THE BIOLOGICAL ROLE OF CALCIUM IN ADULT AND
NEONATAL NEUTROPHIL CHEMOTAXIS

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ABSTRACT

Comparative chemotaxis studies of adult and newborn polymorphonuclear leukocytes (PMNs) were performed by a technique which adapted the micropore filter method to a multiwell chamber. Cord blood neutrophils responded to the chemoattractant FMLP with 60% of adult PMN activity. In order to investigate the defect of neonatal PMN chemotaxis as a calcium-associated phenomenon, PMNs were suspended in a calcium-deprived buffer and in the presence of the calcium channel blocker, verapamil, before being assayed for migration. Adult neutrophils responded to both calcium manipulations with reduced chemotaxis as expected--19% inhibition in calcium-deprived media (p < 0.05), and 32% reduction (p < 0.001) with 10^{-4} M verapamil. Neonatal PMNs, however, increased chemotactic activity in the absence of extracellular calcium--26% (p < 0.05) and to a greater extent in the presence of 10^{-4} M verapamil (p < 0.001). This suggests that the flux of calcium which occurs upon stimulation of the neutrophil by chemotactic factors may, in fact, be responsible for the neonatal defect and that inhibitory concentrations of intracellular calcium may be present in the neonatal cell.
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CHAPTER 1

INTRODUCTION

Although medical and technological advancements have contributed to a dramatic decrease in infant mortality in the last twenty years (Wegman, 1982), infection still imposes serious risk upon the welfare of neonates and infants. Bacterial invasion and proliferation can readily occur in all newborns (Eriksson et al., 1983), but the premature, low birth weight and other compromised neonate is particularly susceptible to life-threatening infection. Studies indicate that infection still occurs in a substantial number of newborn intensive care unit (NICU) patients--5%-25% (Eriksson et al., 1982). Eisenfeld and others (1983) reported postmortem discovery of systemic infection in 41% of neonatal deaths.

Numerous maternal, environmental, and neonatal factors are implicated in the cause of neonatal infection. Maternal sepsis and premature ruptured membranes are two common modes of transmission of microorganisms to the developing newborn. In spite of disinfectants and sterile techniques, nurseries, NICUs, and their staff remain contaminated with ubiquitous organisms which can easily gain entry into the poorly defended newborn. Invasive diagnostic and therapeutic procedures which damage the skin and mucous membranes of the neonate promote microbial colonization (Williams, 1982). Finally, the newborns themselves are less capable of resisting these bacterial
insults because of deficiencies in host defense mechanisms. Newborn susceptibility to many pathogens has remained over the years but currently, neonates demonstrate significant vulnerability to Group B Streptococcus and low virulence organisms like *S. epidermidis* and Candida species (Nelson, 1982; Williams, 1982). Susceptibility to such low virulence organisms is one indication of impaired immune defense in the newborn (La Gamma et al., 1983).

Evidence of diminished resistance to infection in neonates exists in all known areas of host protection, specifically, humoral and cellular immunity as well as in the inflammatory response.

The most obvious example of depressed humoral immunity in the neonate is in antibody production. The newborn is protected from infection primarily by maternal IgG transferred through the placenta and by a relatively small amount of synthesized IgM and even less IgA (Cederqvist, 1981). Generally, neonatal serum gamma globulin levels are about two thirds that of adults (Barrett, 1983). Other serum proteins diminished in the newborn are those of the complement system in which qualitative and quantitative deficiencies in both classical and alternative pathways have been demonstrated. Hemolytic titres of newborn complement components are generally one half of adult levels (Ballow et al., 1974; Drew and Arroyave, 1980; Fireman, Zuchowski, and Taylor, 1969) and reach adult activity by about six months of age (Davis, Vallota, and Forrestal, 1979). Opsonic activity (formation of C3b) by the alternative pathway of complement in neonates appears to be reduced (Feinstein and Kaplan, 1975; Mills, Bjorksten, and Quie, 1979) and probably to a greater extent than opsonic activity.
initiated by the classical pathway (Edwards et al., 1983; Stossel, Alper, and Rosen, 1973). Among other protective chemical substances, lymphokine production as measured by migration inhibition factor and leukocyte inhibition factor is also reportedly low in newborns (Winter et al., 1983), as is the synthesis of immune interferon (Davies, Isaacs, and Levinsky, 1982).

Aspects of cellular immunity in the newborn which have been shown to be different than in the normal adult include an increase in total lymphocyte count, and a decrease in percent of E rosette-forming cells (Wara and Barrett, 1979) and abnormal lymphocyte proliferative responses to certain ubiquitous antigens (Leikin, Whang-Peng, and Oppenheim, 1970). Neonatal T cells have also been shown to elicit diminished delayed hypersensitivity reactions (Uhr, 1960) as well as impaired functions in cytotoxicity (Kohl, 1983).

Finally, there is also evidence of deficient neonatal function in phagocytic or nonspecific processes of host protection. This critical element of immunity comprises the first line of human defense against invading pathogens or toxic substances—the inflammatory response. Abnormalities of neonatal phagocytic cells have been identified in adherence (Anderson, Hughes, and Smith, 1981), aggregation, (Mease, Burgess, and Thomas, 1981) and migration toward a chemical stimulus (Fontan et al., 1981; Tono-Oka et al., 1979). Altered aspects of biochemical killing of microorganisms (Ambruso, Bentwood, and Johnston, 1979; Mills et al., 1979) and of control over the ensuing toxic metabolic products have also been demonstrated (Shigeoka et al., 1981; Strauss et al., 1980). Furthermore, increased production
and release of granulocytes by the bone marrow, in response to
the stimulus of infection, is reportedly impaired in neonates.
Christensen and Rothstein (1980) described depletion of the bone
marrow storage pool in septic newborns, which implies that not only
is there a functional deficiency in phagocytic cells of the neonate,
but the resupply system is also lacking.

These examples of immune and inflammatory defects have been
described in the normal term newborn infant. In the premature,
infected or otherwise stressed infant, many of the above problems are
exacerbated (Cederqvist, 1981; Laurenti et al., 1980; Sacchi et al.,
1982), and some additional difficulties in host defense occur
(Chirico et al., 1983; Miller, 1979; Shigeoka et al., 1979). A
better understanding of these defense mechanisms is therefore essen­
tial to the treatment and prevention of neonatal infection and to
further reduction in neonatal mortality as a consequence of infec­
tion.

The functional defect of neonatal immunity which is the sub­
ject of this study is neutrophil chemotaxis. Directed locomotion by
chemically stimulated cells toward an area of insult is a critical
step in the inflammatory process and therefore in host defense.
There is general agreement among investigators that such migration is
impaired in newborn neutrophils (Anderson, Hughes, and Smith, 1981;
Boner, Zeligs, and Bellanti, 1982; Fontan et al., 1981; Mease et al.,
1980; Tono-Oka et al., 1979).

In addition to the myriad of other physiological processes
including enzyme reactions, bone metabolism, hemostasis, and muscle
contraction, calcium functions in the activation and movement of cells. Studies indicate that there is direct involvement of calcium in chemotaxis—the exact role, however, has not been elucidated. Some investigators have concluded that the primary calcium event influencing neutrophil motility is calcium influx (Boucek and Snyderman, 1976; Snyderman and Goetzl, 1981), and that chemotaxis is depressed if calcium is not included in the extracellular milieu (Estensen et al., 1976). Others report that calcium efflux is essential for optimal chemotaxis (Gallin and Rosenthal, 1974). Since transport of calcium across the cell membrane is known to be an important part of so many physiologic processes of the cell (Elbrink and Bihler, 1975), it seems logical to determine if the abnormality in calcium flux could account for the functional defect in the neonatal neutrophil. Perhaps a qualitative or quantitative abnormality of cellular calcium exists which alters the neonatal cell's ability to recognize or respond to the chemotactic stimulus.

This study compares adult and neonatal neutrophil chemotaxis in the presence and absence of added extracellular calcium and in the presence and absence of a calcium inhibitor, verapamil, in an attempt to determine if there is a calcium defect of newborn chemotaxis.

The following is a review of what is known about the biochemical and physical events leading to normal chemotaxis, the functional defect in the neonatal polymorphonuclear leukocyte (PMN), the role of calcium in chemotaxis, and some properties of calcium channel blockers.
The Mechanism of Chemotaxis

The phenomenon of neutrophil chemotaxis involves recognition of a gradient of chemical signals at the cell membrane, the subsequent triggering of molecular events including energy-forming processes, and the translation of the latter into movement of the cell.

The stimulus for chemotaxis is derived either directly from invading microorganisms or from the interaction of serum factors with antigens or microorganisms (Wolach, 1982). Among the more potent chemotaxins are the synthetic formyl peptides, especially n-formylmethionyl-leucyl-phenylalanine (FMLP) (Schiffman, 1975) and n-formylnorleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine (Niedel, Wilkinson, and Cuatrecasas, 1979), which in part, represent the amino acid sequences coding the initiation of bacterial protein synthesis (Schiffman, 1975). The activated form of the fifth component of serum complement (C$_5$a) is another potent chemoattractant (Shin et al., 1968). Other substances in plasma during the inflammatory response have also been ascribed chemotactic activity. Kallikrein (Kaplan, Kay, and Austen, 1972) and fibrinopeptide B (Kaplan, Goetzl, and Austen, 1973) are two such products that are formed by the initiation of the coagulation and fibrinolytic systems, respectively. Bacterial factors (Ward, Lepow, and Newman, 1968), lymphokines
(Altman, 1978) and oxidized lipids (Turner, Campbell, and Lynn, 1975), including prostaglandins and leukotrienes, have also been reported to stimulate directed migration. Leukotrienes are released from PMNs which have ingested antigens or other particles (Snyderman and Goetzl, 1981). Leukotriene $B_4$ ($LTB_4$) appears to be a potent a chemotactic factor as the formyl peptides and $C_5a$ (Ford-Hutchison, 1980). Other less defined chemoattractants released by PMNs are probably lysosomal protease enzymes which generate activated $C_5a$ (Goldstein, 1974).

Specific receptors have been demonstrated for the two potent chemotaxins, FMLP (Schiffman, Corcoran, and Aswanikar, 1978; Williams et al., 1977) and $C_5a$ from $C_5$ (Chenoweth and Hugli, 1978 and 1980). Binding studies indicate that attachment of these substances to the neutrophil surface proceeds with high affinity and is reversible (Chenoweth and Hugli, 1978; Schiffman, Corcoran, and Aswanikar, 1978; Williams et al., 1977). Ford-Hutchison et al. (1980) suggested that specific receptors on PMNs for leukotriene $B_4$ exist on the neutrophil surface since $LTB_4$ does not interfere with PMN binding of $C_5a$ or FMLP. More recently, Ford-Hutchison (1983) used an aggregation procedure to indicate the presence of specific receptors for $LTB_4$ on the neutrophil. It has been shown with some chemotactic factors that once binding of one of these substances on the PMN surface occurs, further binding of that substance is inhibited (Niedel, Kahane, and Cuatrecasas, 1979). Niedel reported that internalization of bound receptors occurs within five minutes of attachment in human PMNs (Niedel, Kahane, and Cuatrecasas, 1979).
The mechanism by which neutrophils recognize a gradient of a chemoattractant is not clearly understood. McNab and Koshland (1972) suggested a spatial detecting method in which a cell is capable of comparing concentration differences across its length and orienting toward the greatest concentration of the chemotaxon. Several theories concerning modulation of receptors and receptor activity have recently been proposed to explain the regulation of cell sensitivity to chemotactic factors. These primarily deal with alteration of PMN membrane properties which culminate in increased or decreased number, affinity, or distribution of receptors for chemoattractants (Fletcher and Gallin, 1980; Snyderman, Pike, and Kredich, 1980; Sullivan and Zigmond, 1980; Thrall et al., 1980).

The binding of chemotactic factors to neutrophils initiates a host of molecular events including alterations in transmembrane potential (Korchak, 1978; Kuroki, 1982; Seligmann et al., 1980), which could be associated with previously demonstrated changes in cell surface charge (Schaak et al., 1980) and ion flux (Gallin, Durocher, and Kaplan, 1975; Marasco, 1980), particularly Na⁺, K⁺ and Ca²⁺ (Gallin et al., 1978). There is also a transient rise in cyclic adenosine-3',5'-monophosphate (cAMP) (Simchowitz, 1980; Smolen, Korchak, and Weissmann, 1980) associated with calcium influx as reviewed by Snyderman and Goetzl (1981), and a prolonged increase in cyclic GMP (Hill, 1978). The interaction of chemotactic factors with PMN receptors generates fluctuations in composition of the phospholipids in the plasma membrane (Snyderman, Pike, and Kredich, 1980). The activation of phospholipase which occurs upon stimulation
generates the release of arachidonic acid and its metabolites as has been demonstrated in rabbit neutrophils (Hirata et al., 1979). Utilization of glucose via glycolysis and the hexose monophosphate (HMP) shunt are enhanced to provide the energy necessary for movement, and lysosomal enzymes are released (Wolach, 1982).

The physical effects of these molecular events include cellular swelling due to ionic influx (O'Flaherty, Kreutzer, and Ward, 1977) and the polymerization of microfilaments and microtubules (Gallin and Rosenthal, 1974). Microtubules assemble about the centriole and radiate between it and the nucleus which has relocated from its central position, and attach to the microfilaments. Thus the cell is polarized in the direction of the chemoattractant (Zigmond, 1978). A pseudopod forms filled with cortical gel which is composed of microfilaments and attaches to the substrate at the tip. This becomes the "head" of the oriented cell. At the opposite end of the pseudopod, a "tail" forms which is also filled with cortical gel. Movement of the cell occurs as cytoplasm streams forward and into the advancing pseudopod. Granules flow with the cytoplasm to the cell periphery where they make contact, fuse with the plasma membrane, and release their enzymatic constituents (Gallin, Wright, and Schiffmann, 1978). Fusion of the granule membrane with the plasma membrane is one explanation for the temporary increased number of chemoattractant receptors on the cell surface (Fletcher and Gallin, 1980). Retraction fibers composed of cytoplasm and microfilaments in the tail are pulled out as the cell crawls through its environment (Ramsey, 1974).
Immunofluorescence and other techniques have revealed the presence of contractile proteins in mammalian neutrophils pointing out the similarity of chemotaxis to the movement of muscles. Substances which have been identified in the PMN include actin, actin-binding protein (ABP), cofactor for myosin ATPase, myosin, and gelsolin. Actin, which constitutes 10% of the total neutrophil protein, has been identified in pseudopods. Actin-binding protein comprises 1% of the cell protein and is reported to concentrate in PMN lamellipod and tail (Valerius et al., 1982). It crosslinks actin to a lattice work which makes up the gel. Myosin which is found throughout the cytoplasm and possibly in the plasma membrane amounts to 1%-3% of PMN protein. It contracts actin, and its ATPase activity provides the necessary energy for the contraction (Stossel and Pollard, 1973). Actomyosin, gelsolin and cofactor for myosin ATPase have also been identified in smaller quantities in the mammalian PMN (Valerius et al., 1982). Gelsolin, described as the protein which shortens actin segments so that myosin can act on them, is reportedly stimulated by chemotactic factors and increased intracellular calcium (Valerius et al., 1982; Yin, Zaner, and Stossel, 1980). Directional locomotion of the cell occurs as fragments of the actin lattice gel move away from the area of high calcium concentration and sol formation to an area of low calcium concentration or greater rigidity (Snyderman and Goetzl, 1981; Wolach, 1982).

After the neutrophil directs its migration toward the attractant, it can ingest and kill the invading organism with its elaborate microbicidal systems, including digestive enzymes, acidic
phagolysosomes, cationic proteins, hydrogen peroxide, the superoxide radical, myeloperoxidase halogenation, and singlet oxygen formation (Wolach, 1982).

**Functional Defects of the Neonatal PMN**

Numerous studies have examined the functional capacity of the neonatal PMN in conferring protection from pathogenic microorganisms. Reports of these phagocytic capabilities are, however, discrepant primarily due to the variety of assay methods employed.

Measurements of attachment (Pross et al., 1977), ingestion (Miller, 1969), and killing (Quie and Mills, 1979; Shigeoka et al., 1979 and 1981) indicate that the normal newborn neutrophil behaves in a similar manner to the adult cell. PMNs from in vivo or in vitro stressed neonates, however, exhibit defective microbicidal activity (Chirico et al., 1983; Miller, 1979; Shigeoka et al., 1979 and 1981). In vivo stress refers to such conditions as prematurity or infection, while in vitro stress includes experimental use of extreme dilutions of opsonin, for example, in phagocytosis assays (Matoth, 1952; Miller, 1969), or large ratios of bacteria to PMNs in chemiluminescence (Quie and Mills, 1979).

Some authors have reported enhanced resting PMN metabolic HMP shunt activity in the normal neonate (Anderson, Pickering, and Feigin, 1974; Park, Holmes, and Good, 1970) and depressed stimulated HMPS activity in the infected newborn (Anderson, Pickering, and Feigin, 1974). Oxidative metabolism as determined by the generation of superoxide is elevated both in normal as well as stressed newborns.
(Shigeoka et al., 1981; Strauss and Snyder, 1983). It has been suggested that an autooxidative process may be partially responsible for the killing deficiency observed in PMNs of stressed neonates (Shigeoka et al., 1981; Strauss and Snyder, 1981). Strauss and others (1980) reported decreased levels of the HMP enzymes glutathione peroxidase and catalase in neutrophils of stressed newborns. These are some of the detoxifying agents which control the destructive activity of the high energy oxygen molecules. Without a sufficient quantity of these agents, peroxidative membrane damage can occur, causing malfunction or destruction of the host inflammatory cells and the spread of microorganisms. Perhaps this is one reason for the more frequent dissemination of localized infections in the neonate (Williams, 1982) than that which occurs in adults.

Impaired locomotion of neonatal PMNs has been reported with greater consistency than the abnormalities of microbicidal activity. Numerous techniques have been employed to demonstrate the difference between adult and neonatal chemotaxis including micropore filter methods (Miller, 1971; Tono-Oka et al., 1979; Yegin, 1983), direct microscopic observation (Anderson, Hughes, and Smith, 1981; Boner, Zeligs, and Bellanti, 1982; Miller and Cheung, 1982) including cinemicrographic and microvideotape procedures (Miller, 1980), radiolabeled methods (Mease et al., 1980 and 1981), agarose plate procedures (Boner, Zeligs, and Bellanti, 1982; Klein et al., 1977; Tono-Oka et al., 1979), and the in vivo skin window test (Bullock et al., 1969), among others. These and other studies have probed the mechanism of the neonatal chemotactic defect by investigating the physical
and chemical events of the phenomenon. Some of their findings follow.

Miller (1975) reported decreased deformability of neonatal PMNs exposed to chemotactic factors. Change in shape is presumably necessary for a cell not only to squeeze between endothelial cells while migrating from the blood stream to extravascular tissue, but also for the cell to crawl toward a chemical stimulant or foreign particle. Whether or not deformability is requisite for polarization, however, is uncertain. Fontan and others (1981) reported normal orientation by human neonatal PMNs but defective random and stimulated random movement--chemokinesis. Anderson, Hughes, and Smith (1981) demonstrated by phase contrast microscopy a change in configuration of neonatal PMNs exposed to chemotactic factors similar to that which occurred in adult controls, but a reduced ability to form a tail or uropod. They also reported abnormal adhesive responses of neonatal cells to sequential chemoattractant stimulation. According to some authors, adhesion is a necessary preliminary event to migration as a cell must adhere to vascular endothelium before translocating to the tissue space (Craddock et al., 1979). In the study of Anderson, Hughes, and Smith (1981), neonatal PMNs did not increase adherence to a serum-coated glass substrate upon initial single exposure to chemoattractant as did adult control cells. Restimulation with chemotactic factors caused a decrease in adherence in adult cells presumably due to redistribution of adhesion sites to the uropod (Smith and Hollers, 1980). Adhesion under these conditions in neonatal cells, however, was not diminished, and
distribution of adhesion sites (as revealed by locations of PMN binding to albumin-coated latex beads) remained random (Anderson, Hughes, and Smith, 1981). From these studies, it appears that the neonatal PMN defect could be in modulation of cell surface moieties which promote adhesion and possibly locomotion.

The above-mentioned works as well as several other studies indicate that the functional defect of the neonatal PMN may occur at the level of the cell membrane. There does not appear to be an abnormality in detection of the chemotactic signal in newborn neutrophils. Several studies have demonstrated similar binding characteristics toward chemoattractants in the adult and neonatal PMN (Anderson, Hughes, and Smith, 1981; Stark and Kahane, 1983). The abnormality in the newborn is most likely in the response of the neutrophil to ligand attachment. Mease, Burgess, and Thomas (1981) proposed that neonatal PMNs which exhibit irreversible aggregation may not be capable of leaving the microvasculature. This irreversible aggregation could also partially explain the apparent granulocyte storage pool depression which accompanies neonatal sepsis (Christensen and Rothstein, 1980).

Localization of ligand receptor complexes to a specific site on the cell surface requires molecular communication between the cell membrane and cytoskeletal elements. Normal adult neutrophils have demonstrated this "capping" phenomenon when exposed to Concanavalin A (Con A). The microtubule disrupter, colchicine, significantly enhanced the response (Oliver and Zurier, 1976). Neonatal cells, however, did not respond to colchicine with increased Con A capping,
indicating, again, a membrane interaction deficiency (Kimura et al., 1981). Also at the level of the cell membrane, studies of beta-adrenergic receptors in the neonatal PMN have revealed a decrease in number but similar affinity, when compared with adult cells. This diminished number of receptors could depress adenylate cyclase activity which would inhibit the transient rise in cAMP associated with chemotactic stimulation (Roan and Galant, 1982). The finding of increased negative surface charge, decreased deformability, decreased adhesiveness, decreased cell spreading and pseudopod formation in immature granulocytes (Lichtman and Weed, 1972) is an indirect indication that the cell surface defects associated with neonatal chemotaxis may indeed be developmental.

Calcium and PMN Function

Calcium is involved in membrane activation in numerous cell types, where it often acts as a second messenger invoking a variety of physiologic responses. There is strong evidence of an association of calcium with many aspects of the neutrophil inflammatory function including adherence (Gallin, 1980), motility (Boucek and Snyderman, 1976; Estensen et al., 1976; Gallin and Rosenthal, 1974), granule secretion and superoxide generation (Simchowitz and Spilberg, 1979), among others. Calcium has also been linked to several chemotaxis-associated events including membrane ruffling (O'Flaherty and Ward, 1979; Oseas et al., 1980), deformability (Miller and Myers, 1975), alteration in membrane polarization (Mottola and Romeo, 1982), surface charge (Gallin, 1980), and activation of several neutrophil
substances of biologic significance. Activation of phospholipase A₂, for example, contributes to release of arachidonic acid and its chemotactic metabolites (Hirata et al., 1979), particularly LTB₄. Calcium also appears to influence activation of an esterase which may serve a regulatory function in chemotactic factor-receptor interaction (Mehta et al., 1981). Finally, a calcium-related activation of cAMP occurs upon stimulation of PMNs with chemotaxis (Simchowitz et al., 1980; Smolen, Korchak, and Weissmann, 1980). The biologic effects of cAMP elevation are uncertain, however, at present (Snyderman and Goetzl, 1981).

Membrane potential studies have been used (Gallin, Durocher, and Kaplan, 1975; Hoffstein, Korchak, and Smolen, 1981) along with measurements of electrophoretic mobility (Schaak et al., 1980) to demonstrate the change in surface charge which occurs when neutrophils are activated by ligands or chemotactic factors. This change which reflects altered membrane permeability to ions is determined by the rate of uptake of radioactive lipophilic cations or lipophilic cationic dyes by the PMN (Seligmann and Gallin, 1981 and 1982; Seligmann et al., 1980). Exposure of PMNs to chemotactic factors appears to generate a transient depolarization followed by a more prolonged hyperpolarization (Hoffstein, Korchak, and Smolen, 1981; Seligmann and Gallin, 1981 and 1982; Tatham et al., 1980). There is evidence that calcium is associated with the hyperpolarization phase and that the latter represents calcium activation of the Na⁺-K⁺ pump. For example, Seligmann and others (1980) inhibited the hyperpolarization phase with Mg²⁺ EGTA and high concentrations of K⁺. Similarly,
Tatham and others (1980) reported no hyperpolarization phase in the absence of external calcium as well as in the presence of ouabain and EGTA.

Perhaps more direct evidence of the involvement of calcium in chemotaxis is derived from the interaction of contractile proteins in the neutrophil. Calcium activates and regulates gelsolin—the protein which shortens actin segments and decreases cellular rigidity (Stendahl and Stossel, 1980; Stossel and Pollard, 1973; Valerius et al., 1982; Yin, Zaner, and Stossel, 1980).

In spite of intensive investigation the specific mechanism by which calcium influences chemotaxis has yet to be determined. Studies of the flux of calcium ions occurring in stimulated neutrophils have yielded discrepant findings primarily because of the difficulty in directly measuring intracellular calcium as well as calcium transport across membranes. The following pages will discuss some of the evidence of the rise in intracellular calcium in chemotactically stimulated neutrophils and possible sources of this calcium.

Most investigators agree that the interaction of chemotactic factors with neutrophils evokes a rise in the level of intracellular free calcium ion. Boucek and Snyderman (1976) demonstrated this using $^{45}$Ca uptake studies and showing that a similar concentration of calcium transport blocker—LaCl$_3$—inhibited $^{45}$Ca uptake in PMNs and depressed chemotaxis. Ultrastructural analysis of the morphological events in chemotaxis reveal submembraneous precipitates of pyroantimonate at the leading edge of a stimulated neutrophil. Dissolution of precipitate deposition by EGTA and EDTA and microprobe
determinations indicate that the cation associated with the pyroantimonate precipitates is not Mg$^{+2}$ and is most likely Ca$^{+2}$ (Cramer and Gallin, 1979; Hoffstein, Korchak, and Smolen, 1981). Assays of PMN locomotion through micropore filters in a Boyden chamber have shown depressed chemotaxis when calcium was omitted from the extracellular milieu (Estensen et al., 1976; Wilkinson, 1975). In addition, divalent ionophore A23187 has been shown to enhance chemotaxis by the micropore filter method (Estensen et al., 1976), to promote neutrophil degranulation, and to stimulate superoxide generation (Romeo, Zabucchi, and Soranzo, 1975). This indicates that calcium entry into the cell promotes some PMN function. White and others (1983) have recently reported the use of the calcium-sensitive fluorescent probe Quin-2 to demonstrate a rapid increase in intracellular free calcium in stimulated human and rabbit PMNs. The peak level of calcium influx occurred fifteen seconds after stimulation with FMLP, C$_5$$^a$, and LTB$_4$. Hallet and Campbell (1982) employed a calcium-sensitive photoprotein, Obelin, to demonstrate a two-fold increase in intracellular Ca$^{+2}$ within thirty minutes of stimulation in intact rat peritoneal PMNs.

The source of this rise in intracellular free calcium is uncertain. Some of the studies mentioned previously, such as radioactive uptake assays (Boucek and Snyderman, 1976) and micropore filter determinations with and without exogenous cations (Estensen et al., 1976; Wilkinson, 1975) have led to the inference that there is a transmembrane flux of calcium from the exterior of the cell to the interior. Becker described this mechanism as a "concentration
dependent increase in membrane permeability to calcium which occurs in response to the interaction of chemotactic factors with the neutrophil surface (Becker, Naccache, and Sah'afi, 1983).

Elements of these and other studies, however, indicate that calcium is also made available to the cell from intracellular sources. It may be mobilized from storage pools, released from bound forms in the cytoplasm, or it may be associated with the plasma membrane (Sha'afi and Naccache, 1981). Wilkinson (1975) and Estensen et al. (1976) and others have shown that A23187 enhances chemotaxis even in the absence of added extracellular calcium. This could be interpreted as facilitation of intracellular cation exchange by ionophore (Takeshige et al., 1982). Naccache and others (1981) reported from calcium flux studies in rabbit PMNs that chemotactic concentrations of LTB$_4$ generated a rapid increase in the steady-state level of cell-associated calcium. Without LTB$_4$, there was a rapid transient decrease in steady-state calcium. They interpreted from these findings that LTB$_4$ might be responsible for the release of previously unavailable intracellular calcium.

**Calcium Channel Blockers**

A great deal of the understanding of changes in the level of intracellular calcium and of membrane potential has come from studies in muscle physiology, especially cardiac muscle, where calcium and calcium influx play a central role. Among current pharmacologic tools used to study calcium influx are the calcium channel blockers. These are often termed slow channel blockers because of their effect on the slow inward current of cardiac action potential which
accompanies the flow of calcium ions across the membrane of myocardial cells (Antman et al., 1980). These channels have not been specifically identified but have been described as large protein pores in the cell membrane which may be regulated by internal and external gates. The gates may be activated by an ionic concentration gradient across the cell membrane and/or by agonist-receptor interaction (Antman et al., 1980). Godfraind (1982) divides the channels into three types—leak channels, potential operated channels, and receptor operated channels.

The calcium antagonists differ in chemical structure, affinity, potency, in tissue selectivity and in pharmacological effects. They are generally, however, agents which inhibit entry of calcium into a cell by blocking the enhanced membrane permeability which occurs as a result of depolarization or receptor modulation. Godfraind (1982) claimed that calcium antagonists do not affect mobilization of intracellular calcium or calcium efflux. Walus, Fondacaro, and Jacobsen (1981) and Saida and van Breeman (1983), however, reported inhibition of release of calcium from intracellular storage sites in canine and rabbit mesenteric arteries, respectively, by calcium inhibitors.

Some of the calcium channel blockers commonly used as research tools and in treatment of cardiac disorders are verapamil, nifedipine, diltiazem, and nitrendipine. Verapamil represents a pioneer class of these agents. It binds with high affinity to myocardial cells of the slow channel dependent system and is used in the treatment of arrhythmias (Hulthen et al., 1982). Hulthen and others
(1982) demonstrated specific high affinity binding of $[^3H]$ verapamil to frog heart membranes which was dependent on the concentration of calcium in the medium. This binding of $[^3H]$ verapamil could be displaced by verapamil and another class of calcium antagonist, nitrendipine. Binding of the latter, however, was only slightly inhibited by verapamil suggesting that more than one group of binding sites exists for calcium entry blockers in cardiac membranes.

Verapamil has been used to study other cell systems involving calcium flux. Of particular interest are platelet and neutrophil functions both of which possess intracellular contractile elements. Addonizio and others (1982) reported inhibition of serotonin release and platelet aggregation by verapamil. Simchowitz and Spilberg (1979) demonstrated dose-dependent inhibition of superoxide generation in stimulated PMNs with verapamil. Verapamil has also been shown to inhibit aggregation of stimulated human, guinea pig, and rabbit neutrophils and to prevent pseudopod formation. Transmission electron micrographs illustrate the lack of membrane ruffling in activated PMNs which have been exposed to $9 \times 10^{-4}$ M to $9 \times 10^{-6}$ M verapamil (Oseas et al., 1980).

The following study describes a different method of measuring chemotaxis and looks at the role of calcium in adult and neonatal PMN migration by depriving cells of calcium ions in the suspending media and by preincubating cells with the slow channel antagonist, verapamil.
CHAPTER 3
RESEARCH DESIGN

Adaptation of Boyden Technique and Micromethod

Chemotaxis was performed by an adaptation of the Boyden technique (Boyden, 1962) to a micromethod (Falk, 1980) with the use of a multiwell microchemotaxis chamber (Neuro Probe, Inc., Cabin John, MD 20818). This acrylic chamber assembly measures 40 mm by 20 mm and is equipped with an upper plate containing through-holes and a lower plate with wells. The plates are bolted together with 6 mounting screws and combine to form 48 chemotaxis wells in a single apparatus. A silicone gasket fits between the upper and lower plates on top of the membrane filter and serves to seal the unit.

Advantages. An obvious advantage of such an apparatus is the small volumes of both cells and reagents required for an assay. The wells of the bottom (chemoattractant) plate are each filled with 25-30 microliters of solution. The top wells hold 50 microliters of cell suspension each. When a cell concentration of 2 x 10^6 PMNs/ml is used in an assay, 48 determinations can be performed with a total of approximately 5 x 10^6 cells. This is merely 20% of the average yield (2.6 x 10^7) of PMNs isolated from 10 ml of cord blood by the method described below in this study. By comparison, 48 determinations using the modified Boyden technique as performed in this laboratory would require over ten times the number of cells (Hill et al.,
Moreover, only 10 microliters of stock chemoattractant (10^{-3} M FMLP) and approximately 200 microliters of stock calcium antagonist (5.5 \times 10^{-3} M verapamil) were required for each assay in this study.

The micromethod is also more convenient than the modified Boyden technique in that time is saved in assembling, disassembling, cleaning and staining filters as a unit rather than as numerous individual elements. In addition, the multiwell microchamber uses less space in the incubator than multiple chambers.

Finally, the cost provides a significant advantage over the microchamber method. For example, one could perform an assay with only 5 or 6 determinations using Boyden chambers for the same price as a microassay with 48 testing spaces. Membrane filters are also less expensive (approximately 2¢ per determination) for the 48-well method than for the Boyden technique (approximately 20¢ per test).

**Disadvantage.** One of the primary sources of concern of investigators regarding the multiwell technique is the possibility of cross contamination by fluid leakage between the lower plate wells. While the manufacturer has provided slightly raised rims on the wells to avoid this problem, the minimal overfilling of these wells suggested, to prevent bubble formation later when the filter is applied, reduces the efficacy of such rims. Various measures can be taken in the laboratory, however, to alleviate this situation. Wetting the membrane, for instance, helps to curb excessive fluid spread. In addition, the use of blank wells which contain only buffers in the upper and lower portions, provides a neutral barrier between
potentially antagonistic fluids. We have not, however, observed an increase in random migration when such wells are juxtaposed with chemotaxis wells, as might be expected if fluid leakage exists.

Reproducibility. Reproducibility studies of the micromethod were also satisfactory. Chemotaxis values of donors repeated on two to three different occasions over several months varied an average of 16% of the mean (Table 1). This compared with 13% variability observed in a previous study in this laboratory using the modified Boyden technique (Hill et al., 1975).

Variability among replicate samples of the same donor was less with the multiwell technique than in previously reported triplicate studies using the modified Boyden assay (Hill et al., 1975). Micromethod sampling consisted primarily of triplicate samples of the same donor performed in one experiment but in some cases, four and five replicates were performed. The average standard deviation among replicates of 27 adult donors was 6% of the mean. Replicates of 27 cord blood samples averaged 7% of the mean. Similar replicate studies using the modified Boyden method yielded an average standard deviation of 13% of the mean among 14 donors (Hill et al., 1975).

Identical experiments were performed with two microchambers on one occasion. The resulting chemotaxis counts were within 10% of each other thus demonstrating little variability between chambers.

Filters. The polycarbonate filters recommended by the manufacturer were not used in these experiments because of difficulties in handling and cell staining. We chose, instead, to use cellulose acetate membrane filters (5.0 m pore size and 9 in. diameter)
Table 1
PMN Chemotaxis Values of 8 Adults Tested on Separate Occasions

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Chemotaxis value assay number</th>
<th>Mean ± S.D.</th>
<th>S.D. percent of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200, 185, 236</td>
<td>207 ± 26</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>319, 166, 146</td>
<td>210 ± 95</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>190, 111, ---</td>
<td>151 ± 56</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>213, 265, ---</td>
<td>239 ± 37</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>164, 166, ---</td>
<td>165 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>144, 153, ---</td>
<td>149 ± 6</td>
<td>4</td>
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<tr>
<td>7</td>
<td>171, 212, 193</td>
<td>192 ± 21</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>198, 188, ---</td>
<td>193 ± 7</td>
<td>4</td>
</tr>
</tbody>
</table>

S.D. - Standard deviation
(Millipore Corp., Bedford, Mass. 01730) and cut them to fit the microchamber. Due to the thickness of the cellulose acetate filters, longer incubation periods were required than with polycarbonate filter use. We, therefore, increased the incubation time to three hours as routinely used in the modified Boyden assay in this laboratory (Hill et al., 1975).

In summary, the modification of the micromethod appears to be convenient (especially when using neonatal blood or other samples yielding small volumes), inexpensive and reproducible.

Materials and Methods

Specimens. Samples were obtained from the veins of healthy adult donors and from the umbilical cords of placentas of normal term neonates immediately following delivery. Whole blood in Acid Citrate Dextrose (Becton-Dickinson and Co., Rutherford, NJ 07070) was settled approximately 45 minutes with dextran-6%-MW 70,000 (McGaw Laboratories, Irvine, CA 927140), separated on Ficoll Hypaque (Pharmacia, Inc., Piscataway, NJ 08854), and hypotonically lysed to eliminate erythrocytes. Cell yields of approximately 95% PMNs were resuspended to a final concentration of $2 \times 10^6$ PMNs per ml in a buffer containing 135 mM NaCl, 4.5 mM KCl, 1.3 mM MgSO$_4$·6H$_2$O, 1.5 mM CaCl$_2$, 1 mg/ml dextrose, 10 mM Hepes (Sigma Chem. Co., St. Louis, MO 63178), and 1% bovine albumin--fraction V (Sigma) adjusted to pH 7.2. Minimum Essential Medium (Flow Laboratories, McLean, VA 22102) was used as the buffer in one group of experiments. In some experiments, CaCl$_2$ was omitted from cell suspensions. Calcium chloride and albumin were omitted from the buffer when the latter was used to dissolve the
chemotactic factor and the calcium antagonist. In all experiments plastic implements and containers were used to prevent excessive contamination with exogenous calcium.

Chemotactic factor. Stock solutions (10^{-3} M) of N-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) (Sigma) were made in Hepes balanced salt solutions and stored at -70^{0}C. Working solutions were diluted with the same buffer and approximately 30 microliters were placed in the wells on the lower plate for chemotaxis testing. Control wells on this plate contained the buffer without FMLP for random migration determinations.

Calcium antagonist. Verapamil hydrochloride stock liquid (5.5 \times 10^{-3} M) (Knoll Pharmaceutical Co., Whippany, NJ) was stored at 4^{0}C after opening, for no more than 30 days. Dilutions were made in media without calcium or albumin. Neutrophils (2 \times 10^{7}/ml) suspended in media with calcium were diluted 1:10 in appropriate verapamil concentrations and incubated 15 minutes at room temperature before being placed in the chamber.

Chamber loading and chemotaxis evaluation. Membrane filters and a silicone gasket were applied and the chamber assembled. Fifty microliters of cell suspensions with or without verapamil were placed in the upper wells of the apparatus and incubated in a moist chamber at 37^{0}C in a humidified 5\% CO_{2} incubator. After three hours, filters were removed from the chamber and stained with hematoxylin and permanently mounted. Neutrophils which completely migrated through the filter were counted in 10 random fields using a 10X ocular, 45X objective and a 5 mm by 5 mm photographic reticule. Chemotaxis was
expressed as the number of stimulated cells which migrated through the filter minus the number of unstimulated cells migrating through the filter (random migration). Neutrophils remained approximately 95% viable throughout the test period as determined by Trypan Blue exclusion.

Evaluation of significance was determined by the Student t test.
CHAPTER 4

RESULTS

FMLP Dose Response

In order to determine the concentration of formyl peptide (FMLP) stimulant rendering optimal chemotaxis by this newly devised method, dose response studies were performed. In this group of determinations, Minimum Essential media with 10 mM hepes was used for dilutions of FMLP and, with the addition of 1% bovine albumin, for cell suspensions. Subsequent experiments performed with the hepes balanced salt solution previously described (and used in all other assays in this study) generated similar findings.

Neutrophil preparations from four adults were assayed with concentrations of FMLP ranging from $10^{-10}$ M to $10^{-4}$ M. As is shown in Figure 1, adult PMNs demonstrated a response to the chemoattractant at concentrations as low as $10^{-10}$ M. Chemotactic activity increased with increasing doses of FMLP up to $10^{-8}$ M where peak activity occurred. Greater concentrations of the stimulant appeared to diminish the response such that at $10^{-6}$ M FMLP, almost no chemotactic activity was observed.

The same study was performed on neutrophils from four newborn cord blood samples. Figure 1 illustrates the comparative dose response curves and shows that overall chemotaxis in the neonates was lower than that found in adults but that the response of neonatal
Figure 1. Mean Chemotactic Activity of Adult and Neonatal PMNs to Varying Concentrations of N-Formyl-Leucyl-Methionyl-Phenylalanine (FMLP) (S.E.M. = 1 standard error of the mean.)
cells to varying concentrations of FMLP was similar. In the neonate, chemotactic activity was detected at $10^{-10}$ M and increased with higher concentrations of stimulant to peak levels at $10^{-8}$ M FMLP. Activity rapidly dropped at doses greater than $10^{-8}$ M and was not detectable at $10^{-6}$ M. These findings correlate with the reports of others for optimal chemotaxis concentrations of FMLP with the micropore filter method (Cramer and Gallin, 1979).

It appeared from this data that the qualitative response of neonatal neutrophils to the chemotactic stimulant was similar to that of adults but that the magnitude of the response was depressed. As previously mentioned, others have shown through receptor affinity studies that neonatal neutrophils do not lack recognition capabilities with chemoattractants, indicating that the neonatal deficiency probably occurs in the response of the cell to the chemotactic signal (Anderson, Hughes, and Smith, 1981; Stark and Kahane, 1983).

**Adult vs. Neonatal PMN Chemotaxis**

Further studies were performed to quantify the differences between adult and neonatal chemotaxis seen in the FMLP dose response assays. Results appear in Figure 2.

Neutrophils from 24 adult samples were assayed for chemotactic activity with $10^{-8}$ M FMLP. The mean value of chemotaxis was 197 migrating PMNs/10 random high power fields. The standard error was 10 which represents 5% of the mean. Chemotactic activity ranged from 101 to 319. Neither of these two extreme values fell within 2 standard deviations of the mean, but there was no evidence of physical or experimental abnormality which would warrant their exclusion from the
Figure 2. Chemotactic Responses of 24 Adults and 24 Neonates to FMLP (S.E.M. = 1 standard error of the mean; S.D. = standard deviation.)
data. Such variability is an unfortunate but generally recognized handicap with the micropore filter chemotaxis method (Ward, 1978). The remaining samples demonstrated a smaller range of 138 to 265.

Comparative studies of 24 samples of cord blood PMNs run simultaneously with the adult specimens revealed neonatal chemotactic activity which was significantly lower than that found in adults \( (p < .001) \). As is shown in Figure 2 the mean value of chemotaxis in newborn neutrophils was 117 cells/10 random fields compared to 196±10 in adults \( (60\%±5.7 \text{ S.E.M.}) \). The standard error representing 10% of the mean was 12. Variability of chemotaxis among neonatal samples appeared greater than that found in adults as demonstrated by the standard error--5% of the mean in adults and 10% of the mean in neonates. The range of neonatal chemotactic activity \( (12-226) \) was comparable to that observed in adults. Unlike the findings in adult chemotaxis, however, all values of newborn PMN migration represented by this range were within 2 standard deviations of the mean.

It is interesting to note that 18 of the 24 (75%) neonatal samples studied exhibited chemotaxis values equal to or less than \(-1\) standard deviation of the adult mean while only 3 of the 24 (13%) adult determinations fell at or below \( 1\) standard deviation.

Also of interest in this comparative study is the observation that five newborn samples exhibited severely depressed chemotaxis--less than 50 PMNs/10 random fields. In these cases, neonatal chemotaxis averaged less than 25% of adult controls (Table 2). Records of deliveries were thoroughly reviewed on these patients. There was no obvious evidence of abnormality in the delivery or in the physical
Table 2
Low Neonatal Chemotaxis Values and Adult Controls

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Adult chemotaxis</th>
<th>Neonatal chemotaxis</th>
<th>Neonate % of adult control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>173</td>
<td>38</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>101</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
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<td>12</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>222</td>
<td>25</td>
<td>11</td>
</tr>
</tbody>
</table>
status of the neonate at birth that could account for the markedly diminished chemotaxis found in this small population. Nor were there any examples of comparably low chemotactic activity in adult samples. Clearly, then, the magnitude of the chemotactic response in the newborn as demonstrated by this microchamber filter assay is reduced.

**Effects of Extracellular Calcium**

Considering evidence from the literature indicating the involvement of calcium in neutrophil movement as well as other functions, we chose to investigate the role of calcium in chemotaxis as a potential source of the neonatal defect. Two manipulations affecting the calcium environment of the cell were performed. Results are summarized in Figure 3. The heavy line in this illustration indicating 100% chemotaxis represents different control values for each set of experiments.

The effects of exogenous calcium on directed migration, which have generated variable reports in the past, were studied. Neutrophils were suspended in media free of added extracellular calcium. Analysis by mass spectrometry in another laboratory revealed this "calcium free" media to contain a constant but minimal contaminant of approximately 1 μM calcium. Chemotaxis was compared in cells suspended in this calcium-deficient media with control samples of the same specimen suspended in media containing 1.5 mM CaCl₂. As shown in Figure 3, eight adult PMN samples demonstrated an average reduction in chemotaxis of 19% (±5 S.E.M.) in the absence of calcium.
Figure 3. Effects of Calcium Deprivation and Verapamil ($10^{-4}$ M) on Adult and Neonatal PMN Chemotaxis (Chemotaxis is expressed as mean percent of control chemotaxis with calcium and without verapamil; $n$ = number of determinations; significant difference from controls was determined by the Student t test.)
This slight but significant (p < 0.05) inhibition in chemotactic activity has been reported by others (Estensen et al., 1976).

In striking contrast to the adult cells, neonatal PMNs exhibited enhanced chemotaxis when calcium was omitted from the suspending media. The mean level of directed stimulated migration in calcium deprived neutrophils from nine cord blood specimens improved 26% (±9 S.E.M.) over control chemotaxis in the presence of calcium (p < 0.05). In this case, then, there was not merely a quantitative difference between adult and neonatal chemotactic activity as was observed in the responses of adult and newborn PMNs to varying FMLP concentrations. There was in addition a directional difference between adult and newborn PMN reaction to calcium deprivation—depressed chemotaxis in the adult and enhanced chemotaxis in the neonate.

Effects of Calcium Antagonist

We further examined the involvement of calcium flux in chemotaxis by performing the second calcium manipulation in which neutrophils were exposed to the calcium channel blocking agent, verapamil, prior to chemotaxis determination. Results of chemotaxis in both adult and neonatal PMNs preincubated with varying doses of verapamil appear in Figure 4. Concentrations of the calcium antagonist ranged from $10^{-10}$ M to $10^{-4}$ M.

In neutrophils from 20 adult individuals, chemotaxis was inhibited 32% (S.E.M. = 3) by the highest concentration of verapamil—$10^{-4}$ M (p < 0.001). Succeeding dilutions of the drug diminished chemotaxis significantly (p < 0.05), but to a lesser
Figure 4. Mean Chemotactic Activity of Adult and Neonatal PMNs in the Presence of Varying Concentrations of Verapamil (Chemotaxis is expressed as percent of control chemotaxis without verapamil; the chemotactic stimulant is FMLP.)
12% (Table 3). The lowest dose of verapamil ($10^{-10}$ M) did not alter chemotaxis significantly in the adult from control chemotaxis values without verapamil.

Verapamil did not significantly inhibit chemotaxis in the neonate. In fact, newborn neutrophils responded to the highest concentration of verapamil with increased rather than depressed chemotactic activity. Twenty-four cord blood specimens demonstrated an average of 57% enhancement in chemotaxis at a $10^{-4}$ M dose which was statistically significant ($p < 0.001$). Lower concentrations of the drug, however, did not appear to affect chemotaxis significantly (Table 3).

The standard error values appearing in Table 3 illustrate the large variability which occurred in the dose response of neonatal PMNs to verapamil. This was especially seen at concentrations of $10^{-4}$ M and $10^{-5}$ M where standard errors were 18 and 20, respectively. This range of reactivity appeared to be primarily a reflection of the response of the group of neonates which, as previously mentioned, exhibited extremely low chemotaxis. In some of these cases, chemotaxis improved over 300%. An example of the actual values of chemotaxis in such situations was, for instance, 34 PMNs/10 random fields without verapamil, and 111 PMNs/10 random fields with $10^{-4}$ M verapamil. Excluding the population of newborn PMNs exhibiting extremely low chemotaxis, $10^{-4}$ M verapamil enhanced chemotaxis by 33% (S.E.M. = 7.9; $p < 0.001$). This improvement in neonatal chemotaxis did not abolish the 34% deficit in these same samples compared to adults, but it did reduce the deficiency by one half—to 17% (S.E.M. = 6.2, data
Table 3
Effects of Varying Doses of Verapamil on Adult and Neonatal Chemotaxis

<table>
<thead>
<tr>
<th>Dose</th>
<th>10^{-10} M</th>
<th>10^{-9} M</th>
<th>10^{-8} M</th>
<th>10^{-7} M</th>
<th>10^{-6} M</th>
<th>10^{-5} M</th>
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<tbody>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>\bar{x}*</td>
<td>96</td>
<td>---</td>
<td>88</td>
<td>82</td>
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<td>12</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>p</td>
<td>NSD</td>
<td>---</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
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<td>&lt;0.05</td>
<td>&lt;0.001</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonate</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>\bar{x}*</td>
<td>91</td>
<td>91</td>
<td>93</td>
<td>91</td>
<td>90</td>
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<td>24</td>
</tr>
<tr>
<td>p</td>
<td>NSD</td>
<td>NSD</td>
<td>NSD</td>
<td>NSD</td>
<td>NSD</td>
<td>NSD</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\bar{x} = mean
SE = standard error of the mean
n = no. of determinations
p = significance by Student t evaluation
NSD = no significant difference
* = percent of control chemotaxis without verapamil
not shown). It was apparent, then, that alteration of the calcium flux with verapamil (though at a relatively high concentration), enhanced the neonatal chemotactic response while inhibiting adult chemotaxis. The effects of exogenous calcium deprivation and preincubation of neutrophils with verapamil (Fig. 3), then, were similar. On six of the occasions illustrated in Figure 3, both calcium manipulations were performed on adult and neonatal PMNs. The effects generated by verapamil of inhibited chemotaxis in adult PMNs and enhanced chemotaxis in neonatal PMNs were slightly, but significantly, greater ($p < 0.05$) than responses which occurred when cells were deprived of exogenous calcium.

These data provide evidence for the role of calcium not only in chemotaxis in general, but as a potential source of the problem in impaired neonatal chemotaxis.
CHAPTER 5

DISCUSSION

We have devised a convenient, economical, and reproducible method for measuring neutrophil chemotaxis through micropore filters. This procedure is especially useful when the quantity of cells is limited as is the case with neonatal samples. Employing this technique, we have compared adult and neonatal PMN chemotaxis to the stimulant FMLP and have demonstrated, as have others (Tono-Oka et al., 1979), significant reduction in the magnitude of newborn neutrophil chemotaxis. The similarity in dose response of adult and neonatal PMNs to the chemoattractant supports the indication from receptor studies that neonatal cells are able to recognize a chemo­tactic stimulant. The translation of this detection into neutrophil action appears to be where newborn cells are inefficient or, perhaps, undeveloped.

We have also examined the effects of two calcium manipula­tions on adult and neonatal neutrophil chemotaxis and have shown in adults mildly depressed chemotaxis in the absence of added extracel­lular calcium. Others have reported similar findings (Estensen et al., 1976; Wilkinson, 1975). Diminution of migration also occurred when adult cells were treated with the calcium entry blocker, vera­pamil (10^{-4} M). Inhibition also occurred at lower concentrations
(i.e., $10^{-8}$ M to $10^{-5}$ M), although the magnitude of the inhibition was not directly dose-dependent.

The exact mechanism by which verapamil influences calcium translocation in myocardial and smooth muscle cells, for which it is used therapeutically, has not been elucidated. Calcium antagonists, as reviewed by Saida and van Breeman (1983), are thought to exert their effects in several ways--by blocking calcium influx through an altered slow channel, by interfering with the release of intracellular calcium from storage pools, and/or by altering calmodulin. Zelis and Flaim (1981) proposed that verapamil affects vascular smooth muscle through receptor-operated channels which inhibit the release of sarcolemmal calcium. Although the nature of verapamil's action on these cell types is uncertain, its effect of blocking calcium influx in myocardial cells (Lee and Tsien, 1983) and smooth muscle cells (Rosenberger, Ticku, and Triggle, 1979) has been shown. It seems reasonable to assume that the rise in intracellular calcium which occurs upon stimulation of PMNs by chemotactic factors is also inhibited by verapamil.

Calcium is a ubiquitous element in nature. There are numerous sources of calcium in a cell including the plasma membrane, mitochondria, nucleus, and cytoplasm. Inhibition of the entry of exogenous calcium by deprivation or pharmacologic membrane blockage obviously impedes only a small amount of calcium traffic in the cell. The entire scenario of calcium movement in a cell and its effect on cell function cannot, therefore, be explained by these two manipulations. Furthermore, it has been reported that low levels of
extracellular calcium (Becker et al., 1981) and intracellular calcium (Yin, Zaner, and Stossel, 1980) are sufficient to initiate biologic activities of the neutrophil such as locomotion (Yin, Zaner, and Stossel, 1980), degranulation and superoxide generation (Becker, Naccache, and Sah'afi, 1983). Petroski and others (1979) reported that when the concentration of external calcium falls below 5 μM, one third of the intracellular calcium is extruded from rabbit neutrophils. As mentioned, by mass spectrometry, we have estimated exogenous calcium levels at approximately 1 μM in calcium-deprived media (data not shown). Whether the resulting depression in adult chemotaxis is due to a lack of calcium influx from external milieu or from an alteration in the steady-state level of intracellular calcium which occurs when exogenous calcium is diminished (Becker, Naccache, and Sah'afi, 1983) is unknown.

Since a rise in intracellular calcium initiates muscle contraction (Smith et al., 1983), verapamil could be considered to inhibit "contraction" in the adult neutrophil at both high and low concentrations. Inhibition of this contraction, then, may also occur as a result of the depletion of extracellular calcium which generates the compensatory loss of intracellular calcium. In any event, it appears from this study that in adult PMNs, optimal chemotaxis, as determined by the micropore filter method, requires the presence of extracellular calcium. In addition, it appears that blockage of the rise of intracellular calcium which reportedly occurs in PMNs stimulated for chemotaxis causes adult neutrophils to have depressed chemotaxis.
In this study, neonatal neutrophils did not respond in a similar manner to the previously mentioned calcium manipulations as did adult PMNs. Deprivation of exogenous calcium and/or treatment of PMNs with verapamil surprisingly evoked enhanced neonatal chemotaxis. The reason for this finding cannot be explained at present. Perhaps the slow channel entry of calcium in neonatal neutrophils is over-active, thereby allowing excessive amounts of calcium into the newborn cells. Pharmacologic blockage of the activation of this channel or diminution of calcium in the extracellular matrix may then reduce intracellular calcium to normal levels by allowing only essential concentrations of calcium into the neutrophil.

Association of the altered response of neonatal cells to calcium-deprived media with the abnormal response to verapamil cannot be directly made. It may be that the responses of PMNs to verapamil and to calcium deprivation are manifest through the same biologic responses but that the actions of these calcium manipulations on the cell are unrelated.

Perhaps neonatal neutrophils have impaired control over the steady-state level of intracellular calcium such that repressively high concentrations of calcium are generated by chemotactic factor stimulation. Employing the analogy of muscle physiology, these cells would be in a fixed state of contraction. Release of intracellular calcium in the presence of less than 5 μM exogenous calcium (Petroski et al., 1979) might allow neonatal neutrophils to return to a normal steady-state level of calcium and to enter the relaxation phase necessary for normal locomotion. Verapamil may exert a similar
effect upon intracellular calcium and chemotaxis in the neonate by inhibiting the release of calcium from storage sites, and promoting relaxation levels of intracellular calcium. A similar role of other calcium antagonists has been suggested in contraction of mesenteric arteries (Saida and van Breeman, 1983).

The lack of dose-dependent inhibition of chemotaxis in adults and enhancement in neonates, indicates that verapamil may exert more than one effect at the highest concentration. Perhaps inhibition of intracellular calcium mobilization as well as inhibition of calcium influx is occurring. This might explain the profound increase in neonatal chemotaxis at $10^{-4}$ M. At lower concentrations (i.e., less than $10^{-5}$ M verapamil) only slow channel sources of extracellular calcium entry might be inhibited. These doses impose less but significant inhibition of adult chemotaxis and no significant effect on neonatal PMNs which may have excessive levels of intracellular calcium.

Future studies which might aid in the explanation of the opposite behavior of adult and neonatal neutrophils to the calcium manipulations employed in this study include the following:

1. Determinations of the steady-state and stimulated concentrations of free intracellular calcium in newborn and adult neutrophils.

2. Comparative calcium flux studies in adult and neonatal PMNs by radioactive uptake analysis and membrane potential determinations. Use of calcium-specific probes such as quin-2 would make such data more reliable than many previous studies.
3. Kinetic studies of adult and neonatal PMN binding to verapamil and other calcium antagonists to determine whether there exists a divergence in receptor properties.

4. Studies of the effects of these calcium antagonists on chemotaxis as well as other neutrophil functions.
REFERENCES


Simchowitz, L., Fischbein, L. L., Spilberg, I., and Atkinson, J. P.: Induction of a transient elevation in intracellular levels of adeno-


