale University School of Medicine, New Haven, CT). Rabbit antiserum to p47-*phox* was provided by Drs. William M. Nauseef and Robert A. Clark (University of Iowa,

Iowa City, IA).

Preparation and fractionation of neutrophils

Neutrophils, isolated as described previously (26), were divided into three experimental groups: **A**, where the cells were incubated in control buffer (0.1% BSA, 250 U/ml catalase, 50 U/ml SOD in HBSS) at 37^{0}C for 10 min and then diluted with 5 volumes of ice cold control quench buffer (Q) to cool down the cells rapidly and preserve the morphology of the cells; **B**, where the cells were incubated for 10 or 15 min at 37^{0}C with 2 μg/ml dHCB in control buffer and then quenched with Q containing 2 μg/ml dHCB, and **C**, where the cells were incubated with 2 μg/ml dHCB in control buffer for 10 or 15 min at 37^{0}C, stimulated with 1μM fMLF for 5 minutes, and then quenched with Q containing 1 μM fMLF and 2 μg/ml dHCB. The cells were then pelleted and resuspended at 5 x 10^7 cells/ml in cavitation buffers for each respective experimental group (identified by bold letters) as follows: **A**) control cavitation buffer, containing 0.34 M sucrose, 10 mM Hepes, 1mM EDTA, 0.1 mM $MgCl_2$, 1mM ATP, 10 μg/ml chymostatin, and 0.1 mM PMSF (pH 7.4); **B**) control cavitation buffer containing 2μg/ml dHCB in control cavitation buffer, and **C**) control cavitation buffer containing 1μM fMLF and 2 μg/ml dHCB. The resuspended cells were then disrupted by N_2 cavitation (400 psi/15 min at 4^{0}C) and the resulting homogenates were fractionated into a low speed (1000 X g for 5 min) supernatant (1KS) and foam pellet

residue (1KP). The 1KP fraction was resuspended in **A**, **B**, and **C** cavitation buffers, respectively and rehomogenized with 5 strokes in a Dounce homogenizer and again fractionated into 1KS and 1KP. The respective 1KS fractions were pooled, filtered, and fractionated by isopycnic sucrose density gradient sedimentation. Greater than 75-80% of all membrane and granule markers assayed were present in the 1KS fraction.

For each experimental group, a different isopycnic sucrose density gradient was constructed as follows: For group **A**, a control 20 ml gradient composed of 20-55% (weight/weight in 10 mM Hepes, pH 7.4) sucrose was layered on top of a 8 ml 60% sucrose cushion. A 0.5 ml cushion of 15% sucrose was overlaid before application of 11 ml of the pooled 1KS fraction on the gradients. Group **B** was identical to the control except 2μg/ml of dHCB was included in the sucrose solutions before pouring the gradient, and, likewise, group **C** was identical to the control except 1μM of fMLF and 2 μg/ml dHCB were included in the sucrose gradients. The inclusion and maintenance of dHCB and fMLF assured the dHCB and fMLF binding sites were continuously occupied during the entire preparations. The gradients were then sedimented at 167,000 X g for 56 min (4^0C) with slow acceleration and deceleration in a Beckman VAC50 vertical rotor. 1.3 ml fractions were collected from each gradient.

Cell surface labelling

^{125}I-conjugated wheat germ agglutinin (^{125}I-WGA) was prepared as described previously (31). To determine the distribution of plasma membrane, cells treated as in conditions **A**, **B**, and **C** were surface labelled using ^{125}I-WGA (6 X 10^4 cpm or 0.16 mg WGA per 10^8 cells) in appropriate cell resuspension buffer and incubated at 4°C for 5 min. following incubation/stimulation and then washed, and resuspended in the same buffer. ^{125}I-WGA content of the fractions was counted directly using a gamma counter (10/200 Plus, ICN Biochemical Inc.). As reported previously, WGA at these levels does not alter the sedimentation distribution of the plasma membrane markers (26,32).

Biochemical assays

Protein was measured by Pierce protein assay (Pierce, Rockford, IL) using BSA as protein standard. Alkaline phosphatase and myeloperoxidase were measured as described Jesaitis et al. (31). The sucrose density of each fraction was determined by refractive index measurement.

Superoxide generating activity was measured in 1.6 ml microcuvettes utilizing 650 μl reaction buffer [0.5 μM cytochrome C/2 mM $MgCl_2$/10 mM Hepes, pH 7.4 (± 100 U/ml SOD)] and 50 μl of gradient sample (26). The reaction was initiated by the addition of 10 μl of 10 mM NADPH (140 μM final concentration) and monitored at 550 nm. The rate of superoxide dismutase inhibitable cytochrome C reduction was

calculated using ϵ=1.85 mM^{-1} cm^{-1}.

Electrophoresis and Western Blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 20°C using 9% polyacrylamide slab gels or 7-15% gradient gels as described previously (33). Electrophoretic mobility of the samples was compared with the mobility of prestained standard proteins (BRL, Bethesda, MD). Proteins were visualized on the gels by staining with 0.1% Coomassie blue G in 50% methanol/10% acetic acid. Gels were then destained in 25% isopropanol/10% acetic acid, washed in H_2O, and silver stained under basic conditions as described by Wray et. al. (34).

Western blotting was performed as described previously (26). Transfers were blotted with 1μg/ml rabbit IgG or 5μg/ml mouse IgM (anti-actin) for 16 hr at 4°C, followed by alkaline phosphatase conjugated goat anti-rabbit IgG or (anti-mouse IgM) secondary antibodies for 1 hr at 20°C, and developed using an alkaline phosphatase development kit (Kirkegaard & Perry Laboratories, Gaitherburg, MD). Blots were quantitated by video densitometry using an image analysis system [Microcomputer Imaging Device (MCID) with Image Analysis software] (Imaging Research, Inc., Brock University, Ontario, Canada) as described previously (20). The relative density of a sample represents the density of the sample relative to an arbitrary gray scale defined by the image analysis system to cover the total gray shade

range of the image.

Electron microscopy

Cells treated as described for experimental groups **A**, **B**, and **C** were lightly fixed (0.02% glutaraldehyde in HBSS) at 3 x 10^7 cells/ml and layered onto poly-L-lysine (0.05mg/ml) coated polycarbonate filter disks (0.4μm pore size) at room temperature. After the cells were allowed to adhere to the filter for 30 min., the filters were treated with 2% glutaraldehyde in HBSS for 1 hr. at 4^{0}C, washed 3 times with HBSS at 4^{0}C, washed in 0.1M cacodylate (pH 7.4) buffer, and then fixed in 1% osmium in cacodylate buffer for 1 hr. at 4^{0}C. The cells were then dehydrated in a graded series of ethanol. For transmission electron microscopy, the cells were infiltrated with Spurr's resin, polymerized, sectioned, and stained with uranyl acetate and lead citrate. For scanning electron microscopy, dehydrated cells were critical point dried with CO_2, mounted on an aluminum planchet, and coated with carbon followed by gold palladium (35).

Results

Subcellular Fractionation

To compare changes in membrane and NADPH oxidase organization in cells treated with dHCB±fMLF, purified neutrophils were exposed to three experimental conditions as described under Methods [**A**=control, **B**=2 μg/ml dHCB alone, and **C**=2 μg/ml dHCB+1 μM fMLF], and these cells were analyzed

by subcellular fractionation. In all steps of this procedure, including washing, N_2 cavitation, and sucrose density gradient sedimentation, the same respective concentrations of dHCB and fMLF as used for stimulation were maintained to ensure that cellular actin was kept in a depolymerized state and the receptors occupied. The sucrose density profiles were linear between 12-59% for all three groups (not shown) and, thus, activities are plotted as a function of sucrose percentage. The distribution of subcellular markers for each of these conditions (see Figures 2.1-4) shows several interesting differences. As expected, most of the protein in each gradient was present in the cytosolic fractions at the lowest sucrose percentage (Figure 2.1, top panel). In control (group **A**) and dHCB (group **B**) gradients, there were two peaks in the granule containing region representing specific and azurophil granules. A broader distribution of protein was seen in fractions isolated from dHCB+fMLF treated cells (experimental group **C**).

Analysis for azurophil and specific granule markers, myeloperoxidase and lactoferrin, respectively, demonstrated that both types of granules were intact in experimental groups **A** and **B** and sedimented to essentially the same respective densities for both treatment conditions (Figure 2.1, center and bottom panels). For cells treated under condition **C** (dHCB+fMLF stimulated cells), less than 20% of

the myeloperoxidase or lactoferrin detected in **A** or **B** was present, suggesting that it was lost to the extracellular medium by the degranulation process (Figure 2.1). This was confirmed by measuring the myeloperoxidase activity released by intact neutrophils treated with dHCB and fMLF (experimental group **C**). The myeloperoxidase activity measured in the quench buffer of these cells was 77.3±11.4% (mean±S.D., n=3) of the total cellular activity, indicating that indeed condition **C** effectively causes almost complete degranulation of neutrophils.

Plasma Membrane Changes

In completely degranulated cells, the plasma membrane composition should be significantly altered due to membrane addition from the granule envelopes. To investigate changes in plasma membrane density and composition, we analyzed the gradient fractions for the distribution of alkaline phosphatase, a plasma membrane-associated protein in human neutrophils that has been used as a plasma membrane marker in a variety of studies (27). In control cells (experimental group **A**) (Figure 2.2, upper left panel) alkaline phosphatase displayed a peak at 30% sucrose which corresponded to the previously described light plasma membrane fraction (26,27). This distribution did not change considerably upon dHCB treatment (Figure 2.2). In contrast, stimulation of the cells with dHCB+fMLF (experimental group **C**) caused a marked change in the distribution pattern of

this marker enzyme with a broad peak at a higher sucrose density range (27-50%) (Figure 2.2). This broad distribution suggests that the plasma membrane from these cells is much more heterogeneous than in either control or dHCB-treated cells. Although a number of studies have identified alkaline phosphatase as a marker for secretory granules in unstimulated cells (36,37), neutrophils prepared by our methods, which involve exposure to lipopolysaccharide during gelatin sedimentation (38), show no evidence for such a pool. Hence, alkaline phosphatase activity remains a useful marker for the plasma membrane in our studies.

To confirm this significant change in the plasma membrane, surface labelling using ^{125}I-WGA, which binds to the cell surface glycoproteins, was used as an additional method to identify plasma membrane distributions. In control cells (group **A**), the peak of the surface label was at ~30% sucrose; the same position as the alkaline phosphatase peak (Figure 2.2, lower left panel). A shoulder of radioactivity can be observed in the heavier fraction indicating an additional surface membrane at slightly higher densities corresponding to the heavy plasma membrane region (26,27). In dHCB-treated cells (group **B**), the ^{125}I-WGA profile corresponds well with the alkaline phosphatase profile, but may be shifted slightly to higher densities (Figure 2.2, lower left panel). This small change might represent a slightly different distribution of the

glycoproteins and alkaline phosphatase in the surface of dHCB treated cells or other subtle changes in membrane composition, e.g., addition of protein to the membrane. The distribution of the ^{125}I-WGA surface label in subcellular fractions following dHCB+fMLF stimulation (group C) also parallels that of the broad alkaline-phosphatase profile, confirming a major reorganization of the plasma membrane in these degranulated cells (Figure 2.2, lower left panel).

Membrane Cytoskeletal Proteins

The membrane cytoskeleton appears to play an important role in the lateral organization of protein components of the plasma membrane, including chemotactic receptors, GTP-binding proteins, and NADPH oxidase proteins (26,27,39). Therefore, we analyzed the effect of our three experimental treatments of the subcellular distribution of the cytoskeletal proteins actin and fodrin. In all three experimental groups, most actin was localized in the cytosol; however, a significant fraction was also localized in the heavy plasma membrane (Figure 2.2, upper right panel). Quantitative measurement of the membrane-associated actin clearly showed that the actin content associated with the membrane fractions in cells treated with dHCB+fMLF was significantly greater than control and dHCB-treated cells (23.1% of total actin versus 2.7% for control and 5.4% for dHCB-treated cells). These results suggest that dHCB does not inhibit, but actually promotes membrane association of

actin during cell activation with the chemotactic peptide.

Further analysis of the gradient fractions for another component of the membrane cytoskeletal network, fodrin, confirmed the membrane remodelling (Figure 2.2, lower right panel). This protein also appears to be redistributed to the plasma membrane in fractions obtained from cells treated with dHCB+fMLF (experimental group **C**). Quantitative measurement of fodrin in these samples also showed a significant translocation of fodrin from the cytosol to the plasma membrane fractions in cells stimulated with dHCB+fMLF as compared to control and dHCB treated cells (40% of total fodrin versus 13.3% for control and 17.4% for dHCB-treated cells).

The fractions prepared from each of the experimental conditions were analyzed by SDS-PAGE, and the silver stained gels are shown in Figure 2.3. Although the staining patterns were very similar for control (group **A**) and dHCB treated (group **B**) cells (panels A and B, respectively), significant changes in the protein distributions were observed in cells treated with dHCB+fMLF (group **C**) (panel C). Most evident was the loss of granule proteins (due to degranulation) and the presence of greater amounts of actin deeper in the gradient, cosedimenting with the plasma membrane (PM) markers (see Figure 2.2, upper right panel).

Electron Microscopy

To visually determine how these conditions affected plasma

membrane morphology, we analyzed the cells treated under the three experimental conditions using transmission and scanning electron microscopy. In the control cells (group **A**), we found by scanning electron microscopy (SEM) that the cells were rounded and nonpolarized. Their surface was not completely smooth, however, and contained some ruffles and surface invaginations (Figure 2.4, panel A). By transmission electron microscopy (TEM), we found again that control cells were nonpolarized and contained some small surface irregularities (Figure 2.4, D). Also characteristic of these cells were the densely staining granules which were abundant in the cytoplasm. These results (both SEM and TEM for control cells) are consistent with those previously described by Hoffstein et al. (35). In contrast to the control cells, cells treated with dHCB exhibited a dramatically different morphology. By SEM, these cells had a complete loss of plasma membrane topography (Figure 2.4, panel B), and they were almost completely rounded and smooth on the surface, with only minor membrane irregularities still present. This type of membrane morphology was confirmed in cross section using TEM of dHCB-treated cells (Figure 2.4, panel E). Again, the plasma membrane surface was quite smooth with minor ruffled areas. As with control cells, the cytoplasm of these cells was still loaded with the characteristic densely staining granules.

Neutrophils stimulated with dHCB and fMLF displayed a

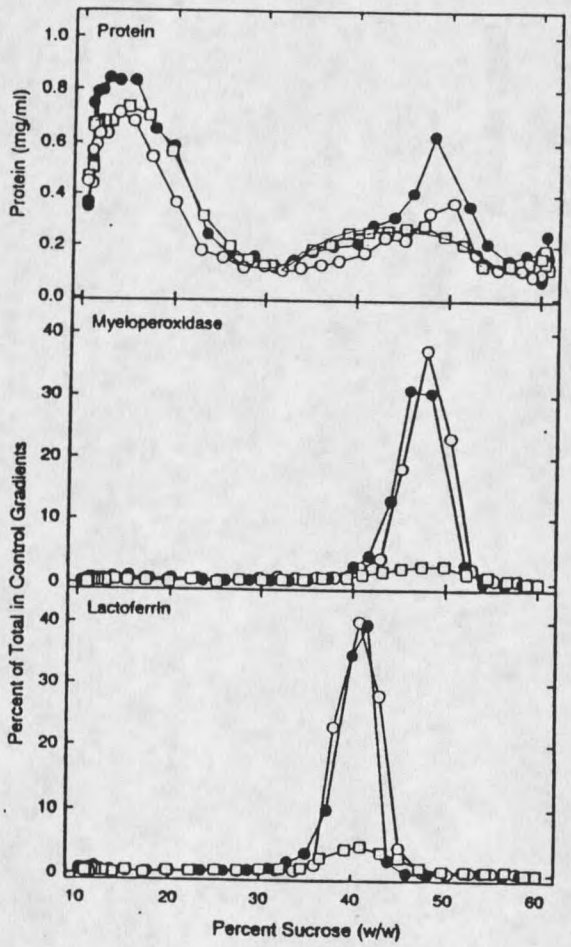

Figure 2.1. Subcellular Distribution of Protein, Myeloperoxidase, and Lactoferrin in Fractionated Human Neutrophils. Control (○), dHCB (●), and dHCB+fMLF (□) treated cells were disrupted by N_2 cavitation and fractionated by isopycnic sucrose density gradient sedimentation as described under Methods. The protein content of each fraction, expressed in mg/ml, is plotted as a function of percent sucrose (top panel). Approximately 85-90% of the protein applied to the gradient was recovered in the fractions. Representative of six comparable experiments. The subcellular distribution of the azurophil granule marker, myeloperoxidase (center panel) and the specific granule marker, lactoferrin (bottom panel) are expressed a percent of total activity recovered in control gradients and are plotted as a function of percent sucrose. Greater than 90% of the activity applied to the gradients was recovered. Representative of six comparable experiments.

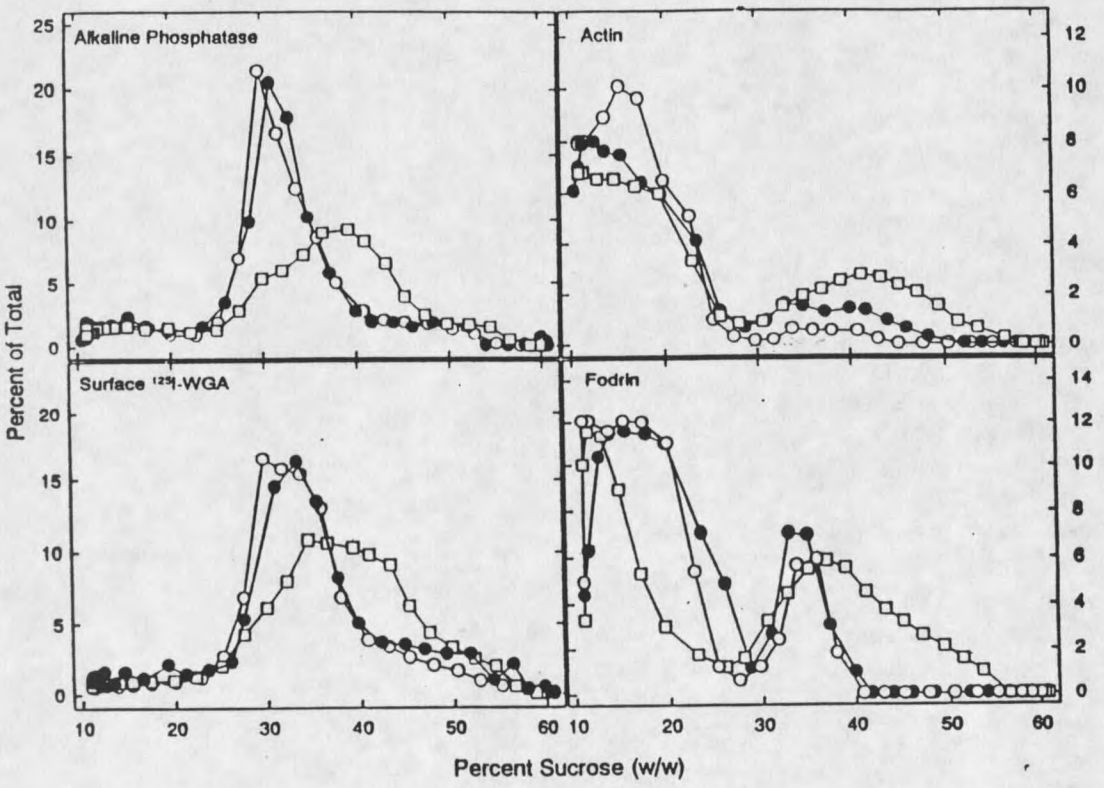

Figure 2.2. Subcellular Distribution of Neutrophil Plasma Membrane Markers. Control (○), dHCB (●), and dHCB+fMLF (□) treated cells were disrupted by N_2 cavitation and fractionated by isopycnic sucrose density gradient sedimentation as described under Methods. The subcellular distribution of alkaline phosphatase (upper left panel) and surface ^{125}I-WGA (lower left panel), expressed as percent of total activity measured in the gradient, is plotted as a function of percent sucrose. Approximately 95-100% of the alkaline phosphatase activity and 93-100% of the radioactivity applied to the gradients was recovered. The total alkaline phosphatase activity or ^{125}I-WGA applied to the gradients varied <10% for each of the three experimental conditions. Representative of six (alkaline phosphatase) or two (^{125}I-WGA) comparable experiments. Gradient fractions were also analyzed by Western blotting for actin (upper right panel) and fodrin (lower right panel) as described. Blots were quantitated using video densitometry as described, and the densities of the actin and fodrin band are plotted for each sample as a percentage of the total actin or fodrin in the gradient. Recovery of these proteins on the gradients was >95%. Representative of six (actin) or two (fodrin) comparable experiments.

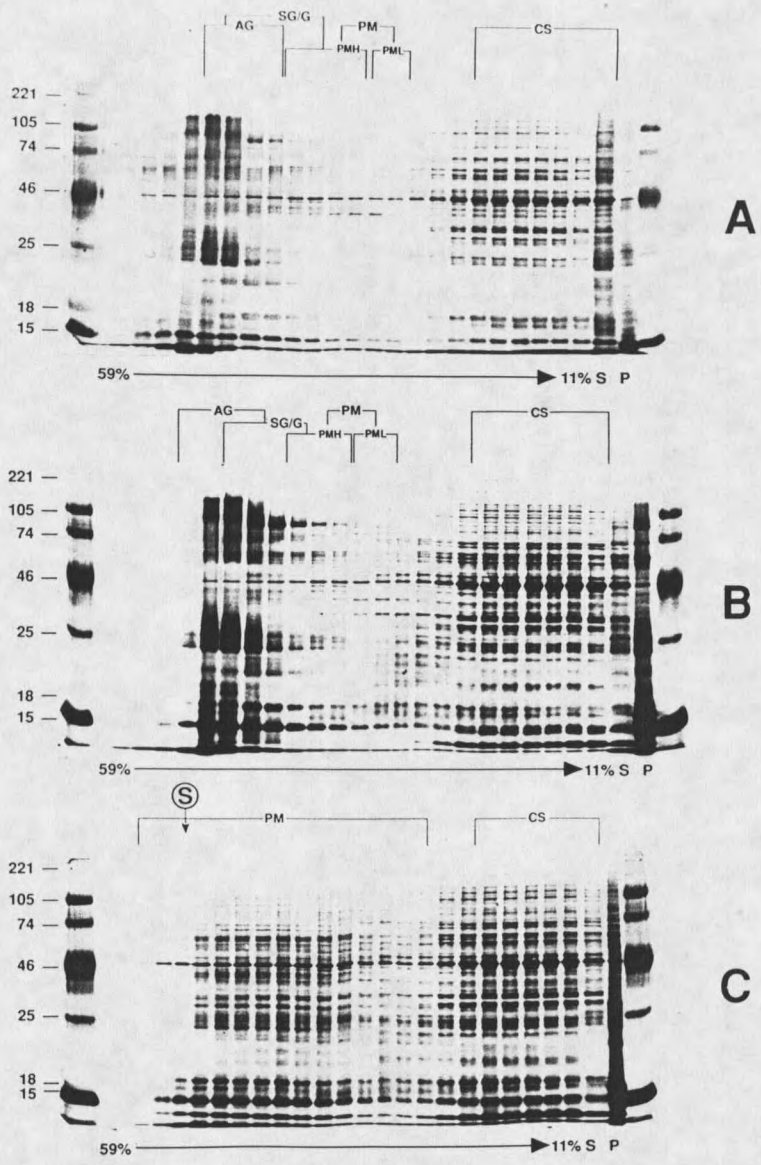

Figure 2.3. SDS-Polyacrylamide Gel Electrophoresis Analysis of Fractionated Human Neutrophils. Control (panel A), dHCB (panel B), and dHCB+fMLF (panel C) treated cells were disrupted by N_2 cavitation and fractionated by isopycnic sucrose density gradient sedimentation as described under Methods. Gradient fractions were solubilized in SDS-containing solubilization buffer and separated on 9% SDS-PAGE gels (4.8×10^4 cell eq./lane). Percent sucrose (%S) of the fractions is indicated under each gel, and P is the 1K pellet. Abbreviations used to identify subcellular organelles in panels A and B are: AG=fractions rich in azurophil granule markers (peak at 48-50% sucrose), SG/G=fractions rich in specific granule/Golgi markers (peak at 40-42% sucrose), PM-H=heavy plasma membrane fractions (peak at 33-35% sucrose), PM-L=light plasma membrane fractions (peak at 29-30% sucrose), and CS=cytosolic fractions (10-25% sucrose). The abbreviations used in panel C are: PM=reorganized plasma membrane (brad distribution at 27-56% sucrose), (S)=location of the peak superoxide generating activity (peak at 49-50% sucrose), and CS=cytosolic fractions (10-25% sucrose). Representative of six comparable experiments.

completely different morphology than both control and dHCB-treated cells. By SEM, these cells became polarized, and we observed plasma membrane ruffles, knobs, extended knobs, and bleb like structures, usually located on one end of the cell (Figure 2.4, panel C). Some of the blebs seemed to be bound by single unit membrane with basal thickening. Analysis of these cells using TEM, confirmed the SEM results and showed again the polarized nature of these cells and the extensive blebbing at one end of the polarized cell (Figure 2.4, panel F). In addition, the majority of the cells in dHCB+fMLF population lacked the densely staining granules in their cytoplasm, confirming our gradient analysis data described above and indicating that indeed these cells were almost completely degranulated under these conditions. In these studies we observed slight heterogeneity among the neutrophil population. In SEM studies, the control cell population contained some cells (~5%) that appeared to be activated, as evidenced by membrane ruffling, whereas the remaining 95% of the cells had normal unstimulated PMN membrane appearance. Following dHCB+fMLF treatment, almost all the cells (>90%) gave the characteristic stimulated appearance shown; however, there were some non-responders that appeared unstimulated. These differences may reflect the heterogeneity which is known to be present in the neutrophil population (40,41).

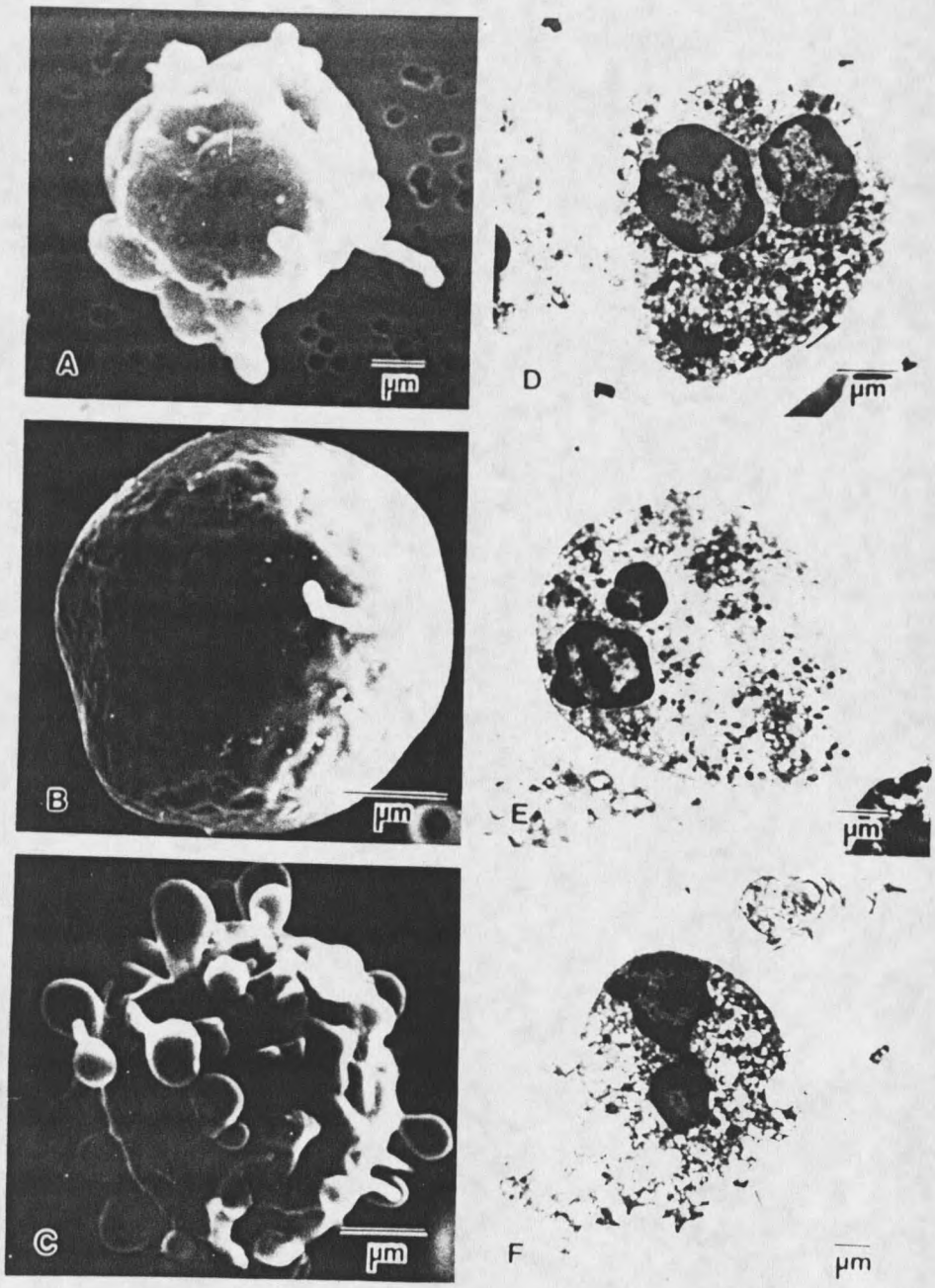

Figure 2.4. Scanning and Transmission Electron Microscopy of Neutrophils Treated with Dihydrocytochalasin b and fMLF. Purified neutrophils were fixed and prepared for electron microscopy as described under Methods. Panels A-C show scanning electron micrographs of representative neutrophils treated with nothing (control group, panel A), treated with 2 μg/ml dHCB (panel B), and treated with 2 μg/ml dHCB and 1 μM fMLF (panel C). Panels (D-E) show transmission electron micrographs of similar cells treated with nothing (control group, panel D), treated with 2 μg/ml dHCB (panel E), and treated with 2 μg/ml dHCB and 1 μM fMLF (panel F). All micrographs are representative of 3 separate experiments. where $\geq$ 20 cells were examined per experiment for each of the three experimental conditions.

NADPH Oxidase Organization

Considering the major change in plasma membrane composition and morphology in cells treated with dHCB and fMLF, we expected a significant reorganization in the cellular distribution of NADPH oxidase components and possible loss of the lateral organization of the O_2^- generating activity and components. Analysis of the gradient fractions from cells stimulated with dHCB+fMLF for NADPH-dependent O_2^- generating activity, however, revealed that some form of membrane organization remained and that O_2^- generating activity was limited to a subfraction of the plasma membrane region, with peak activity at ~50% sucrose (Figure 2.5, bottom panel). These fractions accounted for only ~20% of the total activity generated by whole cells, suggesting that either a small part of the total distribution of oxidase components was properly assembled into an active complex or, more likely, that most of the highly labile O_2^- generating activity was lost during sample preparation.

Analysis of gradient fractions from cells treated with dHCB+fMLF for NADPH oxidase components showed that the distribution of p47-*phox* and cytochrome b paralleled the plasma membrane markers (alkaline phosphatase and ^{125}I-WGA) and membrane skeletal components (actin and fodrin) (for reference see Figure 2.2). In control and dHCB treated cells (groups **A** and **B**, respectively), p47-*phox* was localized

almost completely (≥97% of total) to the cytosol. As we observed previously (42), a small amount of p47-*phox* (<3% of total) was detected in the membranes of control, unstimulated cells. Whether this represents contamination by the cytosol or a functional association with the membrane remains to be determined. Analysis of cytochrome b showed a typical bimodal distribution between plasma membrane (~30% of total) and specific granule (~70% of total) fractions in both control and dHCB-treated cells (Figure 2.5, top and center panels, respectively). In cells treated with dHCB+fMLF (group **C**), a significant fraction of p47-*phox* (~16.9% of total) was translocated from the cytosol to the plasma membrane, where it showed a broad distribution corresponding to the distribution of the other plasma. The majority of cytochrome b was also translocated from the specific granules to the plasma membrane (>99% of total) in cells treated with dHCB+fMLF (Figure 5, center panel), and, as with p47-*phox*, the distribution of cytochrome b followed the broad distribution of the plasma membrane markers. Thus, it is evident from these results that in the fully activated cell not all of the oxidase components associated with the membrane participate in active O_2^- generating complexes. An alternative possibility is that the subfraction of plasma membrane that is producing O_2^- contains a protein (or lipid) component that stabilizes oxidase activity and that this component is not present in

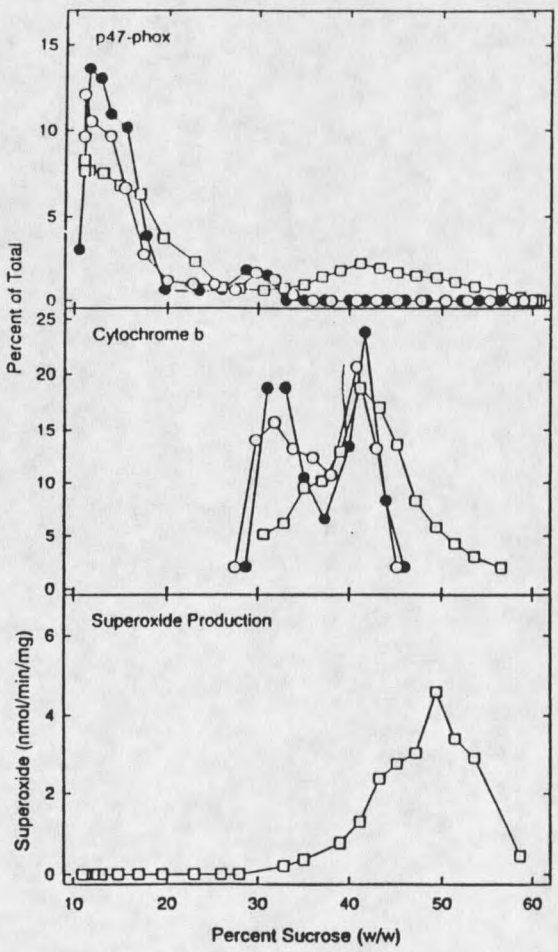

Figure 2.5. Subcellular Distribution of NADPH Oxidase Components and Superoxide Generating Activity in Fractionated Human Neutrophils. Control (○), dHCB (●), and dHCB+fMLF (□) treated cells were disrupted by N2 cavitation and fractionated by isopycnic sucrose density gradient sedimentation as described under Methods. Gradient fractions were separated by SDS-PAGE and analyzed by Western blotting for p47-*phox* (top panel) and cytochrome b light chain (p22-*phox*) (center panel) as described under Methods. Blots were quantitated using video densitometry as described, and the densities of the p47-*phox* and p22-*phox* bands are plotted for each sample as a percentage of the total p47-*phox* or p22-*phox* in the gradient. Recovery of these proteins on the gradients was >95%. Representative of six (cytochrome b) or three (p47-*phox*) comparable experiments. Superoxide generating activity of the fractions was determined spectrophotometrically using a standard cytochrome C assay system as described, and the specific activity is plotted against percent sucrose (bottom panel). Representative of three separate experiments. membrane markers (Figure 5, top panel).

all other regions of the plasma membrane. To investigate this possibility, we pooled plasma membrane fractions from light (27-33% sucrose), heavy (33-38% sucrose), and O_2^- generating (45-52% sucrose, for dHCB+fMLF treated cells only) plasma membrane regions and analyzed them using SDS-PAGE and silver staining. The silver stained gel in Figure 2.6 shows the protein composition of these membrane fractions. Comparison of the actin containing band (~42 kDa) of heavy and light plasma membranes from control (lanes 1 and 2, respectively) and dHCB-treated (lanes 3 and 4, respectively) cells confirmed our previous results (26,27) showing that indeed the heavy membranes (lanes 1 and 3) were enriched in actin compared to the light plasma membranes (lanes 2 and 4). Moreover, actin content was increased in all subfractions of plasma membranes isolated from cells treated with dHCB+fMLF (lanes 6 and 7, respectively) compared to control (lanes 1 and 2) and dHCB-treated (lanes 3 and 4) cells. The O_2^- generating membranes (lane 5) were also highly enriched in actin, consistent with our previous hypothesis that the membrane skeleton plays a role in the structure or function of the NADPH oxidase (26,43).

Comparison of the heavy and light plasma membrane subdomains from control, dHCB, and dHCB+fMLF treated cells showed potentially important differences in protein composition.

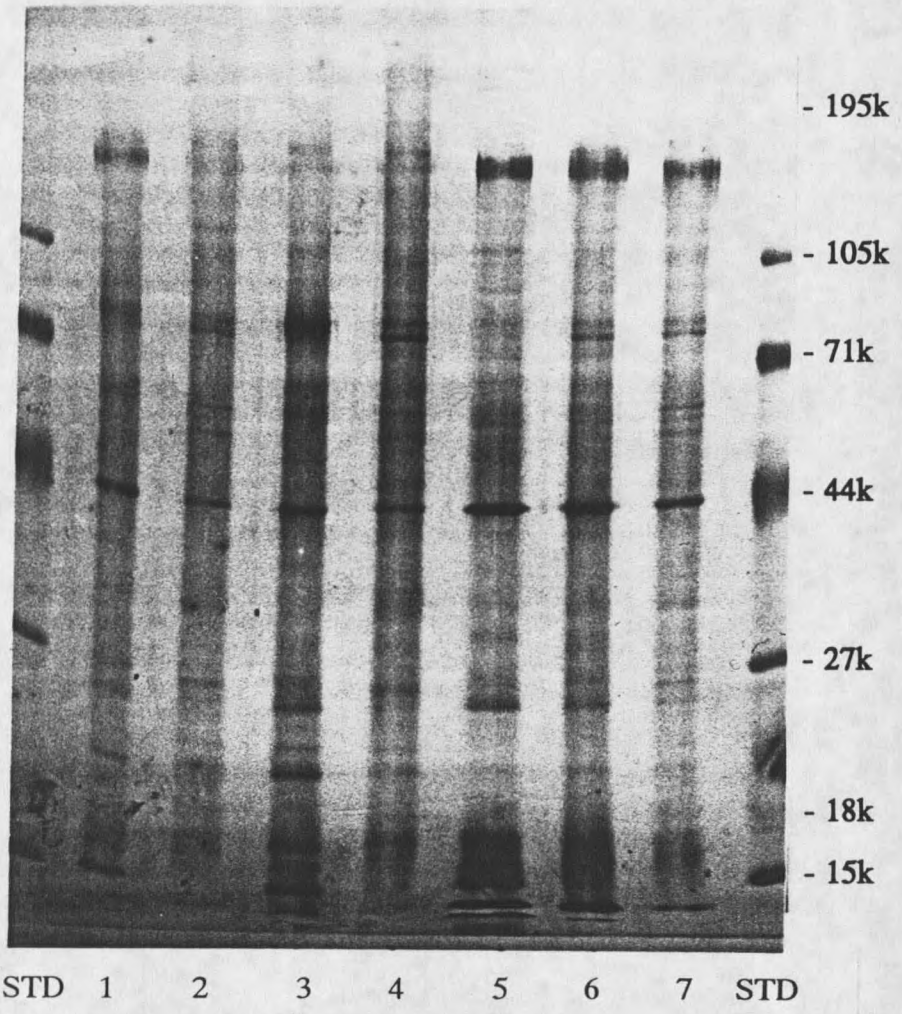

Figure 2.6. Protein Composition of Neutrophil Plasma Membranes. Heavy (34-38% sucrose) and light (28-33% sucrose) plasma membranes prepared from control cells (lanes 1 and 2, respectively), dHCB-treated cells (lanes 3 and 4, respectively), dHCB+fMLF-treated cells (lanes 6 and 7, respectively), and from the O_2^- generating fractions (45-51% sucrose) of dHCB+fMLF-treated cells (lane 5) were solubilized in sample buffer and analyzed by SDS-PAGE on 7-18% polyacrylamide gradient gels (3.8×10^4 cell eq./lane), followed by Coomassie blue and silver staining. Prestained molecular weight standards were used on these gels (STD), and their molecular weights are indicated. Representative of two comparable experiments.

In cells treated with dHCB+fMLF, unique protein bands were present at ~200, 107, and 95 kDa in the O_2^- generating subdomain (lane 5) which were absent in non-O_2^- generating plasma membrane subdomains of similarly treated cells (lanes 6 and 7). Interestingly the light plasma membrane (28-33%) of dHCB-treated cells (lane 4) contains a band at ~200 kDa which is absent in both membrane subdomains of control cells (lanes 1 and 2), suggesting the possibility that dHCB treatment alone might be responsible for the translocation of this protein to the plasma membrane. Further analysis of the significance of these differences is in progress.

Discussion

The studies described here demonstrate that the plasma membrane of dHCB-treated neutrophils undergoes significant remodelling after fMLF stimulation. This remodelling, however, is not surprising since these cells are almost completely degranulated, resulting in the fusion of granule membranes with the plasma membrane. Hence, the remodeled membrane displays new characteristics of heterogeneity and altered lipid and protein composition, as demonstrated here by subcellular fractionation analyses and electron microscopy.

In the studies reported here, we achieved a level of degranulation higher than that previously reported in the literature for cells stimulated with dHCB+fMLF. We observed that such treatment of the cells resulted in an average of

77.3±11.4% release of myeloperoxidase. A similar high level (85%) of degranulation has only been reported when neutrophils were treated with calcium ionophore A23187 and dHCB (44), but never with dHCB and fMLF. However, in these previous studies, the stimulant (e.g., fMLF and dHCB) was only present during the activation step, and then it was removed by washing in the subsequent steps (24,27,30,44-46). In our effort to understand the molecular basis for the effects of dHCB on fMLF-stimulated O_2^- production, we took a slightly different approach. Because the effects of fMLF and dHCB are rapidly reversible after dilution or washing (47), we maintained the stimulant and dHCB levels constant throughout all subsequent steps until the membranes were isolated. This experimental design then allowed us to isolate membranes as close to the state that they were in during activation and eliminated the possibility that removal of the stimuli or perturbants at subsequent steps was allowing the cells to reverse the changes in membrane and cytoplasmic composition that occurred during activation.

The most noteworthy feature of the remodeled neutrophil plasma membrane is its heterogeneity and increased density. The new density range of these membranes is from between 27% (1.113 gm/cm^3) sucrose to 50% (1.230 gm/cm^3) sucrose. This increased density is most likely due to a higher amount of protein associated per unit of membrane, possibly from fusion of intrinsically denser granule membranes or from

newly associated membrane proteins, such as actin (44). Even though dHCB is responsible for actin depolymerization in the cytoplasm, a higher concentration of actin (23.1% of total actin) tends to cosediment with the plasma membrane fractions in cells treated with dHCB+fMLF compared to control or dHCB-treated cells (2.7 and 5.4% of total actin, respectively). These results suggest the possibility that the membrane skeleton might contain a different population of actin than that found in the cytosol that is insensitive to dHCB or that new actin binding sites are generated in the membranes of activated cells. In addition, the increased actin monomer concentration in the cytoplasm might also cause more actin to associate with the membrane skeleton. Previous studies showing that the association of epidermal growth factor receptors with actin in A431 cells is resistant to dHCB at a significantly higher concentration than that used here (10 μg/ml) support the idea of dHCB-insensitive actin pools (48), as do reports by others who identified cytochalasin-insensitive pools of actin in neutrophils (47,49).

It is evident from this and previous work that the membrane skeleton plays a role in NADPH oxidase assembly in stimulated neutrophils (26,43,50,51). Our previous studies analyzing subcellular fractions of neutrophils stimulated with PMA localized O_2^- generating activity to a minor subfraction of the plasma membrane enriched in actin and

fodrin (heavy subdomain) with a peak at ~34-36% sucrose (26). In comparison, the O_2^- generating fractions identified here were also confined to a subdomain of the plasma membrane, although sedimenting much deeper into the gradient (peak at 49-50% sucrose). This increased density may reflect the presence of more total protein, including cytoskeletal proteins, associated with the heavy plasma membrane which would cause it to sediment with the denser fractions of plasma membrane in cells treated with dHCB+fMLF. In both cases (PMA and dHCB+fMLF), however, the O_2^- generating fractions are confined to the heaviest subdomains of the plasma membrane, which are also enriched in actin and fodrin.

Interestingly dHCB, which inhibits actin polymerization, can also inhibit the normal termination of O_2^- production. That is, if the cells are treated with dHCB prior to fMLF treatment, the regulatory control of O_2^- production is perturbed, and the cells generate O_2^- for a longer time and at a higher rate (43). One possible explanation for this is that the enhancement of specific granule mobilization caused by dHCB in these cells increases the concentration of formyl peptide receptors and NADPH oxidase components in the plasma membrane, thus enhancing the signal transduction or effector protein apparatus in the plasma membrane. The effects of dHCB might also alter membrane fluidity by decreasing the cytoskeletal network,

and changes in membrane fluidity have been reported to affect the oxidative burst (52,53). Cytochalasins have also been demonstrated to elevate and prolong intracellular calcium release when cells are stimulated with high concentrations of formyl peptides (54), and elevated calcium levels could also enhance O_2^- production (55,56). Finally, perturbations in the interaction of receptors with the membrane skeleton might alter receptor regulation and termination of activation (27,39).

The detailed picture of cellular events in the assembly of the active oxidase is unknown. Our work demonstrates that, even though all the currently known NADPH oxidase factors are present in the plasma membrane of the neutrophils treated with dHCB+fMLF, not all of the plasma membrane-containing fractions produce O_2^-. One explanation is that there may be some other unidentified factors responsible for facilitating the assembly of these components in the membrane. In the absence of such factors, an active complex might not form or would be unstable. Support for this notion exists in the fact the O_2^- production is stabilized to a greater degree in membranes prepared from particulate stimuli, which may maintain membrane order when the cells are disrupted (57). Some of the unique proteins we have identified in the isolated O_2^- generating fractions may be candidates for this stabilizing role. Future investigation to characterize these proteins might provide

additional information about the structure of the active NADPH oxidase complex in neutrophils.

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CHAPTER 3

REDISTRIBUTION OF MAC-1 ON THE SURFACE OF STIMULATED AND POLARIZED HUMAN NEUTROPHILS MAY BE ASSOCIATED WITH CYTOSKELETAL MODULATION

Introduction

Neutrophils play an important role in host defense against microbial invasion by providing the primary cellular response to acute inflammatory signals. Upon arrival at a site of infection, neutrophils engage microbicidal responses which serve to kill the invading pathogens and in certain situations damage surrounding host tissue. Generation of superoxide radical is one of the responses which plays a major role in killing invading pathogens in the phagosome. It has been shown that the terminal electron donor in NADPH oxidase complex, cytochrome b_{558} forms clusters in the phagosomal membrane suggesting possible sites for O^-_2 generation (1). This clustering might be important in respiratory burst *in vivo* because it would enhance local concentration of oxidants. Our previous studies have shown that the generation of O^-_2 is preserved in subdomains of the plasma membranes of dihydrocytochalasin plus formyl peptide (dHCB+fMLF) stimulated neutrophils, and phorbol myristate acetate (PMA) stimulated cells. The molecular basis for the formation of such stabilized NADPH oxidase assemblies in the plasma membrane of neutrophils remains unknown (2).

Neutrophil integrin Mac-1 has been directly implicated in the regulation of the neutrophil oxidative burst. Adherent neutrophils respond to stimulation by tumor necrosis factor, TNF-α (100ng/ml), by generation of higher amounts of H_2O_2 and O^-_2 compared to cells in suspension (3,4). Neutrophils from patients with leukocyte adhesion deficiency (LAD), which lack Mac-1, fail to show the oxidative burst (3) induced by TNF-α. In addition, cross linking of the subunits of β_2 integrins directly initiates respiratory burst in neutrophils (5). Since Mac-1 is transported in the same vesicles with cytochrome b during translocation from specific granule stores, (6) it may participate in the formation of the NADPH oxidase.

Mac-1 has also been shown to take part directly in various signal transduction pathways. Neutrophil Mac-1 mediates firm adhesion of neutrophils to the vascular endothelium in response to acute inflammatory stimuli, such as certain chemoattractants (FMLF, IL-8, LTB_4), bacterial endotoxin, interleukin-1, TNF-alpha, or lymphotoxin (7-9). The β subunit (CD18) of Mac-1 binds to the endothelial molecule ICAM-1 (see chapter 1) (10), and the α subunit (CD11b) mediates specific ligand binding (11). Direct ligand binding to Mac-1 can raise the intracellular Ca^{2+} level, and pH in the cells (12).

Interestingly, cytoskeletal components have been shown to play an intermediary role in some of the signal

transduction events mediated by the integrins (12,13). Both Mac-1 and NADPH oxidase systems are associated with the cytoskeleton (14-16) and thus might share common regulatory mechanisms involving several small GTP binding proteins (17). Also, work in our laboratory and others has shown that active NADPH oxidase components are in the TX-100 insoluble fractions of the plasma membrane, indicating possible cytoskeletal linkage. Because Mac-1 has also been shown to associate directly with several actin binding proteins (9,18,19), it may also participate in oxidase organization or at least provide clues as to how it might be assembled.

The neutrophil chemoattractant fMLF induces translocation of NADPH oxidase components to the plasma membrane and causes generation of O^-_2. It also induces 5-10 fold increase in surface expression of Mac-1 within 10 to 30 minutes of exposure (20). Whether this excess distribution of Mac-1 on the plasma membrane is restricted in specific structural subdomains (where it could take part in forming stable oxidase complex) or is distributed randomly is not known. In our previous studies using scanning and transmission microscopy we found that neutrophils stimulated with fMLF and the cytoskeletal perturbing agent dHCB undergo a major reorganization of the plasma membrane. Even though the plasma membrane distribution in these gradients was different than unstimulated neutrophil, eg., restricted to

33-31% sucrose for heavy plasma membrane and 28-30 % for light plasma membrane, of special interest is a heavier plasma membrane subdomain where NADPH oxidase activity was preserved (peaked at 50% sucrose). In this study, utilizing high resolution scanning electron microscopy and surface labelling with anti Mac-1 antibody, we found that Mac-1 forms large aggregates in dHCB+fMLF stimulated suspension cells. In adherent cells it is non randomly distributed with predominance in the leading edge of lamellipodia.

Materials and methods

Reagents

The chemicals used in this study were of the highest quality commercially available. Monoclonal antibodies to Mac-1, 13.22, 13.14 and 13.13a, were produced against the heparin ultrogel eluate of neutrophil plasma membrane octyl glucoside extracts prepurified on a lectin affinity matrix. Goat anti-mouse antibody and all scanning electron microscopy (SEM) supplies were purchased from Ted Pella, Inc (city,ST). The rabbit polyclonal antibody R7928, and purified Mac-1 was a gift from Dr. Charles Parkos.

Preparation of the cells

Neutrophils were isolated as described previously (2). Erythrocytes were sedimented at 1Xg using in Dextran 500 or

Gelatin. Cells were treated as described previously (2) for immune electron microscopy to ascertain the resulting morphology. Control cells were experimental group **A**, dHCB (2 ug/ml) treated cells were group **B**, and dHCB (2 ug/ml) + fMLF (1uM) treated cells were group **C**.

Antibody Characterization

Antibodies used in this study were characterized by examining their activity against different purified and non purified antigens. SDS-PAGE was run on NP-40 membrane extracts, purified Mac-1, wheat germ agglutinin (WGA)/sepharose eluate from octyl glucoside extracted neutrophil plasma membranes (contains glycosylated membrane proteins) (21), and R7928 immuno-depleted extracts (specific against alpha subunit of Mac-1).

Electrophoresis and Western Blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 20° C using 9% polyacrylamide slab gels (22). Electrophoretic mobility of the samples was compared with the mobility of prestained standard proteins (BRL, Bethesda, MD).

Western blotting was performed as described previously (23). Transfers were blotted with (2.4μg/ml) monoclonal antibody 13.22, 13.14, or 13.13a (all IgM) overnight at 4°C, followed by incubation with alkaline phosphatase conjugated goat anti-mouse IgM secondary antibodies (1:1000 dilution) for 1 hr at 20°C, and developed using an alkaline

phosphatase development kit (Kirkegaard & Perry Laboratories, Gaitherburg, MD). Blots were scanned with Hewlett-Packard Deskjet scanner and quantitated using Imagequant™ software (24). The relative density of a sample represents the density of the sample relative to an arbitrary gray scale defined by the image analysis system to cover the total gray shade range of the image.

Immunogold labelling

Cells for immune electron microscopy were mounted on either a quartz slide to avoid zinc artifacts produced in high resolution SEM, or on a 0.4 micron polycarbonate filter. The quartz slides had been previously cleaned with acetone followed by an alcohol wash and were precoated with 0.1% BSA.

A 0.1% poly-L-lysine coated polycarbonate filters were used as a matrix to collect cells in suspension after different stimulatory conditions (A, B, and C). The cells were allowed to adhere for 20-30 min at room temperature (RT) in a high moisture chamber, rinsed three times with 0.1M PIPES buffer (pH 7.3) and lightly fixed in 0.02% glutaraldehyde in PIPES (pH 7.3) to retain their morphological characteristics without modifying the cell surface antigenicity. Cells were then washed with 0.1% glycine in PIPES to get rid of excess free aldehyde groups (25). Labelling with the primary (0.024mg/ml) and secondary antibody (1:100 dilution of the commercial

antibody) was done in blocking buffer containing 1% globulin free BSA, and 1% decomplemented human serum in 0.01 M PBS. The cells were then fixed in 2% glutaraldehyde, critical point dried, and coated with carbon, and observed using JEOL 6100 scanning electron microscope. Secondary electrons emitted from the samples were used to generate the cell surface morphology and backscatter electrons to detect the gold particles on them. The images were superimposed on the film to obtain the final composite image.

Results

Our previous neutrophil subcellular fractionation and electron microscopic studies indicated that the plasma membrane undergoes a major reorganization following stimulation with fMLF in the presence of dHCB. Curiously, only a subfraction of the plasma membrane in fully activated cells appeared to be capable of producing superoxide (2) supporting earlier studies by us and others, suggesting that only a fraction of the plasma membrane oxidase components take part in active superoxide generation (23). Because of the close relationship of Mac-1 to the NADPH oxidase, it was important to determine whether Mac-1 is similarly distributed under such stimulatory conditions. Thus we also examined the surface organization of this important membrane protein molecule by high resolution immuno-electron microscopy and subcellular fractionation. For such studies immuno-electron microscopy using gold labelled antibody

provided an excellent tool to visualize the surface morphology.

Subcellular Distribution of Mac-1

To determine the subcellular distribution of Mac-1 in control, dHCB-treated, and dHCB+fMLF-stimulated cells, cells so treated were disrupted by N_2 cavitation and the homogenate separated on linear sucrose density gradient as previously described (2). Antibodies 13.13a, 13.22 were used to identify Mac-1 distribution in such gradients by western-blotting (Fig 3.1). In control cells, the majority of Mac-1 was localized in the specific granule fraction with very little found in the plasma membrane. This distribution (Fig. 3.2a) is consistent with the previous observations (26,27). Treatment with dHCB did not alter the distribution of Mac-1 significantly (Fig 3.2b). However, in stimulated cells (dHCB+fMLF) the distribution was dramatically different (Fig. 3.2c), displaying a broad heterogenous distribution of the plasma membrane (2) with microheterogeneity consistent with membrane subdomains overlapping NADPH oxidase distribution.

Morphology of suspended cells

In parallel experiments, cells from the three experimental groups were adhered to polylysine coated polycarbonate filters to allow observation of their suspension morphology as closely as possible in order to morphologically corroborate fractionation results (2).

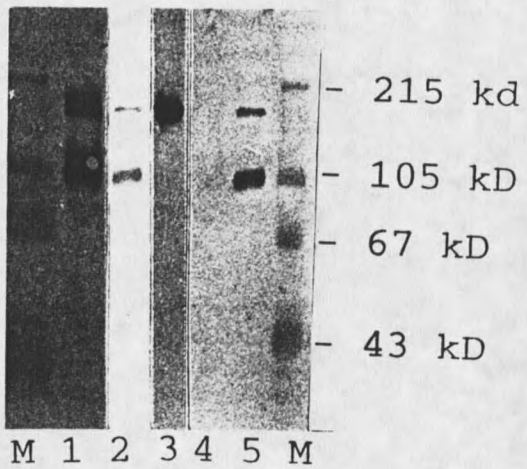

Fig. 3.1. Specificity of monoclonal antibodies 13.22 for Mac-1. (M) lanes are molecular weight markers. Samples from NP-40 solubilized membrane (lane 1, $2X10^7$ cell equivalent), WGA/Sepharose column eluate of neutrophil plasma membrane (WSE) (lane 2, $1X10^7$ cell equivalent), same sample as lane 2 was blotted with R7928 as primary antibody (for positive control), WSE immuno-depleted with R7928 (lane 4, 2.8 ug), and purified Mac-1 (lane 5, 4.5 ug) were separated on SDS-PAGE and transferred to nitrocellulose membrane. The primary antibody used for Lane 1,2,4, and 5 in this western blot was 13.22 (13.22, 13.14, and 13.13a gave identical results). The secondary antibody used was alkaline phosphate conjugated goat anti rabbit IgM antibody (BioRad).

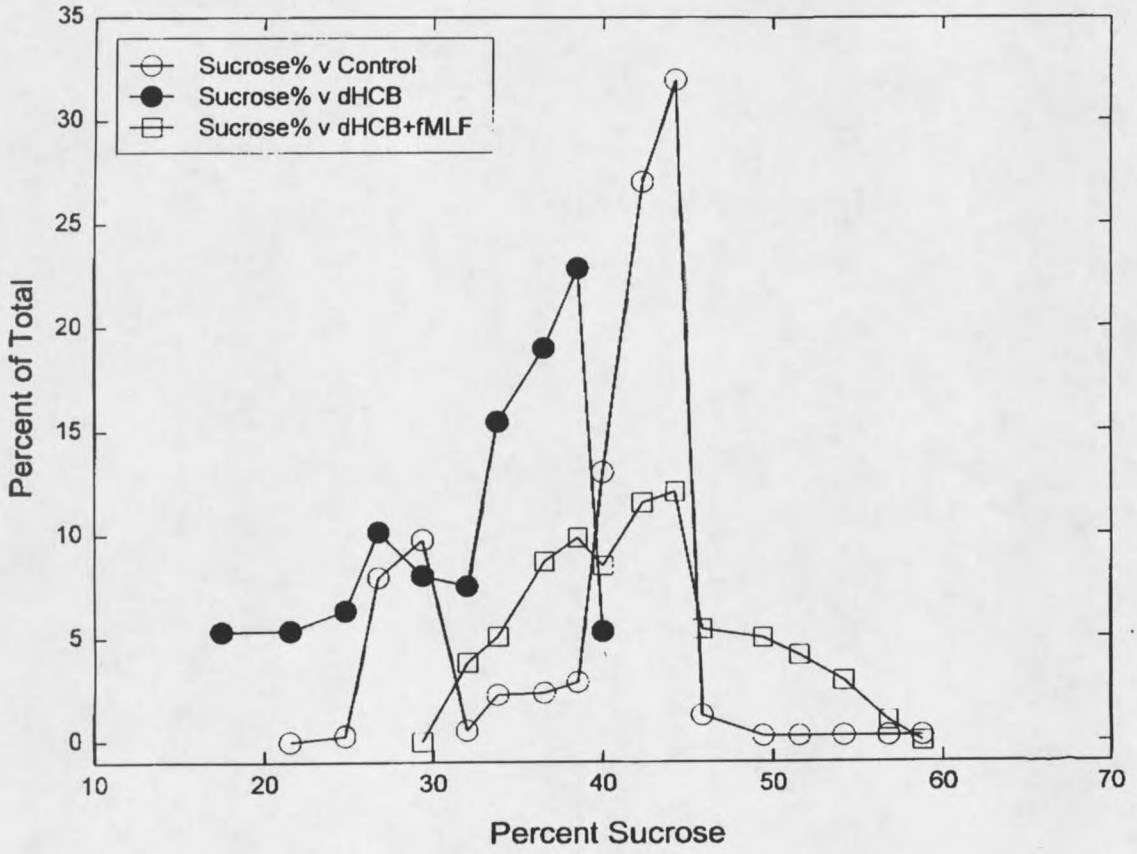

Fig 3.2. Subcellular distribution of Mac-1 in fractionated human neutrophils. Control, dHCB, and dHCB+fMLF treated cells were disrupted by N2 cavitation and fractionated by isopicnic sucrose density gradient sedimentation as described in Mukherjee et. al. (2). Gradient fractions were separated by SDS-PAGE and analyzed by Western blotting for Mac-1 as described under Methods. Blots were quantitated using scanning densitometry as described in Methods, and density of the bands are plotted for each sample as a percent of the total Mac-1 in the gradient. Recovery of this protein on the gradient was >95%. Representative of three comparable experiments.

Control cells (condition **A**) prepared using 2% gelatin exhibited the classical neutrophil morphology of non-polarized cells with some membrane ruffles and surface invaginations (Fig. 3.3 a). This result is consistent with previously described results of Hoffstein et al. (28). The distribution of gold labeled Mac-1 on the surface was uniform, indicating that the cells were not polarized and confirming previous studies (29). In dHCB treated cells (condition **B**), the cells lost the typical ruffled architecture of the plasma membrane and assumed a more rounded smooth shape losing much of the surface features (Fig. 3.3 b). The extent of rounding depended on the time of exposure to dHCB (data not shown). The distribution of gold-labelled Mac-1 on the surface was still found to be random. However, following dHCB+fMLF treatment (condition **C**), the cells assumed a distinct polarized shape (Fig. 3.3 c). As we noted previously, plasma membrane ruffles, knobs, extended knobs, and blebs were present (2). The distribution of gold particles (Mac-1) on the surface also changed dramatically. In condition **C**, there appeared to be a higher density of gold labelled Mac-1 in the ruffled or irregular regions of the plasma membrane. The distribution of gold particles in these regions was highly aggregated and occasionally could be observed as linear arrays (see arrows). A Similar distribution has previously been shown for epidermal cell growth factor receptors (30).

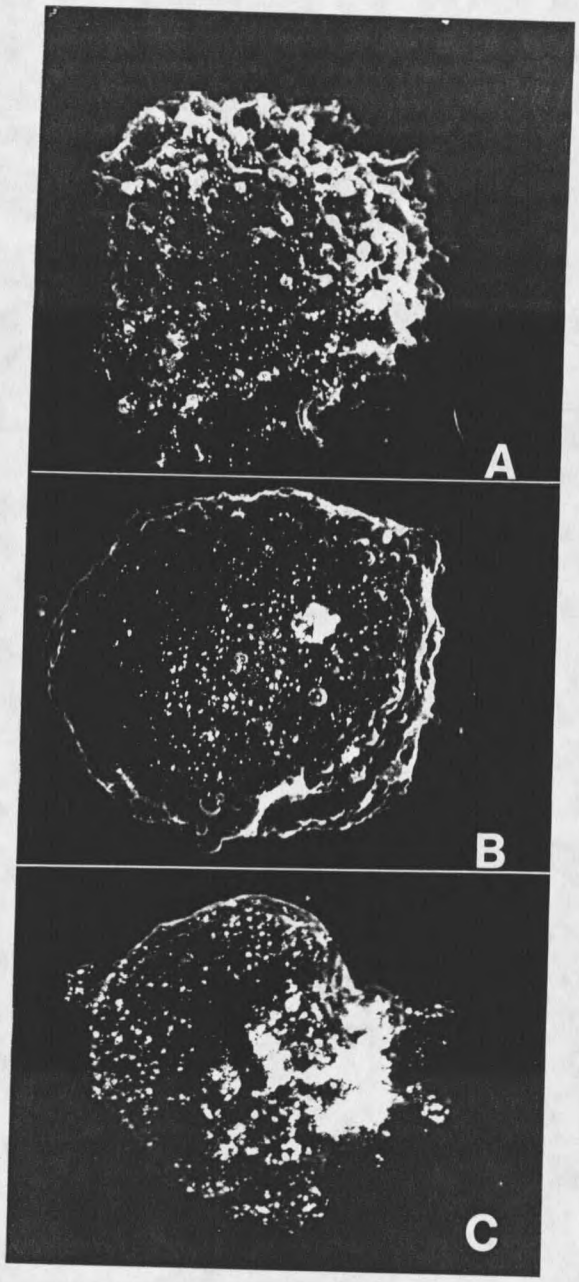

Fig. 3.3. Scanning Electron Microscopy of distribution of Mac-1 on control, dHCB treated and dHCB+fMLF stimulated neutrophils adhered to polycarbonate filters. Purified neutrophils were prepared and labelled for immnuo-electron microscopy as described under methods. Panel A-C shows distribution of Mac-1 on unstimulated neutrophil (control group panel A), neutrophil treated with 2 μl/ml dHCB (panel B), and treated with 2 μl/ml dHCB and 1 μM fMLF (panel C). All micrographs are representative of 3 separate experiments.

Heterogeneity of the neutrophil population in response to different stimuli has been reported previously by Kishimoto et al. (31). In the present studies, >90% of cells treated by conditions **B** and **C** showed the characteristic morphology, while there were also some non-responsive cells (<5%). In addition, less than 5% of the control cells exhibited a morphology similar to that of activated cells as evidenced by membrane ruffling. The photographs included here represent the typical morphology observed for each of the treatments.

Morphology of the adherent cells

In vivo, neutrophils function primarily as adherent cells when activated by acute inflammation (3). To examine the possible surface alterations of neutrophils when they are adherent to a solid matrix and stimulated with fMLF, cells were also allowed to adhere to BSA-coated quartz slides. Stimulated quartz-adherent cells displayed a dramatically different morphology compared to the cells adhered to polycarbonate filters (Fig. 3.4). These cells were considerably spread out on the quartz surface and formed extensive lamellipodial structures in random directions. The gold labelled Mac-1 molecules were mostly restricted to the cellular extensions.

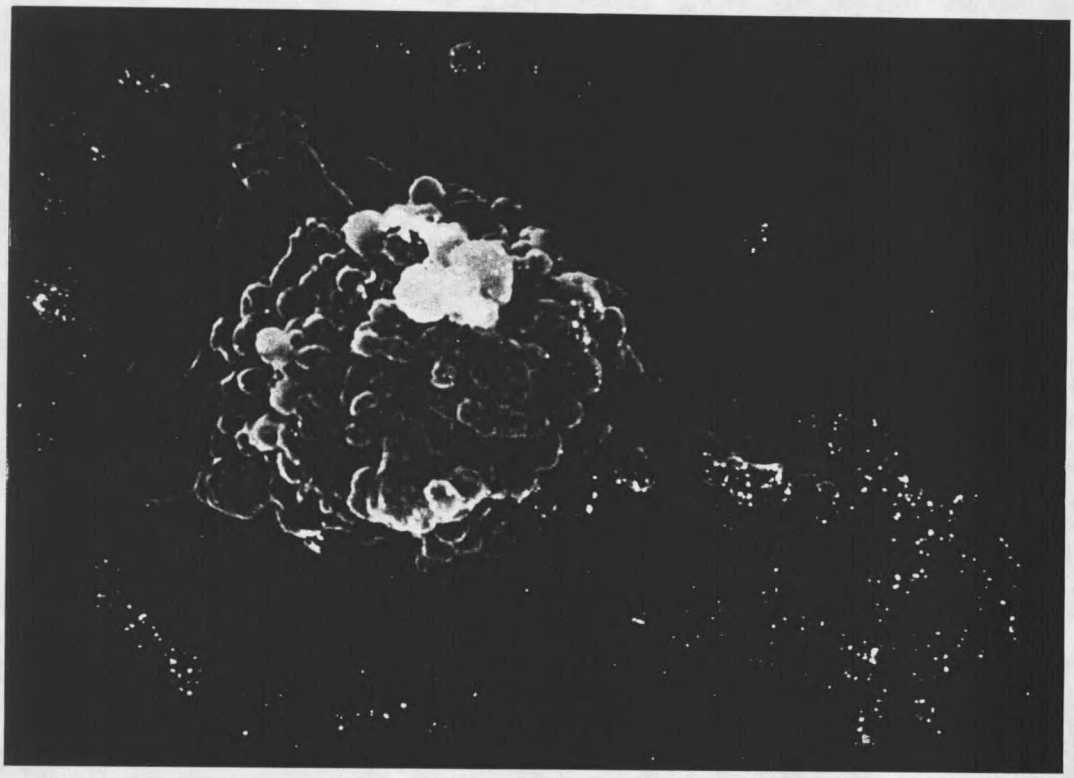

Fig. 3.4. Scanning Electron Microscopy of quartz adherent neutrophils showing the distribution of immunogold labeled Mac-1 on the surface. Purified neutrophils were prepared and fixed for electron microscopy as described in the Methods. The cells were stimulated with 2 μl/ml dHCB and 1 μM fMLF.

Discussion

The neutrophil's ability to defend the host from invading pathogens depends on its efficiency of sensing the inflammatory signals, migrating towards the inflammatory site, and successfully destroying the pathogens. All of these steps require complex regulation and interaction of different surface and intracellular components and involves deployment of the phagocytic adhesion systems.

In this study, we examined the surface distribution of one adhesion molecule and its organizational relationship to stable NADPH oxidase formation in the neutrophil plasma membrane. This relationship might have significant relevance in directing and restricting the respiratory burst to the vicinity of bacteria in the phagosome and thus increasing its effectiveness. Most recently, we found that the components of the NADPH oxidase system were distributed uniformly among plasma membrane fractions of heterogenous density in dHCB+fMLF stimulated cells in subcellular fractionation, but active oxidase complex was stabilized in only a subfraction (2). Our current results suggest that a significant fraction of Mac-1 at the cell surface is organized in a way consistent with formation of specialized plasma membrane domains that may contain the NADPH oxidase.

Cytoskeletal components might be the common link between fMLF mediated assembly of the components of the NADPH oxidase system and the integrin Mac-1. Some of the

components of NADPH oxidase have been shown to associate with various cytoskeletal proteins (9, 19, 32-34) and thus the lateral mobility of these molecules may be governed by the cytoskeletal system. It has also been shown that the sites of integrin mediated adhesion to the extracellular matrix in the plasma membrane act as foci for cytoskeletal assembly (12,13). On the other hand, ligand occupied FPR can induce formation of F-actin in neutrophils and directly bind to it (35-37). Thus the chemotactic peptide can induce cytoskeletal rearrangement via FPR which in turn might affect the surface distribution of Mac-1 and NADPH oxidase components.

In the control cells sedimented on polycarbonate filters, the distribution of Mac-1 was random in a lower magnification of SEM (29). In our high resolution (X 70,000) examination we found that its distribution was restricted to the plasma membrane ruffles. It is possible that the plasma membrane ruffles are the contact points for certain cytoskeleton or membrane skeleton proteins and the plasma membrane; however, it is postulated that only a part of the constitutively expressed or newly inserted membrane Mac-1 is associated with alpha-actinin and thus actin in unstimulated neutrophils (9). Other cytoskeletal proteins could also be involved, however. Research on *Dictyostelium discoideum* has shown that coronin, an actin binding protein which resembles the beta subunit of hetrotrimeric G-

proteins, accumulates at cell surface protrusions which project toward chemoattractants (38). Understanding the cell biology of such proteins may thus provide clues needed to understand the reason for restricted distribution of surface proteins on the cell surface.

Treatment of cells in suspension with dHCB did not seem to alter the total amount of Mac-1 on the cell surface, but as the cells were rounded, Mac-1 seemed to be distributed more randomly. DhCB causes breakdown of intracellular actin filaments, and causes loss of the plasma membrane ruffles, supporting a role for actin on ruffle formation in localization of Mac-1 in unstimulated cells.

Interestingly, cells treated with fMLF in presence of dhCB showed more membrane irregularities than the control cells possibly related to the eight fold increase in membrane bound actin. As there is more G-actin and short actin filaments generated in the cell due to the presence of dHCB, it may be available to bind to membrane sites possibly generated upon treatment with fMLF. Several studies have shown that localized change in the osmomolality in the membrane can initiate membrane shape change (39-41). In contrast to the control cells, the organization of Mac-1 on the surface of dHCB+fMLF stimulated cells is more in the form of large aggregates and linear arrays. This distribution is consistent with that of many types of cell surface proteins which form aggregates or caps when the

cells are stimulated (42). The functional consequences of this aggregation of Mac-1 upon stimulation with fMLF could be important in neutrophil function as it has been shown that Mac-1 is the major factor in binding of fMLF stimulated neutrophils to the vascular endothelium (43). It will be of further interest to attempt correlation of the surface distribution of cytochrome b558, the terminal component of NADPH oxidase on similarly stimulated suspension cells.

Traditionally most of the research on NADPH oxidase assembly has been done with soluble stimuli in suspension conditions. Even though those studies have facilitated our understanding of the system, *in vivo*, neutrophil activation takes place under adherent conditions (3). When the dHCB+fMLF stimulated cells were adhered to BSA coated quartz, a couple of interesting observations were made. These cells were spread out on the coverslip and showed the typical appearance of chemokinetic cells (44), and the gold labelled Mac-1 was now restricted to the cell body and in lamellipodium. The ability of cells stimulated with fMLF in the presence of dHCB to spread on the adherent surface is remarkable.

The mechanism of adhesion dependent respiratory burst is not known. Molecular associations between components of the respiratory burst and integrins has been demonstrated before (45). Although Mac-1 copurifies with the cytochrome b_{558} on heparin ultragel eluate of neutrophil plasma membrane

octyl glucoside extract, there does not appear to be a stable association of the two proteins (Quinn, Parkos, Jesaitis Unpublished). However, a similar cell trafficking pattern has been reported for cytochrome b_{558} and Mac-1, with both proteins being transported in the same vesicle (46). Thus, if both of these molecules are clustered on the same or neighboring subdomains of the plasma membrane and participate in NADPH oxidase targeting of superoxide, the participation of linking structures such as the membrane skeleton must be involved. Thus it would be of great interest to see if superoxide generation is restricted to the vicinity of the adhesion plaques in neutrophils.

Small GTP binding proteins have been shown to play a specific role in cytoskeletal regulation and NADPH oxidase assembly. It has also been shown that in fibroblasts, the small GTP-binding protein, Rac, could regulate membrane ruffling when activated with growth factors (45) Several small GTP-binding proteins of the Ras superfamily, such as Rac1, Rac2, and Rap1A have been implicated in regulating the cytoskeletal architecture and take part in the NADPH oxidase assembly (38,47). In fact, recent work showed that the "effector" domain (amino acids 30-40) of Rac2 is required for both NADPH oxidase assembly and actin assembly (48). Thus these small GTP-binding proteins could be the common regulator between integrin dictated cytoskeletal assembly and formation of the active NADPH-oxidase complex.

In a chemotactic gradient, the mechanism by which neutrophils localize their integrins on the adhesion plaque of the pseudopods are not known. It is possible that a chemotactic stimulus might modulate cytoskeletal architecture and thus affect the distribution of integrin on the cell surface (32). In our experiments Mac-1 was localized on the forward end of the lamellipodium suggesting strongly that stimulation with chemoattractants might help in presenting the integrins on the forward end of the pseudopods. Interestingly, it has been shown that Mac-1 is the major factor in binding of fMLF stimulated neutrophils to the endothelium (43). Thus it is possible that the asymmetric distribution of the adhesion molecules on neutrophils represented by asymmetric distribution of Mac-1 on the cell surface and in subcellular fractionation may also play an important role in directed migration of neutrophils.

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CHAPTER 4

CONCLUSIONS

The function of neutrophils in combatting infection involves invoking several important cellular processes including chemotaxis and phagocytosis. The long term objective of this study was to understand the role of cytoskeleton and cytoskeletal proteins in chemoattractant activated NADPH oxidase activity in the cell. Previous research from our laboratory has shown that the cellular-actin-filament capping agent DHCB can enhance the duration and magnitude of O^-_2 production, suggesting a possible role for actin in the process of O^-_2 generation. Thus the immediate aim of our research was to examine the role of dHCB in this process.

In case of bacterial phagocytosis, the neutrophil has to maximize the efficiency of its NADPH oxidase to compete for the available oxygen with the bacteria. Investigators from our laboratory have shown that the terminal component of NADPH oxidase, cytochrome b_{558}, is found in a clustered architecture in the phagosome (1). It is known that opsonized bacteria carry iC3b on their surface and that it is a ligand for the neutrophil adhesion molecule Mac-1 (2). Thus, it is possible that these proteins might direct NADPH oxidase assembly in neutrophils in the vicinity of points of adhesion. If this hypothesis is correct, then Mac-1 could play a direct role in

the regulation of NADPH oxidase assembly. The secondary goal of this project was to investigate the possible role of Mac-1 in the regulation of O^-_2 generation.

Several signal transduction pathways initiated by occupancy of the chemotactic peptide FPR are known (3), but numerous theories prevail about how occupancy of these receptors triggers the assembly of the NADPH oxidase components. Researchers have identified several components of this oxidase by the use of cell free reconstitution assays, but the architecture and mechanism of assembly of NADPH oxidase in the cell remain unknown. Our work has shown that the presence of these proteins together in the isolated plasma membrane of activated cells is not a sufficient condition for generation of O^-_2 in isolated organelles (4). This finding strongly indicates the presence of yet unknown regulatory factors which could involve both cytosolic or membrane proteins. Such factors could be specific proteins or lipids required to stabilizing the NADPH oxidase. Examination of plasma membrane domains, whether they exist in control cells or are generated when the cells are stimulated, might hold the answer to regulation of directed O^-_2 production in phagocytosing neutrophils. Interestingly, treatment with PMA, opsonized zymosan, or dHCB+fMLF preserves oxidase activity in the plasma membrane. Such treatment also involves degranulation and alteration of the plasma membrane and its cytoskeletal sub-structure. Analysis of these changes

supports a role for the cytoskeleton in preserving membrane domains that retain active NADPH oxidase.

We studied the molecular composition and structure of membranes from stimulated, and control neutrophils in order to determine whether reorganization of the membrane takes place during activation. We observed that the plasma membranes from dHCB+fMLF stimulated cells undergo a drastic restructuring. Our results suggested new hypothesis to explain dHCB induced enhancement of superoxide production. Previously, we believed that actin depolymerization destabilizes lateral order in the activated plasma membrane. Based on our present results we now think that this treatment, in fact, favors stabilization of a new lateral order by promoting actin binding to the membrane. The effect is not due to the action of dHCB itself because the concentration of membrane associated actin was only slightly higher in dHCB treated cells as compared to control cells. We believe that upon stimulation, there are more actin binding sites generated in the membrane. As dHCB makes more G-actin and short actin filaments or actin dimers available in the cells, those sites get occupied, resulting in higher levels of membrane bound actin. This new actin may form networks that stabilize the order of newly inserted and other plasma membrane proteins. This new lateral order probably is vital in the stabilization of supramolecular assemblies such as the NADPH oxidase.

The subcellular fractionation and the ultrastructural

morphology of dHCB+fMLF stimulated cells also raised some interesting issues concerning the mechanism of pseudopod formation, cellular orientation in chemotaxis, and pathogen contact. When applied without stimulation, dHCB caused the neutrophils to round up and lose their membrane ruffles; however, the addition of fMLF to these cells caused the generation of asymmetric plasma membrane protrusions (see chapter 2). Force generation by actin polymerization cannot be responsible for this process as sufficient amounts of dHCB to disrupt F-actin are present. Instead the result supports the theories of Oster et al. (5), who proposed that localized alteration of osmomolality (due to presence of high local concentration of monomeric actin on or near the membrane) may be responsible for outward bulging of the membrane. When coupled with FPR-directed microtubule driven clustering (6), and sweeping of FPR-linked actin filaments, this mechanism could provide the answer to oriented asymmetry of cells up fMLF chemoattractant gradient.

In vivo, neutrophils are activated under adherent conditions, in part through cytoskeleton-anchored Mac-1. Under these conditions, the integrity of cytoskeletal network appears to be important for proper orientation of the cells in a chemotactic gradient or maintain proper contact with a pathogen. How the distribution of Mac-1 is maintained in a highly ordered distribution in stimulated-adherent cells after treatment with 2 ug/ml dHCB is not fully understood. One

possible explanation is that the actin structure in the plasma membrane are not sensitive to dHCB. It is also possible that cytoskeletal proteins other than actin take part in anchoring Mac-1 to specific surface domains and the absence of actin filaments is not enough to disrupt the cytoskeletal association of Mac-1.

As expected, many new avenues of research were generated by our studies. It is now clear that the plasma membrane of dHCB+fMLF-stimulated cells is not homogenous, but contains different structural and functional domains with significant actin content. It will be of great interest to morphologically locate the NADPH complex on the membrane of intact cells and to see if higher amounts of actin and Mac-1 are associated with those structures. We also were able to observe the distribution of Mac-1 on the dorsal surface of neutrophils (see chapter 3). It would be interesting to fix the cells on BSA coated quartz, shear the dorsal surface of the cells, and immunocytochemically localize Mac-1, actin and cytochrome relative to adhesion plaques. Our prediction is that they will colocalize and thus provide strong evidence for a cytoskeleton-grounded supermolecular structure containing both Mac-1 and NADPH-oxidase. Such a finding would then provide direct evidence for adhesion targeted superoxide production by activated human neutrophils. Indeed much remains to be learned about the membrane skeleton architecture in neutrophil host defence process. The studies discussed in

this thesis should accelerate the acquisition of a molecular understanding of these important functions.

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