A MORPHOMETRIC AND FUNCTIONAL STUDY OF THE EARLY EVENTS OF HUMAN NEUTROPHIL ACTIVATION

By

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ABSTRACT

A MORPHOMETRIC AND FUNCTIONAL STUDY OF THE EARLY EVENTS OF HUMAN NEUTROPHIL ACTIVATION

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The purpose of these studies was to investigate the early morphological and functional responses of human neutrophils to activation. Cells were exposed to the synthetic chemoattractant N-formylmethionyl leucyl phenylalanine (fMLP), the calcium ionophore A23187 or a temperature transition from 4C to 37C. Neutrophils were fixed and evaluated with light microscopy for shape change. Cell-free supernatants were harvested from activated cells and assayed for evidence of primary, secondary and tertiary extracellular granule release. Quantitation and characterization of intracellular granules and morphometric measurements were made on transmission electron micrographs of unactivated and activated cell profiles. In addition, light and electron microscopic evaluations were performed on fMLP and A23187 treated neonatal neutrophils.

The results from these investigations suggest that 1) neutrophilic peroxidase negative granules may be heterogeneous in terms of composition, 2) peroxidase negative granules may undergo sequential or selected release, and 3) the release of these granules is coincident with the earliest visible indication of cellular shape change and may be associated with a transient, apparently regulated increase in exposed cell surface membrane.

The following sequence of events is proposed by these studies to occur early in adult neutrophil activation: 1) Brief activation of neutrophils mediates cellular ruffling presumably associated with the selected release of a population of peroxidase negative granules containing large quantities of lactoferrin. 2) These granules merge with the plasma membrane resulting in granule-plasma membrane fusion thereby mediating an increase in exposed surface membrane. 3) An activation induced increase in cellular volume, seen in the ultrastructural morphometric determinations made in these studies as an increase in cross-sectional area, subsequently occurs. This volume increase provides a "compensation" for the increase in exposed surface membrane which, by the methods used in this study, appeared to be regulated and returned to basal levels.

To my Mom, Dad and siblings who may not have always agreed or understood, never gave up.

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And finally, I am most grateful to those who helped me enjoy a life beyond graduate school.

> "If the study to which you apply yourself has a tendency to weaken your affections, and to destroy your taste for those simple pleasures in which no alloy can possibly mix, then that study is certainly unlawful, that is to say, not befitting the human mind."
> Mary Shelley, *Frankstein*.

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INTRODUCTION

By migrating to a site of injury and clearing foreign material and tissue debris, activated neutrophils represent one of the major defensive cell types of the human body. Substances generated from bacterial invasion or from damaged tissue can act as activating agents, or chemoattractants, which bind to select neutrophil membrane receptors initiating activation (Murphy, 1970). *In vitro*, activation can be dissected and studied by exposing isolated neutrophils to purified or synthetic chemoattractants.

The binding of the synthetic chemotactic tripeptide N-formylmethionyl leucyl phenylalanine (fMLP) to specific neutrophil membrane receptors has been found to induce morphological and functional cellular alterations which are defined as activation. Activation events have been shown to include cellular shape changes (Smith et.al., 1979; Zigmond, 1978), enhanced adherence to substrata (Hoover et.al., 1980; Smith et.al., 1979; Tonnesen et.al., 1984), chemotaxis (Zigmond, 1978) extracellular granule release (Brentwood and Henson, 1980; Dewald et.al., 1982; Hibbs et.al., 1983; Petrequin et.al., 1985; Wright and Gallin, 1979) and modifications in the quality, in terms of increased expression of membrane components, (Arnaout et.al., 1984; Berger et.al., 1984; Fearon and Collins, 1983; Fletcher and Gallin, 1980; Lanier et.al., 1985; Zigmond et.al., 1982) and quantity of exposed plasma membrane (Hoffstein et.al., 1982).

Intracellularly, a consequence of chemoattractant binding is an increase in cytosolic free calcium through mobilization of intracellular calcium stores as well as increased calcium influx from the external environment (Naccache et.al., 1985). The calcium ionophore A23187 has been reported to increase intracellular calcium levels by both of these mechanisms while by-passing receptor ligation. Exposure of neutrophils to A23187 has been found to mediate cellular shape change (unpublished personal observation), enhanced adherence (Gallin et.al., 1978), extracellular granule release (Hoffstein and Weissmann, 1978) and alterations in the quality of exposed plasma membrane (Arnaout et.al., 1984; Todd et.al., 1984).

Several of the cellular alterations describing activation can be mediated in the absence of conventional activating agents or calcium ionophores. Temperature transitions have been reported to spontaneously induce actin polymerization (Howard and Oresajo, 1985), enhance adhesion (Charo et.al., 1985), mediate apparent extracellular granule release (Corcino et.al., 1970; Dewald et.al., 1984; Goldstein et.al., 1974; Wright and Gallin, 1979) and cause an increase in the expression of surface membrane components (Berger et.al., 1984; Fearon and Collins, 1983; Springer et.al., 1986).

The studies presented in this report investigated morphological and functional cellular responses occurring after a brief exposure of neutrophils to fMLP, following treatment with A23187 and in response to a temperature transition.

LITERATURE REVIEW

The purpose of this section is to provide foundational information concerning the morphological and functional events of neutrophil activation addressed in this study. The events of activation are discussed first. The synthetic chemotactic tripeptide fMLP and the calcium ionophore A23187 as initiators of activation are examined in parts II and III, respectively. Part IV discusses a temperature transition from 4C to 37C as an initiator of neutrophil responses. Because neutrophils isolated from umbilical cord blood are reported to exhibit select deficiencies in functional responses to activation, they were investigated in this study. Neonatal neutrophils are discussed in part V of this section.

I. Events of Neutrophil Activation

A. Shape Change

In most tissues, the polarity of individual cells is reflective of the overall architecture and function of the tissue. Neutrophils respond to chemoattractants generated in vivo (through bacterial invasion, cleavage of complement proteins or tissue degradation) by adhering to endothelial cells adjacent to the origin of the stimuli and migrating out of the microcirculation into specific extravascular sites of tissue destruction and infection (Murphy, 1970). While cells in the process of directional migration exhibit an apparent acquired functional and morphological polarity (Zigmond, 1978), polarity in neutrophils has been reported to be an intrinsic characteristic independent of both adhesion and directed migration (Smith et.al., 1979).

Viewed with phase contrast optics (Figure 1), nonactivated neutrophils in suspension are spherical. Within seconds after exposure to chemotactic factors the cells begin to exhibit shape change responses. The first visual indication of shape change is a ruffling of the cell membrane. Thin, sheet-like membrane protrusions, or pseudopods, are randomly extended over all regions of the cell surface giving the cells a ruffled appearance. With continued exposure to the chemoattractant the cells may lengthen to exhibit bipolar configurations displaying defined front and rear regions. Pseudopods are restricted to the front end of the cell, the other end is smooth, constricted and may have an knob-like structure or uropod. The mid-region is also smooth, without membrane extensions or ruffles (Zigmond, 1978). The fact that a bipolar shape change response can

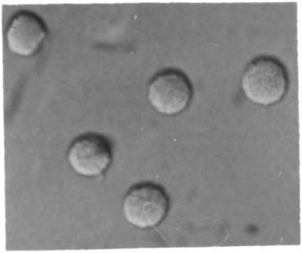
Figure 1
NEUTROPHIL SHAPE CHANGE: LIGHT MICROSCOPY

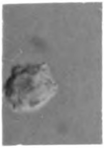
Upper panel: Unactivated isolated adult neutrophils.

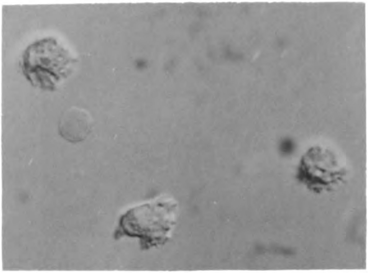
Middle panels: Activated neutrophils (fMLP); three ruffled cells and one bipolar cell. Note knob-like uropod on bipolar cell seen on lower portion of the middle panel.

Lower panel: Bipolar configurations with uropods.

Bar = 10um







take place in the absence of a substratum or a chemotactic gradient suggests that neutrophils have an intrinsic polarity which can be expressed when cells are activated in suspension (Smith et.al., 1979).

Shape change responses are maintained while the cells are exposed to the chemoattractant but are reversed by washing the cells free of the activator. Active glucose utilization is required for shape change but the effects of blocked glucose metabolism are also reversible. Shape change responses are reported to be cation (Ca++ and Mg++) independent but to require intact microfilaments (Heerdt et.al., 1984; Smith et.al., 1979).

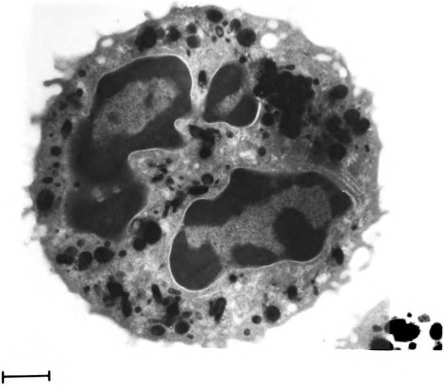
Activation of neutrophils has been shown to cause polymerization of actin, the major component of microfilaments (Rao and Varani, 1982). Detergent insoluble cytoskeletal preparations from unactivated cells are composed almost exclusively of cross-linked actin filaments, actin binding proteins and a small amount of myosin. Within seconds after chemoattractant receptor binding the quantity of polymerized actin increases, appearing to be localized to the developing pseudopods (Fechheimer and Zigmond, 1983; Howard and Oresajo, 1985). Activation induced actin polymerization is chemoattractant dose and time dependent, subsiding within approximately 60 seconds (Rao and Varani, 1982; White et.al., 1982).

Ultrastructural changes accompany activated neutrophil shape change (Figure 2). Microfilaments in nonactivated spherical neutrophils are typically arranged in a uniform submembranous meshwork. Granules appear to be randomly distributed throughout the cytoplasm. The centriole is generally localized between the nuclear

Figure 2
NEUTROPHIL SHAPE CHANGE: TEM

(Figure 2 is comprised of three pages)
1. Examples of unactivated isolated adult neutrophils. Note the nuclear lobes in the center of cells, electron dense peroxidase positive granules and more electron lucent peroxidase negative granules dispersed throughout the cytoplasm.

Bars = lum



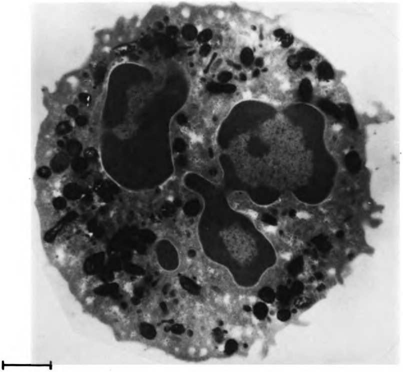


Figure 2 (continued)

2. Examples of fMLP ruffled adult neutrophils. Note nuclear lobes in central region of the cells, agranular zone of pseudopod, peroxidase positive and negative granules dispersed around the nuclear lobes.

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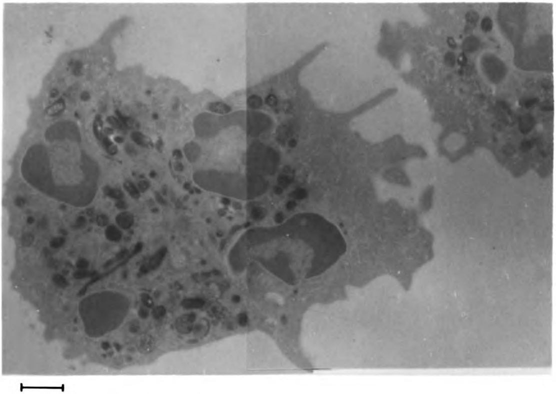


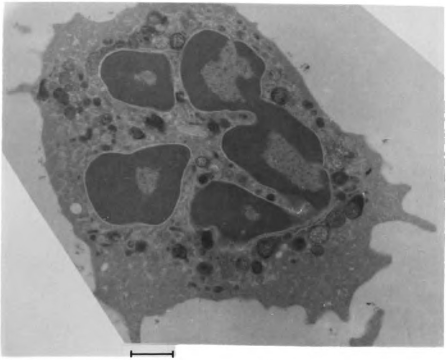
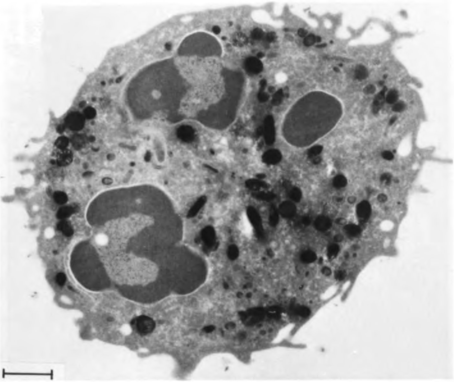
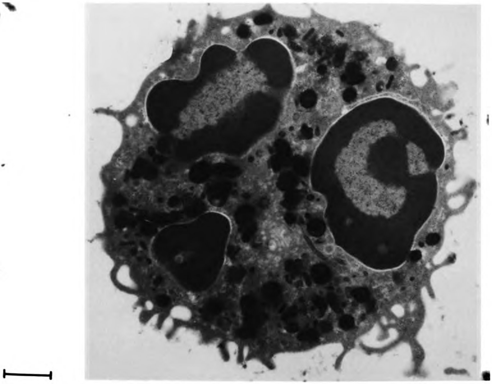


Figure 2 (continued)

3. Examples of A23187 ruffled adult neutrophils. Note nuclear lobes and centrioles in each cell section and peroxidase positive and negative granules. Also notice that the membrane extensions and agranular zones exhibited on A23187 ruffled cells are finer and not as expansive as those mediated by fMLP.

Bars = lum





lobes and from here microtubules radiate toward, but rarely make contact with the cytoplasmic side of the plasma membrane. As cells begin to change shape the newly formed pseudopods appear as agranular zones containing a fine meshwork of filamentous material. In the activated neutrophil, as in the spherical cell, the centriole remains within the central region of cell, between the nuclear lobes. Microtubules radiate toward but do not contact the plasma membrane (Anderson et.al., 1982; Oliver and Berlin, 1982).

As neutrophils become bipolar, the agranular-zone is localized to the front of the cell, the nucleus to the mid-region with the granules dispersed generally around the lobes. Neither the nuclear lobes nor the granules extend into the uropod. Using anti-tubulin antibody and indirect immunofluorescent techniques, Anderson and coworkers (1982) demonstrated that microtubules lengthen along the axis of cellular polarity and shorten perpendicular to polar axis. The mean number of microtubules in chemotactically activated neutrophils has been reported to be significantly greater than the number seen in the nonactivated cells, suggesting microtubule involvement in activated shape change. Microtubule assembly appears to be transient. Several minutes after activation the number of microtubules returns to baseline levels (Goldstein et.al., 1973). The uropod of bipolar cells is organelle-free, composed of microfilaments and frequently a core of intermediate filaments (Oliver and Berlin, 1982).

B. Enhanced Adherence

Neutrophil adherence to artificial substrata or to cultured endothelial cells is glucose, temperature and extracellular Ca++ dependent but is independent of polymerization of microfilaments

(Charo et.al., 1985; Smith et.al., 1979). Chemotactic factors have been reported to enhance adhesion not only by increasing the number of cells adhering to a surface (Hoover et.al., 1980; Smith et.al., 1979; Tonnesen et.al., 1984) but also through increasing the strength of cellular adhesion (Charo et.al., 1985). Smith and coworkers (1979) reported that, in contrast to shape change responses, chemoattractant mediated enhanced adhesion is not reversible. The augmented adhesion induced by exposing neutrophils to fMLP was not removed by washing the cells free of the chemoattractant. In addition, although neutrophils exposed to 2-deoxyglucose (2-DOG) demonstrated a reversible inhibition of shape change responses, normal adhesion functions were not restored when the 2-DOG was removed (Heerdt et.al., 1984).

A degree of neutrophil adhesion is a prerequisite for cell motility (Charo et.al., 1986). Cells in the process of adhering appear to first flatten against the substratum and then round up and extend pseudopods oriented in the direction of eventual migration (Zigmond and Sullivan, 1979). Adhesion sites exhibit polarity forming at the front of the cell and being transported to the tail as translocation occurs. As the cell migrates new adhesion sites are formed at the pseudopod region and again transported to the rear of the cell (Smith and Hollers, 1981).

Ultrastructurally, localized cytoskeletal-adherent membrane associations appear to be generated upon adhesion. In an elegant investigation of the cytoplasmic side of neutrophil plasma membranes during adherence, Boyles and Bainton (1979) demonstrated that during neutrophil adhesion complexes of dense globular centers with radiating networks of thin branched filaments are formed. These regions were located exclusively on the adherent membranes, never being found on the ventral side of cells. Pseudopods extending out from cells, in the process of forming new adhesion sites, were generally free of these regions. The extent of formation of central regions and the associated filaments was determined to be temporally related to cell adhesion. As cells became more adherent the branched filaments spread to cover the entire cytoplasmic side of the adherent membrane. The nature of these regions is unknown but the evidence strongly suggests that they are intimately involved with the adhesion of cell activation.

C. Migration

Neutrophils undergoing directed migration, or chemotaxis, demonstrate morphological and functional polarity based on the concentration gradient of the chemoattractant (Smith and Hollers, 1981; Sullivan et.al., 1981; Zigmond, 1978). In a chemotactic gradient the majority of cells are oriented in the same direction suggesting an acquired polarity. Pseudopods are directed toward the higher concentration leading the way as the cells migrate up the gradient. If the gradient is reversed so that the higher dose of chemotactic factor is toward the uropods, the cells reorient along the gradient, pseudopods again toward the higher concentration (Zigmond, 1977).

Conditions simulating _in vivo_ chemotaxis have been developed _in vitro_ by a number of methodologies including migration of neutrophils toward a chemoattractant on plastic (Zigmond, 1977), under agarose, through porous filters (Gallin and Quie, 1978), through cultured endothelial cells (Beesley et.al., 1979) and through endothelial cells grown on porous filters (Taylor et.al., 1981). Manipulations of these <u>in vitro</u> chemotaxis conditions have indicated that maximum neutrophil chemotactic responses require extracellular Ca++, glucose, and polymerized microfilaments and microtubules (Caner, 1965; Wilkson, 1974; Zigmond, 1978).

D. Extracellular Granule Release

Mature human neutrophils contain an assortment of cytoplasmic granules which have been classified by morphology, cytochemical techniques and enzyme content into at least three discrete types designated as primary (or azurophilic), secondary (or specific) and tertiary (sometimes referred to as C-particles). The granules appear at different stages during granulocyte maturation, contain different enzymes (Baggiolini and Dewald, 1985; Bainton and Farquhar, 1968; Scott and Horn, 1979) and respond differently in terms of release dynamics (Brentwood and Henson, 1980; Dewald et.al., 1982; Hibbs et.al., 1983; Lew et.al., 1986; Petrequin et.al., 1985; Wright and Gallin, 1979). A summary of granule components is shown is Table 1.

1. Primary Granules:

The primary granules are the first type detectable in maturing granulocytes, appearing in the promyelocyte stage. Mature primary granules contain neutral proteinases (elastase and cathepsin G), acid hydrolases (N-acetyl-B-glucosaminidase, cathepsins B and D, B-glucuronidase, B-glycerophosphatase and a-mannosidase) and microbicidal enzymes (myeloperoxidase and lysozyme) (Baggiolini and Dewald, 1985; Bainton and Farquhar, 1968). Recently, three small structurally related peptides, referred to as defensins, were localized in the primary granules of human neutrophils (Ganz et.al.,

Table 1
SUBCELLULAR LOCALIZATION OF GRANULAR COMPONENTS

Type of Component	Primary Granules	Secondary Granules	Tertiary Granules
Antimicrobial	Myeloperoxidase Lysozyme Defensins	Lysozyme	
Neutral Proteinases	Elastase Cathepsin G	Collagenase	Gelatinase Plasminogen Activator
Acid Hydrolases	N-acetyl-B-glucosaminidase Cathepsin B Cathepsin D B-glucuronidase B-glycerophosphatase a-mannosidase		N-acetyl-B-glucosaminidase Cathepsin B Cathepsin D B-glucuronidase B-glucerophosphatase a-mannosidase
Other		Lactoferrin Vitamin B12 binding protein	Proton Pump ATPase

1985; Selsted et.al., 1985). Defensins were found to have antimicrobial, antifungal and antiviral activities in vitro.

Ultrastructurally, primary granules can be stained very electron dense through a peroxidase mediated oxidation of diaminobenzidine (DAB). In neutrophils the myeloperoxidase contained in primary granules provides the enzymatic activity for this reaction, resulting in the deposition water insoluble end products of DAB oxidation in primary but not secondary granules (Mitsui, 1960). Thus, at an ultrastructural level, primary granules are more electron dense and are distinguishable from secondary granules (Figure 2)..

2. Secondary Granules:

Secondary or specific granules are unique to neutrophils, appearing as promyelocytes mature into myelocytes (Bainton and Farquhar, 1968). Secondary granules are reported to contain approximately 2/3 of the cell's total lysozyme, vitamin B-12 binding protein, the neutral proteinase collagenase, and the glycoprotein lactoferrin (Baggiolini and Dewald, 1985).

a. Lysozyme:

Lysozyme is a cationic antimicrobial enzyme found in tissues, secretions, plasma, monocytes and neutrophils (Grossowicz et.al., 1979; Leffell and Spitznagel, 1972; Pryzwansky et.al., 1978). Lysozyme's microbicidal activities are the result of it's ability to lyse many gram-positive bacteria by catalyzing the hydrolysis of bacterial cell walls (Grossowicz et.al. 1979; Nerurkar, 1981). Gordon et.al. (1979) have reported that lysozyme may be involved in the modulation of activated neutrophil functions by serving as a negative feed back mechanism. Purified human lysozyme was found to significantly inhibit directional migration and the generation of toxic oxygen radicals from neutrophils activated with several different agents.

b. Lactoferrin:

Lactoferrin is a ubiquitous single chain, iron-binding glycoprotein found in secretions bathing mucosal surfaces, plasma and secondary neutrophilic granules (Cramer et.al., 1985; Hetherington et.al., 1983; Leffell and Spitznagel, 1972; Lima and Kierszenbaum, 1985; Pryzwansky et.al., 1978). Each lactoferrin molecule is capable of binding 2 Fe 3+ molecules. The iron molecules are released biphasically at acid pH, the first between pH 6.5 and 5.5, the second between pH 4.5 and 3.5 (Moguilevsky et.al., 1985). In association with it's iron-binding capacity, lactoferrin is bacteriastatic. At neutral pH lactoferrin may limit the iron available to bacteria by binding it and not releasing it (Arnold et.al., 1977; Bullen and Armstrong, 1979). In contrast, by releasing iron molecules to the neutrophil's toxic oxygen radical generating system, lactoferrin is thought to enhance hydroxyl radical production (Ambruso and Johnston, 1981; Fantone and Ward, 1982; Lima and Kierszenbaum, 1985).

Additionally, exogenous lactoferrin has been found to modulate neutrophil adhesion activities. Human neutrophils bind lactoferrin in a saturable fashion, with each cell capable of binding approximately 1.35 X 10 6 molecules. At doses ranging from 4 to 15ug of lactoferrin/10 million neutrophils (equivalent to approximately 3 X 10^6 to 11.5 X 10^6 molecules/cell) lactoferrin was found to induce a dose dependent decrease in cell surface charge (Boxer et.al., 1982c). Such decreased surface charges have been associated with enhanced hesion activities (Harlan, 1985). Addition of exogenous lactoferrin isolated cells has been reported to induce neutrophil aggregation, crease adherence of neutrophils to cultured endothelial cells (Oseas .al., 1981), enhance neutrophil-alveolar macrophage adhesion through specific macrophage receptor (Campbell, 1982) and augment adherence neutrophils to nylon wool fibers (Boxer et.al., 1982c).

In vivo, the adhesion mediating activities of lactoferrin ve been shown to induce a transient neutropenia. In a hamster cheek uch model, Boxer and coworkers (1982a) visualized increased utrophil adherence to endothelial cells following the infusion of ctoferrin. Enhanced adherence was found to coincide with systemic utropenia. By promoting adhesion to endothelial cells and at the ame time enhancing toxic hydroxyl radical generation, lactoferrin has een implicated in *in vitro* endothelial cell damage and *in vivo* dema formation (Vircellotti et.al., 1985).

Despite the possible deleterious affects on endothelium, ctoferrin appears to plays an important role in the body's defense gainst disease. Several patients suffering from recurrent bacterial nfections have been found to lack lactoferrin. Neutrophils isolated rom these patients showed a variety of defects in cell adherence and ydroxyl radical production. Ultrastructurally, these neutrophils ere found to be deficient in secondary (peroxidase negative) granules nd morphometrically they appeared chemotactically activated and nresponsive to further activation (Boxer et.al., 1982b; enton-Gorius et.al., 1980; Strauss et.al., 1974).

Collagenase:

The neutral protease collagenase is also a constituent of the secondary granules of neutrophils (Murphy et.al., 1977). Intracellularly this metallo-enzyme occurs in a latent form, probably as an inhibitor-enzyme complex. Weiss and coworkers (1985) have reported that approximately 50% of the collagenase secreted from activated human neutrophils is in an active, rather than a latent form. These investigators determined that cells auto-activated the enzyme through the production of hypochlorous acid (HOCl). In the presence of hydrogen peroxide (generated from respiratory burst activity) myeloperoxidase (contained in primary granules) catalyzes the peroxidation of chloride to HOCl. HOCl in turn was found to activate latent collagenase. The remaining latent enzyme was found to be activateable with organomercurials. Activation of latent collagenase with mercurials results in a decrease in molecular weight thought to be caused by removal of the inhibitor from the enzyme-inhibitor complex (Murphy et.al., 1980).

Activated collagenase specifically cleaves native type I collagen into characteristic fragments 3/4 and 1/4 the size of the original collagen molecule (Cawston and Barrett, 1979). Collagenase activity is dependent upon divalent cations, being inhibited with EDTA or 1,10-phenanthrolein (Murphy et.al., 1980). Cathepsin G and elastase, serine proteases found in primary neutrophilic granules (Baggiolini and Dewald, 1984; Tanaka et.al. 1985), have been reported to destroy collagenase activity (Murphy et.al., 1980).

3. Tertiary Granules:

Fractionation of human neutrophil homogenates by zonal differential sedimentation has revealed the presence of a third population of small particles referred to as C-particles or tertiary granules. Although morphologically still unidentified in whole cells, the fractionation technique indicated that tertiary granules are considerably smaller than either primary or secondary granules (Dewald et.al., 1982). Recent evidence suggests that a proton pump ATPase, responsible in part for the acidification of phagolysosomes following neutrophil ingestion of foreign materials, is associated with tertiary granules (Mollinedo and Schneider, 1984; Mollinedo et.al., 1986). These granules also contain a number of acid hydrolases also found in peroxidase positive granules including cathepsin B and D, beta-glucuronidase, beta-glycerophosphatase, alpha-mannosidase and N-acetyl-beta-glucosiminidase (Baggiollini and Dewald, 1985). Additionally, the metallo-proteinase gelatinase has been reported to be localized exclusively in neutrophilic tertiary granules (Sopata and Dancewicz, 1974). Monocytes contain little if any gelatinase (Dewald et.al., 1982), and cultured macrophages do not exhibit gelatinase activity (Vartio, 1985).

a. Gelatinase:

Similar to collagenase, gelatinase is a metallo-proteinase, occurring as a latent enzyme-inhibitor complex. Like collagenase, a portion of the latent enzyme is thought to be auto-activated by neutrophils through the production of HOCl (Peppin and Weiss, 1986). Gelatinase activation with organomercurials is associated with a decrease in molecular weight, suggesting that enzyme-inhibitor complex disruption accompanies enzyme activation. The latent form of gelatinase is thought to be inactivated by elastase in such a way that it cannot subsequently be activated. Unlike collagenase, the serine proteinase cathepsin G has been found to activate, rather than prevent the activation, of latent gelatinase. Gelatinase activity is inhibited by EDTA or 1,10-phenanthrolein but is not affected by serine proteinase inhibitors (Dewald et.al., 1982; Hibbs et.al., 1985; Murphy et.al., 1980; Sopata, 1982).

Once activated gelatinase acts synergistically with collagenase to degrade type I collagen. Collagenase, as mentioned, cleaves collagen at a single site producing 3/4 and 1/4 collagen fragments. These fragments spontaneously denature at body temperature making them accessible to further degradation by gelatinase. Although gelatinase does not directly cleave native type I collagen it is capable of direct degradation of type V collagen (Hibbs et.al., 1985; Murphy et.al., 1980).

Gelatinase secretion from human neutrophils appears to take place prior to secondary or primary granule release. Dose response determinations of extracellular granule release mediated by several different activating agents indicated that gelatinase was released following exposure of cell to low doses of the activators. Secondary and primary granule release were not detected until higher concentrations of activators were used (Dewald et.al., 1982; Hibbs et.al., 1983; Petrequin et.al., 1985).

Hibbs and associates (1985) have recently reported that secreted gelatinase consists of three molecular weight species. All three forms were found to be immunologically identical and proteolytically active against gelatin.

4. Subpopulations of Neutrophilic Granules

There is evidence indicating that subpopulation of the three types of cytoplasmic granules just discussed may exist. Peroxidase positive-primary granules in human neutrophils can be subdivided into populations differing in morphology (Pryzwansky and Brenton-Gorius, 1985), enzyme composition and extracellular release dynamics (Kinkade et.al.,1983; Pember and Kinkade,1983).

Ultrastructural studies of DAB treated neutrophils revealed three morphologic forms of peroxidase positive granules; large spherical granules with strong homogeneous peroxidase activity, somewhat elongated granules with weaker heterogeneous peroxidase activity and small peroxidase reactive granules appearing in clusters or chains. By high voltage electron microscopy these small granules appeared to be interconnected. (Pryzwansky and Brenton-Gorius, 1985).

Subpopulations of primary granules differing in enzyme content have been identified using centrifugation techniques. Continuous sucrose density centrifugation of neutrophilic primary granule fractions has been found to yield two populations of granules with high and low densities (Kinkade et.al., 1983; Pember and Kinkade, 1983). Three distinct forms of myeloperoxidase (MPO), reported to differ in enzymatic activity, extractability (Pember et.al., 1982), amino acid composition (Morita et.al., 1986) and localization have been isolated from the granule subpopulations obtained from a single neutrophil donor. MPO forms II and III were confined to the low density granules while the high density granules contained all three forms of MPO (Kinkade et.al., 1983; Pember and Kinkade, 1983).

Studies of extracellular release of the different forms of MPO suggest that selective secretion of primary granule subpopulations occurs. MPO II and III, contained within low density granules, were found to be secreted by human neutrophils activated with fMLP in the presence of cytochalasin B, while extracellular MPO I, localized in the high density subpopulation, was not detected (Kinkade et.al., 1983; Pember and Kinkade, 1983).

Similar to MPO, the neutral proteinase elastase is a primary granule constituent found to exist in different forms. Garcia et.al. (1985) have identified at least three isoenzymes of elastase, differing in carbohydrate content and extractability. Unlike MPO, the distribution of the different forms of elastase appeared to be equivalent in both high and low density primary granule subpopulations, and the isoenzymes were not selectively secreted in response to neutrophil activation with fMLP plus cytochalasin B.

Distinct subpopulations of peroxidase negative granules have been identified in neutrophils isolated from ruminant blood (Baggiolini et.al., 1985; Genarro et.al., 1983). During maturation of these cells three types of granules were identified. As in human neutrophils, peroxidase positive granules formed first (Bainton and Farquhar, 1968). These were followed by an intermediate type of peroxidase negative granule and finally classical peroxidase negative secondary granules were formed. The intermediate granules were reported to be larger than the later peroxidase negative granules, had more acutely defined membranes and contained lactoferrin exclusively. Lysosomal hydrolases, peroxidase and serine- or metallo-proteinases were not detected in the intermediate granules. Similar to secondary granules, these intermediate granules were secreted in response to soluble stimuli (A23187) independently of primary granule release (Gennaro et.al., 1983).

There are reports indicating that distinct forms of secondary granule lactoferrin may be divided into select subpopulations of cells. Broxmeyer and coworkers (1980) reported that a specific form of human neutrophil lactoferrin inhibited the Granulocyte-Macrophage Colony Stimulating Activities (GM-CSA) from endogenous bone marrow derived GM-CSA producing cells. Further, these investigators found that the type of lactoferrin exhibiting these inhibitory properties was contained in a specific population of circulating cells. Isolated neutrophils were separated into subpopulations based on the capacity of the cells to bind antibody coated erythrocytes (EA). Neutrophils binding EA were referred to as Fc receptor positive (FcR+) while those not binding EA were FcR-. Compared to FcR- cells, FcR+ neutrophils demonstrated superior adhesiveness, aggregation, chemotaxis and phagocytic capacity of opsonized particles. The difference in FcR expression was not due to cell maturity as aging did not mediate a conversion from one type to the other. In addition, conversion could not be achieved with neutrophil activation (Klemnper and Gallin, 1978). Quantitatively, both FcR+ and FcR- populations contained equivalent amounts of lactoferrin, approximately 3.4 ug/million cells. However, qualitatively the lactoferrin from the subpopulations of neutrophils differed in function. FcR+ cells were found to contain an active form of lactoferrin which bound to human monocytes and inhibited the GM-CSA. FcR- neutrophils, on the other hand, contained an inactive type of lactoferrin which demonstrated negligible binding to monocytes and did not affect the GM-CSA. Additionally, lactoferrin extracted from FcR- cells interfered with the binding of active lactoferrin to monocytes and consequently blocked the inhibitory effect of FcR+ lactoferrin on GM-CSA.

Although neutrophils generally function as defensive cells, essential for maintaining health, they can be involved in the exacerbation of disease states. As mentioned, lactoferrin has been implicated in endothelial cell damage and subsequent edema formation (Vircellotti et.al., 1985). Neutrophils migrating into sites of inflammation may also contribute to tissue destruction by releasing their proteolytic enzymes (Muirden and Leyden, 1984). Gelatinase and collagenase have the capacity to alter extracellular matrix materials. Collagenase is thought to be critical in initiating the extracellular collagen degradation seen in rheumatoid arthritis (Spalding et.al., 1986), and has, in fact, been reported to play a key role in the development of inflammatory disease states affecting every organ system in the body (Weiss et.al., 1985). Acting in concert with collagenase to further degrade the extracellular matrix, gelatinase may contribute to tissue destruction.

Laskin and coworkers (1986) have recently demonstrated that collagen degradation products have the potential of being as chemotactically active for human neutrophils as fMLP. Therefore, infiltrating neutrophils not only have the capacity to degrade collagen matrixes, but the act of degradation has the potential of recruiting more neutrophils to the site.

In vitro, limited extracellular granule release has been implicated in increased chemotatic peptide binding (Fletcher and Gallin, 1980; Gallin et.al., 1978) and enhanced neutrophil adhesion and migration (Gallin et.al. 1978; Wright and Gallin, 1979). While extracellular Ca++ is not essential for limited granule secretion,

secretion does require the mobilization of intracellular calcium stores (Smolen et.al., 1981). Release is inhibited by the presence of 2-DOG but these affects are reversible (Heerdt et.al., 1984; Smolen et.al., 1980). Granule release is not modulated by protein synthesis inhibitors (Gallin et.al., 1978). Disruption of microfilament formation with the fungal metabolite cytochalasin B has been shown to enhance activated extracellular granule release (Henson and Oades, 1973; Skosey et.al., 1973; Zurier et.al., 1973).

E. Plasma Membrane Modifications

1. Qualitative membrane changes: Up-regulation of membrane components

A correlation between up-regulation of chemotactic peptide receptors and secondary granule release has been reported, suggesting that these granules may act as intracellular pools for the receptors (Fletcher and Gallin, 1980). Recent reports indicate that neutrophil activation induces up-regulation of other surface moieties as well, possibly also through the mobilization of intracellular pools (Fearon and Collins, 1983; Thrall et.al., 1980; Zigmond et.al., 1982). Activated cells were found to exhibit increased membrane expression of the related glycoproteins (GPs), Mo 1, also known as OKM 1 and Mac-1 (Berger et.al., 1984) and p150,95 (Springer et.al., 1986). These GPs represent a major portion of the components found on the surface of human neutrophils. Structurally the GPs are double chain noncovalently associated alpha-1 beta-1 complexes (Sanchez-Madrid et.al., 1983). A 95 kilodalton beta chain is common to both while the alpha chain varies. A third structurally related GP, the lymphocyte function associated antigen-1 (LFA-1), shares the common beta chain while expressing a unique alpha chain. Similar to Mo 1 and p150,95, LFA-1 has been identified on leukocyte membranes (Gallin, 1985; Sanchez-Madrid et.al., 1983). However, while the LFA-1 expressed on the surface of monocytes has been reported to increase when these cells are activated (Mentzer et.al., 1986), LFA-1 is not up-regulated on neutrophils in response to activation (Lanier et.al., 1985). Functionally the Mo 1, p150,95 and LFA-1 glycoproteins are thought to be involved in some form cellular adhesiveness (Anderson et.al., 1985; Anderson et.al., 1986).

The alpha chain of Mo 1 is approximately 160KD in size and contains the antigenic epitopes. Monoclonal antibodies directed against specific portions of the alpha chain have been found to block the adherence of iC3b coated particles to granulocytes, suggesting that the Mo 1 antigen is closely related to, if not identical with, the iC3b receptor (CR3) found on granulocyte membranes (Anderson et.al., 1986; Arnaout et.al., 1983; Arnaout et.al., 1984; Dana et.al., 1984; Sanchez-Madrid et.al., 1983). This GP has also been associated with cellular-substratum adhesion and subsequent chemotaxis (Anderson et.al., 1986). In addition to being found on granulocytes, Mo 1 has been reported on monocyte and natural killer cell membranes (Eddy et.al., 1984; Gallin, 1985; Sanchez-Madrid et.al., 1983).

The p150,95 GP consists of the common 95KD beta chain and a 150KD alpha chain. p150,95 has been identified on neutrophil and monocyte membranes (Gallin, 1985; Sanchez-Madrid et.al., 1983). Functionally this GP is thought to be involved in adhesion activities of these cells (Anderson et.al., 1986; Harlan et.al., 1985; Lanier et.al., 1985; Springer et.al., 1986). Wright et.al. (1985) presented preliminary evidence suggesting that p150,95 may be the CR2 receptor expressed on the surface of phagocytic cells. CR2 is reported to bind iC3b, C3dg and C3d cleavage fragments of C3 (Arnaout and Colten, 1984) and act as the receptor for the Epstein-Barr virus (Frade et.al., 1985).

LFA-1, identified on the surfaces of lymphocytes, thymocytes and activated macrophages as well granulocytes (Gallin, 1985; Sanchez-Madrid et.al., 1983), has been reported to participate in the adhesion between target cells and cytotoxic T lymphocytes or natural killer cells (Gallin, 1985), homotypic adherence of B lymphocytes (Mentzer et.al., 1985) and monocytes (Mentzer et.al., 1986), T-cell dependent antibody production (Fischer et.al., 1986; Howard et.al., 1986) and the interactions between activated macrophages and tumor cells (Strassman et.al., 1986). The characteristic alpha chain of LFA-1 is approximately 170KD in size (Gallin, 1985).

Although intracellular localization of the GP subunits has not been demonstrated ultrastructurally, Arnaout et.al. (1984) and Todd et.al. (1984) have implicated secondary granule release with increased surface expression of the Mo 1 antigen. Examination of whole cells and subcellular fractions using electrophoretic and immunological techniques indicated that Mo 1 was associated predominately with secondary granule membranes. These investigators proposed that during activated secondary granule release Mo 1 was transported to the plasma membrane through granule-plasma membrane merger. O'Shea and coworkers (1983) also presented evidence suggesting that secondary granules may act as the intracellular pool for CR3 by reporting that a patient with secondary granule deficiency was unable to increase cell surface expression of the CR3 receptor in response to neutrophil activation.

Additional evidence indicating that secondary granules may represent the intracellular source of the Mo 1 antigen was provided by the myloid cell lines KG1 and THP-1. These cells do not contain secondary granules and do not express Mo 1 on their surfaces (Arnaout et.al., 1984).

Petrequin and associates (1985) recently presented evidence suggesting that the Mo 1 GP may be more closely associated with tertiary than with secondary granules. Neutrophils activated with 1nM or 100nM fMLP for 5 minutes were reported to exhibit a 3 fold increase in cell surface Mo 1 expression and concomitant extracellular gelatinase release without significant release of secondary granule vitamin B12 binding protein or primary granule myeloperoxidase.

2. Quantitative membrane changes: Increased plasma membrane expression

Evidence suggests that activated granule release may not only produce qualitative alterations in neutrophil plasma membrane, in terms of up-regulation of certain moieties, but may also contribute to a quantitative alteration in exposed surface membrane. Hoffstein and coworkers (1982) reported that new membrane was added to neutrophil surfaces following 10 seconds exposure of cells to 100nM fMLP. The rapidity of membrane addition suggested that an intracellular pool of preformed membrane was being mobilized to the cell surface. Furthermore, quantitation of intracellular granules at an electron microscopic level suggested that the source of this pool was the secondary granules.

II. fMLP as an Initiator of Neutrophil Activation

The tripeptide N-formylmethionyl leucyl phenylalanine (fMLP) was originally synthesized based on the finding that low molecular weight peptides from rapidly growing bacteria activated leukocytes (Keller and Sorkin, 1967; Schiffmann et.al., 1975a; Schiffmann et.al., 1975b; Schiffmann et.al., 1978; Showell et.al., 1967). Competitive binding experiments using synthetic peptides and bacteria culture filtrates indicated that fMLP was structurally related to the naturally occurring bacterial substances, both activating cells through a common leukocyte receptor (Aswanikumar et.al., 1976; Aswanikumar et.al., 1977; Schiffmann et.al., 1975a).

Membrane receptors for fMLP have been identified on pulmonary macrophages (Spilberg et.al., 1981), monocytes (Weinberg et.al., 1981) and neutrophils (Aswanikumar et.al., 1977; Sha'afi et.al., 1978; Vitkauskas et.al., 1980; Williams et.al., 1977; Zigmond and Sullivan, 1979). The receptors on human neutrophils have been isolated and were found to be glycoproteins approximately 40,000 to 70,000 daltons in size (Goetzl et.al., 1981; Niedel et.al, 1980). As a population, isolated neutrophils are reported to exhibit heterogeneity in terms of fMLP binding characteristics and subsequent functional responses (Berkow and Baehner, 1985; Gallin, 1984; Klempner and Gallin, 1978; Seligmann et.al., 1981; Seligmann et.al., 1984).

Exposure of neutrophils to fMLP mediates a number of morphological and functional cellular responses indicative of activation. These responses include shape change (Smith et.al., 1979; Zigmond, 1978), enhanced adhesion to a substratum (Hoover et.al., 1980; Smith et.al., 1979; Tonnesen et.al., 1984), migration (Zigmond,

1978) extracellular granule release (Brentwood and Henson, 1980; Dewald et.al., 1982; Wright and Gallin, 1979), modifications in the expression of surface membrane moieties (Arnaout et.al., 1984; Berger et.al., 1984; Fearon and Collins, 1983; Lanier et.al., 1985; Zigmond et.al., 1982) and alterations in the quantity of exposed surface membrane (Hoffstein et.al., 1982).

III. The Calcium Ionophore A23187 as an Initiator of Neutrophil Activation

Ionophores are small hydrophobic molecules which dissolve into the lipid bilayer of cell membranes and transport specific ions down their concentration gradients into the cell cytosol (Alberts et.al., 1983). The ionophore calcimycin, or A23187, specifically transports divalent cations such as Ca++.

Increased cytosolic free Ca++ concentration is one of the first measurable events to occur following neutrophil receptor-ligand binding. After a lag of approximately 3 seconds, cells treated with 100nM fMLP have been reported to rapidly take up extracellular calcium (Korchak et.al., 1984) as well as mobilize intracellular Ca++ stores (Naccache et.al., 1985; von Tschnarner et.al., 1986). Thus, the result of A23187 treatment of neutrophils is a receptor independently induced increase in intracellular Ca++.

In neutrophils, as in other cells, calcium is believed to act as a secondary messenger in activation. Neutrophils treated with A23187 have been found to change shape (unpublished personal observation), exhibit enhanced adhesion (Gallin et.al., 1978), extracellular granule secretion (Hoffstein and Weissmann, 1978) and up-regulation of the CR3 receptor (Arnaout et.al., 1984; Todd et.al., 1984).

IV. Temperature Transition as an Initiator of Neutrophil Activation

Neutrophils exposed to an increase in temperature have been reported to exhibit responses somewhat analogous to the morphological and functional events of cellular activation.

A. Actin Polymerization

As mentioned previously, neutrophil activation has been shown to induce polymerization of actin (Oliver and Berlin, 1982; Anderson et.al., 1982), and microfilament formation is required for cellular shape change (Smith et.al., 1979). Howard and Oresajo (1985) recently reported that temperature mediated actin polymerization takes place in human neutrophils. The relative F-actin content of cells incubated at various temperatures ranging from 4C to 37C was assessed by fluorescence activated cell sorter analysis of phallicidin stained cells. Compared to cells held at 4C, these investigators detected a 4 fold increase in F-actin content following 37C incubation.

B. Enhanced Adherence

Charo et.al. (1985) reported that a temperature increase from 4C to 37C induced enhanced neutrophil adhesion to albumin coated plastic or cultured endothelial cells. The response was determined to be temperature dependent, as cells exposed to either substrata and maintained at 4C did not demonstrate augmented adherence.

C. Extracellular Granule Release

Apparent temperature mediated extracellular granule release has been reported. Goldstein and associates (1974) and Wright and Gallin (1979) detected lysozyme in the cell-free supernatants of neutrophils incubated at 37C for 30 minutes, with no additional release seen after

60 minutes incubation. Release was determined to be dependent upon extracellular Ca++, plateauing at 1mM calcium chloride. Spontaneous temperature dependent vitamin B12 binding protein release has been reported by Corcino et.al. (1970). These investigators found significant amounts of extracellular vitamin B12 binding protein in supernatants from neutrophils incubated at 37C for 5 minutes. Vitamin B12 binding protein was not detected in supernatants from cells incubated up to 5 hours at 4C. Dewald and coworkers (1982) also reported that vitamin B12 binding protein was released from cells after 5 minutes incubation at 37C, but only from neutrophils which were isolated from buffy coat cells held at 4C for 24 hours. Freshly isolated neutrophils did not spontaneously release vitamin B12 binding protein. Neutrophils isolated from buffy coats aged for 24 hours at 4C were also found to spontaneously release gelatinase when warmed at 37C. Dramatic, highly significant release occurred within 5 minutes incubation at 37C. As was determined for spontaneous vitamin B12 binding protein release, neutrophils isolated from freshly drawn blood did not demonstrate spontaneous temperature dependent gelatinase release (Dewald et.al., 1982; Baggiloini and Dewald, 1984).

In contrast to activation induced extracellular granule secretion, cytochalasin B has been reported to have no affect on temperature mediated granule release (Skosey et.al., 1973; Zurier et.al., 1973).

D. Plasma Membrane Modifications

1. Qualitative membrane changes: Up-regulation of membrane components

Recent evidence indicates that qualitative alterations in neutrophil membrane can occur without exposing the cells to conventional activating agents. Incubation of human neutrophils at 37C has been reported to produce spontaneous increases in surface expression of CR1, the C3b receptor, (Fearon and Collins, 1983) CR3 (Berger et.al., 1984; Fearon and Collins, 1983) and the p150,95 GP (Springer et.al., 1986).

V. Neonatal Neutrophils

Human newborns are more susceptible to frequent and serious bacterial infections than adults. The evidence suggests that this susceptibility is, at least in part, due to depressed neutrophil functions. Depressed activity is not the result of an aberration in chemoattractant sensory mechanisms but is thought to be related to decreased responses of neonatal cells to activation (Anderson et.al, 1981; Anderson et.al., 1984; Ambruso et.al., 1984; Becker-Freeman et.al., 1984; Krause et.al., 1982).

Recently, Krause and coworkers (1986) reported that isolated neonatal human neutrophils represent a heterogeneous population of cells. These investigators identified a subpopulation of cells which, like adult cells, expressed quantitatively less of a specific surface membrane component and were less motile than the major population of cells. Compared to isolated adult cells, this subpopulation represented a significantly larger portion of isolated neonatal cells. Krause et.al. (1986) further suggested that the depressed functional responses of neonatal neutrophils may be partially due to the relatively larger proportion of this subpopulation of cells.

Depressed responses are transient, subsiding within several months after birth. Krause and coworkers (1982; 1986) reported that the activities of neutrophils isolated from neonates and from infants 3 days to 2 months of age were significantly diminished when compared to adult cells. However, between 2 and 4 months of age cell activities were markedly increased and continued to increase until approximately 17 years of age.

A. Shape Change

Neonatal neutrophils were found to express relatively normal intrinsic polarity, but defective acquired polarity. Neutrophils isolated from umbilical cord blood and activated in suspension exhibited ruffled configurations within seconds. Although bipolar forms were seen with continued exposure to the chemoattractant, compared to adult shape change responses, fewer neonatal cells exhibited knob-like tail structures or uropods (Anderson et.al., 1981). On the other hand, neonatal neutrophils demonstrated a marked depression in the ability to orient, or express acquired functional polarity. Anderson et.al. (1984) reported that while more than 90% of adult cells demonstrated proper orientation in a chemotatic gradient by exhibiting pseudopods directed toward the higher concentration of chemoattractant, only 70% of neonatal cells oriented properly. Additionally, when the gradient was reversed, those neonatal cells that did orient were unable to reorient.

B. Adherence

Neonatal neutrophils also exhibit abnormal adherence associated activities. While baseline adhesion of newborn neutrophils was reported to be comparable to adult levels, chemoattractant mediated enhanced adhesion was diminished (Anderson et.al., 1981; Becker-Freeman et.al., 1984). An inability of newborn cells to modulate adherence sites may be associated with depressed activation mediated adhesion. In suspension, neonatal neutrophils exposed to chemotactic factors did not demonstrate a normal capacity to transport latex bead-bound adhesion sites from pseudopods to the tail of polarized cells (Anderson, et.al. 1981).

C. Migration

The abnormalities in newborn neutrophil orientation and adhesion are reflected in impaired motile responses. The motility of neonatal cells has been found to be significantly reduced in response fMLP, C5 cleavage products and low molecular weight chemoattractants isolated from <u>E. coli</u> culture fluid (Anderson et.al. 1981; Krause et. al. 1986; Nunoi, et.al.1983).

D. Extracellular Granule Release

Neutrophils isolated from umbilical cord blood have been reported to contain myeloperoxidase and beta-glucuronidase and lysozyme in quantities comparable to those found in adult neutrophils. On the other hand, lactoferrin levels have been found to be significantly less (Ambruso et.al., 1984; Becker-Freeman et.al., 1984). The levels of lactoferrin in lysates prepared from neonatal cells are reported to be approximately 50% of those found in adult lysates. Compared to adults cells, neonatal cells exposed to fMLP, A23187 or the tumor promoter PMA secreted equivalent amounts of myeloperoxidase and lysozyme but approximately 50% less lactoferrin (Becker-Freeman et.al., 1984).

E. Plasma Membrane Modifications

1. Quantitative: Up-regulation of membrane components

Compared to adult cells, neonatal neutrophils have been reported to exhibit depressed baseline levels of the adhesion associated GP Mo 1. Neonatal neutrophils demonstrated dose dependent up-regulation of the alpha and beta subunits following activation with fMLP or exposure to A23187 or PMA, however, the degree of expression was significantly diminished when compared to activated adult neutrophils (Becker-Freeman et.al., 1984).

As discussed above, Mo 1 and lactoferrin have been implicated in neutrophil adhesion and hydroxyl radical production. The low levels of Mo 1 expression and lactoferrin content in neonatal cells may contribute to the increased susceptibility and severity of bacterial infections seen in newborns (Becker-Freeman et.al., 1984).

MATERIALS AND METHODS

Reagents

The following reagents were routinely used throughout these studies. Unless otherwise indicated, complete PBS, phosphate buffered saline containing 1.0mM calcium chloride, 0.15mM magnesium chloride, pH 7.4 (Gibco) made 5mM glucose was used as reagent dilution buffer and neutrophil suspension buffer. Elutriation buffer was PBS, 0.25mM EDTA. Stock solutions of 1mM fMLP, cytochalasin B at 5mg/ml (Sigma) and 5mM A23187 (Calbiochem) were made in 100% dimethyl sulfoxide (DMSO) and stored at -35C until use. The highest concentration of DMSO present in reactions with neutrophils, 0.01% (v/v), had no affect on the cells or the assay systems. Cells were fixed for light and electron microscopy with diluted electron microscopy grade glutaraldehyde (Ladd).

Neutrophil Isolation: Adult

For these studies, human neutrophils were isolated from whole blood obtained by venipuncture, with informed consent, from male and female donors ranging from 21 to 68 years of age. Blood was anticoagulated with buffered sodium citrate and neutrophils were purified using one of three methods; cold counterflow centrifugal elutriation, cold ficoll/hypaque discontinuous density gradient or ficoll/hypaque discontinuous density gradient performed at room temperature (see Appendix A).

The counterflow centrifugal elutriation technique utilized a JE10-X elutriation rotor, J6M centrifuge (Beckman) and Masterflex Pump with 7016-20 head (Barnstead). Blood samples subjected to elutriation were mixed with Hespan (6.0% hetastarch in 0.9% sodium chloride, American Hospital Supply) to sediment erythrocytes. Leukocyte rich plasma was diluted with Ca++,Mg++-free PBS (Gibco) and pumped into the elutriation chamber at approximately 45ml/minute, centrifuging at 1420 rpm, 10C. After the sample had been loaded, cold elutriation buffer was pumped into the chamber at 45ml/minute for 10 minutes. The pump speed was then increased to approximately 55ml/minute for 10 minutes followed by 64ml/minute for 30 minutes. Samples from chamber effluent were taken at 2.5 minute intervals for Coulter Counter determinations of erythrocyte content. When erythrocyte counts fell below 40,000/ml, the pump was stopped and centrifuge speed decreased to 1000 rpm. After 4 minutes the centrifuge was stopped, the separation chamber removed and the isolated neutrophils were transferred to tubes pretreated with normal human serum. Cells were adjusted to 10 million/ml in complete PBS and held on ice until use.

For ficoll/hypaque isolation of neutrophils in the cold, citrated blood was diluted with an equal volume of Ca++,Mg++-free PBS at room temperature and carefully layered over one-half the volume of cold ficoll/hypaque solution (Histopaque type 1083, Sigma) in glass tubes. Tubes were centrifuged at 1000 rpm for 20 minutes at 4C resulting in the formation of three discrete regions. The upper plasma region and mid-ficoll/hypaque interface region, containing mononuclear cells, were aspirated and discarded. The erythrocyte-granulocyte pellets were resuspended to twice their volume with cold Ca++,Mg++-free PBS, transferred to normal human serum-coated tubes containing Hespan, at one-tenth of the cell volume, and mixed. The cell isolation tubes were maintained at 4C for approximately 30 minutes to allow erythrocyte sedimentation. The upper granulocyte-containing layer was removed and washed twice by centrifugation and resuspension of cell pellet with cold Ca++,Mg++-free PBS. Any contaminating erythrocytes were removed by hypotonic lysis. One ml of ice cold sterile distilled water was added to the cell pellet while mixing for 20 seconds. An equal volume of cold 2X saline was then added to restore tonicity, followed by Ca++,Mg++-free PBS. Isolated neutrophils were washed once with Ca++,Mg++-free PBS followed by two washes with complete PBS. Cells were adjusted to 10 million/ml and held on ice until use.

Neutrophils isolated at room temperature were obtained from ficoll/hypaque gradients performed as above with the exception that all centrifugations and reagents were at room temperature. Isolated cells were maintained at room temperature until use.

Neutrophil Isolation: Neonatal

Neonatal neutrophils used in these studies were isolated from anticoagulated whole blood samples obtained from umbilical cords with informed parental consent. Neutrophils were isolated in the cold using the ficoll/hypaque gradient technique and held on ice until use.

Isolated Neutrophils: Purity and Viability

Isolated cells were approximately 95% neutrophils (determined by polychromatic dye stained smears) containing no more than 3% erythrocytes. Viability of the cells, determined by trypan blue exclusion, exceeded 97%. An aliquot of isolated cells was mixed with an equal volume of 0.04% trypan blue (w/v) in PBS and held for approximately 5 minutes at room temperature. At least 200 cells were examined with light microscopy for exclusion of the dye. Neutrophils were used within two hours of isolation.

Neutrophil Morphology: Light Microscopy Shape Change Determinations

Determinations of neutrophil shape change responses were made with light microscopy on cells fixed in suspensions with an equal of volume 2% buffered glutaraldehyde (v/v diluted with Ca++,Mg++-free PBS). At least 200 fixed cells were examined with phase contrast or Normarski optics and classified as "spherical", "ruffled" or "bipolar" as described (Smith et.al., 1979). Round cells with a smooth contour were considered to be spherical. Neutrophils that were basically round in shape but exhibiting membrane protrusions, or pseudopods, were classified as ruffled. Bipolar cells appeared ovoid or triangular in shape (Figure 1). After warming, approximately 90% of fixed isolated adult and neonatal neutrophils exhibited round configurations.

Dose response fMLP mediated cell shape change was determined. Neutrophil suspensions were prewarmed for 5 minutes at 37C. Warmed fMLP was added, with mixing, to obtain final concentrations from 1pM to 100nM. Thirty-seven degree incubation conditions continued for an additional 5 minutes at which time the cells were fixed with glutaraldehyde.

Kinetic shape change responses were determined on neutrophils activated with fMLP. Cell suspensions were warmed by incubation for five minutes at 37C. Prewarmed fMLP was then added, with mixing, to achieve final concentrations of 1nM or 100nM. At 10-20 second intervals for up to 5 minutes aliquots of the cell-activator mixture were removed and immediately fixed with glutaraldehyde.

Neutrophil shape change in response to exposure to the calcium ionophore A23187 was determined by prewarming the cells for 5 minutes at 37C, followed by the addition of warm A23187 to obtain final concentrations of either 100nM or 250nM. Samples of treated cells were fixed 30 seconds, 1,3,5,7 and 10 minutes after the addition of ionophore.

Cells exposed to a temperature transition were isolated in the cold transferred from an ice bath to a 37C water bath. At time points from 0 to 60 minutes at 37C, sample aliquots were taken and cells were fixed.

Results of shape change determinations are expressed as the percent of total cells evaluated exhibiting spherical, ruffled or bipolar configurations.

Adherence

The effect of fMLP on adherence of adult neutrophils to a serum coated substratum was evaluated as described (Smith et.al., 1979). Briefly, the adherence chambers consisted of two milled brass plates supporting two 25mm round cover glasses separated by a rubber gasket. One of the cover glasses was pretreated with 2% normal human serum (v/v in complete PBS) for 2 minutes and washed with complete PBS. The other cover glass was simply washed with PBS. Suspensions of 1 million neutrophils were exposed to 1nM or 100nM fMLP at room temperature and immediately loaded into the chambers. Cells were allowed to settle onto the serum treated glass for 400 seconds and random fields were counted using inverted phase contrast optics to obtain "total number of cells". The chambers were flipped over. Cells not adhering to the serum coated substratum fell off and become attached to the untreated glass. The chambers were flipped again and random fields of cells adhering to the serum-treated glass cover slip were counted again to obtain "number of adhering cells". Percent adherence of fMLP activated and nonactivated cells was calculated as follows:

$$\frac{(\text{Total number of cells - Number of cells adhering})}{\text{Total number of cells}} \text{X100}$$

Chemoattractant mediated adherence is expressed as percent over nonactivated baseline adherence.

Chemotaxis

Evaluations of fMLP mediated cell motility were made using blind-well chambers (Neuroprobe). The lower compartments were filled with 10nM fMLP, made 2% (w/v) HSA (essentially globulin-free, Sigma), or 2% HSA alone to determine baseline migration. The compartments were covered with a 13mm, 3um pore-size nitrocellulose filter (Schleicher and Schuell). Two hundred ul of isolated adult neutrophils, at 2 million/ml in complete PBS made 2% HSA, were loaded into the upper compartment. The chambers were incubated for 30 minutes at 37C. Filters were removed, stained, cleared (Maderazo and Wornick, 1978) and motility was evaluated with light microscopy by determining the maximum distance migrated by at least 2 cells, the "leading front" technique (Zigmond and Hirsch, 1973). Chemotaxis is expressed as percent migration over baseline level.

Extracellular Granule Release

Cell-free supernatants were assayed to indicate extracellular release of granule components from adult neutrophils in response to fMLP activation or exposure to A23187 or a temperature transition.

Granule release mediated by fMLP was determined on supernatants from neutrophils activated with 1nM, 10nM or 100nM fMLP. One million neutrophils/ml were preincubated for 5 minutes at 37C. Warm fMLP was added and the incubations were continued for 20 seconds or 10 minutes.

A23187 mediated extracellular granule release determinations were made on supernatants harvested from 1 million neutrophils exposed to the ionophore. Prewarmed cell suspensions (5 minutes at 37C) were exposed to 100nM or 250nM A23187 for 1 to 10 minutes at 37C.

Extracellular granule release conditions were terminated by immersing the reaction tubes into an ice bath for approximately 5 minutes. Baseline release determinations were made on supernatants from nonactivated cell suspensions incubated for 5 minutes at 37C.

Granule release in response to a temperature transition was determined on neutrophils isolated in the cold or at room temperature. Cells were adjusted to 1 million/ml in complete PBS and incubated at 37C. At several time points, ranging from 1 to 60 minutes, the reaction tubes were transferred to an ice bath. Baseline line release was determined on supernatants from cell suspensions maintained on ice or at room temperature.

Cell-free supernatants were harvested from fMLP, A23187 and temperature mediated granule release reaction tubes by centrifugation at 1000 rpm for 10 minutes at 4C.

The total cellular content of granule components were determined from cell lysates. Neutrophils were treated with 0.1% triton X-100, sonicated approximately 20 seconds on ice (Heat Systems-Ultrasonics) and freeze-thawed at least once. Lysates were centrifuged at 5000 rpm for 60 minutes at 4C to remove cellular debris before assaying.

Granule Component Assays

Cell-free supernatants were assayed to indicate extracellular release from primary, secondary and tertiary neutrophilic granules.

Primary Granule: Beta-Glucuronidase

Beta-glucuronidase was assayed as an indicator of primary granule release. The assay used is based on the enzymatic hydrolysis of phenolphthalein glucuronide at acid pH (Fishman, 1965). The phenolphthalein liberated as a result of the reaction generated a pink color at alkaline pH which was quantitated spectrophotometrically at 540nm. Standard curves were generated with solutions of phenolphthalein (Sigma) diluted in complete PBS to provide 10ug to 200ug of phenolphthalein/ml. One hundred ul of standard solutions, cell-free supernatants or lysates were added to 0.8ml 0.1M acetate buffer, pH 4.5. One hundred ul of substrate, 100mM phenolphthalein glucuronide, pH 4.5 (Sigma), was added, the tubes were mixed and incubated at 37C for 17 to 20 hours. The pH of the reaction mixtures was then adjusted to approximately 10.3 by the addition of 2.5ml glycine solution, (217mM glycine, 217mM NaCl and 300mM NaOH, pH approximately 11.20) 1.0ml 5% (w/v) trichloroacetic acid (TCA) and 1.5ml distilled water. The tubes were mixed and the optical density (O.D). was read at 540nm against a water blank. Beta-glucuronidase activity is expressed in Fishman units, defined as the amount of enzyme which liberates 1ug phenolphthalein from 100mM substrate solution in 1 hour at pH 4.5. Extracellular primary granule beta-glucuronidase release is expressed as percent of total Fishman units detected in neutrophil lysates prepared from 1 million cells.

Secondary Granule: Lactoferrin

The glycoprotein lactoferrin was assayed as an indicator of secondary granule release. An indirect double-sandwich enzyme-linked immunoassay (ELISA) was used (Hetherington et.al., 1983). Sodium phosphate buffer (0.01M, pH 7.4) containing 0.5M NaCl was used throughout as the assay buffer. Assay buffer made 0.05% Tween 20 (polyoxethylenesorbitan monolaurate, Sigma) was used as the plate washing buffer. Except were indicated, all incubations were performed at room temperature for 30 minutes. Ninety-six well, flat bottom polystyrene plates (NUNC Immunoplate I, Gibco) were incubated with 100ul/well of goat anti-human lactoferrin (Nordic) diluted 1:2000 in 0.1M carbonate/bicarbonate buffer, pH 9.0 (coating buffer), at 4C overnight. Coated plates were washed 3 times for 3 minutes each with washing buffer. Washed, coated plates could be held in plastic bags at 4C for up to one week prior to use without affecting the lactoferrin ELISA. One hundred ul/well of diluted sample was added to prepared plates and incubated. Plates were washed (3 times 3 minutes each wash), and 100ul/well of the IgG fraction of rabbit anti-human lactoferrin (Cappel) diluted 1:3000 in assay buffer was added. Plates were incubated, washed and 100ul/well of peroxidase conjugated swine anti-rabbit IgG (Dako) diluted 1:1000 with assay buffer was added. After incubation and washes, a peroxidase substrate was added. Two hundred ul of freshly prepared 0.4mg/ml orthophenylene diamine hydrochloride (Kodak) made 0.025% hydrogen peroxide in 0.05M citrate buffer, pH 5.0, were added to each well. The peroxidase reaction was allowed to develop for 30 minutes at room temperature, then stopped by the addition of 50ul/well 2.5N sulfuric acid. ELISA plates were spectrophotometrically read on a microplate reader, Dynatech or Titertek, at 490nm or 492nm respectively. Lactoferrin standard curves were generated for each granule release assay using human milk lactoferrin (Sigma) at dilutions ranging from 6.25ng/ml to 200ng/ml and plotting ng of lactoferrin/ml against O.D. at 490nm or 492nm. The amount of lactoferrin released into the supernatants or contained in cell lysates was determined from the standard curve correcting for initial sample dilutions. Extracellular release results are expressed as percent of total lactoferrin detected in lysates prepared from 1 million neutrophils.

Secondary Granule: Lysozyme

In addition to lactoferrin release, evidence of extracellular secondary granule release was evaluated by assaying cell-free supernatants for the enzyme lysozyme. The lysozyme assay is based on kinetic bacteriolytic activities of the enzyme (Neurkar, 1981). Suspensions of substrate bacterium, Micrococcus lysodeikticus (Sigma), were made in 0.066M potassium phosphate buffer, pH 6.24. The amount of bacteria/ml of buffer was adjusted so that the O.D. at 450nm, read against air, was between 0.600 and 0.700. One hundred ul of lysozyme standard (egg white lysozyme Grade I, Sigma) providing from 12 units/ml to 100 units/ml or 100ul of supernatant or cell lysate was added to 2.5ml of substrate in a polystyrene cuvet and mixed. O.D. readings of the mixture were made one minute after the addition of test solutions and at one minute intervals thereafter for five minutes. The average change in O.D./minute mediated by the lysozyme standards were calculated and plotted against units/ml to generate standard curves. The amount of lysozyme contained in assay samples were determined by calculating the average change in O.D./minute and interpolating units/ml from standard curves. Extracellular granule release of lysozyme is expressed as the percent of total enzyme detected in cell lysates prepared from 1 million neutrophils.

Secondary Granule: Collagenase

Some cell-free supernatants were also assayed for collagenase to indicate extracellular secondary granule release. Collagenase activity was assessed using a soluble [14C]-collagen substrate. Labeled collagen was prepared from calf skin type I collagen as described below for use in determination of gelatinase activity, with the exception that dialyzed labeled collagen was lyophilized and redissolved to half the volume with 0.01M acetic acid.

The collagenase assay employed was a combination of several published methods (Cawston et.al., 1979; Murphy et.al., 1980; Sellers and Reynolds, 1977). Supernatants and lysates were first treated with 1mM phenylmethanesulfonyl fluoride (PMSF, Sigma) exactly as described below for the material used in gelatinase determinations. Prior to assaying, latent collagenase was activated by incubating PMSF-treated samples with 1mM p-chloromercuricbenzoate (Sigma) and 10mM $CaCl_2$ in a total volume of 225ul at pH 8.4, for 60 minutes at 37C. The samples were cooled to room temperature and soluble [14C]-collagen, also at room temperature, was added to provide approximately 45,000 cpm per reaction tube. Samples were incubated at room temperature for 24 hours. Uncleaved substrate was pelleted by centrifuging assay tubes at room temperature for 15 minutes at 5000 rpm. Cpm were determined on 100ul of assay supernatant added to 10 ml scintillation fluid (Hydrofluor, National Diagnostics). One unit of collagenase is defined as the amount of enzyme that degrades lug of substrate/minute. Collagenase release is expressed as percent of total activity found in lysates from 1 million neutrophils.

Tertiary Granule: Gelatinase

The tertiary granule component gelatinase was assessed by the ability of cell-free supernatants and cell lysates to enzymatically cleave labeled gelatin. [14C]-gelatin was prepared by heat denaturation of labeled calf skin type I collagen (Calbiochem). Briefly, type I collagen was solubilized in 0.01M acetic acid at lmg/ml with stirring at 4C. The pH was adjusted to 9.0 with 0.1M sodium borate buffer and lmCi of [14C]-formaldehyde (ICN)/72mg collagen was added. The mixture was stirred on ice for 60 seconds followed by the addition of four 225ul aliquots of 0.01M sodium borohydride. After an additional 30 minutes of mixing on ice and one 500ul aliquot of 0.01M sodium borohydride, the mixture was loaded into dialysis tubing. The [14C]-collagen was dialyzed exhaustively at 4C against 0.01M acetic acid to remove any unbound [14C]. The final preparation was stored at 4C in the presence of 0.02% sodium azide.

The gelatinase assay used combined several published methods for determination of the enzyme (Dewald et.al., 1982; Hibbs et.al., 1985; Murphy et.al., 1980). Harvested supernatants or prepared lysates were first treated with lmM PMSF in 50mM Tris-HCl, 50mM NaCl, pH 7.6, for 60 minutes at 37C to block serine proteinases. Treated samples were frozen at -35C until they were assayed. Latent gelatinase was pre-activated by incubating PMSF-treated samples with 1mM p-aminophenylmercuric acetate (Sigma) and 10mM $CaCl_2$ in a total volume of 225ul at pH 8.4, for 1 hour at 37C. Immediately before use [14C]-collagen was denatured by heating at 60C for 20 minutes. A portion of labeled gelatin providing at least 10,000 cpm was added to each assay tube at 37C and the incubation was continued for 1 hour. The reaction was terminated by precipitating uncleaved [14C]-gelatin with ice cold TCA. 50ul of 90% (w/v) TCA was added to each assay tube, the tubes were mixed and incubated on ice for 30 minutes. The tubes were then centrifuged in the cold at 5000 rpm for 15 minutes and 100ul samples of assay supernatants were removed, added to 10ml scintillation fluid and counted for 0.1 minute. One unit of gelatinase activity is defined as the amount of enzyme degrading lug of substrate/minute. Extracellular gelatinase release is expressed as percent of total gelatinase detected in lysates prepared from 1 million neutrophils.

Electron Microscopy: Neutrophil Processing

Ultrastructural evaluations of neutrophil morphology and intracellular granule characterization and quantitation were made on micrographs of adult and neonatal neutrophils activated with fMLP or exposed to A23187 and on adult cells exposed to a temperature transition.

To standardize the population of cells to be used in the morphometric measurements it was essential that the region of each cell being sectioned was representative of the whole cell regardless of the orientation of sectioning. Therefore, it was critical that the cells were not bipolar in configuration but were spherical or ruffled in shape.

Chemotactic peptide mediated ruffling conditions, determined with light microscopy of adult and neonatal neutrophils, were used to activate cells for transmission electron microscopy. Cells were prewarmed for 5 minutes at 37C in complete PBS. Warm fMLP was added to obtain final concentrations of either 1nM or 100nM and the incubation conditions were continued for 20 seconds.

Adult and newborn cells were exposed to the calcium ionophore to induce cell ruffling. Prewarmed neutrophils were incubated for 5 minutes at 37C with either 100nM or 250nM A23187. Spherical nonactivated control cells for fMLP and A23187 morphometric determinations were neutrophils incubated at 37C for 5 minutes.

Adult neutrophils treated with a temperature transition were isolated in the cold and incubated at 37C for 5 or 30 minutes. Baseline control cells were maintained on ice.

For transmission electron microscopy, cells were fixed with warm fixative, 1.8% (v/v) glutaraldehyde buffered with 0.1M cacodylate, pH 7.4 (Electron Microscopy Sciences), made 1.0% (w/v) sucrose. Fixation continued for 1 hour at 37C followed by up to 96 hours at 4C. After washing with 0.01M sodium cacodylate, pH 7.4, made 7.0% (w/v) sucrose, cells were resuspended in Karnovsky's diaminobenzidine (Graham and Karnovsky, 1966), 3-3'diaminobenzidine tetrahydrochloride (DAB, Sigma) at 0.5mg/ml in 0.05M tris-HCl, pH 7.6, made 0.01% (v/v) hydrogen peroxide and 1% (w/v) sucrose. Following 30 minutes room temperature incubation with DAB, neutrophils were washed 3 times with 0.05M acetate-veronal buffer (A-V buffer), pH 7.4 and postfixed with 1% (v/v) osmium tetroxide (Electron Microscopy Sciences) in A-V buffer made 5% sucrose, for 1 hour. Cells were washed twice with A-V buffer and stained enbloc for 1 hour with 0.5% (w/v) uranyl acetate dissolved in A-V buffer made 4% sucrose. After dehydration in graded ethanol and transition with propylene oxide, samples were embedded in either Epon/Araldite or LX-112 resin (Ladd). Gold-silver sections were cut using glass or diamond knives, picked up on acid-alcohol cleaned copper grids and stained with saturated aqueous uranyl acetate counterstained with Reynold's lead citrate. Sections were examined using a JEOL 100S or 100CX transmission electron microscope at 60KV or a Siemens Elmiskop 101 electron microscope at 80KV. All microscopes were initially and periodically calibrated for magnification using a shadow cast replica of diffraction line grading mounted on a copper grid (Pellco).

In an attempt to standardize the ultrastructural granule quantitations and morphometric measurements, negatives were taken only if a sectioned cell represented the central region of a neutrophil. Sections were considered to be through the central region if the cell profile contained at least three nuclear lobes or the centriole. Cells fulfilling these criteria were photographed at approximately 7,000X magnification.

Ultrastructural Evaluation of Granule Release:

Intracellular Granule Characterization and Quantitation

Intracellular granule characterizations were made based on cytochemical staining. As indicated above, neutrophils processed for use in ultrastructural analyses were treated with DAB and hydrogen peroxide prior to osmium tetroxide postfixation. The DAB reaction stained primary granules electron dense making them distinguishable from the relatively more electron lucent secondary granuls (Figure 2). Peroxidase positive (primary) and negative (secondary) granules were counted on electron micrographs from neutrophils activated with fMLP, exposed to A23187 or a temperature transition and nonactivated control cells. Intracellular granule quantitation results are expressed as the average number of peroxidase positive and negative granules per neutrophil profile.

Extracellular Granule Release/Intracellular Granule Loss

To associate extracellular and intracellular evidence of granule secretion, release was expressed as a ratio of the amount of a particular granule component detected extracellularly per intracellular granule lost. To calculate the ratios, extracellular release was first re-expressed on a per neutrophil basis. The mean quantity of each granule component detected in cell-free supernatants was converted from percent of total to ng or units and divided by 1 million to generate an average extracellular release per cell. The release per cell values were then divided by the average number of granules lost (peroxidase positive or negative depending on the granular origin of the component) in response to the particular treatment of the cells as determined by intracellular granule counts made on micrographs. Since tertiary granules have not been identified ultrastructurally, the assumption was made that they may be peroxidase negative and gelatinase released/peroxidase negative granule was calculated. The resulting extracellular granule release/intracellular granule loss ratios were then corrected for nonactivated baseline release.

Ultrastructural Evaluation of Morphology:

Morphometric Determinations

For ultrastructural evaluations of neutrophil morphology, measurements of micrographs were made on a Houston graphics tablet interfaced to an Apple IIe computer equipped with software by Optomax. The graphics tablet was first calibrated so that the final magnification of each electron micrograph was equivalent to 18,000X. Perimeter measurements were made directly from micrographs by tracing the cell profile with a cursor. The Optomax program calculated the area from the perimeter measurements. Surface-to-volume ratios (S/V) were determined to describe morphometric measurements of two dimensional sectioned cells in three dimensional terms. S/V were calculated from perimeter measurements and calculated area based on the following geometric constants for circles and spheres:

Circle:

Perimeter $= 2\pi r$

Area $= \pi r^2$

Sphere:

Surface Area $= 4\pi r^2$

Volume $= 4\pi r^3/3$

$$S/V = 3/2(P/A)$$

Exposed surface membrane was then calculated from S/V using 397um ^{3} as the mean neutrophil volume (Korchak and Weissmann, 1978; Hoffstein, et.al. 1982). Perimeter and area measurements are expressed in arbitrary units.

Presentation of Data

Results are expressed as mean ± 1 standard deviation. Statistical significance was determined utilizing the Student's t-test using pooled estimator of common variance ^{2} and (n 1 + n 2) - 2 degrees of freedom. Deviations from baseline determinations were considered significant if the calculated p values were <0.01.

RESULTS

Two series of experiments were performed using the chemotactic tripeptide fMLP as a specific, receptor mediated, initiator of neutrophil activation. The first experiments examined the events of cellular activation in terms of visible shape change from spherical to bipolar forms, enhanced adhesion, chemotaxis and extracellular granule secretion. The second series of experiments specifically investigated those events occurring early in fMLP mediated activation.

fMLP Mediated Shape Change: Dose Response:

Neutrophils were found to be very sensitive to fMLP in terms of a visible shape change. Results of 5 minutes dose response shape change experiments are shown in Table 2. The cells responded to concentrations of fMLP as low as 1pM by exhibiting ruffled configurations. Approximately 70% of cells activated with 1nM fMLP for 5 minutes at 37C had become bipolar. The percentage of bipolar cells remained at about 60% to 70% with increasing doses of fMLP.

fMLP Mediated Shape Change: Kinetic Response:

Not only were neutrophils morphologically sensitive to pM amounts of fMLP, they were also found to respond rapidly to the chemoattractant. Results of kinetic shape change determinations made on cells treated with fMLP are shown in Table 3 and Figure 3.

Table 2
fMLP MEDIATED SHAPE CHANGE: DOSE RESPONSE

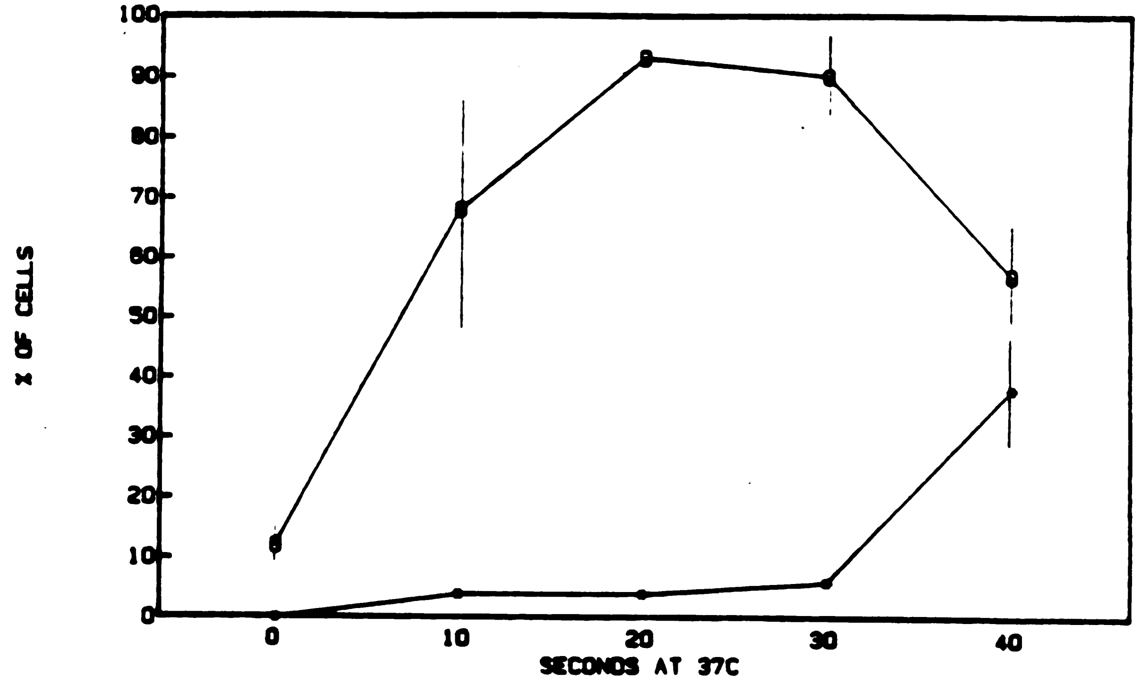
nM fMLP	% Ruffled forms	% Bipolar forms	n
None	12±5	0+1	20
0.001	76±8	2±2	5
0.005	75±3	2±1	5
0.01	77±7	2±2	5
0.05	82±8	3+2	3
0.1	71±23	6±7	4
0.5	56±13	42+16	4
1.0	25±8	70±8	8
5.0	24±8	72±4	3
100	42±3	60±3	2

Neutrophils were prewarmed to 37C, exposed to fMLP at various concentrations for 5 minutes at 37C.
Results are expressed as mean±standard deviation.

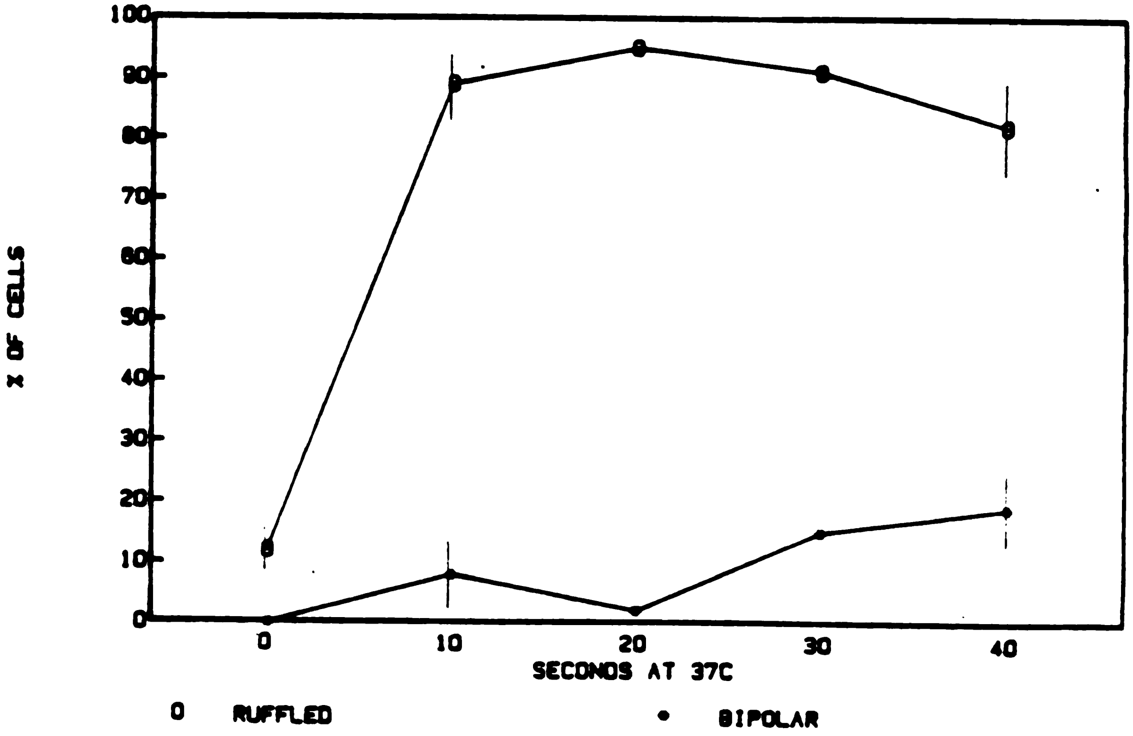
Table 3
fMLP MEDIATED SHAPE CHANGE: KINETIC RESPONSE

Seconds Exposure:	% Ruffled forms	% Bipolar forms	n
1nM fMLP			
10	68+30	4±4	7
20	93±3	4±3	16
30	90±10	6±9	2
40	57±14	38±16	12
60	36±18	63±19	5
90	14±3	84±2	3
120	11+7	86+8	7
300	25±8	70±8	8
100nM fMLP			
10	89+8	8±10	3
20	95±2	2±2	7
30	91	8	1
40	82±13	15±14	3
60	66±34	19±10	3
300	42±3	60±3	2
Baseline : Cells warmed to 37C	12±5	0±1	20

Neutrophils were prewarmed 5 minutes at 37C prior to exposure to fMLP.
Results expressed as mean±standard deviation



1 NM FMLP



100 NM FMLP

Figure 3
fMLP MEDIATED SHAPE CHANGE: KINETIC RESPONSE
Data from Table 3

Approximately 90% of the cells exposed to 1nM fMLP exhibited ruffled configurations within 20 to 30 seconds. By 40 seconds approximately 40% of the cells had progressed to bipolar forms. After 2 minutes, 90% of the cells had become bipolar. Neutrophils activated with 100nM fMLP for 10 to 40 seconds also exhibited ruffled configurations. However, with longer incubation periods, cells activated with the higher dose of fMLP tended to remain ruffled rather than progressing to bipolar forms.

fMLP Mediated Adherence:

Compared to nonactivated baseline adhesion, neutrophils exposed to fMLP at room temperature demonstrated enhanced adherence to an artificial substratum. 21.34 ± 4.20% or 29.24 ± 6.77% more cells adhered to serum coated glass surfaces following approximately 6.5 minutes treatment with 1nM or 100nM fMLP, respectively (mean ± standard deviation, n=4).

fMLP Mediated Chemotaxis:

Chemotaxis was performed and analyzed by the "leading front" technique as described in the Methods section. Neutrophils in chemotaxis chambers containing 10nM fMLP (in 2% HSA) in the lower compartment were found to have migrated 24.36 ± 8.46% farther into the filter then control cells in chambers with 2% HSA in the lower compartment (mean ± standard deviation, n=5).

fMLP Mediated Extracellular Granule Release:

The chemotactic tripeptide was also found to mediate extracellular granule release. Neutrophils were prewarmed for 5 minutes at 37C and exposed to various concentrations of fMLP for 10 minutes at 37C. Cell-free supernatants were harvested and assayed for primary, secondary and tertiary granule components. The results of dose response fMLP mediated granule release are shown in Table 4 and Figure 4. fMLP induced lactoferrin and gelatinase secretion in an apparently dose responsive fashion. Lactoferrin release was significant following 10 minutes exposure to 1nM, 10nM and 100nM fMLP. Significant amounts of gelatinase were detected extracellularly following 10 minutes activation of cells with 10nM or 100nM fMLP. Neither lysozyme nor beta-glucuronidase were detected in significant quantities.

fMLP Mediated Extracellular Granule Release:

Effect of Cytochalasin B:

The effect of cytochalasin B (CB) on fMLP mediated extracellular release from primary, secondary and tertiary granules was then investigated. Neutrophils were pretreated with 5ug/ml of CB for 5 minutes at 37C before fMLP was added. The incubations continued for 10 minutes at 37C, and cell-free supernatants and lysates prepared from CB treated cells were assayed for granule components. CB did not interfere with any of the component assays (data not shown). As seen in the lower portion of Table 5, the total content of granule components detected in lysates prepared from CB treated cells did not differ significantly from the amounts detected in lysates from cells without CB pretreatment (shown in the lower portion of Table 4). Table 5 and Figure 4 show that, compared to release without pretreatment, CB had no influence 1nM fMLP mediated lactoferrin, gelatinase or beta-glucuronidase release. On the other hand, CB appeared to enhance 1nM fMLP mediated lysozyme secretion. Pretreatment of cells augmented 10nM and 100nM fMLP induced lysozyme,

Table 4
fMLP MEDIATED EXTRACELLULAR GRANULE RELEASE

Release Condition:	Granule Component: Lactoferrin	Lysozyme	Gelatinase	B-glucuronidase
Baseline	2.10±0.64(13)	4.81±1.31(6)	5.81±3.75(8)	0 (2)
nM fMLP				
1	4.55±1.70(3)*	4.44±0.73(3)	11.39±4.50(4)	0 (1)
10	6.15±1.68(3)*	6.00±1.64(3)	17.94±10.15(3)*	0 (1)
100	9.85±3.95(5)*	8.82±3.86(3)	18.67±5.50(6)*	1.43±2.48(3)

Neutrophils were prewarmed at 37C for 5 minutes.
fMLP was added, incubations continued for 10 minutes.
Granule release is expressed as percent of total based on the amount of enzyme contained in lysates from 1 million neutrophils:
  Lactoferrin: 2.76±0.63 ug n=16
  Lysozyme: 258.09±44.52 Units n=8
  Collagenase: 5.13 X 10^{-3}±0.69 X 10^{-3} Units n=6
  Gelatinase: 0.331±0.069 Units n=13
  B-glucuronidase: 19.32±5.76 Units n=6
Mean±standard deviation
Baseline: Cells warmed 5 minutes at 37C
(Number of experiments in duplicate)
* $p<0.01$ vs baseline

Table 5
THE EFFECT OF CYTOCHALASIN B ON fMLP MEDIATED EXTRACELLULAR GRANULE RELEASE

Release Conditions:	Granule Component:			
	Lactoferrin	**Lysozyme**	**Gelatinase**	**B-Glucuronidase**
Baseline	1.78±0.28(4)	5.52±1.85(2)	10.39±6.56(2)	0 (2)
nM fMLP				
1	2.62±1.51(2)	9.43±0.89(2)	10.00±8.58(2)	0 (2)
10	19.03±5.87(2)	16.10±0.00(2)	74.06±39.51(2)	0 (1)
100	22.36±3.55(2)	17.10±1.42(2)	108.43±7.44(2)	28.91±5.22(2)

Neutrophils were preincubated with 5ug/ml of CB for 5 minutes at 37C.
fMLP was added, incubations continued for 10 minutes.
Release is expressed as percent of total based on the amount of component detected in lysates prepared from 1 million neutrophils in the presence of 5ug/ml CB.

Lactoferrin:	2.71±0.67 ug	n=5
Lysozyme:	202.29±25.23 Units	n=6
Gelatinase:	0.352±0.15 Units	n=2
B-glucuronidase:	23.05±3.53 Units	n=2

Baseline: Cells incubated for 5 minutes at 37C with 5ug/ml CB.
Mean±standard deviation
(Number of experiments)

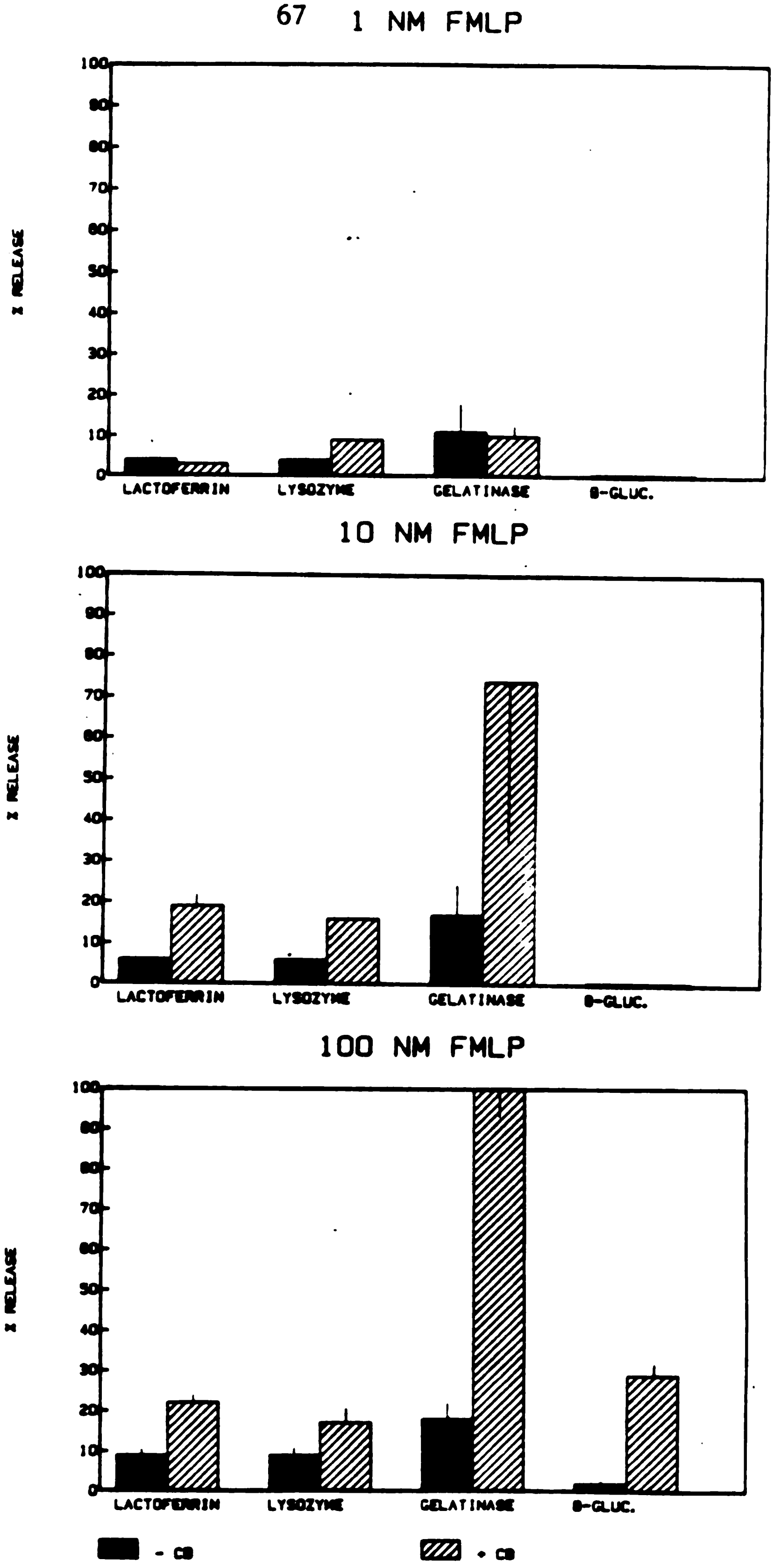


Figure 4
EFFECT OF CB ON fMLP MEDIATED EXTRACELLULAR GRANULE RELEASE
Data from Tables 4 and 5

lactoferrin and gelatinase release. The primary granule enzyme beta-glucuronidase was detected extracellularly following activation of CB pretreated cells with 100nM fMLP for 10 minutes.

The data presented above, from the first set of experiments investigating fMLP as an initiator of activation events, concurs with the well documented finding that the synthetic chemotactic tripeptide mediates visible changes in neutrophil morphology from spherical to bipolar configurations (Smith et.al., 1979), enhanced adherence (Smith et.al.,1979), chemotaxis (Zigmond, 1978) and extracellular granule release which is augmented by pretreating the cells with CB (Dewald et.al., 1982).

Those events occurring early in fMLP mediated activation were examined in the next series of experiments. The earliest visible indication of cell activation, ruffling, was used as the criteria for selecting the early fMLP activation conditions.

The fMLP mediated shape change determinations shown above indicated that neutrophils began to ruffle within seconds following exposure to low doses of fMLP (Tables 2 and 3, Figures 3). To determine whether functional events of activation were detectable early in fMLP mediated activation, cell-free supernatants from fMLP ruffled cells were analyzed for extracellular granule release.

Early fMLP Mediated Extracellular Granule Release:

Extracellular release of secondary granule lactoferrin was detected early following exposure of neutrophils to fMLP. Cells were prewarmed for 5 minutes at 37C and treated with 1nM, 10nM or 100nM fMLP for 20 seconds to produce cell ruffling. Cell-free supernatants were harvested and assayed for evidence of extracellular granule

Table 6
EARLY fMLP MEDIATED EXTRACELLULAR GRANULE RELEASE

Release Conditions:	Granule Component:				
	Lactoferrin	Lysozyme	Collagenase	Gelatinase	B-glucuronidase
Baseline	2.10±0.64(13)	4.81±1.31(6)	2.38±1.91(2)	5.81±3.75(8)	0 (2)
nM fMLP					
1	3.97±0.82(9)*	4.83±1.90(6)	4.34±0.12(3)	5.45±2.77(8)	0.93±1.16(3)
10	3.67±1.13(3)*	6.74±2.54(3)	N.D.	5.49±2.72(4)	0 (1)
100	4.35±0.89(9)*	8.15±3.53(6)	3.60±1.89(3)	5.37±2.29(8)	0.93±1.16(3)

Neutrophils were prewarmed for 5 minutes at 37C.
fMLP was added, incubations continued for 20 seconds.
Results are expressed as percent of total based on the amount of enzyme contained in lysates prepared from 1 million neutrophils (See Table 4).
Baseline: Cells warmed 5 minutes at 37C.
Mean±standard deviation
N.D.: Not Determined
(Number of experiments)
* $p<0.01$ compared to baseline

secretion. The results of these experiments are shown in Table 6 and Figure 5. The mean quantity of lactoferrin detected in supernatants from neutrophils exposed to each concentration of fMLP was significantly greater than baseline levels ($p<0.005$ for 1nM, 10nM and 100nM fMLP). Twenty seconds activation of cells with 1nM, 10nM or 100nM fMLP did not induce significant lysozyme, collagenase, gelatinase or beta-glucuronidase release.

Early fMLP Mediated Extracellular Granule Release:

Effect of Cytochalasin B:

Next, granule release experiments were performed to determine whether CB had an influence on early fMLP mediated extracellular granule release. The results of these experiments are shown in Table 7 and Figure 5. Neutrophils were pretreated with CB as described above, fMLP was added and the incubations continued for 20 seconds. As previously indicated, CB had no affect on the granule assays or on the total amount of granule components detected in cell lysates. Compared to release without pretreatment, CB had no affect on 20 seconds 1nM fMLP mediated secondary or tertiary extracellular granule release. However, lactoferrin and gelatinase release were enhanced following 20 seconds exposure of CB pretreated cells to 10nM or 100nM fMLP. Lysozyme release was not altered.

In terms of early neutrophil responses to activation, 20 seconds exposure of cells to 1nM or 100nM fMLP induced cell ruffling and caused significant increases in extracellular lactoferrin. To further investigate these early events of fMLP mediated activation, cells were examined ultrastructurally. First, intracellular granule characterizations and quantitations were made on electron micrographs

Table 7

THE EFFECT OF CYTOCHALASIN B ON EARLY fMLP MEDIATED EXTRACELLULAR GRANULE RELEASE

Release Conditions:	Granule Component:		
	Lactoferrin	**Lysozyme**	**Gelatinase**
Baseline	1.78±0.28(4)	5,52±1.85(2)	10.39±6.56(2)
nM fMLP			
1nM	3.05±1.87(2)	5.66±0.89(2)	10.56±6.33(2)
10nM	19.57±2.85(2)	6.30±0.00(1)	77.40±21.25(2)
100nM	20.20±1.90(2)	12.26±4.89(2)	109.41±1.99(2)

Neutrophils were preincubated with 5ug/ml of CB for 5 minutes at 37C.
fMLP was added and incubations continued for 20 seconds.
Granule release is expressed as percent of total amount of component contained in lysates prepared from 1 million neutrophils in the presence of 5ug/ml of CB (See Table 5).
Baseline: Cells incubated for 5 minutes at 37C with 5ug/ml of CB.
Mean±standard deviation
(Number of experiments)

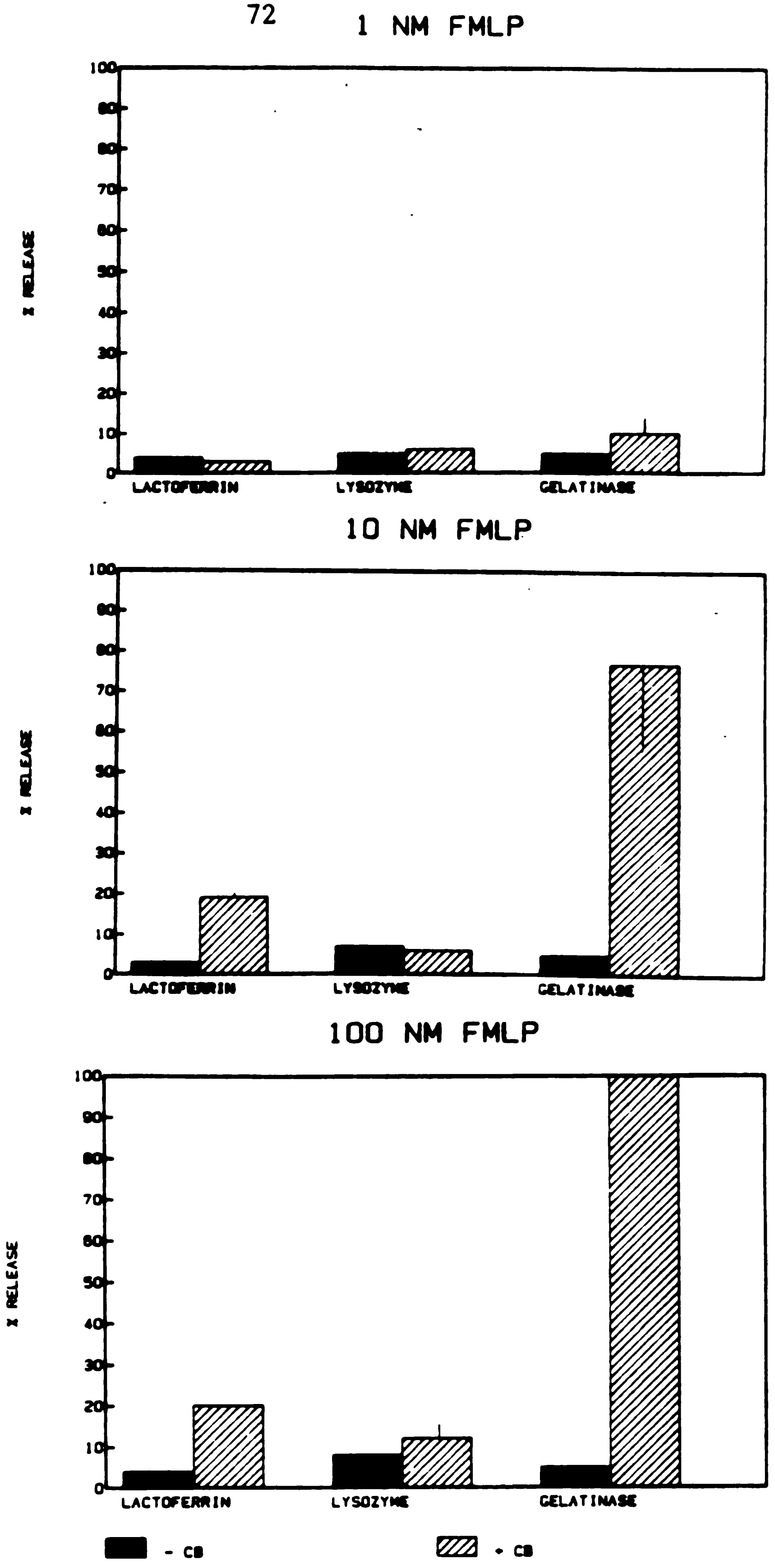


Figure 5
EFFECT OF CB ON EARLY fMLP MEDIATED EXTRACELLULAR GRANULE RELEASE
Data from Tables 6 and 7

of ruffled neutrophils to confirm the biochemical results indicating extracellular granule secretion by demonstrating a depletion in intracellular granules.

Ultrastructural Evaluation of Early fMLP Mediated Activation: Intracellular Granule Quantitation:

Neutrophils were warmed to 37C and exposed to 1nM or 100nM fMLP for 20 seconds. Activation was terminated by the addition of TEM fixative. Fixed cells were processed and photographed as described in the Methods section. Intracellular granules were characterized and counted based on peroxidase staining.

Granule quantitations made on electron micrographs of control and fMLP activated cells are shown in Table 8 and Figure 6 A. The early events of fMLP mediated activation induced a significant decrease in the average number of peroxidase negative granules in cells treated with either 1nM or 100nM fMLP ($p<0.005$ vs baseline for both concentrations of fMLP) while not significantly affecting the number of intracellular peroxidase positive granules ($p>0.25$ and $p<0.10$ for 1nM and 100nM fMLP, respectively). The average number of peroxidase negative granules/cell profile lost following activation with either 1nM or 100nM fMLP for 20 seconds was almost identical, 11.4 and 12.0, respectively (not significantly different, $p>0.25$).

fMLP Mediated Extracellular Granule Release/Intracellular Granule Loss:

To further investigate the granule secretion occurring early in activation, calculations associating extracellular granule release with intracellular peroxidase negative granule depletion were made as described in the Methods section. The results of early fMLP mediated

Table 8
ULTRASTRUCTURAL GRANULE QUANTITATION OF EARLY fMLP MEDIATED ACTIVATION

Granules/Cell Profile:

	Peroxidase Positive	Peroxidase Negative
Baseline (40)	74.2±13.7	60.2±11.1
nM fMLP		
1 (22)	72.6±14.5	48.9±9.8*
100 (25)	79.5±11.3	48.2±10.0*

Neutrophils were prewarmed for 5 minutes at 37C.
fMLP was added, incubation continued for 20 seconds.
Pooled data from 2 neutrophil donors.
Baseline: Nonactivated cells which were incubated for 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline determination

neutrophil granule release/granule loss are shown in Table 9. Correcting for spontaneous release, neutrophils activated with fMLP for 20 seconds released an average 4.84 X 10^{-6} ng of lactoferrin/granule. The results from assays of cell-free supernatants for lysozyme (Table 6) indicated that 20 seconds activation with either dose of fMLP did not cause significant lysozyme release. Expressed as corrected release/granule (Table 9), 1nM fMLP did not induce lysozyme release above baseline levels while 100nM caused the release of 0.72 X 10^{-6} units/granule. fMLP mediated extracellular collagenase release also did not differ significantly from baseline release (Table 6). Correcting for background release, activation of neutrophils with fMLP for 20 seconds caused an average release of 0.71 X 10^{-11} units of collagenase/granule. If tertiary granules are peroxidase negative, none of the granules released early in fMLP activation contained gelatinase.

These results suggested first, that the lactoferrin detected in supernatants from ruffled cells was granular in origin and second, that these granules contained lactoferrin exclusively or relatively high concentrations of lactoferrin. To investigate a correlation between the alteration in cell shape and the secretion of granules seen early in fMLP activation, quantitative morphometric determinations were made on electron micrographs of fMLP ruffled cells.

Ultrastructural Evaluation of Early fMLP Mediated Activation: Morphometric Determinations:

Neutrophils were activated as described above for ultrastructural intracellular granule quantitation. Morphometric measurements were

Table 9

fMLP MEDIATED EXTRACELLULAR GRANULE RELEASE/INTRACELLULAR PEROXIDASE NEGATIVE GRANULE LOSS

	Release/Cell	Release/Perox.Neg.Granule	Corrected Release/Granule
A. Lactoferrin :	(average ng)		
Baseline	5.79×10^{-5}		
1nM	10.95×10^{-5}	9.60×10^{-6}	4.52×10^{-6}
100nM	12.00×10^{-5}	9.99×10^{-6}	5.16×10^{-6}
B. Lysozyme :	(average Units)		
Baseline	1.24×10^{-5}		
1nM	1.24×10^{-5}	1.09×10^{-6}	0.00
100nM	2.10×10^{-5}	1.75×10^{-6}	0.72×10^{-6}
C. Collagenase :	(average Units)		
Baseline	1.22×10^{-10}		
1nM	2.26×10^{-10}	1.89×10^{-11}	0.91×10^{-11}
100nM	1.85×10^{-10}	1.54×10^{-11}	0.52×10^{-11}
D. Gelatinase :	(average Units)		
Baseline	1.92×10^{-8}		
1nM	1.80×10^{-8}	1.58×10^{-9}	-0.10×10^{-9}
100nM	1.77×10^{-8}	1.47×10^{-9}	-0.12×10^{-9}

Cells were prewarmed 5 minutes at 37C, fMLP was added, incubation continued for 20 seconds.
Average total component/neutrophil was calculated as described in Methods section.

Lactoferrin:	2.76×10^{-3}	ng
Lysozyme:	2.58×10^{-4}	Units
Collagenase:	5.13×10^{-9}	Units
Gelatinase:	3.31×10^{-7}	Units

Extracellular release/peroxidase negative granule was calculated as described in Methods section.
Baseline: Cells incubated 5 minutes at 37C.

made on electron micrographs of unactivated and fMLP activated cells as described in the Methods section. The results of these measurements are presented in Table 10 and Figure 6 B. The perimeters of neutrophils activated with either concentration of fMLP were found to have increased significantly when compared to control cells ($p<0.005$). Neutrophils activated with 1nM fMLP for 20 seconds exhibited a mean increase in perimeter of 25.5%, cells exposed to 100nM fMLP for 20 seconds increased an average of 17%. These increased perimeter measurements are probably reflective of the ruffling occurring early in fMLP initiated cell activation. Additionally, compared to baseline determinations, neutrophils activated for 20 seconds with 1nM fMLP demonstrated an average increase in exposed surface membrane of 17% ($p<0.005$). While activation of cells with 100nM fMLP for 20 seconds had no influence on the quantitation of exposed plasma membrane ($p>0.25$), it did produce a significant increase in calculated cross-sectional area. Area determinations made on profiles of cells activated with 100nM fMLP for 20 seconds averaged 14.6% greater than baseline ($p<0.005$). In contrast, the average area determination of cell profiles from 1nM fMLP treated cells was not significantly different than the baseline value ($p<0.025$).

Within 20 seconds following exposure to 1nM or 100nM fMLP, neutrophils were found to have changed shape, released significant quantities of lactoferrin extracellularly and lost a significant number of intracellular peroxidase negative granules. Additionally, in response to the lower dose of fMLP, cells were found to have significantly more surface membrane, while cells exposed to 100nM fMLP

Table 10

ULTRASTRUCTURAL ANALYSIS OF EARLY fMLP MEDIATED ACTIVATION: MORPHOMETRIC QUANTITATIONS

	Perimeter	Area	S/V	Calculated Membrane(um^2)
Baseline (71)	62.36±13.65	68.70±13.48	1.39±0.32	552.06±128.01
nM fMLP				
1 (52)	78.24±18.23*	74.59±15.36	1.63±0.49*	648.23±194.77*
100 (42)	72.83±18.89*	78.75±18.25*	1.43±0.41	569.99±164.67

Neutrophils were prewarmed for 5 minutes at 37C.
fMLP was added, incubation continued for 20 seconds at 37C.
Pooled data from 5 neutrophil donors.
Baseline: Cells incubated 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline

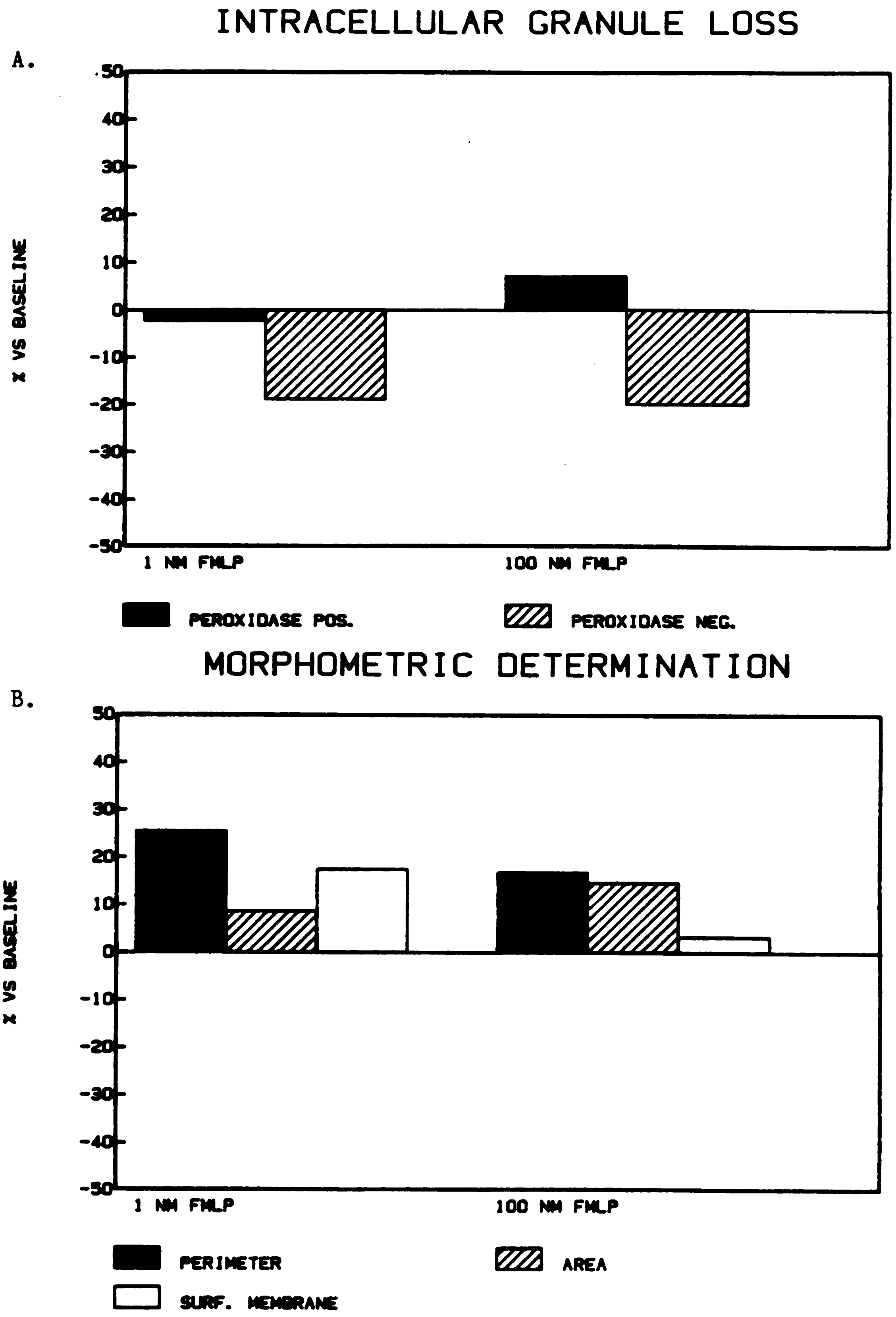


Figure 6
ULTRASTRUCTURAL EVALUATION OF EARLY fMLP MEDIATED ACTIVATION
Data from Tables 8 and 10 expressed as % deviation from baseline

displayed significantly larger cross-sectional areas.

As previously mentioned, activation of neutrophils with fMLP causes a rapid rise in intracellular free Ca++ (Korchak et.al., 1984; von Tscharner et.al., 1986) which is thought to act as a secondary messenger in cell activation (Arnaout et.al., 1984; Gallin et.al., 1978; Hoffstein and Weissmann 1978; Todd et.al., 1984). Neutrophils were exposed to the calcium ionophore A23187 to determine whether the morphological and functional alterations detected early in fMLP mediated activation could be duplicated by increasing intracellular Ca++ in the absence of receptor ligation. A23187 treated cells were found to exhibit responses similar to, but not identical with, those detected early in fMLP activation.

A23187 Mediated Shape Change:

Visible shape change responses of neutrophils exposed to A23187 were made with light microscopy. As seen in Table 11 and Figure 7, approximately 90% of the neutrophils became ruffled within 1 minute after exposure to 100nM or 250nM A23187 and maintained this configuration for up to 10 minutes. Ionophore treated cells did not progress to bipolar forms.

A23187 Mediated Extracellular Granule Release:

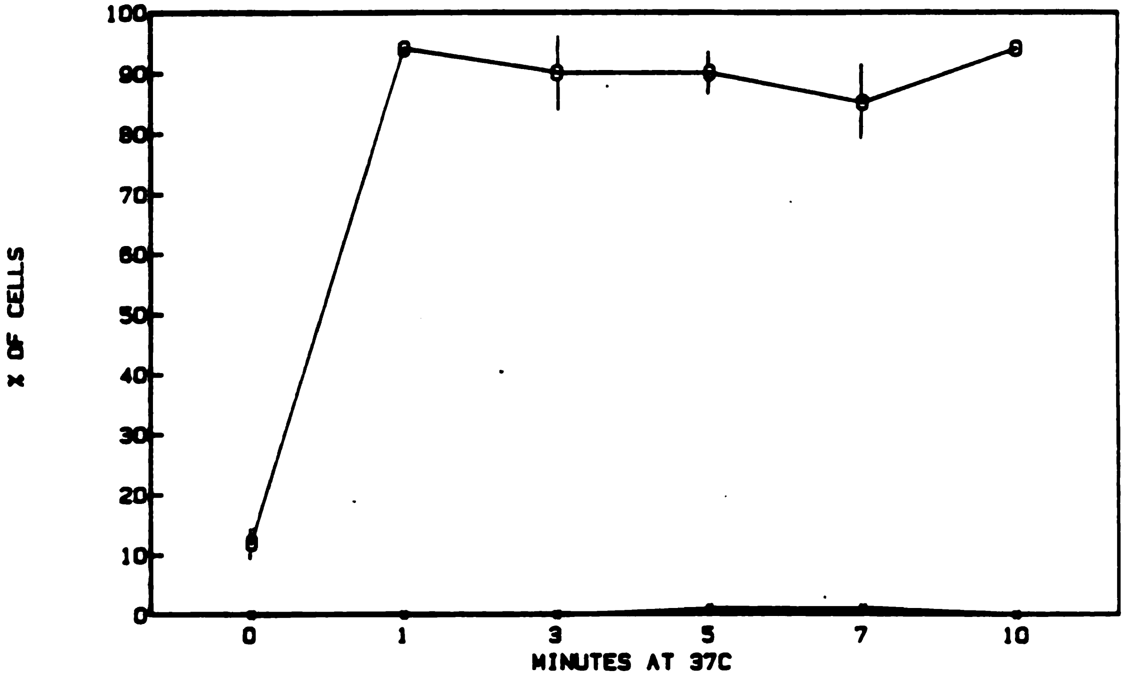
To determine whether extracellular granule release accompanied ionophore mediated neutrophil ruffling, kinetic granule release experiments were performed. As seen in Table 12 and Figure 8, significant amounts of extracellular lactoferrin and lysozyme and gelatinase were detected following 5 and 10 minutes exposure of neutrophils to either 100nM or 250nM A23187. Release appeared to plateau after 5 minutes. Collagenase was also released from cells

Table 11
A23187 MEDIATED SHAPE CHANGE

Minutes Exposure	% Ruffled forms	% Bipolar forms	n
Baseline	12±5	0±1	20
A23187: 100nM			
0.5 min.	15±5	0	3
1.0	94±4	0	3
3.0	90±10	0	3
5.0	90±7	1±1	3
7.0	85±12	1±1	3
10.0	94±3	0	3
A23187: 250nM			
0.5 min.	15±1	0	3
1.0	91±7	0	3
3.0	85±10	1±0	3
5.0	93±4	0	3
7.0	81±4	1±1	3
10.0	92	1	2

Neutrophils were prewarmed to 37C then exposed to A23187 as indicated
Baseline: Neutrophils were not exposed to ionophore but were incubated 5 minutes at 37C and fixed.
Results are expressed as mean±standard deviation

100 NM A23187



250 NM A23187

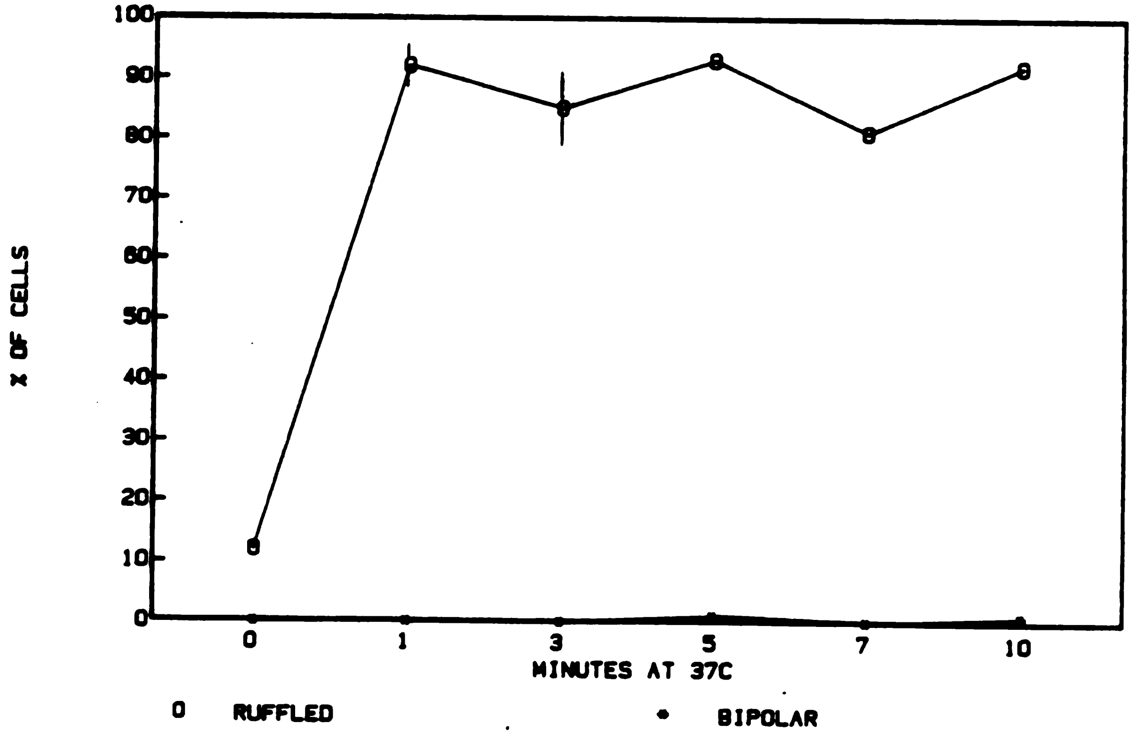


Figure 7
A23187 MEDIATED SHAPE CHANGE
Data from Table 11

Table 12
A23187 MEDIATED EXTRACELLULAR GRANULE RELEASE

Release Conditions:	Granule Component:				
	Lactoferrin	Lysozyme	Collagenase	Gelatinase	B-glucuronidase
Baseline	2.10±0.64(13)	4.81±1.31(6)	2.38±1.91(2)	5.81±3.75(8)	0 (2)
A23187					
100nM:0.5 min.	8.00±2.83(2)	9.17±0.04(2)	N.D.	8.21±0.44(2)	N.D.
1.0	10.75±1.06(2)	8.10±3.54(4)	N.D.	12.80±4.35(2)	N.D.
3.0	16.56±3.98(2)	9.73±3.04(3)	N.D.	22.15±6.15(2)	N.D.
5.0	18.55±4.96(9)*	13.18±3.09(6)*	6.61±2.12(2)	24.91±7.91(9)*	1.66±1.81(3)
7.0	23.06±5.39(2)	14.78±1.44(2)	N.D.	23.49±7.30(2)	N.D.
10.0	22.72±6.43(5)*	13.04±1.40(4)*	N.D.	24.22±6.26(6)*	0 (1)
250nM:0.5 min.	6.01±0.22(2)	7.83±2.50(2)	N.D.	5.65±1.74(2)	N.D.
1.0	8.79±1.99(2)	7.75±2.54(4)	N.D.	14.95±0.22(2)	N.D.
3.0	14.85±3.60(2)	10.06±2.46(4)	N.D.	22.75±2.75(2)	N.D.
5.0	21.99±5.96(9)*	13.54±2.36(7)*	7.17±0.75(2)	35.15±11.59(8)*	2.95±2.42(3)
7.0	20.19±2.81(2)	12.03 (1)	N.D.	23.89±9.41(2)	N.D.
10.0	21.83±5.95(7)*	18.86±4.65(4)*	N.D.	33.23±8.63(9)*	1.78±0.42(3)

Neutrophils prewarmed 5 minutes at 37C, A23187 added, incubations continued as indicated.
Release is expressed as percent of total (See Table 4).
Baseline: Cells incubated 5 minutes at 37C.
Mean±standard deviation
N.D.:Not Determined
(Number of experiments)
* $p<0.01$ vs baseline release

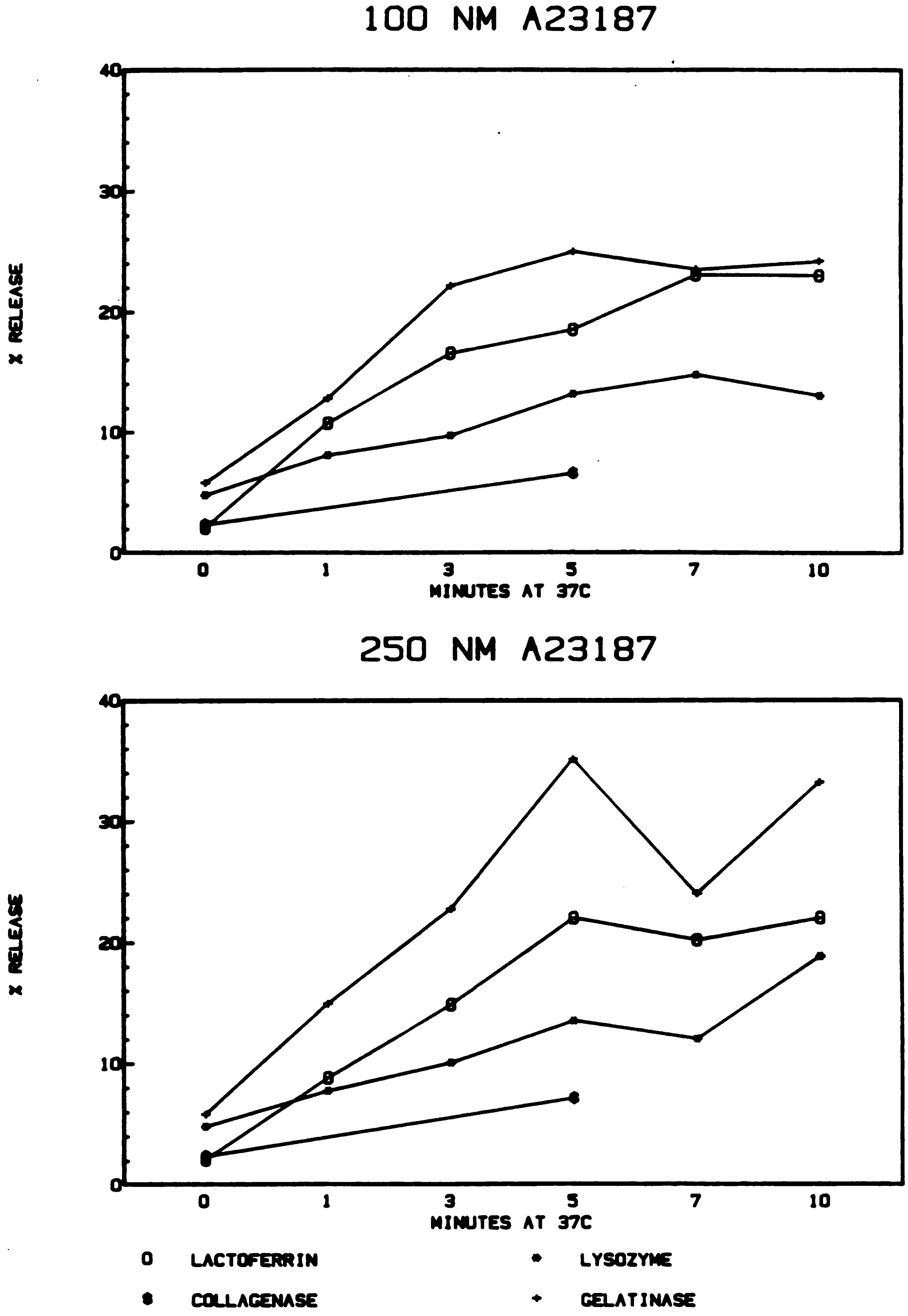


Figure 8
A23187 MEDIATED EXTRACELLULAR GRANULE RELEASE: KINETIC RESPONSE
Data from Table 12

after 5 minutes treatment with ionophore. Primary granule beta-glucuronidase release was not detected.

Effect of Cytochalasin B on A23187 Mediated Extracellular Granule Release:

Granule release experiments were then performed to determine whether CB modulated the extracellular granule secretion mediated by A23187. Neutrophils were preincubated with 5ug/ml of CB for 5 minutes at 37C and then exposed to 100nM or 250nM of the ionophore for various time periods. The results of these experiments, shown on Table 13, indicate that compared to release without pretreatment, CB had no influence on the quantity of lactoferrin released following exposure to either dose of A23187 for either 5 or 10 minutes. In contrast, cells pretreated with CB appeared to release more lysozyme, gelatinase and beta-glucuronidase than neutrophils not treated with CB. Extracellular release plateaued after 5 minutes exposure of pretreated cells to the ionophore. Figure 9, A and B, shows the effect of CB on extracellular granule release occurring following 5 minutes exposure of cells to either 100nM or 250nM A23187, respectively.

Ultrastructural Evaluation of A23187 Mediated Granule Release: Intracellular Granule Quantitation:

Next, intracellular granule quantitations were made to further investigate A23187 mediated granule release from ruffled cells. The results of peroxidase positive and negative granule counts made on cell profiles of ionophore treated cells are shown in Table 14 and Figure 10 A. Five minutes treatment of neutrophils with either concentration of A23187 induced a significant decrease in the mean number of intracellular peroxidase negative granules ($p<0.005$ for

Table 13
THE EFFECT OF CYTOCHALASIN B ON A23187 MEDIATED EXTRACELLULAR GRANULE RELEASE

Release Conditions:	Granule Component:			
	Lactoferrin	Lysozyme	Gelatinase	B-Glucuronidase
Baseline	1.78±0.28(4)	5.52±1.85(2)	10.39±6.56(2)	0 (2)
A23187				
100nM:0.5 min.	N.D.	32.01±0.52(2)	N.D.	N.D.
1.0	N.D.	44.24±3.14(2)	N.D.	N.D.
3.0	N.D.	48.69±3.15(2)	N.D.	N.D.
5.0	19.05±1.47(2)	52.64±7.35(2)	65.23±51.22(2)	30.40(1)
7.0	N.D.	54.38±14.0(2)	N.D.	N.D.
10.0	25.17±7.14(2)	66.73±3.49(2)	91.05±18.31(2)	N.D.
250nM:0.5 min.	N.D.	49.94±2.10(2)	N.D.	N.D.
1.0	N.D.	44.36±4.37(2)	N.D.	N.D.
3.0	N.D.	50.17±1.05(2)	N.D.	N.D.
5.0	24.06±5.24(2)	62.28±6.29(2)	78.71±29.95(2)	30.40(1)
7.0	N.D.	60.31±0.00(2)	N.D.	N.D.
10.0	27.26±10.14(2)	57.34±6.30(2)	89.50±4.41(2)	N.D.

Neutrophils were preincubated with 5ug/ml of CB for 5 minutes at 37C.
A23187 was added, incubations were continued as indicated in the table.
Release is expressed as percent of total (See Table 5).
Baseline: Cells incubated with 5ug/ml of CB 5 minutes at 37C.
Mean±standard deviation
N.D.: Not Determined
(Number of experiments)

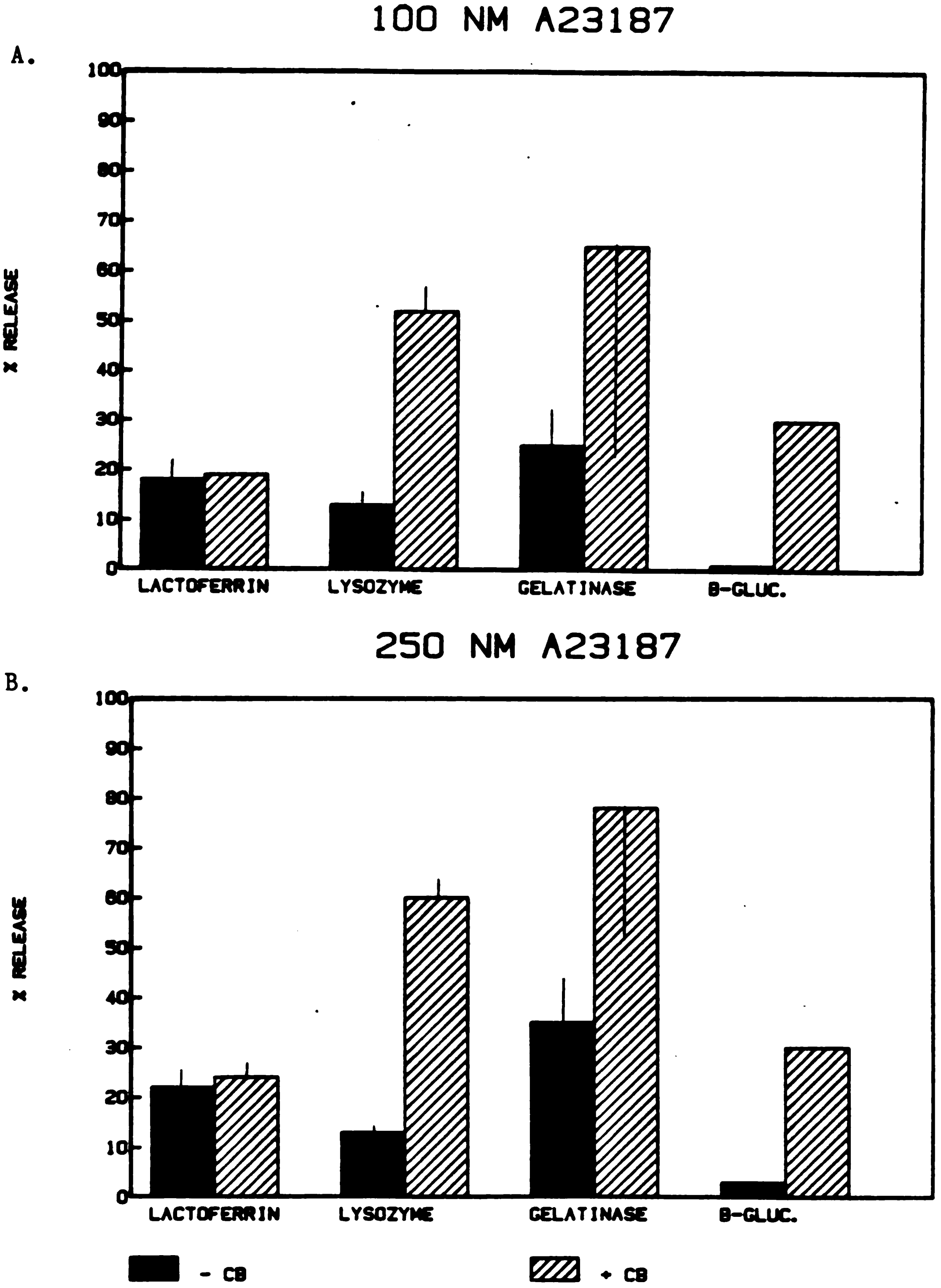


Figure 9
EFFECT OF CB ON A23187 MEDIATED EXTRACELLULAR GRANULE RELEASE
Data from Tables 12 and 13, 5 minutes exposure to A23187

TABLE 14
ULTRASTRUCTURAL GRANULE QUANTITATION: A23187 TREATED NEUTROPHILS

Granules/Cell Profile:

	Peroxidase Positive	Peroxidase Negative
Baseline (40)	74.2±13.7	60.2±11.1
nM A23187		
100 (37)	75.6±13.4	54.1±8.3*
250 (17)	73.1±11.3	46.5±7.4*

Cells were prewarmed for 5 minutes at 37C, A23187 was added and incubation continued for 5 minutes.
Pooled data from 2 neutrophil donors.
Baseline: Neutrophils not treated with A23187 but incubated 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline determination

100nM and 250nM A23187) while not affecting the number of peroxidase positive granules ($p>0.25$). Exposure of neutrophils to 100nM or 250nM ionophore caused an average loss of 6 or 14 peroxidase negative granules/cell profile, respectively. The granule depletion mediated by 250nM A23187 was significantly greater than that induced by 100nM A23187 ($p<0.005$).

A23187 Mediated Extracellular Granule Release/Intracellular Granule Loss:

To associate the results from ionophore mediated extracellular granule secretion and intracellular granule depletion, ratios of release/granule loss were calculated as described in Methods. Table 15 indicates that 100nM A23187 induced the secretion of approximately two fold more of each secondary granule component/peroxidase negative granule then cells treated with 250nM A23187. Neutrophils exposed to 100nM A23187 for 5 minutes released an average of 1.8 times more lactoferrin, 2.1 times more lysozyme and 2.0 times more collagenase than cells treated with 250nM A23187 for 5 minutes. Making the assumption that tertiary granules may be peroxidase negative, 100nM treated cells lost 1.5 times more gelatinase/granule that 250nM A23187 treated cells.

These results appear to suggest that the granules secreted from A23187 ruffled neutrophils contain lactoferrin, lysozyme, collagenase and gelatinase. Further, the granules secreted from cells ruffled with the lower dose of A23187 appear to contain quantitatively more of each component.

Ultrastructural Evaluation of A23187 Mediated Shape Change:

Morphometric Determinations:

Table 15

A23187 MEDIATED EXTRACELLULAR GRANULE RELEASE/INTRACELLULAR PEROXIDASE NEGATIVE GRANULE LOSS

	Release/Cell	Release/Perox.Neg.Granule	Corrected Release/Granule
A. Lactoferrin : (average ng)			
Baseline	5.79 X 10^{-5}		
100nM	51.15 X 10^{-5}	83.71 X 10^{-6}	74.23 X 10^{-6}
250nM	60.73 X 10^{-5}	44.13 X 10^{-6}	39.92 X 10^{-6}
B. Lysozyme : (average Units)			
Baseline	1.24 X 10^{-5}		
100nM	3.40 X 10^{-5}	5.57 X 10^{-6}	3.54 X 10^{-6}
250nM	3.49 X 10^{-5}	2.54 X 10^{-6}	1.64 X 10^{-6}
C. Collagenase : (average Units)			
Baseline	1.22 X 10^{-10}		
100nM	3.39 X 10^{-10}	5.55 X 10^{-11}	3.55 X 10^{-11}
250nM	3.67 X 10^{-10}	2.67 X 10^{-11}	1.78 X 10^{-11}
D. Gelatinase : (average Units)			
Baseline	1.92 X 10^{-8}		
100nM	8.24 X 10^{-8}	13.49 X 10^{-9}	10.34 X 10^{-9}
250nM	11.62 X 10^{-8}	8.45 X 10^{-9}	7.05 X 10^{-9}

Cells were prewarmed 5 minutes at 37C, A23178 was added, incubations continued 5 minutes at 37C.
Average total component/neutrophil was calculated as described in Methods (See Table 9).
Release/granule loss were calculated as described in Methods section.
Baseline: Cells were incubated for 5 minutes at 37C.

To correlate the morphological and functional events of A23187 mediated activation described above, morphometric measurements were made on electron micrographs of ionophore treated cells. The data presented in Table 16 and Figure 10 B show that, compared to baseline, neutrophils treated with either 100nM or 250nM A23187 for 5 minutes exhibited significantly increased average perimeters ($p<0.005$). Exposure of cells to 100nM or 250nM ionophore induced mean perimeter increases of approximately 10% and 20%, respectively. Increased perimeters are probably reflective of A23187 mediated cell ruffling. Neutrophils treated with the lower dose of A23187 also exhibited a significant increase in mean cross-sectional area (18.4% greater than baseline determination, $p<0.005$). In contrast, the average area determination from profiles of 250nM A23187 treated cells was not significantly different from baseline ($p<0.025$). Neither dose of the ionophore demonstrated a significant influence on the amount of exposed plasma membrane. Cells treated with 100nM A23187 for 5 minutes were found to expose an average 8.0% less surface membrane than nonactivated baseline cells, while the surface of neutrophils treated with 250nM A23187 increased an average 9.25% ($p<0.025$ vs baseline for both).

Compared to adult cells, neutrophils isolated from umbilical cord blood have been reported to contain approximately 50% of the normal adult quantities of secondary granule lactoferrin while secondary granule lysozyme and the primary granule enzymes myeloperoxidase and beta-glucuronidase are comparable to adult levels (Becker-Freeman, et.al. 1984; Ambruso, et.al. 1984).

Since both fMLP and A23187 treated adult neutrophils exhibited

Table 16
ULTRASTRUCTURAL ANALYSIS OF A23187 TREATED NEUTROPHILS: MORPHOMETRIC QUANTITATIONS

	Perimeter	Area	S/V	Calculated Membrane(um^2)
Baseline (71)	62.36+13.65	68.70±13.48	1.39±0.32	552.06±128.01
nM A23187				
100 (51)	68.74± 9.28*	81.34±9.90*	1.28±0.18	507.58±72.04
250 (26)	75.61±15.11*	75.11±10.49	1.52±0.28	603.11±113.76

Neutrophils were prewarmed for 5 minutes at 37C, A23187 was added, incubation continued for 5 minutes.
Pooled data from 5 neutrophil donors.
Baseline: Cells incubated 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline

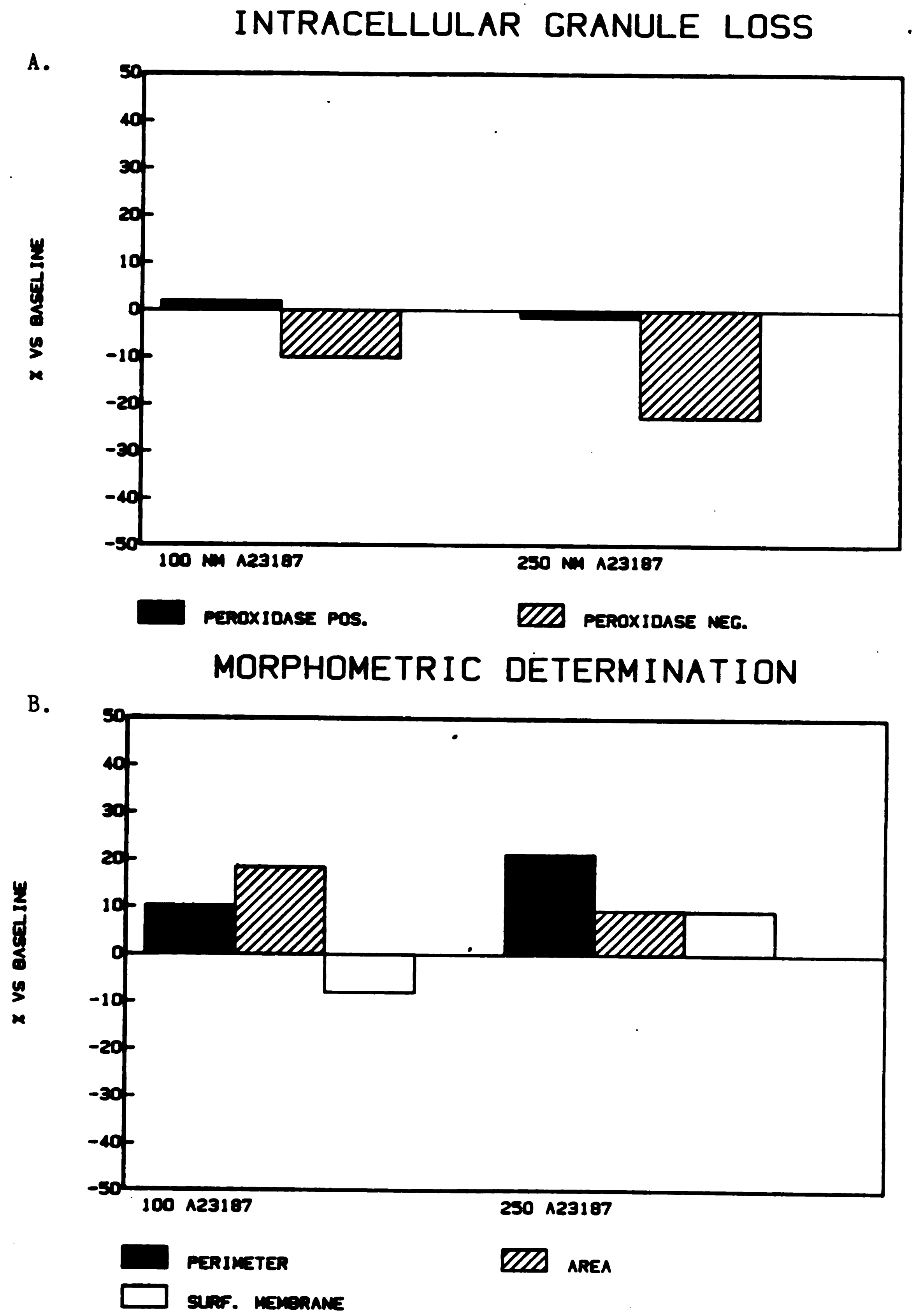


Figure 10
ULTRASTRUCTURAL EVALUATION OF A23187 TREATED NEUTROPHILS
Data from Tables 14 and 16 expressed as % deviation from baseline

increased levels of extracellular lactoferrin which appeared accompany cellular ruffing, intracellular peroxidase negative granule depletion and, in one instance, increased exposed surface membrane, neonatal neutrophils seemed to provide a unique approach to investigate whether lactoferrin-containing granules may be intimately involved in these phenomena.

Neonatal Neutrophils: fMLP Mediated Shape Change:

Results of visible shape change responses of neonatal neutrophils initiated by fMLP are shown in Table 17 and Figure 11. Neutrophils isolated from umbilical cord blood samples were found to respond very quickly to activation with fMLP. Approximately 90% of the cells were ruffled within 20 to 30 seconds after exposure to 1nM fMLP and 60% formed bipolar configurations by 40 seconds.

Neonatal Neutrophils: Ultrastructural Evaluation of Early fMLP Mediated Activation: Intracellular Granule Quantitation:

Intracellular granule characterizations and quantitations were then made on electron micrographs of newborn neutrophils to determine whether a loss of intracellular granules accompanied fMLP mediated ruffling. The conditions used to treat neonatal cells were identical to those used for the ultrastructural determinations made on adult cells. The cells were fixed, processed for electron microscopy and photographed based on the same criteria utilized for adult cells. The results of intracellular granule counts are shown in Table 18 and Figure 12 A. Twenty seconds exposure of newborn cells to fMLP had no affect on intracellular peroxidase positive granule quantitation ($p<0.25$ and $p>0.25$ for 1nM and 100nM fMLP, respectively). Neonatal cells treated with 1nM fMLP did not exhibit a highly significant

Table 17
NEONATAL NEUTROPHILS: KINETIC SHAPE CHANGE RESPONSE TO 1nM fMLP

Seconds Exposure	% Ruffled forms	% Bipolar forms	n
20	84±7	1±1	5
30	85±3	6±4	5
40	65±7	25±10	5
Baseline : Cells warmed to 37C	13±10	1±1	5

Neutrophils were prewarmed 5 minutes at 37C prior to exposure to fMLP
Results are expressed as mean±standard deviation

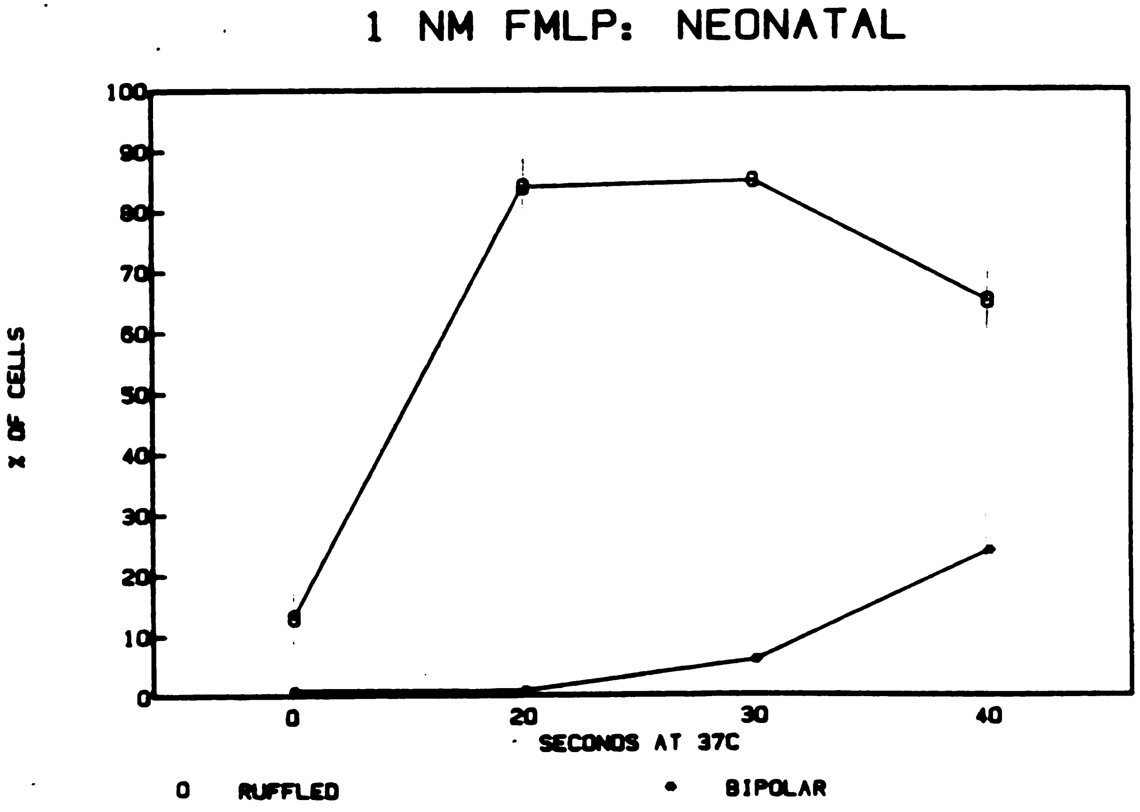


Figure 11
NEONATAL NEUTROPHILS: KINETIC SHAPE CHANGE RESPONSE TO 1nM fMLP
Data from Table 17

Table 18
NEONATAL NEUTROPHILS: ULTRASTRUCTURAL GRANULE QUANTITATION OF EARLY fMLP MEDIATED ACTIVATION

Granules/Cell Profile:

	Peroxidase Positive	Peroxidase Negative
Baseline (38)	84.5±15.4	59.0±10.1
nM fMLP		
1 (40)	80.6±16.7	53.2±13.2
100 (32)	84.2±12.2	50.2±8.4*

Neutrophils were warmed for 5 minutes at 37C, fMLP was added, incubation continued for 20 seconds.
Pooled data from 5 neonatal neutrophil donors.
Baseline: Cells incubated 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline determination.

decrease in peroxidase negative granules ($p<0.025$). However, cells exposed to 100nM fMLP for 20 seconds did exhibit a significant reduction in peroxidase negative granules/cell profile ($p<0.005$).

Neonatal Neutrophils: Ultrastructural Evaluation of Early fMLP Mediated Activation: Morphometric Determinations:

Next, to determine whether the intracellular granule loss detected following 20 seconds activation of newborn cells with 100nM fMLP correlated with morphometric alterations, electron micrographs of neonatal neutrophils were measured. As shown in Table 19 and Figure 12 B, none of the morphometric quantitations made on cell profiles of fMLP ruffled newborn cells differed from the baseline determinations.

Comparisons of the results from the ultrastructural morphometric measurements of neonatal and adult cells exposed to fMLP are shown in Table 20. Nonactivated neonatal neutrophils were found to be comparable in perimeter, significantly larger in area and to expose significantly less plasma membrane than nonactivated adult cells. Following activation with fMLP, the mean area determinations of neonatal and adult cells were comparable, but the exposed plasma membrane of newborn cells was still significantly less. The average perimeter measurements of 1nM fMLP activated neonatal neutrophils were significantly less than those of identically treated adult cells ($p<0.005$), while the mean perimeter determination of newborn neutrophils activated with 100nM fMLP for 20 seconds were comparable to those of adult cells.

As described above, adult cells treated with the calcium ionophore exhibited responses similar those seen early in fMLP activation. Morphological studies were made of neonatal cells treated

Table 19
NEONATAL NEUTROPHILS: ANALYSIS OF EARLY fMLP MEDIATED ACTIVATION: MORPHOMETRIC QUANTITATIONS

	Perimeter	Area	S/V	Calculated Membrane (um2)
Baseline (38)	61.12±11.62	78.28±11.78	1.19±0.27	474.78±108.88
nM fMLP				
1 (39)	55.99±8.43	74.34±9.40	1.13±0.14	450.68±57.55
100 (28)	67.08±11.98	83.07±12.70	1.22±0.17	484.11±68.77

Neutrophils were prewarmed 5 minutes at 37C, fMLP was added, incubation continued for 20 seconds.
Pooled data from 5 neonatal neutrophil donors.
Baseline: Cells incubated 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline determination

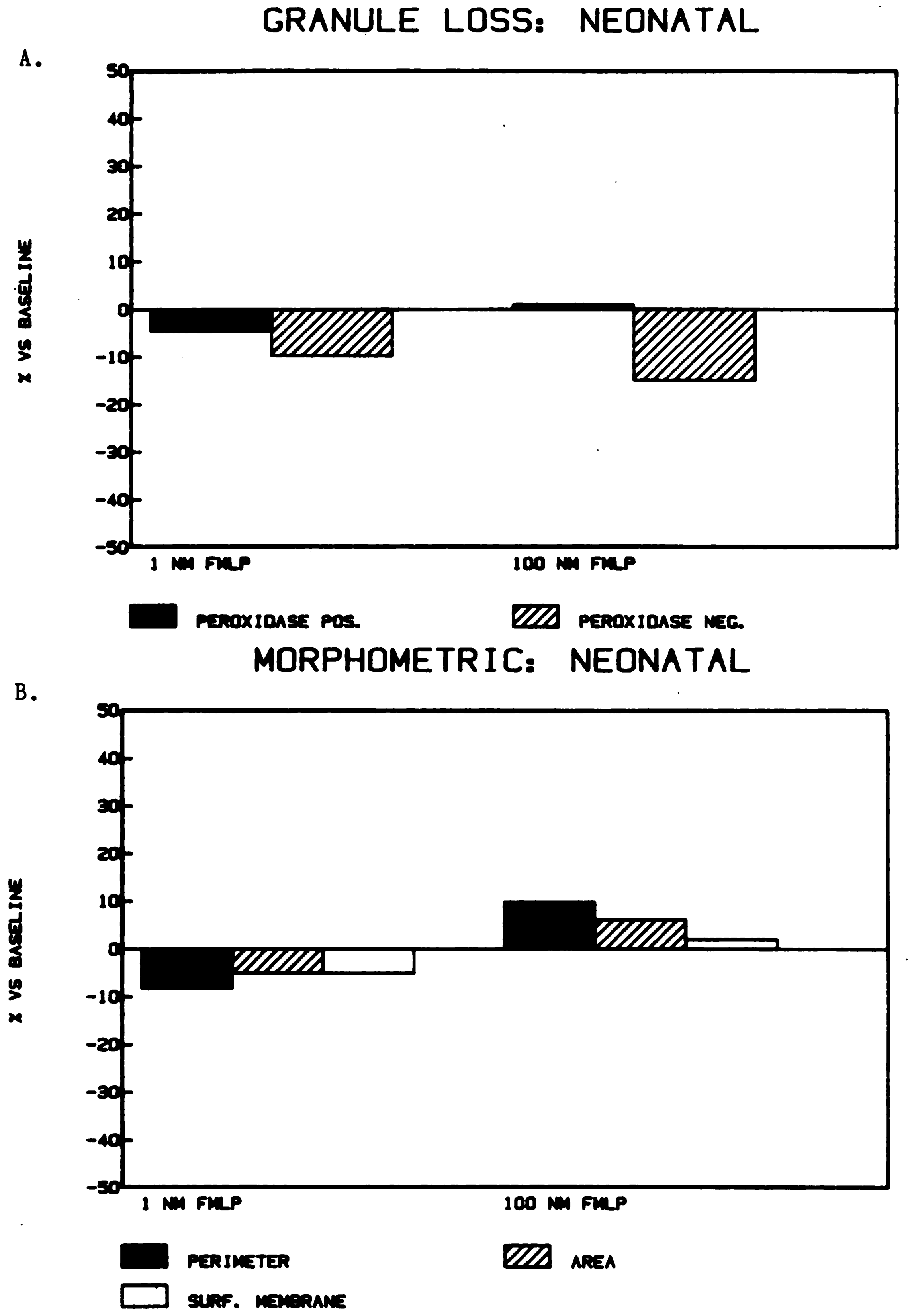


Figure 12
ULTRASTRUCTURAL EVALUATION OF EARLY fMLP MEDIATED ACTIVATION
Data from Tables 18 and 19 expressed as % deviation from baseline

Table 20
NEONATAL vs ADULT NEUTROPHILS: EARLY fMLP MEDIATED ACTIVATION: MORPHOMETRIC DETERMINATIONS

	Perimeter	Area	S/V	Calculated surface membrane
Baseline	=	↑	↓	↓
nM fMLP				
1	↓	=	↓	↓
100	=	=	↓	↓

Results are expressed as significant differences between mean determinations made from neonatal and adult cell profiles ($p<0.01$).
=: No difference
↑: Significant increase, neonatal vs adult
↓: Significant decrease, neonatal vs adult
Baseline: Cells incubated for 5 minutes at 37C and fixed.

with A23187 to determine if these cells also responded to the ionophore in a way that mimicked the early events of fMLP cell activation.

Neonatal Neutrophils: A23187 Mediated Shape Change:

Visually, neonatal neutrophils exposed to 100nM or 250nM of the calcium ionophore for 5 minutes at 37C were found to be ruffled with less than 5% of the cells exhibiting spherical or bipolar forms (data not shown).

Neonatal Neutrophils: Ultrastructural Evaluation of Treatment with A23187: Intracellular Granule Quantitation:

To determine whether changes in intracellular granule content accompanied A23187 mediated ruffling, granule characterizations and quantitations were made. Prewarmed neonatal neutrophils were treated with 100nM or 250nM A23187 for 5 minutes at 37C. The cells were fixed, processed for electron microscopy and photographed as described above. The results of peroxidase positive and negative granule quantitation are shown in Table 21 and Figure 13 A. Both concentrations of ionophore induced a significant depletion in intracellular peroxidase negative granules ($p<0.01$ and $p<0.005$ for 100nM and 250nM A23187, respectively) while not influencing peroxidase positive granule quantitation ($p>0.25$ and $p<0.25$ for 100nM and 250nM A23187, respectively). Cells treated with 100nM A23187 lost an average of 6 peroxidase negative granules/cell profile, while cells treated with 250nM ionophore lost, on average, 9 granules/profile.

Neonatal Neutrophils: Ultrastructural Evaluation of Treatment with A23187: Morphometric Determinations:

Morphometric measurements were then made on electron micrographs

Table 21
NEONATAL NEUTROPHILS: ULTRASTRUCTURAL GRANULE QUANTITATION OF A23187 TREATED CELLS

Granules/Cell Profile

	Peroxidase Positive	Peroxidase Negative
Baseline (38)	84.5±15.4	59.0±10.1
nM A23187		
100 (32)	82.8±17.0	52.7±9.7*
250 (40)	80.2±15.0	50.1±9.3*

Neutrophils were warmed for 5 minutes at 37C, A23187 was added, incubation continued for 5 minutes.
Pooled data from 5 neonatal neutrophil donors.
Baseline: Cell incubated for 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
*** p<0.01 vs baseline**

of ionophore treated newborn neutrophils to investigate a correlation between A23187 mediated visible ruffling, intracellular granule depletion and morphometric alterations. The results of these measurements are shown in Table 22 and Figure 13 B. Compared to baseline measurements, 5 minutes exposure to 100nM or 250nM A23187 caused significant increases in cell perimeter ($p<0.01$ and $p<0.005$, respectively) and exposed surface membrane ($p<0.005$ for both 100nM and 250nM A23187).

Comparisons of the results from morphometric determinations made on A23187 treated adult and neonatal cell profiles are shown in Table 23. As indicated above, nonactivated neonatal cells were found to be significantly larger in area and expose significantly less surface membrane than adult neutrophils. Compared to ionophore treated adult cells, identically treated neonatal neutrophils were comparable in perimeter, calculated area and exposed plasma membrane determinations.

As indicated previously, temperature has been reported to cause functional responses in neutrophils (Berger et.al., 1984; Charo et.al.,1985; Corcino et.al., 1970; Dewald et.al., 1984; Fearon and Collins, 1983; Goldstein et.al., 1974; Howard and Oresajo, 1985; Springer et.al., 1986; Wright and Gallin, 1979). In this investigation, neutrophils were exposed to a temperature transition to determine whether temperature alone could induce functional and morphological responses similar to those seen early in fMLP mediated activation and following exposure of cells to A23187.

Temperature Transition Mediated Shape Change:

The effect of a temperature transition on neutrophil shape was investigated with light microscopy. Neutrophils were isolated in the

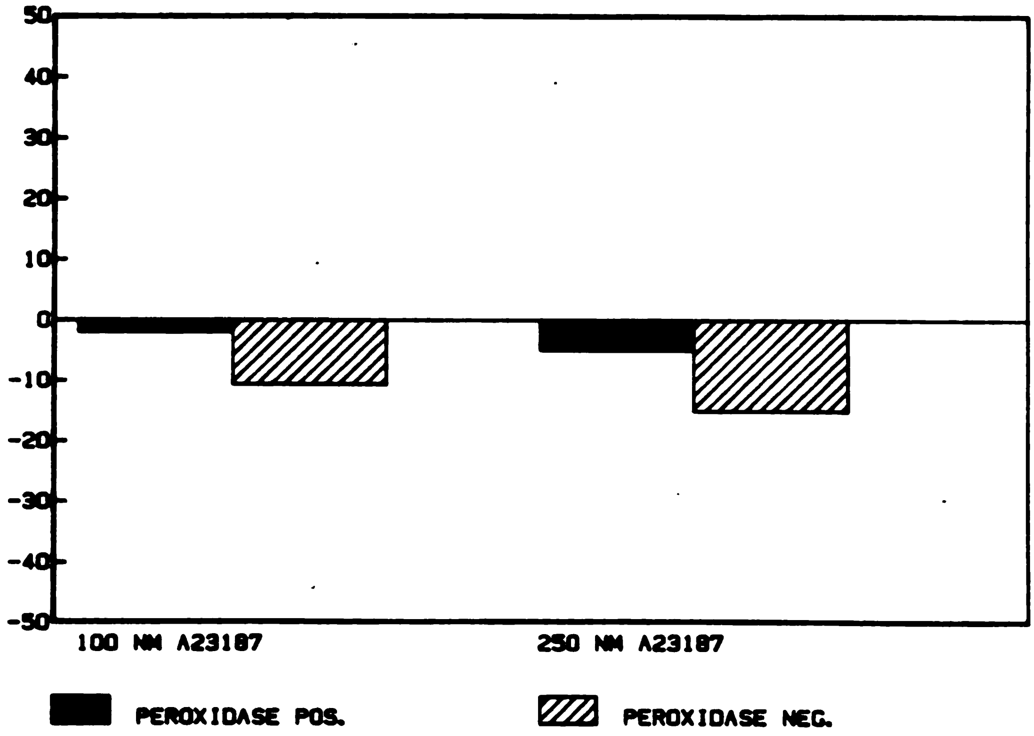
Table 22
NEONATAL NEUTROPHILS: ULTRASTRUCTURAL MORPHOMETRIC QUANTITATIONS OF A23187 TREATED CELLS

	Perimeter	Area	S/V	Calculated Membrane (um^2)
Baseline (38)	61.12±11.62	78.28±11.78	1.19±0.27	474.78±108.88
nM A23187				
100 (31)	67.61±10.16*	75.50±11.63	1.36±0.23	541.71±92.53*
250 (39)	72.56±12.90*	77.44±11.87	1.43±0.30	567.32±118.45*

Neutrophils were prewarmed 5 minutes at 37C, A23187 was added, incubation continued for 5 minutes.
Pooled data from 5 neutrophil donors.
Baseline: Cells incubated 5 minutes at 37C before fixing.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline determination

GRANULE LOSS: NEONATAL

A.



MORPHOMETRIC: NEONATAL

B.

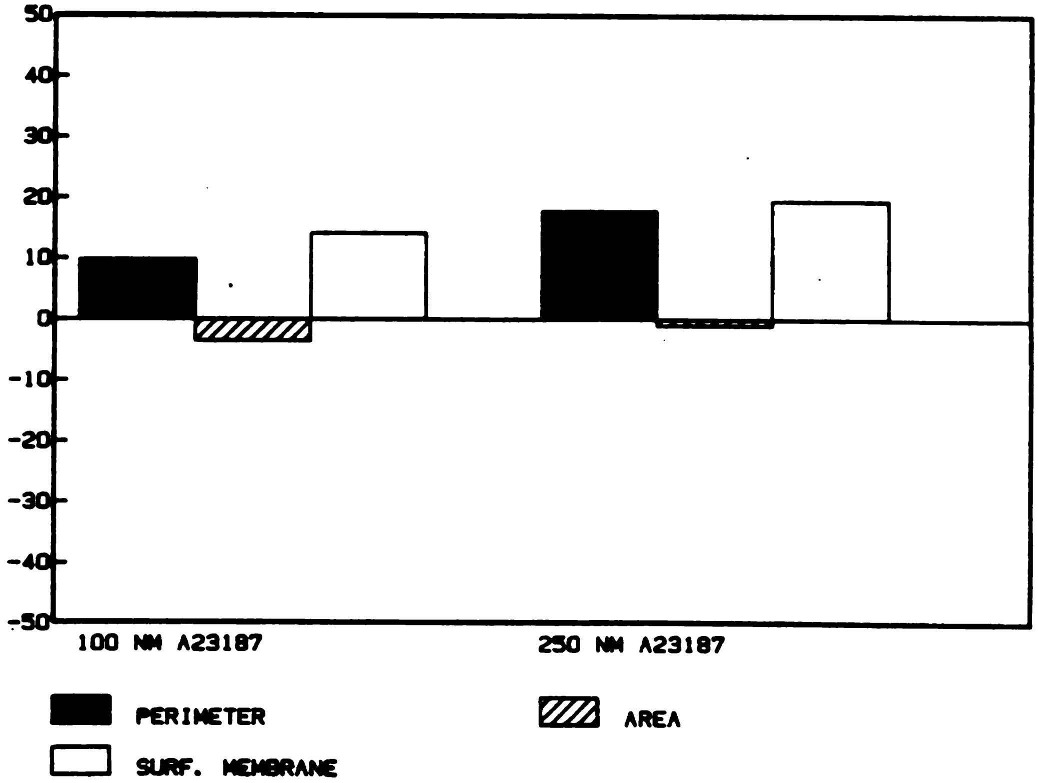


Figure 13
ULTRASTRUCTURAL EVALUATION OF A23187 TREATED NEUTROPHILS
Data from Tables 21 and 22 expressed as % deviation from baseline

Table 23
NEONATAL vs ADULT NEUTROPHILS: EXPOSURE TO A23187: MORPHOMETRIC DETERMINATIONS

	Perimeter	Area	S/V	Calculated surface membrane
Baseline	=	↑	↓	↓
nM A23187				
100	=	=	=	=
250	=	=	=	=

Results are expressed as significant differences between mean determinations made from neonatal and adult cell profiles ($p<0.01$)
=: No difference
↑: Significant increase, neonatal vs adult
↓: Significant decrease, neonatal vs adult
Baseline: Cells were incubated for 5 minutes at 37C and fixed

cold and maintained at approximately 4C until use. A sample of the cell suspension was fixed on ice to serve as the baseline for shape change determinations. Additional samples were transferred to a 37C water bath and fixed at time points ranging from 1 to 60 minutes. Although the cells fixed on ice clearly were not ruffled, approximately 80% of them were found to exhibit membrane extensions. In contrast to ruffles these extensions were short and blunt rather than the thin, spiked, sheet-like membrane protrusions typical of ruffles (Figure 14). Table 24 and Figure 15 show the results of shape change determinations made on cells incubated at 37C. The membrane extensions detected on cold cells disappeared after the cells were warmed for approximately three minutes at 37C (Figure 14). Warmed cells took on a typical spherical appearance, and remained spherical for up to 60 minutes.

The temperatures established during the transition were determined to approximate the temperature at which the membrane extension were resolved. Tubes containing neutrophil suspensions were transferred from ice to a 37C water bath. At various time points the temperature was recorded. As seen in Table 25, a dramatic increase was seen in the first 30 seconds with the maximum temperature being reached within about five minutes. At 3 minutes, the incubation time at which 85% of the cells exhibited spherical shapes (Table 24 and Figure 15), the temperature of the cell suspension was approximately 34C.

Temperature Transition Mediated Extracellular Granule Release:

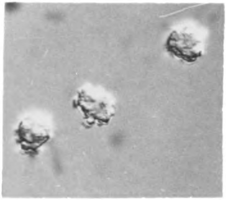
In contrast to fMLP mediated activation, warming neutrophils isolated in the cold induced the resolution, rather than the

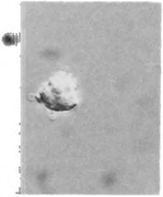
Figure 14
NEUTROPHILS FIXED IN THE COLD: LIGHT MICROSCOPY

Upper panels: Adult neutrophils isolated and fixed in the cold: Note short, blunt membrane extensions occasionally appearing as blebs.

Lower panel: Cells isolated in the cold were warmed for 5 minutes at 37C prior to fixation. Note the loss or resolution of the membrane extensions seen on cells fixed in the cold.

Bar = 10um





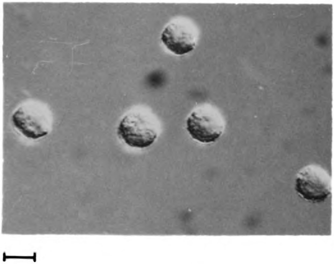


Table 24
TEMPERATURE TRANSITION MEDIATED SHAPE CHANGE

Minutes at 37C	% with membrane extensions	% Spherical	% Ruffled	% Bipolar	n
0 (Baseline)	81±11	18±11	0±0	0±0	14
3	0±0	84±12	16±12	0±0	4
5	0±0	88±11	12±5	0±1	20
10	0±0	83±10	15±8	2±3	10
15	0±0	84±11	14±9	2±3	10
30	0±0	89±5	8±4	1±2	10
45	0±0	91±2	8±3	1±1	5
60	0±0	90±5	8±3	2±3	12

Baseline: neutrophils fixed on ice.
Mean±standard deviation

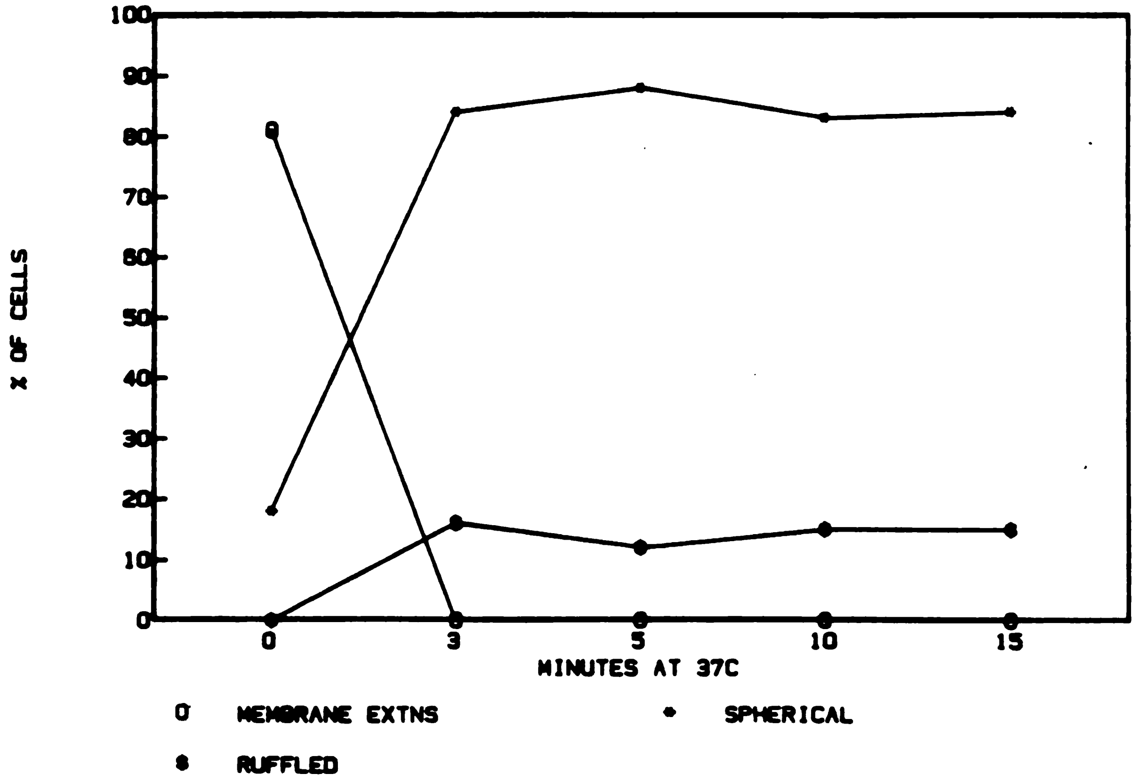


Figure 15
TEMPERATURE TRANSITION MEDIATED SHAPE CHANGE
Data from Table 24

Table 25
TEMPERATURE GRADIENT ESTABLISHED DURING TEMPERATURE TRANSITION

Minutes at 37C:	Temperature (Degrees C)
0.0	0.50±0.00
0.1	13.83±0.23
1.0	21.12±0.23
1.5	27.66±0.23
2.0	30.66±0.23
2.5	32.83±0.23
3.0	34.16±0.23
3.5	34.83±0.23
4.0	35.50±0.00
4.5	36.16±0.23
5.0	36.33±0.23
6.0	36.50±0.00
7.0	36.50±0.00

The results are expressed as mean±standard deviation of three determinations.

formation, of membrane extensions. Since extracellular granule release appeared to be coincident with the ruffling seen early in fMLP activation and following treatment of cells with A23187, supernatants from cells exposed to a temperature transition were assayed to determine if granule release also accompanied membrane extension resolution.

Neutrophils isolated in the cold were incubated at 37C for various time periods. Cell-free supernatants were harvested and evaluated for evidence of granule release. The results of these experiments, shown in Table 26, indicate that significant amounts of lactoferrin were released within minutes after warming the cells. Significant quantities of extracellular lysozyme were also detected following at least 45 minutes incubation of cells at 37C. Extracellular collagenase and gelatinase were not detected in significant quantities in response to the temperature transition.

The data in Table 26 indicate that spontaneous lactoferrin and lysozyme release were temperature dependent. Neutrophils were routinely used within two hours after isolation. An aliquot of cells was held on ice for an additional three hours to determine if spontaneous release was due solely to the aging of isolated cells. The amount of lactoferrin and lysozyme in cell-free supernatants from cells maintained on ice for up to 5 hours after isolation was equivalent to the amount found in supernatants of cells used within two hours after isolation (i.e., baseline release; $p>0.25$ for lactoferrin and lysozyme).

Temperature Transition Mediated Extracellular Granule Release: Affect of Neutrophil Isolation Technique:

Table 26
TEMPERATURE TRANSITION MEDIATED EXTRACELLULAR GRANULE RELEASE: NEUTROPHILS ISOLATED IN THE COLD BY ELUTRIATION TECHNIQUE

Minutes at 37C:	Granule Component:			
	Lactoferrin	Lysozyme	Collagenase	Gelatinase
0 (Baseline)	0.69±0.17(9)	2.24±1.68(5)	4.22±0.25(2)	4.07±3.68(6)
1	1.07±0.21(8)*	2.53±1.75(5)	3.79±0.29(2)	3.78±1.67(5)
3	1.74±0.56(9)*	3.71±1.25(5)	N.D.	7.28±6.78(3)
5	2.13±0.54(9)*	4.67±1.32(5)	2.38±1.91(2)	6.82±6.45(6)
7	2.34±0.73(8)*	3.95±2.63(5)	N.D.	7.93±9.50(3)
10	2.28±0.60(9)*	3.66±1.83(5)	3.85±2.25(2)	8.71±4.27(3)
15	2.24±0.84(9)*	3.17±1.33(5)	5.51±2.90(2)	9.26±7.83(5)
30	2.80±0.63(9)*	4.21±1.31(5)	3.97±2.92(2)	9.30±4.66(5)
45	3.00±0.84(7)*	7.17±4.72(4)*	N.D	9.59±7.56(2)
60	3.34±1.44(9)*	6.24±3.90(5)*	3.80±1.33(2)	6.64±4.46(5)
3hr/OC	0.82±0.30(5)	3.39±1.73(5)	N.D.	0 (1)

Granule release is expressed as percent of total amount of component detected in lysates prepared from 1 million neutrophils isolated in the cold by elutriation:

Lactoferrin: 2.68±0.38 ug n=12
Lysozyme: 262.18±48.72 Units n=6
Collagenase: 5.13 X 10^{-3}±0.69 X 10^{-3} n=6
Gelatinase: 0.346±0.070 Units n=10

Mean±standard deviation
N.D.: Not Determined
(Number of experiments)
* p<0.01 vs baseline release

The finding that significant quantities of extracellular lactoferrin were detected very early in response to cell warming was further investigated. The neutrophils routinely used in these studies were isolated in the cold by elutriation. To determine whether temperature mediated early lactoferrin release was unique to neutrophils isolated by this technique or isolated in the cold, two alternative methods were used to obtain cells. Neutrophils were isolated by the ficoll/hypaque gradient technique performed at room temperature or at 4C. Cells isolated in the cold were held on ice until use, those isolated at room temperature were maintained at this temperature. Thirty-seven degree incubations were carried out as before, the cell-free supernatants were harvested and assayed for the presence of granule components. The results of temperature transition on granule release from neutrophils isolated on ficoll/hypaque gradients centrifuged in the cold are shown in Table 27. Neither the total amount of granule components detected in cell lysates (shown in the lower portion of Table 27) nor the amount detected in supernatants of warmed cells differed significantly from the quantities found in neutrophils isolated by the elutriation technique. Spontaneous release was, again, temperature dependent and not due to aging of the cells. In addition, the primary granule enzyme beta-glucuronidase was not detected in the cell-free supernatants from neutrophils isolated in the cold by ficoll/hypaque gradients.

In contrast, the lower portion of Table 28 indicates that while lysates prepared from neutrophils isolated at room temperature contained comparable amounts of the granule constituents when compared to lysates from cells isolated in the cold (by either elutriation or

Table 27
TEMPERATURE TRANSITION MEDIATED EXTRACELLULAR GRANULE RELEASE:
NEUTROPHILS ISOLATED IN THE COLD BY FICOLL/HYPAQUE GRADIENT TECHNIQUE

Minutes at 37C:	Granule Component:			
	Lactoferrin	Lysozyme	Gelatinase	B-glucuronidase
0 (Baseline)	0.77±0.24(5)	0(1)	6.50±5.12(2)	0 (2)
1	1.24±0.48(3)	N.D.	4.81±3.78(2)	0 (1)
3	1.81±0.42(3)*	2.11(1)	7.84 (1)	N.D.
5	2.17±0.90(4)*	6.00(1)	7.07±5.69(2)	0 (2)
7	2.70±1.18(3)*	N.D.	10.12 (1)	N.D.
10	2.21±0.93(3)*	2.11(1)	11.97±3.98(2)	0 (1)
15	2.70±1.41(5)*	8.04(1)	5.26±3.14(2)	0 (1)
30	2.34±0.53(5)*	3.90(1)	6.45±6.52(2)	0 (1)
45	2.79±1.06(3)*	13.80(1)	10.84 (1)	N.D.
60	2.95±1.31(5)*	13.80(1)	6.01±5.13(2)	0 (1)
3hr/OC	0.95(1)	6.00(1)	N.D.	N.D.

Release is expressed as percent of total component detected in lysates prepared from 1 million cells isolated in the cold by the ficoll/hypaque technique:

Lactoferrin:	2.85±0.73 ug n=4
Lysozyme:	245.82±40.05 Units n=2
Gelatinase:	0.281±0.035 Units n=3
Beta-glucuronidase:	19.44±5.80 Units n=3

Mean±standard deviation
N.D.: Not Determined
(Number of experiments)
* $p<0.01$ vs baseline
\# $p<0.01$ vs release from neutrophils isolated in the cold by the elutriation technique (Table 26).

ficoll/hypaque techniques), room temperature isolation apparently abolished spontaneous lactoferrin and lysozyme release in response to a 37C incubation. As shown in Table 28 and Figure 16, significant amounts of lactoferrin and lysozyme were not detected in the supernatants of cells isolated at room temperature and warmed. However, there was an indication that cells isolated at room temperature may have spontaneously released gelatinase. Extracellular beta-glucuronidase was not detected.

Temperature Mediated Extracellular Granule Release:

Combined Results from Neutrophils Isolated in the Cold:

Since neutrophils isolated in the cold, either by elutriation or by ficoll/hypaque gradient techniques, appeared to be identical in terms of early lactoferrin and later lysozyme release, data from cold isolated cells was combined and is shown in Table 29 and Figure 17. Compared to cells maintained on ice, neutrophils incubated at 37C for 1 minute released significant amounts of lactoferrin ($p<0.005$). From the data describing the establishment of temperature gradients, shown in Table 25, 1 minute at 37C corresponded to approximately 21C. At approximately 34C, established in 3 minutes incubation at 37C, the amount of extracellular lactoferrin was significantly greater than that found in the supernatants of cells held on ice ($p<0.005$), as well as the quantity detected in supernatants of cells at 21C ($p<0.005$). By 5 minutes incubation at 37C maximum temperature had almost been reached and extracellular lactoferrin had begun to plateau. The quantity detected in supernatants at the time points tested during the period from 5 to 60 minutes at 37C did not change significantly.

Significant amounts of extracellular lysozyme were found

Table 28
TEMPERATURE TRANSITION MEDIATED EXTRACELLULAR GRANULE RELEASE:
NEUTROPHILS ISOLATED AT ROOM TEMPERATURE

Minutes at 37C:	Granule Component:			
	Lactoferrin	Lysozyme	Gelatinase	B-glucuronidase
0 (Baseline)	0.96±0.31(4)	1.92±1.46(3)	4.02±0.95(3)	0(1)
1	0.94±0.37(4)	2.50±1.04(3)	27.18±8.54(3)#*	0(1)
3	0.88±0.13(3)#	2.60±1.46(3)	N.D.	N.D.
5	0.90±0.28(4)#	1.92±1.04(3)#	14.24±3.48(3)#*	0(1)
7	0.78±0.15(3)#	2.50±1.04(3)	N.D.	N.D.
10	1.16±0.50(4)#	1.92±1.33(3)	11.32±3.82(3)	0(1)
15	1.53±0.52(4)	3.64±1.27(3)	20.04±6.44(3)*	0(1)
30	1.38±0.60(4)#	2.50±1.04(3)	15.28±6.18(3)	0(1)
45	N.D.	2.50±1.45(3)	N.D.	N.D.
60	1.39±0.67(4)#	1.25±0.00(3)#	11.92±1.69(2)*	0.14 (1)

Release is expressed as percent of total component detected in lysates prepared from 1 million neutrophils isolated at room temperature.

Lactoferrin:	2.85±0.22 ug n=9
Lysozyme:	272.50±22.45 Units n=4
Gelatinase:	0.423±0.01 Units n=3
Beta-glucuronidase:	12.77 Units n=1

Mean±standard deviation
N.D.: Not Determined
(Number of experiments)
* p<0.01 vs baseline
\# p<0.01 vs release from cells isolated in the cold (Table 29).

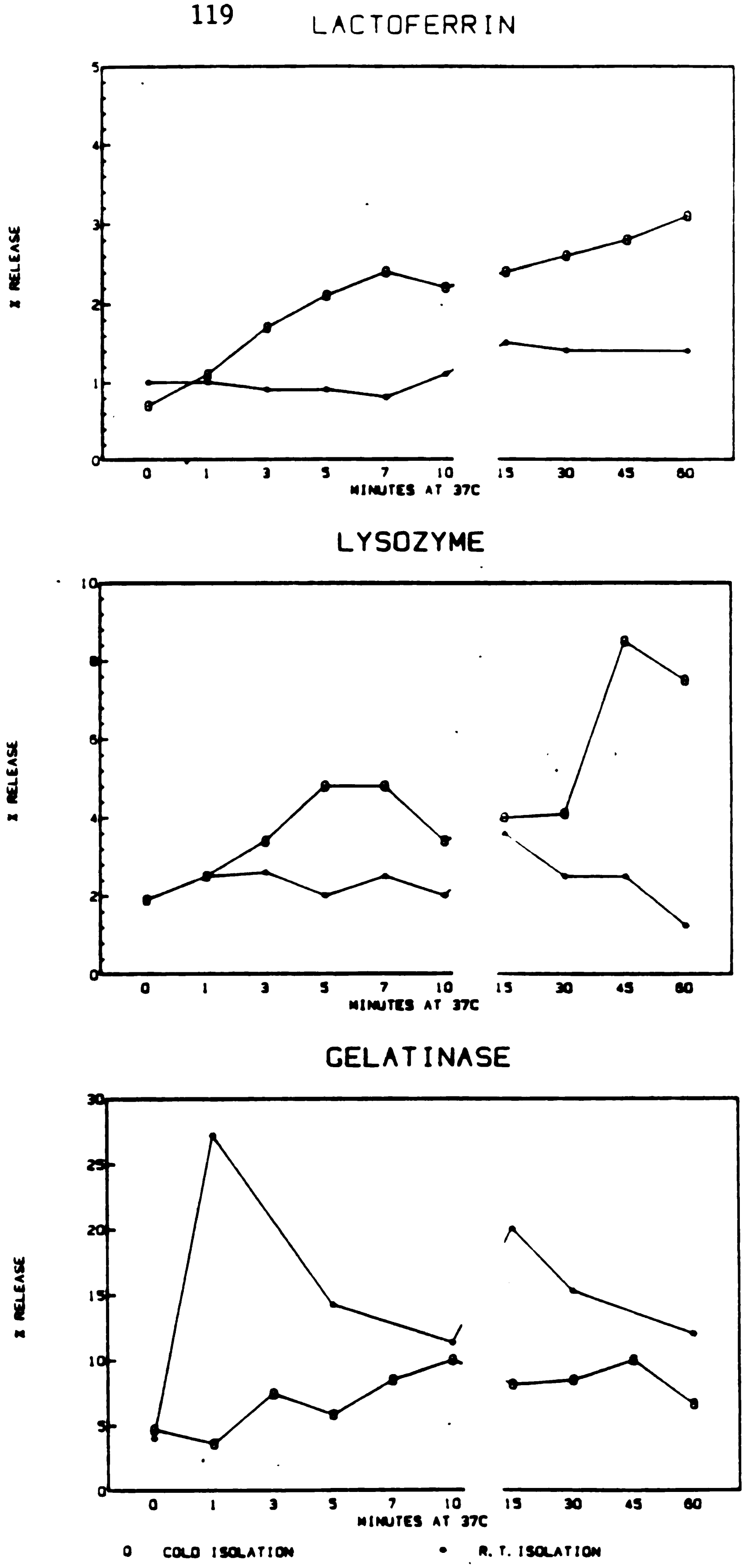


Figure 16
EFFECT OF ISOLATION TEMPERATURE ON TEMPERATURE MEDIATED EXTRACELLULAR GRANULE RELEASE
Data from Tables 28 and 29

Table 29
TEMPERATURE MEDIATED EXTRACELLULAR GRANULE RELEASE:
COMBINED RESULTS FROM NEUTROPHILS ISOLATED IN THE COLD BY ELUTRIATION
OR FICOLL/HYPAQUE GRADIENT TECHNIQUES

Minutes at 37C:	Granule Component:				
	Lactoferrin	Lysozyme	Collagenase	Gelatinase	B-glucuronidase
0 (Baseline)	0.70±0.20(14)	1.86±1.75(6)	4.22±0.25(2)	4.68±3.83(8)	0 (2)
1	1.10±0.30(11)*	2.53±1.75(5)	3.79±0.29(2)	3.59±1.69(7)	0 (1)
3	1.72±0.50(12)*	3.44±1.29(6)	N.D.	7.42±5.54(4)	N.D.
5	2.10±0.64(13)*	4.81±1.31(6)	2.38±1.91(2)	5.81±3.75(8)	0 (2)
7	2.40±0.84(11)*	3.95±2.63(5)	N.D.	8.47±7.84(4)	0 (1)
10	2.21±0.64(12)*	3.40±1.76(6)	3.85±2.25(2)	10.01±4.04(5)	0 (1)
15	2.37±1.07(14)*	3.98±2.13(6)	5.51±2.90(2)	8.12±6.81(7)	0 (1)
30	2.59±0.58(14)*	4.16±1.18(6)	3.97±2.92(2)	8.48±4.84(7)	0 (1)
45	2.87±0.84(10)*	8.50±5.05(5)*	N.D.	10.01±5.39(3)	0 (1)
60	3.15±1.26(14)*	7.50±4.66(6)*	3.80±1.33(2)	6.63±4.04(7)	1.12±1.58(2)
3hr/0C	0.80±0.26(6)	3.82±1.87(6)	N.D.	4.85 (1)	N.D.

Release is expressed as percent of total component detected in lysates from 1 million neutrophils (Combined totals Tables 26 and 27):

Lactoferrin:	2.76±0.63 ug n=16
Lysozyme:	258.09±44.52 Units n=8
Collagenase:	5.13 X 10^{-3}±0.69 X 10^{-3} Units n=6
Gelatinase:	0.331±0.069 Units n=13
B-glucuronidase:	19.32±5.76 Units n=6

Mean±standard deviation
N.D.:Not Determined
(Number of experiments)
* $p<0.01$ vs baseline

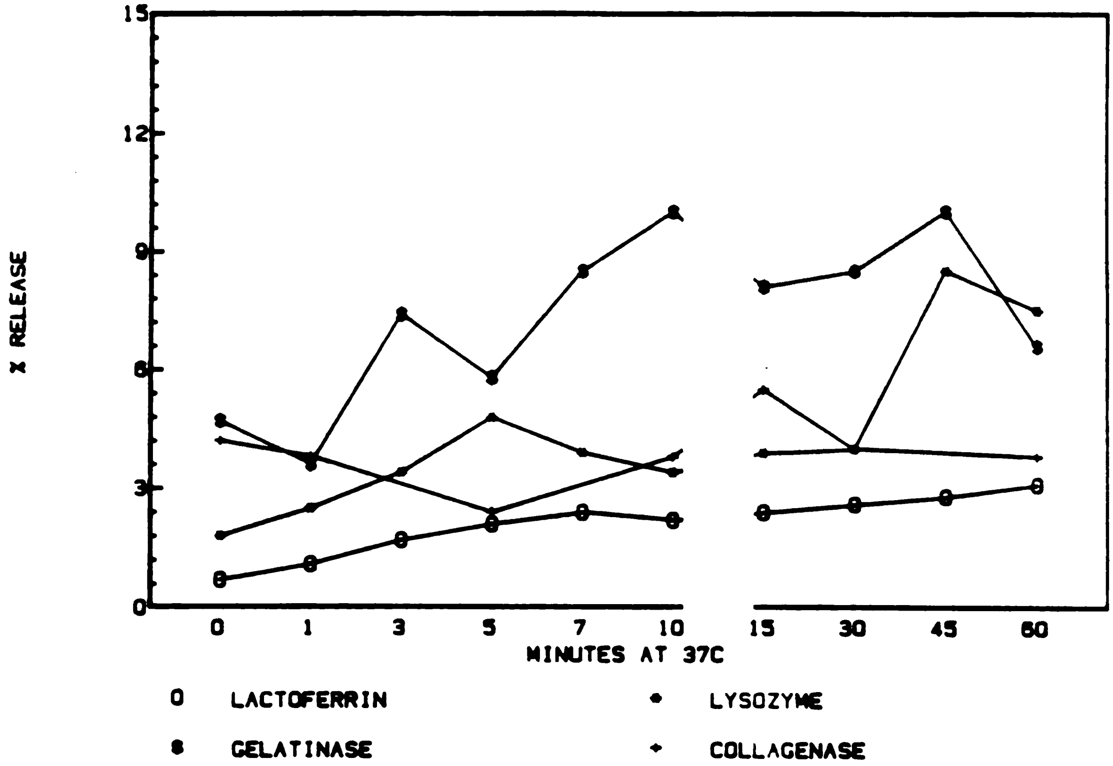


Figure 17
TEMPERATURE TRANSITION MEDIATED EXTRACELLULAR GRANULE RELEASE
Data from Table 29

following 45 minutes incubation of cells at 37C. Since the maximum temperature was achieved by approximately 5 minutes (Table 25), lysozyme was detected after the neutrophils had been maintained at 37C for about 40 minutes.

The resolution of the membrane extensions seen on cold fixed cells appeared to be coincident with the detection of significant amounts of extracellular lactoferrin. By 3 minutes incubation at 37C, corresponding to approximately 34C, 85% of the neutrophils exhibited spherical forms and the mean extracellular lactoferrin levels were approximately 2.5 fold greater that baseline.

Temperature Transition Mediated Lactoferrin Release:

Effect of Various Chemicals:

To investigate a possible mechanism for spontaneous temperature dependent lactoferrin release, cells were pretreated with several chemicals. Neutrophils used to study the influence of cations on release (Ca++,Mg++-free and EDTA) were isolated in the presence of 0.25mM EDTA, cells pretreated with 2-DOG were isolated with glucose-free media. Cells were preincubated in Ca++,Mg++-free PBS or with 5mM EDTA, 5mM 2-DOG or 10ug/ml cycloheximide for twenty minutes on ice. To control for possible interference with the lactoferrin ELISA, standard curves of human milk lactoferrin were generated with the inclusion of the chemicals at the concentrations used in the release experiments. None of the chemicals affected the assay (data not shown). As shown in the lower portion of Table 30, the average amount of lactoferrin contained in lysates prepared from cells treated with the agents or maintained in cation-free buffer was comparable to the amount contained in lysates from cells held in complete PBS.

Table 30

THE EFFECT OF VARIOUS CHEMICALS ON TEMPERATURE TRANSITION MEDIATED LACTOFERRIN RELEASE

Minutes at 37C:	Test Chemical: Ca++,Mg++-free	5mM EDTA	5mM 2-DOG	10ug/ml Cycloheximide
0 (Baseline)	0.84±0.24(3)	0.85±0.24(4)	0.49(1)	0.90±0.40(3)
1	1.28±0.36(3)	1.03±0.38(4)	1.00(1)	N.D.
3	2.03±0.58(3)	1.38±0.28(4)	1.78(1)	N.D.
5	2.47±0.75(3)	1.65±0.53(4)	1.91(1)	2.85±0.84(3)
7	2.81±0.99(3)	1.43±0.42(4)	1.96(1)	N.D.
10	2.60±0.75(3)	1.50±0.36(4)	2.00(1)	N.D.
15	2.79±1.26(3)	1.45±0.50(4)	2.11(1)	2.75+0.44(3)
30	3.04±0.68(3)	1.71±0.21(4)*	2.10(1)	2.87±0.60(3)
45	3.37±0.99(3)	1.57±0.40(4)*	2.30(1)	N.D.
60	3.70±1.50(3)	1.71±0.41(4)	2.45(1)	3.45±0.70(3)

Cells preincubated with test chemical 20 minutes on ice.

Release expressed as percent of total lactoferrin in lysates prepared from 1 million cells incubated with test agent for 20 minutes on ice followed by 60 minutes at 37C:

Ca++,Mg++-free PBS 2.35 ± 0.39 ug n=3
5mM EDTA: 2.71 ± 0.67 ug n=5
5mM 2-deoxyglucose: 2.93 ± 0.92 ug n=2
10ug/ml cycloheximide: 2.15 ± 0.21 ug n=3

Baseline: Cells held on ice with the chemical for the duration of the experiment, 80 minutes.

Mean±standard deviation

N.D.: Not Determined

(Number of experiments)

* $p<0.01$ vs release from neutrophils in complete PBS

Under the conditions used, 5mM EDTA demonstrated significant inhibition of lactoferrin release following 30 and 45 minutes incubation of cells at 37C ($p<0.005$ and $p<0.01$, respectively).

Temperature Transition Mediated Extracellular Granule Release: Effect of Cytochalasin B:

Next, experiments were performed to determine whether CB influenced temperature mediated extracellular granule release. Neutrophils were preincubated with 5ug/ml of CB for 20 minutes on ice. The cell suspensions were then incubated at 37C for various time periods. Supernatants and prepared lysates were evaluated for lactoferrin, lysozyme and gelatinase content. As seen in Table 31, CB had no affect on the total cellular content or spontaneous temperature dependent release of lactoferrin, lysozyme or gelatinase.

The Effect of Various Chemicals on Temperature Transition Mediated Shape Change and Cell Viability:

In terms of visual shape change, none of the chemicals tested altered the morphological responses mediated by the temperature transition. Neutrophils were preincubated with the chemicals for 20 minutes on ice followed by incubation at 37C. Membrane extensions were found on cells fixed in the cold but were resolved if the cells warmed and then fixed (data not shown).

To be certain that the lactoferrin detected extracellularly in response to the temperature transition was not due to cell death, viability determinations (by trypan blue exclusion) were made on neutrophils treated with the chemicals or held in complete PBS for 20 minutes on ice followed by incubation 37C for 60 minutes. Cell viability exceeded 96% for PBS control cells as well as for cells

Table 31

THE EFFECT OF CYTOCHALSIN B ON TEMPERATURE TRANSITION MEDIATED EXTRACELLULAR GRANULE RELEASE

Minutes at 37C:	Granule Component:		
	Lactoferrin	Lysozyme	Gelatinase
0 (Baseline)	0.85±0.18(4)	4.05±1.60(2)	0 (2)
1	1.22±0.44(4)	3.75±0.21(2)	4.49±1.71(2)
3	1.87±0.66(4)	4.30±1.26(2)	18.56±7.92(2)
5	1.78±0.28(4)	5.52±1.85(2)	10.39±6.56(2)
7	2.43±0.78(4)	5.15±1.33(2)	13.78±6.17(2)
10	2.32±0.66(4)	3.01±0.13(2)	12.15±6.29(2)
15	2.61±0.38(4)	6.01±1.16(2)	10.70±7.45(2)
30	3.18±0.86(4)	12.00±7.30(2)	14.76±8.01(2)
45	2.91±0.92(4)	5.76±0.81(2)	12.64±8.66(2)
60	3.03±0.87(4)	5.03±0.22(2)	13.87±7.82(2)
3hr/OC	N.D.	N.D.	7.85±1.14(2)

Neutrophil suspensions were preincubated with 5ug/ml of CB for 20 minutes on ice.
Results are expressed as percent release based on total contained in cell lysates prepared from 1 million cells in the presence of 5ug/ml CB (Table 5).
Baseline: Release from cells held on ice with 5ug/ml of CB for duration of experiment, 80 minutes.
Mean±standard deviation
N.D.: Not Determined
(Number of experiments)
* $p<0.01$ vs release from neutrophils without CB.

treated with each chemical tested.

To further investigate temperature mediated cellular responses, ultrastructural studies were done on neutrophils exposed to the temperature transition.

Ultrastructural Evaluation of the Temperature Transition: Quantitation of Intracellular Granules:

In attempt to determine whether intracellular granule depletion accompanied the detection of extracellular lactoferrin early and lysozyme later in the temperature transition, intracellular granule quantitations were made on neutrophils fixed on ice or following incubation at 37C. The results, shown in Table 32 and Figure 18 A, indicate that the average number of peroxidase positive granules counted on profiles of cells held at 37C for 5 and 30 minutes was not significantly different from cells held on ice ($p<0.05$ and $p>0.25$, respectively). Additionally, although loss of intracellular peroxidase negative granules was detected in response to the temperature transition (approximately 6 granules/cell profile after 5 minutes and 7 granules/profile following 30 minutes incubation), the loss was not highly significant ($p<0.025$ and $p<0.05$ for 5 and 30 minutes incubation at 37C, respectively).

Ultrastructural Evaluation of the Temperature Transition: Morphometric Determinations:

Ultrastructurally, the membrane extensions seen with light microscopy on neutrophils isolated in the cold appeared to be thin protrusions of plasma membrane containing a meshwork of fine filamentous material. In most cases these structures appeared to bend back toward the cell body forming loops. In some cell sections,

Table 32
ULTRASTRUCTURAL GRANULE QUANTITATION: TEMPERATURE TRANSITION

Granules/Cell Profile:

Minutes at 37C:	Peroxidase Positive	Peroxidase Negative
0 (Baseline) (17)	70.6±8.5	59.1±8.3
5 (16)	65.4±6.9	53.4±5.7
30 (7)	73.4±10.8	52.0±6.2

Neutrophils were isolated from a single donor
Baseline: Cells fixed on ice.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline determination

contact between the tip of an extension and the cell body could be seen. Generally the region between the cell body and the membrane loop was devoid of cellular material. However, in some instances there were smaller membrane protrusions contained within the primary loop. These structures were not numerous on an individual cell and seemed to be regionally localized. The remaining plasma membrane was very smooth (Figure 19).

To determine whether cells exposed to the temperature transition exhibited quantitative morphometric changes correlating with the detection of extracellular lactoferrin and the modest depletion of intracellular granules, electron micrographs of neutrophils fixed on ice or warmed for 5 or 30 minutes at 37C were measured.

The data shown in Table 33 and Figure 18 B indicate that the conditions which mediated temperature dependent shape change and lactoferrin release had no influence on ultrastructural morphometric determinations. Mean cell perimeter, calculated area, surface-to-volume ratios and exposed plasma membrane determinations made of profiles from neutrophils incubated at 37C for either 5 or 30 minutes were identical to those made from cells held on ice.

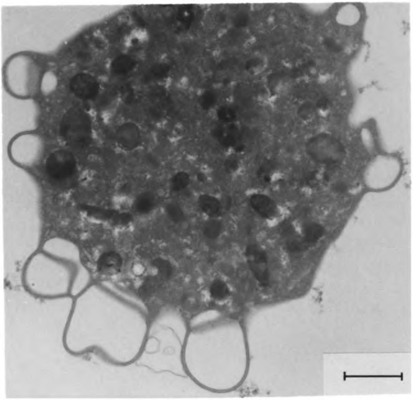
Summary of Results:

The results of these investigations indicate 1) that the morphological event of neutrophil ruffling and the functional event extracellular granule secretion are associated, 2) that these events of activation produce quantitative morphometric alterations and 3) that these morphometric alterations may be specifically related to the activation induced release of a particular type of granule containing relatively high concentrations of lactoferrin.

Figure 19
NEUTROPHILS FIXED IN THE COLD: TEM

(Figure 19 is comprised of 3 pages)
1. First panel: Grazed cell showing delicate membrane loops.
Second panel: Note fine membrane loops on upper and left portion of the cell and blunt protrusion on right side of the cells. Also notice that the remaining cell surface is relatively smooth.

Bars = 1um



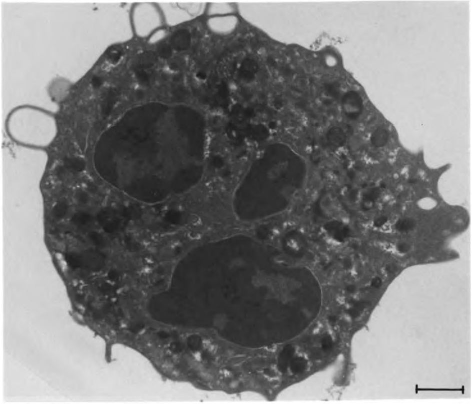
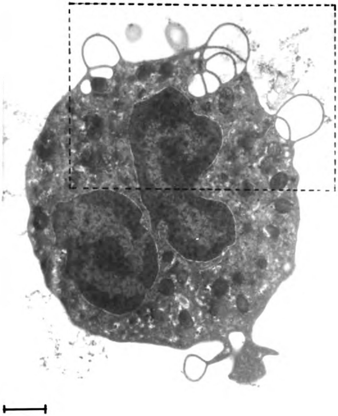


Figure 19 (continued)

2 and 3. The panels shown on the following two pages are sections of a cell isolated and fixed in the cold. Note the intricate membrane looping and what appears to be possible membrane shedding.

Bars = 1um



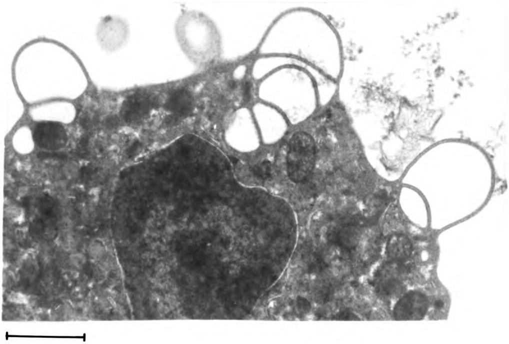


Table 33
ULTRASTRUCTURAL MORPHOMETRIC QUANTITATIONS: TEMPERATURE TRANSITION

Minutes at 37C:	Perimeter	Area	S/V	Calculated Membrane(um 2)
0 (Baseline) (19)	60.87±8.20	65.11±8.56	1.41±0.25	559.77±105.20
5 (17)	62.43±6.05	65.36±7.44	1.45±0.14	576.44±54.39
30 (9)	62.53±7.73	66.17±5.96	1.45±0.23	576.44±90.91

Neutrophils were isolated from a single donor
Baseline: Cells fixed on ice.
Mean±standard deviation
(Number of micrographs analyzed)
* $p<0.01$ vs baseline measurements

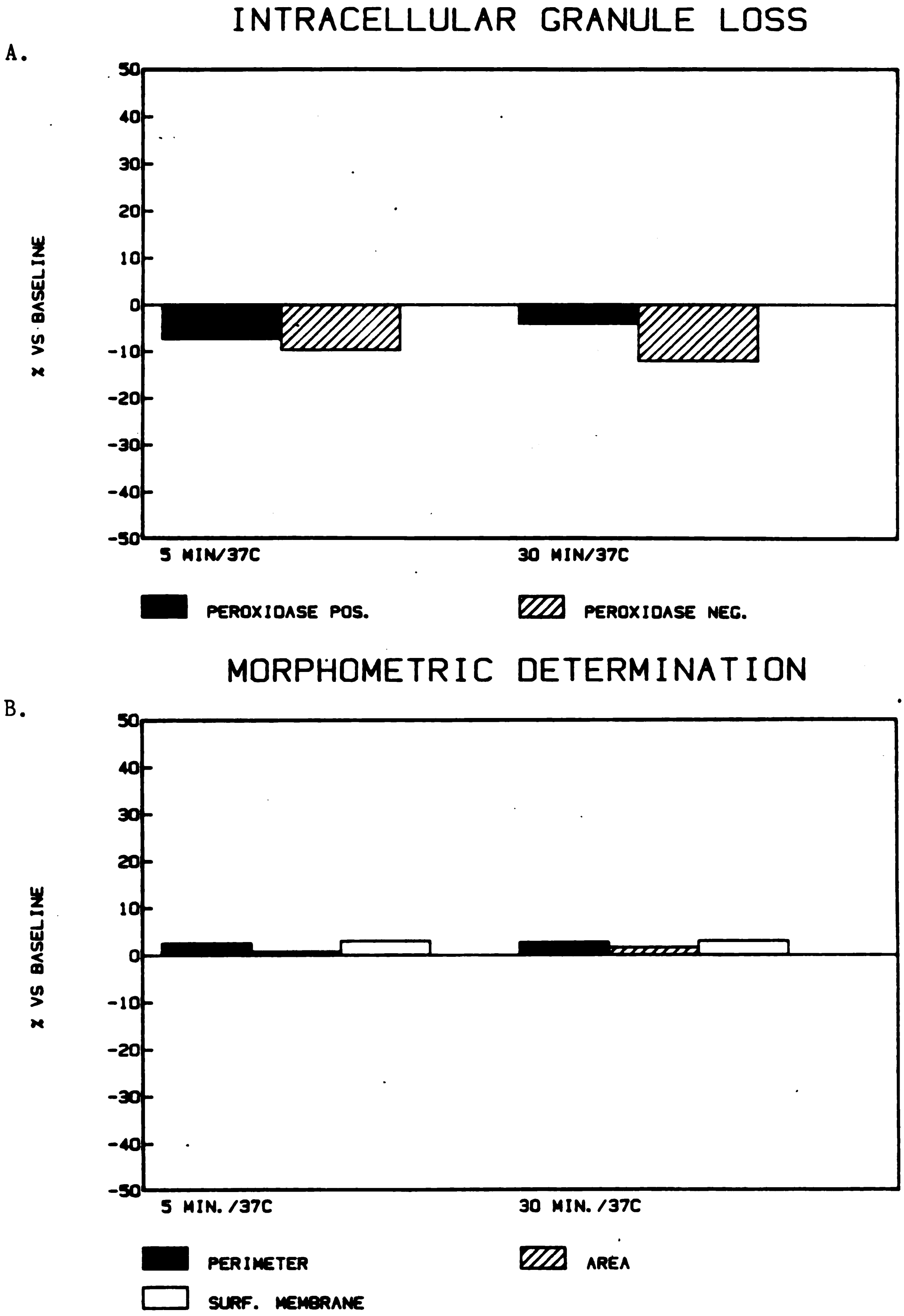


Figure 18
ULTRASTRUCTURAL EVALUATION OF TEMPERATURE TRANSITION
Data from Tables 32 and 33 expressed as % deviation from baseline

DISCUSSION

The purpose of these studies was to investigate the early morphological and functional responses of human neutrophils to activation. Cells isolated from healthy adult donors were exposed to the synthetic chemotactic tripeptide fMLP or the calcium ionophore A23187, fixed and evaluated with light microscopy for qualitative shape change. Cellular ruffling was considered to represent the earliest visible response to activation and was used as the criteria for selecting the experimental conditions utilized for the functional studies. Early functional responses to activation were evaluated next by assaying supernatants from ruffled cells for evidence of extracellular primary, secondary and tertiary granule release. Characterization and quantitation of intracellular granules were then made on electron micrographs of nonactivated spherical and ruffled cells to confirm extracellular granule secretion and to gain additional insight into this event. Finally, morphometric measurements were made on electron micrographs of spherical and ruffled cells to correlate morphological and functional events of early activation. In addition to adult cells, neutrophils were isolated from umbilical cord blood samples and exposed to fMLP or A23187. Intracellular granule characterization and quantitation, and morphometric determinations were made on electron micrographs of spherical and ruffled neonatal cells. Morphometric and functional cellular responses resulting from a temperature transition from 4C to 37C were also studied.

The results from these investigations of early morphological and functional responses of neutrophils to activation suggest that 1) the peroxidase negative granules in adult neutrophils represent a heterogeneous population differing in composition, 2) a population of peroxidase negative granules may undergo sequential or selected release, and 3) the release of these granules is coincident with the generation of cellular shape change and may be associated with a transient or apparently regulated increase in exposed plasma membrane.

Evidence Suggesting Heterogeneity of Peroxidase Negative Granules Differing in Composition and Release Dynamics

Adult neutrophils exposed to 1nM or 100nM fMLP for 20 seconds, or 100nM or 250nM A23187 for 5 minutes exhibited ruffled configurations (Tables 3, 11 and Figures 3, 7). Transmission electron micrographs of ruffled and nonactivated spherical cells, analyzed for intracellular peroxidase positive and negative granules, revealed that both activators mediated a significant loss of peroxidase negative granules while not affecting peroxidase positive granule content. Twenty seconds exposure of cells to 1nM or 100nM fMLP induced a mean loss of approximately 11 granules/cell profile (Table 8). Cells treated with the calcium ionophore for 5 minutes lost an average of 6 granules/cell profile following exposure to 100nM A23187 or 14 granules/profile in response to 250nM A23187 (Table 14). In terms of extracellular granule release, lactoferrin was detected in significant quantities in cell-free supernatants harvested from neutrophils activated for 20 seconds with 1nM or 100nM fMLP (Table 6). Cells exposed to 100nM or 250nM A23187 for 5 minutes were found to have released not only more lactoferrin than fMLP activated cells but significant amounts of lysozyme and gelatinase as well. Collagenase was also detected in the supernatants of ionophore but not fMLP treated cells (Table 12). To associate the extracellular granule secretion from ruffled cells mediated by fMLP or A23187 with intracellular granule loss, release/granule ratios were calculated.

Several assumptions were made in calculating the extracellular granule release/intracellular granule loss ratios shown in Tables 9 and 15; 1) Even though isolated neutrophils are reported to be a heterogeneous population (Berkow and Baehner, 1985; Gallin, 1984; Klempner and Gallin, 1978; Krause et.al., 1986; Seligmann et.al., 1981; Seligmann et.al., 1984), it was assumed that each preparation of isolated neutrophils represented a population of cells having, on average, equivalent inherent abilities to respond to fMLP or A23187 in terms of granule release; 2) It was assumed that all peroxidase negative granules were identical with respect to their ability and probability of being released in response to the stimuli; 3) Assumptions were made that all peroxidase negative granules were identical in regard to their composition; and 4) that peroxidase negative granules did not partition into unique intracellular regions in response to fMLP or A23187.

Taking these assumptions into consideration, the calculated extracellular granule release/intracellular granule loss ratios support the hypothesis that the peroxidase negative granules in adult neutrophils may represent a heterogeneous population differing in content and release dynamics. The ratios calculated from neutrophils exposed to fMLP suggest that the granules lost following 20 seconds activation with 1nM or 100nM fMLP contained lactoferrin exclusively, or contained lactoferrin in relatively greater quantities than the other secondary granule components evaluated (Table 9). Although treatment of neutrophils with the ionophore apparently did not cause the loss of more peroxidase negative granules/cell profile, neutrophils treated with 100nM or 250nM A23187 for 5 minutes released quantitatively more of each component/granule than fMLP activated cells. Compared with neutrophils activated with fMLP, ionophore treated cells released at least 8 fold more lactoferrin, at least 1.5 times more lysozyme and at least 1.7 times more collagenase/peroxidase negative granule. In addition, assuming that tertiary granules may be peroxidase negative, neutrophils exposed to A23187 released approximately 7 fold more gelatinase/granule than fMLP activated cells (compare Tables 9 and 15).

By examining the amounts of each component released/granule in response to A23187 compared to fMLP (i.e., approximately 1.6 fold more lysozyme and collagenase and approximately 7 fold more lactoferrin and gelatinase) it appears that lysozyme and collagenase are packaged together and lactoferrin and gelatinase may be granularly localized with one another. Interestingly, Hibbs and coworkers (1986) have recently suggested that lactoferrin and gelatinase are indeed intracellularly associated. Using immunogold labeling techniques these investigators reported that lactoferrin and gelatinase were co-localized in peroxidase negative granules. These findings may suggest that 1) lactoferrin is a tertiary granule component and tertiary granules are peroxidase negative, 2) gelatinase is a secondary granule component or 3) a novel peroxidase negative granule containing lactoferrin and gelatinase exists in human neutrophils.

The calcium ionophore induced extracellular granule release determinations, shown in Table 12 and Figure 8, indicate that secretion plateaued following 5 minutes exposure of neutrophils to A23187 and that release was not dose responsive. The amounts of each granule component released following 5 and 10 minutes treatment of cells with either 100nM or 250nM A23187 were identical. In contrast, intracellular granule determinations indicated that cells treated with 250nM A23187 for 5 minutes lost approximately twice as many peroxidase negative granules as cells treated with 100nM A23187 for 5 minutes (6 vs 14 granules/cell profile; $p<0.005$ for granule loss in response to 100nM A23187 compared to 250nM A23187 mediated granule loss; Table 14). Consequently, the release/granule calculated for cells exposed to 100nM ionophore for 5 minutes are about twice those calculated for 5 minutes exposure of cells to 250nM A23187. These results could be explained as suggesting selected or sequential peroxidase negative granule release. The granules released in response to 250nM of the calcium ionophore may have contained components not evaluated, or relatively less of those components evaluated in these studies.

The results of experiments performed to investigate the affect of cytochalasin B on granule release also suggest heterogeneity of secondary granules with populations differing in release dynamics. The capacity of CB to enhance granule secretion was found to depend upon the conditions used to mediate secretion as well as the particular granule component being released. Pretreatment of neutrophils with CB enhanced extracellular lactoferrin and gelatinase release occurring within 20 seconds after 1nM or 100nM fMLP activation, but had no apparent affect on lysozyme release (Figure 5). In contrast, CB had no influence on calcium ionophore mediated lactoferrin release, but did enhance lysozyme and beta-glucuronidase release initiated by 5 or 10 minutes exposure of cells to 100nM or 250nM A23187 (Table 13 and Figure 9). Gelatinase release induced by the ionophore also appeared to be enhanced by pretreating the cells with CB. However, the large standard deviation seen after cells were exposed to 100nM or 250nM A23187 for 5 minutes (65.25 ± 51.22% and 78.71 ± 29.95%, respectively, n=2 for both; Table 13) makes this conclusion nebulous. Large variability in gelatinase release, such as those just indicated, are occasionally seen in the literature. Dewald et.al. (1982) reported an average gelatinase release of 25.0 ± 20% following exposure of human neutrophils to 300nM A23187 (without CB pretreatment) for 10 minutes (n="at least 3").

Thus, the data from intracellular and extracellular evaluations of granule secretion suggest that relatively high concentrations of lactoferrin may be contained in a population of peroxidase negative granules which are selectively released from ruffled neutrophils.

Since neutrophils isolated from umbilical cord blood samples have been found to contain approximately 50% of the normal adult levels of lactoferrin but equivalent amounts of lysozyme (Ambruso et.al., 1984; Becker-Freeman et.al., 1984), they provided a unique approach to further investigate the possible existence and release characteristics of a population of peroxidase negative granules containing primarily lactoferrin.

In these studies, transmission electron micrographs of unactivated neonatal cell profiles were found to contain both peroxidase positive and negative intracellular granules in quantities comparable to those found in unactivated adult cells (compare baseline determinations on Table 8 or 14 with baseline on Table 21). Ambruso and coworkers (1984) reported that while neonatal and adult neutrophils contained an equivalent number of intracellular primary granules, neonatal cells contained fewer secondary and more "unidentifiable" granules than adult cells. These investigators classified granules by electron density but did not treat fixed cells with DAB to specifically stain peroxidase positive primary granules. Since primary granules are reported to be morphologically heterogeneous (Prywansky and Brenton-Gorius, 1985), this may have made granule classification difficult, possibly accounting at least in part for the "unidentifiable" granules and may explain the discrepancy in our findings.

The results of intracellular granule quantitations made from micrographs of newborn neutrophils suggested that neonatal and adult granule secretion following 1nM fMLP mediated cell ruffling differed. While 20 seconds activation of adult neutrophils with 1nM fMLP induced a significant depletion in peroxidase negative granules when compared to unactivated cells ($p<0.005$; Table 8 and Figure 6 A), identical treatment of neonatal cells did not significantly affect the number of intracellular peroxidase negative granules ($p<0.025$; Table 21 and Figure 13 A). However, in terms of granule quantitation in response to the other experimental conditions used in these studies, neonatal and adults responded identically. Intracellular peroxidase negative granule depletion following 20 seconds exposure of newborn and adult cells to 100nM fMLP or 5 minutes treatment with 100nM or 250nM A23187 were significant when compared to unactivated control values ($p<0.005$).

The results from early activation mediated intracellular granule depletion from neonatal neutrophils could indicate that the presence of relatively large quantities of lactoferrin in a population of peroxidase negative granules may, in some manner, impart an increased susceptibility or sensitivity to these granules being released. Since newborn cells are partially deficient in lactoferrin, their granules may lack sufficient levels of lactoferrin to confer such a sensitivity. This could explain the finding that a highly significant decrease in peroxidase negative granules was not seen following 1nM fMLP activation of neonatal neutrophils but was detected in identically treated adult cells.

Neutrophils exposed to a temperature transition also suggest that a population of granules containing lactoferrin exclusively, or in relatively large quantities compared to other secondary granule constituents, may exist and be selectively released when cells are warmed. Significant quantities of lactoferrin were detected extracellularly within 1 minute after cells isolated and maintained at 4C were transferred to 37C (Table 29). Additionally, as previously reported (Goldstein et.al., 1974; Wright and Gallin, 1979) extracellular lysozyme was found to increase approximately 3.5 fold when cells were warmed, but not until the cells had been maintained at 37C for approximately 40 minutes (Table 29).

Temperature dependent increased levels of extracellular lactoferrin were not due to aging of cells or cell death (determined by trypan blue exclusion), but did require that the cells be isolated in the cold. However, the manner in which neutrophils were obtained in the cold was irrelevant. Cells isolated with either ficoll/hypaque gradients (Table 27) or elutriation (Table 26) performed in the cold released comparable quantities of lactoferrin when they were warmed. In contrast, neutrophils isolated at room temperature and warmed to 37C did not spontaneously release lactoferrin (Table 28).

Berkow and coworkers (1983) reported that, compared to neutrophils isolated with ficoll/hypaque gradients, cells obtained by the elutriation technique secreted significantly more vitamin B12 binding protein, lysozyme and beta-glucuronidase when exposed to fMLP in the presence of CB. These findings contrast the data presented here, which indicated that as long as cell isolation was done in the cold, the technique played no apparent role in either temperature dependent (Tables 26 and 27) or activated extracellular granule release (data not shown). It should be noted that as an initial step in the ficoll/hypaque procedure, Berkow et.al. (1983) sedimented the erythrocytes in whole blood with dextran for 90 minutes at 25C, while the blood sample used for elutriation of neutrophils was immediately chilled to 4C.

In agreement with published reports (Skosey et.al., 1973; Zurier et.al. 1973) the inhibition of microfilament polymerization with CB had no significant influence on temperature dependent extracellular granule release. Inhibitors of glucose metabolism and protein synthesis played no role in temperature mediated lactoferrin release, suggesting that this response was energy independent and that the secreted lactoferrin was preformed. In addition, lactoferrin release was apparently independent of extracellular calcium. Neither the inclusion of 5mM EDTA nor the exclusion of extracellular divalent cations significantly affected early temperature mediated release (Tables 30 and 31). Interestingly, the time points at which 5mM EDTA significantly inhibited lactoferrin release (30 and 45 minutes) correspond to the approximate incubation periods when significant amounts of lysozyme were detected in cell-free supernatants. Goldstein and coworkers (1974) reported that spontaneous temperature dependent extracellular lysozyme release was dependent upon extracellular Ca++. It is conceivable that at these longer time points 5mM EDTA may have inhibited the release of a population of granules containing lactoferrin as well as relatively high concentrations of lysozyme.

Ultrastructural evaluations of cell profiles from warmed neutrophils suggested that the spontaneously released lactoferrin was granular in origin. Compared to the granule quantitations made on micrographs of neutrophils maintained on ice, cells warmed for 5 or 30 minutes at 37C lost an average of 6 or 7 peroxidase negative granules/cell profile, respectively (Table 32). While this granule loss was not highly significant ($p<0.025$), the percentage of extracellular lactoferrin release, while small, was significant; approximately 2.0% of total cellular lactoferrin was detected in cell-free supernatants after 5 minutes and 2.6% after 30 minutes incubation of cells at 37C ($p<0.005$ for both; Table 29). In terms of release/granule, this represents approximately 9.87×10^{-6} ng of lactoferrin/granule lost. Neutrophils activated with either 1nM or

100nM fMLP for 20 seconds exhibited a significant loss of peroxidase negative granules, about 11/cell profile (Table 8), and secreted approximately 4% of the total cellular content of lactoferrin (Table 6). Thus, a two fold increase in lactoferrin release was accompanied by two fold increase in intracellular granule loss. Additionally, as shown in Table 9, cells activated with fMLP were determined to have released an average of 9.79×10^{-6} ng of lactoferrin/peroxidase negative granule lost, a value comparable to that calculated for temperature mediated lactoferrin release/granule loss.

The data presented above appear to suggest that a population of peroxidase negative lactoferrin-containing granules exists in human neutrophils and are selectively released early in response to cellular activation or in response to a temperature transition from 4C to 37C.

As previously mentioned, neutrophils isolated from both adult peripheral blood and umbilical cord blood are reported to represent a heterogeneous population of cells (Berkow and Baehner, 1985; Gallin, 1984; Klempner and Gallin 1978; Krause et.al., 1986; Seligmann et.al., 1981; Seligmann et.al., 1984). Neutrophil subpopulations were not addressed in the studies presented here. It is feasible that the results suggesting the existence of a population of lactoferrin-containing peroxidase negative granules which are selectively released early in activation, might instead suggest a difference in the response of neutrophil subsets to the experimental conditions used. Conceivably, distinct forms of lactoferrin localized in discrete neutrophil populations, as described by Broxmeyer and coworkers (1980), could provide an alternative explanation for the results presented in this report. In these terms, lactoferrin might be selectively secreted from a subpopulation of cells, rather than a subpopulation of granules, based on an intrinsic susceptibility of the particular cell population to brief exposure to fMLP, treatment with A23187 or a temperature change. Additionally, the distinct forms of lactoferrin described by Broxmeyer et.al. (1980) could be specifically packaged in various quantities with other secondary granule components depending on the type of lactoferrin. Thus, different types of peroxidase negative granules containing various amounts of lactoferrin would be localized in discrete populations of cells rather than a population of lactoferrin-containing granules being found in all cells.

Evidence Suggesting that Granule Release is Coincident with Shape Change and an Apparent Regulation of Membrane Exposure

As just described, the conditions used in these studies to activate neutrophils with fMLP or A23187 induced cellular ruffling and coincident granule secretion, suggesting that these events are associated. Furthermore, the results from the ultrastructural morphometric measurements suggested that under specific circumstances ruffling and granule secretion may cause an apparent increase in exposed plasma membrane.

Hoffstein and associates (1982) reported evidence indicating that activation of human neutrophils produced a significant increase in exposed cell surface membrane. In those studies, morphometric measurements were made on cell profiles of neutrophils activated with fMLP, fixed, reacted with DAB and processed for transmission electron microscopy. As in the studies presented here, only micrographs of cell profiles exhibiting at least three nuclear lobes or the centriole were used in morphometric determinations. These investigators found that neutrophils exposed to 100nM fMLP for 10 seconds exhibited an average 25% increase in exposed surface membrane and a coincident 30% depletion of intracellular peroxidase negative granules. The activation conditions had no affect on intracellular peroxidase positive granule quantitations. The increased cell surface membrane was attributed to the merger of peroxidase negative granule and plasma membranes.

In the studies presented here, intracellular granule and morphometric determinations were made on neutrophils activated for 20 seconds with either 1nM or 100nM fMLP. The results from measurements made on micrographs of neutrophils exposed to the lower concentration of fMLP showed that these cells expressed an average 17.4% more surface membrane than unactivated control cells (Table 10 and Figure 6 B). Additionally, these conditions caused a significant loss of intracellular peroxidase negative granules (an average depletion of 19% compared to unactivated cells) while not affecting peroxidase positive granule content (Table 8 and Figure 6 A). These results appear to indicate, as suggested by Hoffstein et.al. (1982), that the increased cell surface membrane was mediated by peroxidase negative granule-plasma membrane merger. However, while neutrophils treated with 100nm fMLP for 20 seconds also lost an average of 19% of their intracellular peroxidase negative granules, the calculated exposed surface membrane of these cells was not significantly different from unactivated cells ($p>0.25$; Table 10 and Figure 6 B).

The results from these investigations and the study reported by Hoffstein et.al. (1982) suggest that increased plasma membrane, evidently initiated by the merging of peroxidase negative granule and plasma membranes during activated granule secretion, is a dose dependent, kinetically transient and apparently regulated response to activation. Neutrophils activated for 10 seconds with 100nM fMLP or 20 seconds with 1nM fMLP exhibited significant peroxidase negative granule depletion and increased exposed surface membrane. However, while exhibiting comparable granule depletion, neutrophils activated with 100nM fMLP for 20 seconds did not demonstrate an increase in exposed plasma membrane.

The proposed transient and regulated increase in exposed plasma membrane in response to conditions mediating cellular ruffling and granule secretion may also be suggested by the results from ultrastructural morphometric and granule quantitations made on calcium ionophore treated cells. While neutrophils exposed to 100nM or 250nM A23187 for 5 minutes exhibited a significant depletion in intracellular peroxidase negative granules (Table 14 and Figure 10 A), they did not exhibit significant alterations in exposed surface membrane (Table 16 and Figure 10 B). Possibly, during the 5 minutes treatment with ionophore the cells underwent a "regulation"-like response. This response could have returned a transient increase in exposed plasma membrane, proposed to have been initiated by granule-plasma membrane fusion, back to basal levels before the cells were fixed for electron microscopy.

The next series of experiments, made on ruffled neonatal neutrophils, may implicate lactoferrin-containing granules in cellular morphology. The results from the ultrastructural evaluations performed on neonatal cell profiles suggest that although visibly identical, neonatal and adult neutrophils differ morphometrically. When viewed with light microscopy fixed neonatal and adult neutrophils appeared to be identical, with approximately 90% of unactivated cells displaying a spherical shape. However, as shown in the baseline comparisons on Tables 20 and 23, unactivated neonatal and adult cells were found to be morphometrically different. While the mean perimeter determinations of unactivated newborn cells did not differ from perimeter measurements made of adult cell profiles, neonatal cells exhibited significantly larger average area and less exposed surface membrane. Since 2 dimensional area measurements may be considered representative of 3 dimensional volume, this finding suggests that unactivated neonatal cells are larger in volume but smoother, possibly due to fewer or less extensive membrane protrusions, than adult cells.

In terms of visual assessment of activation mediated shape change, adult and neonatal cells exhibited similar responses. Neutrophils isolated from adults or newborns ruffled within 20 seconds following exposure to 1nM fMLP (compare Table 3 and 17). In addition, the cells were found to ruffle within 1 minute and maintain ruffled configurations for up to 10 minutes following exposure to A23187 (Table 11, neonatal data not shown). However, examination of the ultrastructural data from activated neonatal cells suggests that neonatal and adult neutrophils differ in their early responses to activation and further, that lactoferrin-containing peroxidase negative granules may be intimately involved in alterations in cell morphology. In contrast to adult cells, neonatal neutrophils exposed to 1nM fMLP for 20 seconds did not exhibit a highly significant loss of intracellular granules (Table 18 and Figure 12 A) nor an increase in perimeter or exposed plasma membrane (Table 19, Figure 12 B). However, after 20 seconds activation with 100nM fMLP, neonatal cells, like adult cells, demonstrated a significant loss of intracellular peroxidase negative granules (Table 18, Figure 12 A) while not exhibiting significant alterations in exposed surface membrane. Additionally, unlike adult cells, 100nM fMLP treated newborn cells did not exhibit increased average perimeter measurements (Table 19 and Figure 12 B). If increased perimeter is representative of an activated ruffling response (as suggested by the morphometric perimeter measurements made on fMLP and A23187 activated adult cells, Tables 10 and 16), then the lack of significant perimeter alterations in neonatal cells may suggest that, even though visibly comparable, the fMLP mediated ruffling response of neonatal neutrophils differs mechanistically from the adult response. Further, since newborn neutrophils have depressed lactoferrin levels (Ambruso et.al., 1984; Becker-Freeman et.al., 1894), it could implicate the proposed lactoferrin-containing granules in early activation mediated neutrophil shape change.

In summary, comparing the results from granule depletion and morphometric measurements of adult and neonatal cells briefly activated with fMLP suggest that 1) a population of lactoferrin-containing granules exists in normal adult cells, 2) this population of granules is selectively secreted prior to other peroxidase negative granules and 3) these granules are intimately involved in fMLP mediated shape change responses. The hypothesis that lactoferrin-containing granules may be involved in the morphometric alterations seen in activated normal adult neutrophils appears to corroborated by Boxer and coworkers (1982b). These investigators continued examinations of 25 year old male initially identified by Strauss and associates (1974). This patient was described to have suffered from severe, recurrent bacterial infections since birth. Functionally, his isolated neutrophils exhibited depressed adherence associated activities (adhesion to cultured edothelium, aggregation and chemotaxis, and an impaired ability to decrease cell-surface charge in response to activation) and abnormal hydroxyl radical production. Ultrastructurally, his neutrophils were found to contain a large number of intracellular granules, the majority of which were peroxidase positive. Secondary granules were reported to develop later in neutrophil maturation that normal and appeared small and generally deficient in content in mature cells. Determinations of total granule components in lysates prepared from the patient's cells revealed normal levels of lysozyme and beta-glucuronidase but only about 3.5% of the normal level of lactoferrin. Morphometric measurements were made on electron micrographs of unactivated neutrophils and cells briefly exposed to 100nM fMLP. Surface-to-volume ratios of normal control cells were reported to increase approximately 36% in response to activation. In contrast, the S/V determined for unactivated and fMLP activated lactoferrin deficient cells were identical. The results presented in Table 19 of this report show that S/V calculated for 20 seconds 1nM and 100nM fMLP activated neonatal cells were also identical to unactivated baseline levels. Therefore, published data and the data presented in this report appear to indicate that lactoferrin-containing peroxidase negative granules may be involved in normal activation mediated morphometric changes in neutrophils.

Evidence indicating that a population of lactoferrin-containing peroxidase negative granules may be involved in an apparent regulation of exposed plasma membrane is suggested from the results of the ultrastructural quantitations made of calcium ionophore treated newborn cells. As indicated above, neonatal cells exposed to A23187 demonstrated a visual ruffling response and intracellular peroxidase-negative granule loss which was comparable to that found in identically treated adult cells (compare Tables 14 and 21). While ionophore treated adult neutrophils did not exhibit significant changes in exposed surface membrane compared to nonactivated determinations (Table 16, Figure 10 B), identically treated neonatal cells did. Newborn cells exposed to 100nM or 250nM A23187 for 5 minutes exhibited significant increased surface membrane (Table 22 and Figure 13 B). Although unactivated neonatal cells were found to exhibit less surface membrane than unactivated adult cells, the morphometric determinations of neonatal cells after 5 minutes treatment with 100nM or 250nM A23187 were equivalent to adult determinations (Table 23). Therefore, neonatal cells responded to A23187 exposure by depletion of intracellular granules and, in contrast to adult cells, a coincident increase in exposed surface membrane. Secretion of a population of peroxidase negative granules containing primarily lactoferrin, which in some fashion is associated with regulation of exposed surface membrane, may help to explain the finding that A23187 treated neonatal cells exhibited increased exposed plasma membranes while adult cells did not.

Thus, the ultrastructural evaluations of morphology and granule quantitation of fMLP and A23187 treated neonatal neutrophils provide circumstantial evidence suggesting an association between the secretion of a population of lactoferrin-containing granules and the generation and apparent regulation of increased exposed plasma membrane.

In contrast to fMLP and A23187 mediated cellular shape change, neutrophils exposed to a temperature transition from 4C to 37C were found to loose, rather than generate, membrane extensions. Shape change determinations of cells isolated and fixed in the cold revealed that approximately 80% of the cells exhibited short, blunt membrane extensions. As cells isolated in the cold were warmed, these structures disappeared (Table 24, Figure 15). The resolution of the membrane protrusions was found to coincide with the detection of significant quantities of lactoferrin in cell-free supernatants with both events occurring at approximately 22C. It is tempting to speculate that temperature dependent lactoferrin release was associated with the change in neutrophil morphology seen when cold cells were warmed, analogous to fMLP or A23187 mediated ruffling and granule secretion. However, although neutrophils treated with a temperature transition exhibited visual morphological alterations, unlike fMLP or A23187 treated cells, none of the morphometric determinations made on electron micrographs of cells incubated for 5 or 30 minutes at 37C differed significantly from cells maintained at 4C (Table 33 and Figure 18 B).

The results from the ultrastructural morphometric and granule quantitations made on neutrophils exposed to the temperature transition may indicate that a critical level of lactoferrin–containing granule release is required to produce the proposed transient increase in exposed surface membrane. As indicated previously, adult neutrophils briefly activated with 1nM fMLP exhibited a loss of approximately 19% of their intracellular peroxidase negative granules, and an apparent concomitant increase in exposed plasma membrane (Figure 6 A and B). The temperature transition had no affect what so ever on the morphometric determinations (Table 33) and yet caused the loss of approximately 10% of the total intracellular peroxidase negative granules (Figure 18 A). The lactoferrin content of these granules was determined to be comparable to the lactoferrin/granule calculated for adult cells activated with fMLP. If these granules are responsible for initiating an increase in exposed surface membrane in adult cells, perhaps the extent of release in response to the temperature transition was not sufficient to generate such an increase.

Alternatively, increased surface membrane may require a true activation initiated response, suggesting that granule secretion alone may not be sufficient to cause alterations in the quantity of surface membrane.

SPECULATIONS

Early Lactoferrin Containing-Granule Release and Coincident Up-Regulation of Surface Membrane Moieties

As indicated previously, recent reports have shown that activation of neutrophils causes increased expression of certain surface membrane components possibly though the mobilization of intracellular pools of these components (Fearon and Collins, 1983; Lanier et.al., 1985; Thrall et.al., 1980; Zigmond et.al., 1982). Secondary granule membranes have been implicated as the intracellular pool for at least some of these membrane moieties (Arnaout et.al., 1984; Fletcher and Gallin,1980; Todd et.al.,1984). Therefore, low level secondary granule secretion, resulting in granule-plasma membrane merger, could be the mechanism for the up-regulation of these components (Arnaout et.al., 1984).

A temperature transition from 4C to 37C has been reported to mediate spontaneous temperature dependent increases in CR1, the C3b receptor, and CR3, the iC3b receptor, analogous to the Mo 1 antigen (Berger et.al., 1984; Fearon and Collins, 1983). These investigators found two-fold increases in surface expression of each receptor after neutrophils, which had been isolated in the cold, were incubated for approximately 6 minutes at 37C. Treatment of cells with protein synthesis inhibitors had no affect on the spontaneous up-regulation of the receptors suggesting that the receptors were preformed and localized in an intracellular pool (Berger et.al. 1984). Temperature mediated increase did not represent maximal expression of the receptors as additional up-regulation occurred when the warmed cells were exposed to fMLP (Berger et.al., 1984) or C5a des arg, a chemotactically active cleavage product of the fifth component of complement (Fearon and Collins, 1983). Spontaneous increased expression of CR1 and CR3 was specific for these membrane glycoproteins since the temperature transition had no affect on the concentration of other cell surface components (Berger et.al. 1984; Fearon and Collins 1983).

Charo and coworkers (1985) have reported that spontaneous temperature dependent enhanced adherence occurs in human neutrophils. Cells maintained at 4C were allowed to adhere to albumin coated plastic or cultured human umbilical vein endothelial cells at 37C. Enhanced neutrophil adherence was seen after 2 minutes and plateaued after 16 to 20 minutes. Increased adherence was not detected when the experiments were performed at 4C. Since CR3 (Anderson et.al. 1986; Arnaout et.al. 1983; Dana et.al. 1984; Gallin 1985) and lactoferrin (Boxer et.al., 1982a; Boxer et.al. 1982c; Oseas et.al., 1981) have been implicated in neutrophil-substratum adherence activities the spontaneous temperature dependent adherence reported by these investigators may have been mediated by a coincident spontaneous up-regulation of CR3 and extracellular lactoferrin release.

Although secondary granule membranes have been implicated as the intracellular pool of CR1 and CR3 (Arnaout et.al., 1984; Todd et.al., 1984) O'Shea and coworkers (1985) have reported evidence suggesting that the CR1 and CR3 antigens are localized in distinct intracellular pools. Neutrophils isolated from a patient with secondary neutrophilic granule deficiency were activated with fMLP and evaluated for expression of the complement receptors. Increased binding of labeled anti-CR1 was found while the binding of anti-CR3 did not increase. Neutrophils isolated from a normal donor and identically activated with fMLP exhibited up-regulation of both CR1 and CR3. Normal cells were also disrupted and fractionated on sucrose density gradients. The fractions were then evaluated for the ability to bind labeled anti-CR1 and anti-CR3. The plasma membrane fraction reacted with antibodies against both receptors while the secondary granule fraction only bound anti-CR3. Neither anti-CR1 nor anti-CR3 bound to the primary granule fraction. These findings suggested that the intracellular pool of CR1 was not only distinct from the pool for CR3, but also suggested that intracellular CR1 may not be associated with classical secondary granule membranes.

Berger and associates (1985) have reported that the mechanism for spontaneous CR1 and CR3 up-regulation differs. Temperature dependent increased expression of CR1 was found to be independent of extracellular Ca++ occurring in the presence of 5mM EDTA. However, the mobilization of intracellular Ca++ was determined to be necessary for up-regulation. In contrast, up-regulation of CR3 required both extracellular Ca++ and mobilization of intracellular Ca++ stores.

Additional evidence suggesting that the intracellular pools of CR1 and CR3 may not be identical and further suggesting that lactoferrin may not be directly associated with the pool for CR3 is provided by the human promyelocytic leukemia cell line, HL-60. When induced with retinoic acid (RA) or dimethylsulfoxide (DMSO) HL-60 cells differentiate into neutrophil-like cells expressing granulocyte surface receptors (Atkinson and Jones, 1984; Hoyle and Freer, 1984; Pommier et.al., 1984). While both RA and DMSO have been found to induce lysozyme production, neither agent has been reported to mediate the production of lactoferrin (Harris and Ralph, 1985; Olsson et.al., 1981). DMSO has also been reported to induce surface expression of Mo 1 (i.e., CR3) (Pommier et.al., 1984).

In consideration of the published reports described above, the data presented in this study may suggest that lactoferrin is closely associated the intracellular pool of the CR1 receptor. Kinetically, the reported temperature dependent increased surface expression of CR1 and the lactoferrin release presented here appear to be identical, both occurring and plateauing within 6 minutes at 37C. The reported CR1 up-regulation and the early temperature dependent lactoferrin release presented in this investigation both occurred in the presence of 5mM EDTA suggesting that extracellular calcium was not essential for either event. While some reports propose that both CR1 and CR3 are associated with secondary granules there is evidence indicating that they are localized in distinct intracellular pools. Since the data presented here appear to indicate that a population of selectively or sequentially secreted lactoferrin-containing peroxidase negative granules exists in normal adult neutrophils, such granules may represent the distinct, yet secondary granule pool of CR1. This hypothesis requires further investigation.

Mechanism for Apparent Regulation of Increased Exposed Surface Membrane

The data presented in these investigations suggest that the release of lactoferrin-containing peroxidase negative granules may be associated with an alteration in the quantity of exposed cell surface membrane. In these studies surface-to-volume ratios were determined to describe the 2 dimensional perimeter and area measurements made from electron micrographs in 3 dimensional terms. The calculated S/V was then multiplied by a mean neutrophil volume determination (Korchak and Weissmann, 1978) to estimate the amount of surface membrane exposed on a whole cell. The results from the ultrastructural determinations made in these studies suggested that an increase in exposed surface membrane may require a critical level of peroxidase negative lactoferrin-containing granule loss, was transient and that neutrophils have a mechanism for apparently regulating the amount of exposed membrane.

This apparent "regulation" of surface membrane may actually be a "compensation" response which is initiated by cell activation. This possibility appears to be suggested by examining the perimeter and area measurements made from cell profiles (also see Appendix B). The calculated exposed surface membrane depends upon a relationship between surface area and volume, or in two dimensional terms, perimeter and cross-sectional area. Therefore, a change in perimeter or area relative to one another produces an alteration in the calculated S/V ratio and consequently a change in the calculated exposed surface membrane. For example, the mean perimeter determination made from micrographs of adult neutrophils activated for

20 seconds with 1nM fMLP was significantly larger than the determination made from nonactivated cell profiles, while the mean area measurement was not significantly different. Thus, the perimeter increased relative to the area and the calculated S/V was larger than the control value. Consequently, the calculated exposed surface membrane for adult cells activated with 1nM fMLP for 20 seconds was greater than that for nonactivated control cells (Table 10). In contrast, while the average perimeter measurement of neonatal neutrophils exposed to 1nM fMLP was not significantly different from control, it was less. The mean area measurement of these cells was equivalent to control values. Since the perimeter decreased relative to the area, the calculated S/V was smaller than the baseline value. Thus, the calculated exposed surface membrane of neonatal cells treated with 1nM fMLP was less than that of unactivated control newborn cells (Table 19). Adult cells treated with 100nM fMLP for 20 seconds or 100nM A23187 for 5 minutes might provide an example of exposed membrane "regulation". In these cells, both perimeter and area measurements were significantly larger than control values. Since both perimeter and area increased relative to each other, the calculated S/V ratio was comparable to control and no significant difference was detected in exposed surface membrane determination (Tables 10 and 16).

In each fMLP and A23187 experimental condition examined, adult cells responded by increasing in perimeter (probably reflective of ruffling) and, to a degree, their cross-sectional area (Figures 6 B and 10 B). Therefore, the results from the morphometric determinations could indicate that an initial result of activation mediated neutrophil ruffling and coincident granule release may be granule membrane and plasma membrane fusion resulting in increased exposed surface membrane. By subsequently increasing in cross-sectional area neutrophils may compensate for, or apparently regulate, this increase in exposed plasma membrane.

The morphometric measurements made on electron micrographs of A23187 treated neonatal neutrophils demonstrates a lack of this proposed compensation response. As shown in Table 22, the mean perimeter determinations of newborn cells treated with 100nM or 250nM A23187 for 5 minutes were significantly increased over baseline measurements, while the average area determinations were identical to baseline levels. Therefore, the S/V increased significantly, as did the calculated exposed membrane determination.

In 2 dimensional measurements, as those made on the electron micrographs, area determinations are representative of cell volume. Therefore, an increase in the cross-sectional area of a cell profile over baseline levels may be indicative of an increase in volume. Hsu and Becker (1975) and O'Flaherty and associates (1977) have reported that neutrophil activation causes an increase in cell volume. However, these determinations were made with electronic particle analyzers which do not take into consideration activation mediated cell ruffling or bipolar configurations. Conceivably, cellular shape changes could have been misrepresented by these analyzers as changes in cell volume. A recent report (Grinstein et.al., 1986) assessed the volume of activated human neutrophils by three independent methods, forward angle light scattering, isotopic water space determinations and electronic sizing, and concluded that cell swelling did indeed occur in response to neutrophil activation with fMLP. Maximum increases of approximately 21% over the basal cell volume were found to occur within three minutes exposure of cells to 10nM fMLP at 37C, with half-maximum increases in about 30 seconds. Activation mediated volume increases were inhibited by amiloride (a K+ sparing diuretic which inhibits extracellular Na+/intracellular H+ exchange) or by replacing extracellular Na+ with K+ or a non-membrane permeating organic cation, N-methyl-D-glucamine+. These results suggested that sodium ions play a critical role in activation mediated neutrophil volume alterations.

Exposure of human neutrophils to fMLP has been reported to cause increased Na+ fluxes. Simchowitz (1985a) determined that labeled extracellular Na+ passively diffused in and out of resting human neutrophils through a oubain- and amiloride-insensitive ion channel. (Ouabian blocks the Na+/K+ pump by inhibiting Na+/K+ ATPase.) When the cells were exposed to 100nM fMLP Na+ influx was enhanced approximately 30 fold. Influx was found to be extracellular Na+ concentration dependent, saturable, sustained for at least 60 minutes after exposure of cells to fMLP, membrane voltage independent and oubain insensitive. fMLP was also found to stimulate the efflux of Na+ from cells preincubated with labeled Na+, with 50% maximum occurring at approximately 2.5 minutes following cell exposure to 100nM fMLP.

Despite Na+ efflux, the net intracellular Na+ concentration has been reported to increase in response to neutrophil activation. Using flame photometry, Simchowitz (1985a) and Grinstein et.al. (1986) reported that intracellular Na+ levels increased approximately 2 fold and plateaued within 5 minutes after exposure of cells to 100nM fMLP, with 50% maximum concentration reached in about 60 seconds (Simchowitz 1985a). While amiloride had no affect on Na+ fluxes of resting cells, it inhibited activation induced Na+ in- and effluxes. Additionally, monensin, an ionophore which facilitates the exchange of extracellular Na+ for intracellular H+, was found to mimic fMLP induced Na+ fluxes (Simchowitz 1985a). The results from these studies suggested that fMLP activated an amiloride-sensitive Na+/H+ exchange or counter transport mechanism which was dormant in nonactivated, resting human neutrophils.

Such a Na+/H+ counter transport system has been reported by Grinstein and Furuya (1984; 1986) and Simchowitz (1985b) to mediate a biphasic cytoplasmic acidification/alkalinization response in activated neutrophils. To measure intracellular pH changes these investigators pre-loaded cells with fluorescein derivatives which are quenched as the pH is lowered. Within 30 seconds following cell activation with 10nM or 100nM fMLP the cytoplasmic pH was found to drop 0.10–0.15 units from a resting level of approximately 7.20. Such cytoplasmic acidification is thought to be associated with respiratory burst activities (Grinstein and Furuya, 1986). Within 60 seconds after cell activation the pH increased by approximately 0.20 units over the resting level, resulting in cytoplasmic alkalinization. Amiloride blocked the alkalinization suggesting that the pH alterations were caused by the same Na+/H+ counter transport mechanism proposed to be mediating the increased intracellular Na+ concentration and volume increases found in activated neutrophils.

In summary, the studies described above propose that within 30 seconds following exposure of human neutrophils to nM quantities of fMLP the cell cytoplasm becomes acidic and an amiloride sensitive Na+/H+ counter transport mechanism is activated. Intracellular H+ is exchanged for extracellular Na+ which results in cytoplasmic alkalinization and a concomitant increase in intracellular Na+ concentration. Osmotically obliged water is thought to enter the cells as a result of the increased intracellular Na+, mediating cell swelling. Increased cellular volume was detected as early as 30 seconds after the addition of 10nM fMLP, cytoplasmic alkalinization within 60 seconds (Grinstein and Furuya, 1984; Grinstein and Furuya, 1986; Grinstein et.al., 1986).

The activation mediated cross-sectional area increases found in the studies presented in this report may be explained by the Na+/H+ counter transport mediated events proposed above. The area determinations made from profiles of adult neutrophils activated for 20 seconds with 1nM or 100nM fMLP showed average increases of 8.6% or 14.6%, respectively (Table 10 and Figure 6 B). These values are within the 10% half-maximal volume increases reported by Grinstein et.al. (1986) to be reached approximately 30 seconds after exposure of human neutrophils to 10nM fMLP.

CONCLUSIONS

The purpose of these studies was to investigate the early morphological and functional responses of human neutrophils to activation. The results from evaluations of cellular shape change made with light and electron microscopy, and granule secretion investigated both biochemically and ultrastructurally suggest that 1) a population of peroxidase negative granules containing relatively high concentrations of lactoferrin exists in adult neutrophils, 2) this population of granules undergoes sequential or selected release, and 3) the release of these granules is coincident with the earliest visible indication of cellular shape change (i.e. ruffling) and may be associated with a transient, apparently regulated increase in exposed cell surface membrane.

As summarized in Figure 20, the following sequence of events is proposed by these studies to occur early in adult neutrophil activation: 1) Brief activation of neutrophils mediates cellular ruffling and the coincident mobilization of a select population of peroxidase negative granules containing large quantities of lactoferrin. 2) These granules merge with the plasma membrane releasing lactoferrin into the extracellular environment. The resulting granule-plasma membrane fusion provides a mechanism whereby specific surface membrane components (for which the intracellular pool is the membrane of secondary granules) are up-regulated and the quantity of exposed surface membrane is increased. 3) An activation induced increase in cellular volume, seen in the ultrastructural morphometric determinations made in these studies as an increase in cross-sectional area, subsequently occurs. This volume increase provides a "compensation" for the increase in exposed surface membrane which, by the methods used in this study, appears to be regulated and returned to basal levels.

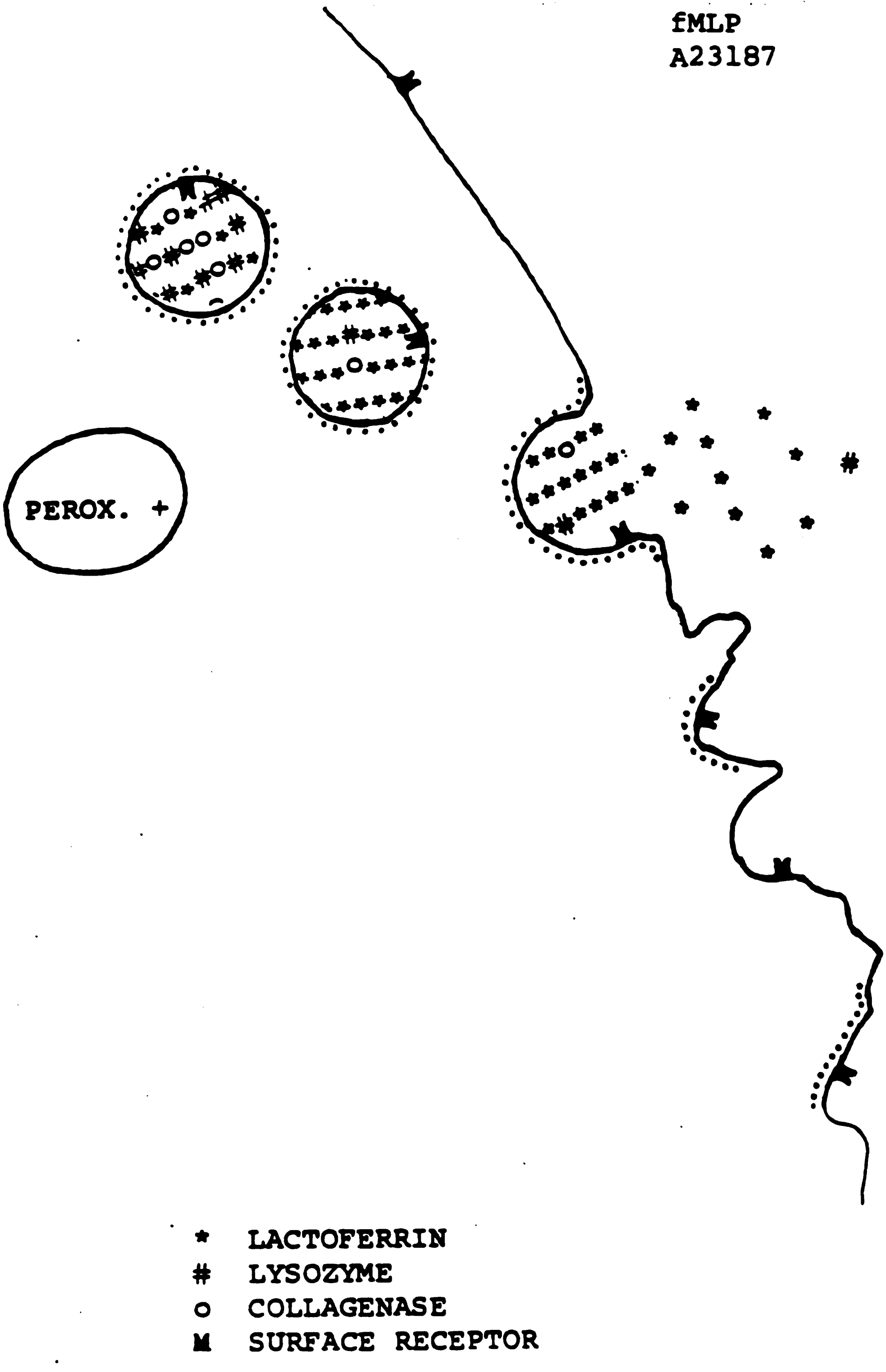


Figure 20
PROPOSED EARLY EVENTS OF NEUTROPHIL ACTIVATION

APPENDICES

APPENDIX A

Neutrophil Isolation by Counterflow Centrifugal Elutriation

The counterflow centrifugal elutriation isolation technique is based on two opposing forces, centrifugal and centripetal, occurring simultaneously within a separation chamber. The centrifugal field is generated by a spinning rotor, the viscous drag of fluid flowing in the opposite direction creates the opposing centripetal force. As the rotor is spinning leukocyte-enriched blood samples are loaded into the separation chamber at a given flow rate. Maintaining this flow rate, elutriation buffer is pumped into the chamber. Due to the opposing forces each cell tends to migrate to a zone where its sedimentation, due to centrifugal forces, is balanced by centripetal force, the flow rate of elutriation buffer through the chamber. By watching the elutriation process through a view port situated above the spinning rotor the formation of an erythrocyte-rich region in the upper portion of the separation chamber can be seen. The flow rate of buffer through the chamber is then increased. As each cell again tends to migrate to the zone where its sedimentation is balanced, lower density erythrocytes are washed from the chamber. By continuing to increase the flow rate of buffer successive populations of larger cells or cells of greater density can be elutriated from the separation chamber. Samples of the elutriant are monitored as the flow rate increases and the process is stopped before neutrophils are washed from the chamber. The chamber is removed from the centrifuge and the isolated neutrophils are the collected.

APPENDIX B

Morphometric Determinations Made of Hypothetical Neutrophils Utilizing Digitizing Tablet

Measurements of hypothetical cells were made for two reasons; 1) to assure that the Houston graphics tablet and Optomax software used in the ultrastructural morphometric measurements of this study were sensitive enough to detect subtle changes in neutrophil morphology and 2) to attempt to better understand the process of neutrophil ruffling. Hypothetical cells were paper cut-outs approximating the size of cells at 18,000 X magnification. The "cells" were made to structurally represent electron micrograph profiles of unactivated spherical cells and neutrophils in the process of ruffling. Two possible mechanisms of cellular ruffling were mimicked with hypothetical cells; 1) membrane redistribution, where theoretically any "new" membrane expressed on the cell surface as a pseudopod would be at the expense of a loss or redistribution of membrane at some other site and 2) pure membrane addition where "new" membrane was simply added to the cells membrane (for example, through granule and plasma membrane fusion). For each mechanism a series of 12 measurements representing a process of neutrophil shape change from spherical to ruffled were made.

The membrane redistribution mechanism was assumed to cause a form of ruffling in which cellular perimeter changed but cellular area did not. To this end, pieces were cut out of the initial hypothetical "spherical cell" and pasted onto the "cell surface membrane". As the cells progressed in the ruffling process, more pieces were cut out and pasted on. The net result was a "cell" with both invaginations and evaginations. Morphometrically this process produced a series of determinations which exhibited an increase in perimeter but remained fairly constant in area (see Table B 1). Figure B 1 shows the computer print out of every third digitized "cell" in the series of measurements.

The process of membrane addition as a mechanism of cellular ruffling was thought to roughly simulate the event of extracellular granule release resulting in granule—plasma membrane fusion. To achieve a theoretical representation of this process, pieces of the hypothetical spherical cell were again cut out and pasted on the "cell" surface to mimic pseudopod formation but this time the cut out regions were returned to a smooth contour. By this mechanism, evaginations were created but invaginations were not. Therefore, both cellular area and perimeter should have exhibited an increased. Table B 2 shows the determinations made from the series of hypothetical cells used to mimic neutrophil ruffling caused by membrane addition. Figure B 2 is the corresponding computerized printout of every third digitized "cell" in this series.

The results from these hypothetical cell determinations indicated several points. In terms of evaluating the methodology used in these studies, the results from the hypothetical cells indicated that subtle morphological alterations in cell shape could be detected with the graphics tablet and software. Additionally, the results from these

Table B 1
HYPOTHETICAL CELL MORPHOMETRIC DETERMINATIONS: MEMBRANE REDISTRIBUTION

Figure	Area	Perimeter	S/V
1	84.18	34.57	0.615
2	86.99	51.99	0.89
3	86.99	55.13	0.95
4	87.68	69.75	1.19
5	87.49	71.02	1.21
6	88.61	82.79	1.40
7	89.05	95.66	1.61
8	89.00	105.20	1.77
9	88.69	113.24	1.91
10	89.11	120.72	2.03
11	76.55	121.80	2.37
12	76.62	123.61	2.42

Table B 2
HYPOTHETICAL CELL MORPHOMETRIC DETERMINATIONS: MEMBRANE ADDITION

Figure	Area	Perimeter	S/V
1	89.29	33.07	0.55
2	90.57	37.76	0.62
3	91.58	41.18	0.67
4	91.75	44.92	0.73
5	92.54	47.84	0.77
6	93.14	50.95	0.82
7	94.44	54.94	0.87
8	95.86	60.56	0.95
9	96.08	62.06	0.97
10	96.70	63.41	0.98
11	96.76	65.22	1.011
12	96.93	65.73	1.017

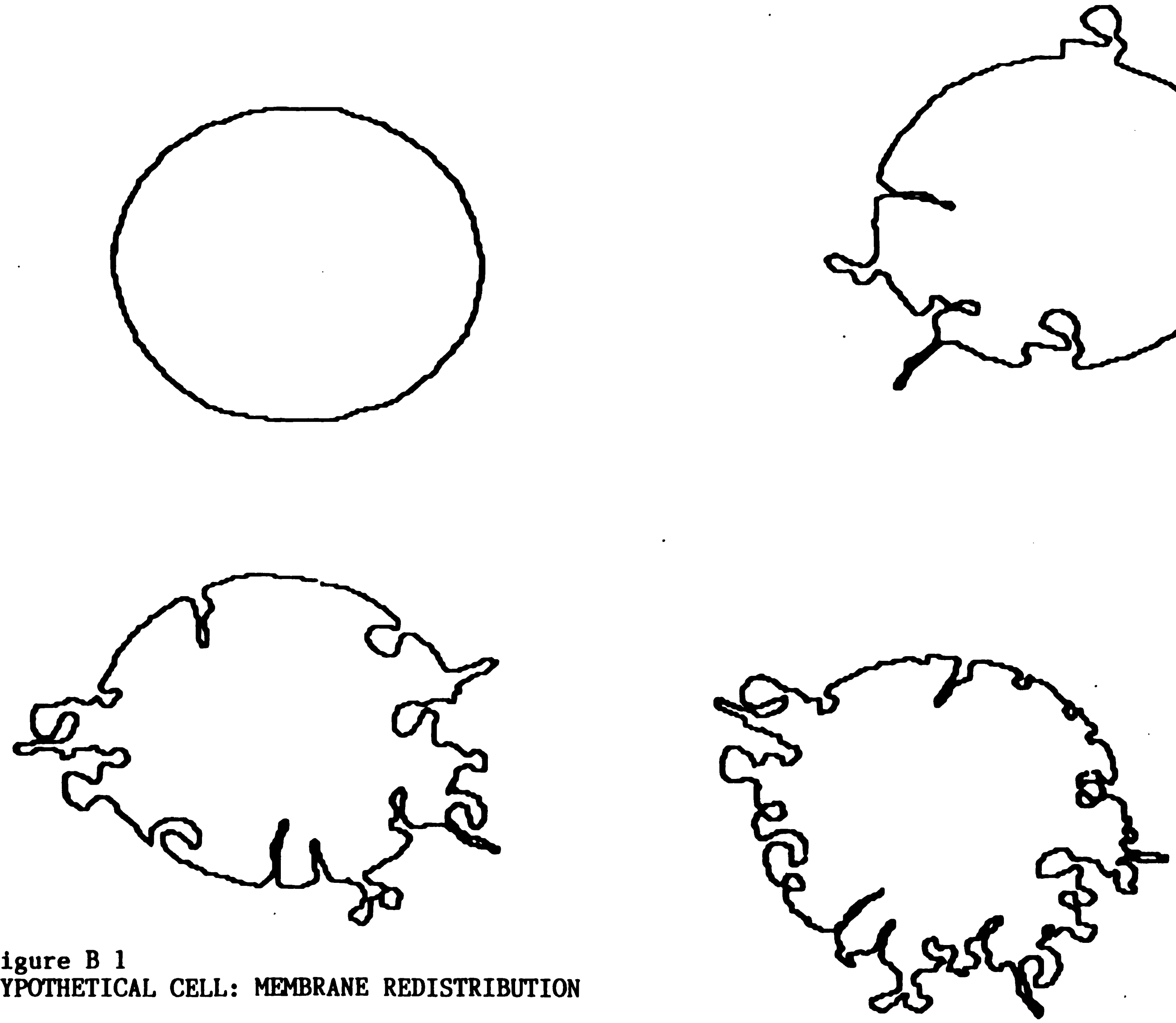


Figure B 1
HYPOTHETICAL CELL: MEMBRANE REDISTRIBUTION

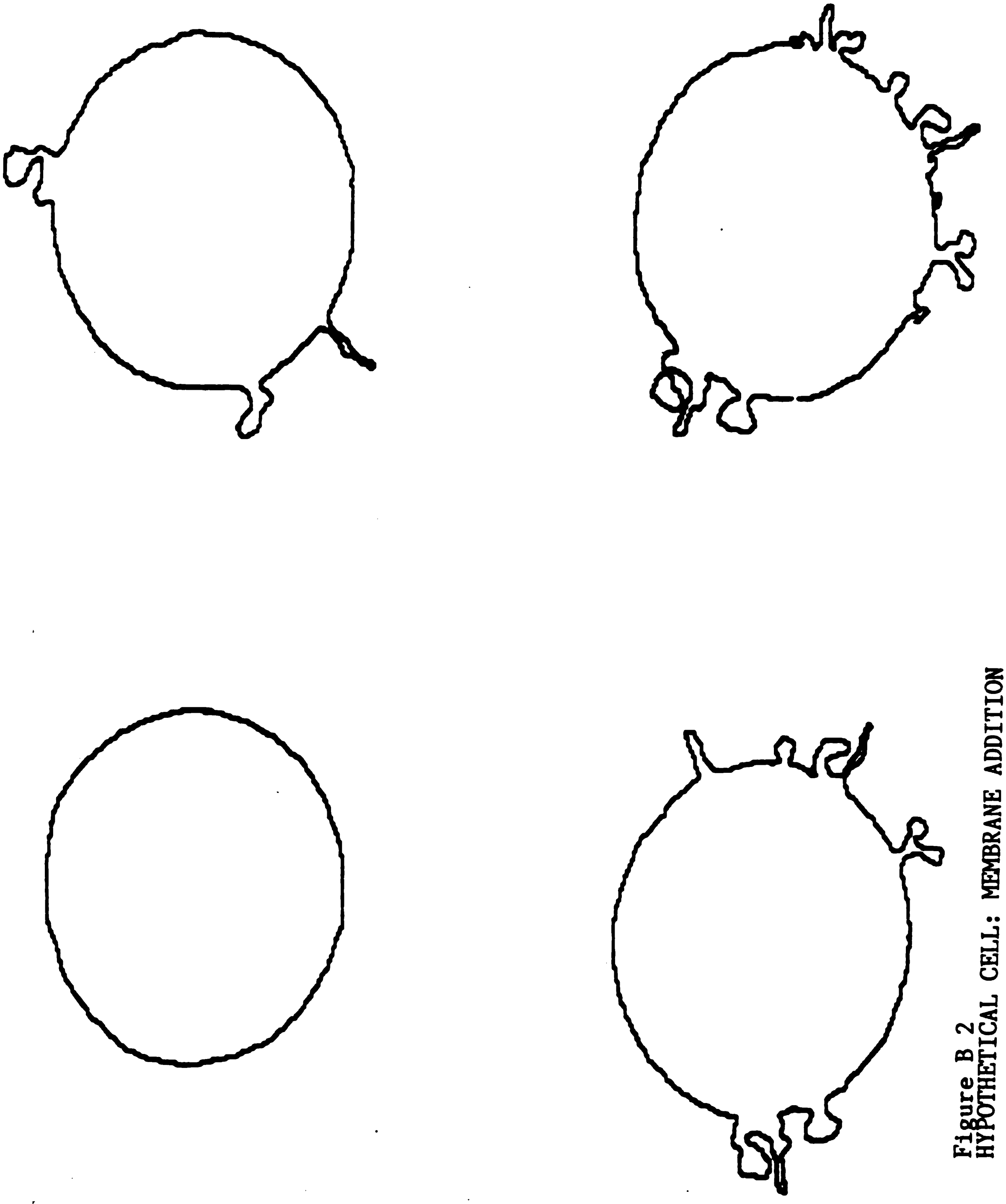


Figure B 2
HYPOTHETICAL CELL: MEMBRANE ADDITION

determinations, when compared with those made from electron micrographs of actual nonactivated and ruffled cells, seem to provide some insights into the process of neutrophil ruffling. First, the hypothetical cell results appear to indicate that ruffling of adult neutrophils is probably more complex than a membrane redistribution. As seen in Tables 10 and 16, and Figures 6 B and 10 B, brief exposure of cells to fMLP or A23187, respectively, mediated an increase in cross-sectional area as well as a significant increase in cell perimeter. The hypothetical cell determinations representing membrane redistribution on the other hand, show an increase in perimeter without a concomitant increase in area (Table B 1). Furthermore, the results from hypothetical determinations representing membrane addition could to support the hypothesis that activation mediated neutrophil ruffling also mediates an increase in cell volume and further, that increased cell volume may be a "compensation" response to a transient increase in exposed surface membrane. As seen in Table B 2, the hypothetical "cells" exhibited increase in perimeter and, albeit slight, an increase in area (8.2% in figure # 12). These changes, relative to one another, resulted in an increase in calculated S/V (85% in figure # 12). In contrast, while both the perimeter and area determinations made on electron micrographs of neutrophils exposed to fMLP or A23187 (Tables 10 and 16 and Figures 6 B and 10 B, respectively) also increased in both perimeter and area, the area increase relative to the perimeter was greater (reflected in S/V compared with baseline spherical determinations) and, with the exception of 1nM fMLP, did not result in an alteration in exposed surface membrane. The increase area detected in activated adult cells, therefore, could have involved more than the addition of surface membrane from membrane fusions.

Ionophore treated neonatal cells may represent cellular ruffling due to the membrane addition mechanism. As seen in Table 22 and Figure 13 B, neonatal cells treated with 100nM or 250nM A23187 for 5 minutes exhibited increased perimeter and exposed surface membrane possibly mediated by membrane fusions (significant depletion in peroxidase granules was found; Table 21 and Figure 13 A) without a concomitant increase in cellular area.

These results provide additional support indicating that the mechanisms of neutrophil ruffling and increased surface membrane are more complex than granule-plasma membrane fusion.

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