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HUMAN NEUTROPHIL INTERACTION WITH IMMUNE COMPLEXES: EFFECTS OF IMMUNE COMPLEXES ON THE EXPRESSION OF C3b (CRI), C3bi (CRIII), AND Fc RECEPTORS, AND ON THE SYNTHESIS OF LEUKOTRIENE-B4 BY NEUTROPHILS.

presented by

ELAHE TORABI

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HUMAN NEUTROPHIL INTERACTION WITH IMMUNE COMPLEXES: EFFECTS OF IMMUNE COMPLEXES ON THE EXPRESSION OF C_3b (CRI), C_3bi (CRIII), AND Fc RECEPTORS, AND ON THE SYNTHESIS OF LEUKOTRIENE-B_4 BY NEUTROPHILS

By

Elahe Torabi

A DISSERTATION

Submitted to
Michigan State University
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ABSTRACT

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Human peripheral blood neutrophils obtained from healthy adults were examined in vitro. The expression of C_3b, C_3bi and Fc receptors on the surfaces of isolated neutrophils, as well as neutrophils in whole blood was determined using monoclonal antibodies and immunofluorescent techniques. In addition, the effects of chemotactic dipeptide N-formyl-L-methionyl-L-phenylalanine (f-Met-Phe), leukotriene B_4 (LTB_4), a temperature transition (i.e., 4°C---> 37°C), and immune complexes (ICs) on expression of neutrophils receptors were examined. Furthermore, the release of LTB_4 by neutrophils after interaction with ICs was studied.

It was shown that unactivated neutrophils in whole blood exhibit a minimal number of C_3b, C_3bi receptors, while expressing a large number of receptors for Fc on their surface. Each of the chemotactic factors, f-Met-Phe, LTB_4, and the temperature transition significantly enhanced the expression of C_3b, C_3bi and Fc receptors on the plasma

membranes of neutrophils in whole blood. When neutrophils were isolated by the standard isolation procedure, the expression of C_3b and C_3bi receptors were only enhanced significantly on cell surfaces upon stimulation with f-Met-Phe or LTB_4. No significant increased expression of Fc receptors was observed on the isolated neutrophil surface.

The interaction of ICs with neutrophils was studied. Neutrophils in whole blood avidly bound and ingested the insoluble ICs. However, the uptake of soluble ICs by neutrophils was 1-5% compared to the insoluble ICs uptake. The interaction of ICs with neutrophils depressed the expression of C_3b and Fc receptors, in contrast to the expression of the C_3bi receptor which was significantly enhanced. In addition, soluble and insoluble ICs induced the synthesis of LTB4 from the endogenous arachidonic acid via the 5-lipoxygenase pathway. The interaction between the Fc receptor and the Fc portion of the antibody molecule in ICs was required for the release of LTB_4 by neutrophils.

The results suggest that ICs modulate the surface receptors associated with the immune adherence, and may be responsible for the depressed locomotion and phagocytic activity of neutrophils observed in some patients with inflammatory diseases. The LTB_4 released by neutrophils upon the interaction with ICs could potentiate the inflammatory reaction by increasing leukocytic infiltration.

Dedicated to my husband, Gregory C. Bader, my son, Alexander, my daughter, Sarah, and my parents, Zinat Amel and Ali Torabi, whose moral support, understanding, and encouragement made this work possible.

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TABLE OF CONTENTS

SECTION I

THE EFFECTS OF CHEMOTACTIC PEPTIDE, LEUKOTRIENE B_4, AND TEMPERATURE TRANSITION ON THE EXPRESSION OF C_3b, C_3bi AND Fc RECEPTORS ON HUMAN NEUTROPHILS

SECTION II

THE EFFECTS OF SOLUBLE AND INSOLUBLE IMMUNE COMPLEXES ON THE EXPRESSION OF C_3b, C_3bi, AND Fc RECEPTORS ON HUMAN NEUTROPHILS IN WHOLE BLOOD

SECTION III

THE EFFECTS OF SOLUBLE AND INSOLUBLE IMMUNE COMPLEXES ON THE SYNTHESIS OF LEUKOTRIENE B_4 BY HUMAN NEUTROPHILS

LIST OF TABLES

TABLE **PAGE**

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

INTRODUCTION

Human neutrophils are highly specialized for several biological functions. One of their primary biological roles includes ingestion, neutralization, and digestion of microorganisms. In addition, neutrophils have also been implicated in causing damage to extracellular tissues observed in various types of inflammatory tissue injury (Issekutz, 1984; Issekutz et al., 1983; Ward et al., 1983). This tissue damage is thought to result from the release of mediators such as, lysosomal enzymes, Platelet - activating factor (PAF), leukotriene-B_4 (LTB_4), and perhaps others by neutrophils (Fanton and Ward, 1982; Williams, 1981).

Activation of neutrophils involved in these processes appears to result from the interaction of a stimulant (ligand) with a cell surface receptor (Becker, 1986; Cochrane, 1984). Different activating factors such as chemotactic peptides, leukotriene-B_4, calcium ionophore A23187, and temperature transition have been reported to activate neutrophils (Fearon and Collins, 1983; Ford-Hutchinson, 1981; Hoffstein and Weissman; Painter at al., 1984; Schiffmann and Wahl, 1975). Neutrophil activation can be accompanied by cellular chemical changes (e.g., consumption of oxygen, oxidative metabolism of glucose, generation of superoxide anion, production of metabolites of arachidonic acid, release of lysosomal enzymes, etc.) and/or cellular physical changes (e.g., changes in cellular shape, expression of membrane components, enhanced adhesiveness, directional migration, and phagocytosis, etc.)(Becker, E., 1986; Fearon and Collins, 1983; Ford-Hutchinson, 1983; Smith et al., 1979; Wright and Gallin, 1977; Zigmond, S.H., 1978).

While the membrane and cytoplasmic events that control neutrophil functions have been poorly understood, recent studies have provided some new insights in this area. It has been shown that the plasma membrane of neutrophils contain receptors for the Fc portion of immunoglobulin G(IgG), formylated peptides, leukotriene B_4, and the complement components (i.e., C_3 and C_5)(Fearon, 1980; Fleit et al., 1982; Goetzl and Hoe, 1979). These receptors are important in the performance of many of the functions of these cells.

At least two distinct receptors for the third component of complement (i.e., C_3) have been demonstrated on the neutrophil surface; one receptor recognizing C_3b, the product of C_3 activation, and has been termed the C3b receptor, or CRI (i.e., C_3b/C_4b receptor) (Fearon, 1984; Wong, et al., 1983), while the second receptor recognizing C_3bi, the product of C_3b cleavage by the serum proteins factors H and I, and has been termed CRIII, or the C_3bi receptor (Ross & Lambris, 1982; Yoon & Fearon, 1985). The

C_3bi, a cell surface glycoprotein, is present on human neutrophils, monocytes, and null cells (Todd, Nadler & Schlossman, 1981) and appears to be involved in some of the neutrophil adherence reactions (e.g., adherence to a substratum, aggregation, orientation, motility, phagocytosis and cytotoxicity) (Arnaout et al., 1983; Beller et al., 1982; Dana et al., 1984). A cooperative interaction between Fc and CRI receptors involving the endocytic process of neutrophils has been suggested in studies by Jack and Fearon (1984).

The capacity of neutrophils to respond to microorganisms or other particles appears dependent on the number of receptors expressed on their plasma membrane. Defects in neutrophil functions, associated with the deficiency or abnormal expression of complement receptors, has been described in several patients and neonates (Anderson et al., 1984; Arnaout et al., 1984; Crowely et al., 1980; Thompson et al., 1984) Studies have shown an increased expression of complement receptors on the neutrophil surface upon activation (Berger et al., 1984, 1985; Fearon and Collins, 1983). An enhanced expression of neutrophil C_3b receptors has been reported to occur in vivo with patients experiencing intravascular complement activation secondary to hemodialysis or thermal injury (Lee et al., 1984; Moore et al., 1986). Therefore, it appears that these receptors play an important role in neutrophil functions.

It is believed that much of the tissue injury seen in inflammatory diseases (e.g. rheumatoid arthritis, vasculitis, systemic lupus erythematosus, chronic inflammatory bowel disease, and certain forms of glomerulonephritis) is mediated by the formation and deposition of immune complex in tissues. The interaction of immune complexes, complement, and neutrophils is believed to be important in the pathogenesis of immune complex diseases (Starkebaum et al., 1982). The interaction between immune complexes and neutrophils proceeds in several steps; 1) adherence, 2) phagocytosis, 3) cellular metabolic alteration, and 4) release of mediators from neutrophils (Goetzl et al., 1984; Lucisano and Mantovani, 1984). The phlogestic potential of immune complexes relates to several factors; the ability of the immune complexes to, a) activate the complement system, b) stimulate neutrophils to release granule contents, platelet-activating factor, superoxide anion, and hydrogen peroxide, and c) induce the release of leukotrienes from monocytes/macrophages (Camussi et al., 1981; Gimbron et al., 1984; Johnson and Ward, 1981; Ringertz et al., 1982; Rouzer et al., 1980).

Leukotrienes are a group of mediators of inflammation (Samuelsson, 1983). These are formed within neutrophils after stimulation with an activating agent, formyl-methionyl-leucyl-phenylalanine (fMLP) or the calcium ionophore via the lipoxygenase pathway (Jubiz et al., 1982; Stenson and Parker, 1979). Leukotriene-B_4 has been identified as a potent mediator of leukocyte function. Leukotriene-B_4 is a potent chemokinetic and aggregating agent for neutrophils and has been shown to cause degranulation, superoxide generation, receptor expression, and mobilization of membrane-associated calcium (Lew et al., 1984; Naccache et al., 1981; Shaafi et al., 1982; and Volpi et al., 1984).

The study presented herein in this thesis investigated, 1) the effects of the chemotactic factor N-formyl-L-methionyl-phenylalanine (f-Met-Phe), leukotriene-B_4, and temperature transition on the expression of C_3b (CRI), C_3bi (CRIII or MO1), and Fc receptor on human neutrophils in whole blood as well as isolated neutrophils, 2) the modulation of these receptors on human neutrophil surfaces upon interaction with soluble and insoluble immune complexes and 3) the release of leukotriene-B_4 by human neutrophils after interaction with immune complexes. A whole blood assay was developed, and the expression of surface receptors was examined using monoclonal antibodies, immunofluorescent techniques, and flow cytofluorography. The immune complexes were composed of human serum albumin and an IgG fraction of rabbit anti-human albumin. A radioimmunoassay (RIA) was employed to measure the amount of leukotriene-B_4 released into the extracellular media by human neutrophils following exposure to immune complexes.

The results from this study showed that the interaction of human neutrophils with immune complexes induced the synthesis of leukotriene-B_4 from endogenous arachidonic acid. In addition, this interaction enhanced the expression of C_3bi (CRIII) receptors and depressed the expression of Fc and C_3b (CRI) receptors on neutrophil surfaces in whole blood. The interaction between the Fc receptor on the neutrophil surface and the Fc portion of the antibody in the immune complex was required, but not sufficient for the release of LTB_4 from neutrophils. The mechanism is currently unknown, but the presence of serum was required. Leukotriene-B_4, as well as f-Met-Phe and temperature transition (i.e., 4^oC --> 37^oC) induced a significant expression of C_3b, C_3bi and Fc receptors on neutrophils in whole blood. These results suggest that the interaction of human neutrophils with immune complexes may potentiate the inflammatory reaction by the release of leukotriene-B_4 and may contribute to the depressed locomotion and phagocytic activities seen in some patients with inflammatory diseases.

REVIEW OF LITERATURE

The purposes of this review are to 1) provide information concerning morphology, structure and physiology of human neutrophils, 2) introduce information regarding the activating agents for neutrophils, 3) discuss events of activation which occur following neutrophil activation, and 4) present information to facilitate an understanding of the physiopathology of immune complexes and neutrophils, along with their involvements in inflammatory reactions.

I. NEUTROPHILS: Composition of cytoplasmic granules, plasma membrane receptors and cytoskeletal proteins

Neutrophils are polymorphonuclear cells which are produced in the bone marrow, mature there into cells containing a variety of enzymes and potent mediators sequestered in granules, and then emigrate and circulate in the blood. Release of neutrophils from the marrow into blood sinuses is generally related to their maturation (Weiss, 1970). However, other factors such as, blood flow (Dornfest et al., 1862), number of neutrophils in blood, bacterial products (Bishop et al., 1968), and "colony stimulating factors" (Athens et al., 1961; Parker and Metcalk, 1974), also influence neutrophil release from the marrow.

In the peripheral blood, neutrophils constitute approximately 60-70% of the blood leukocytes. The neutrophil is an end cell, incapable of cell division. The most prominent feature of a mature neutrophil is the highly lobulated nucleus. The cytoplasm contains numerous membrane-bound granules, few ribosomes and mitochondria, scant rough endoplasmic reticulum, diminished Golgi complex, and is rich in glycogen. The paucity of mitochondria and the abundance of glycogen in neutrophils reflect the predominance of the anaerobic mode of metabolism, which permits neutrophils to function in the poorly oxygenated environment of damaged tissues.

A. Cytoplasmic granules

Based on morphological and histochemical examination of the mature neutrophil, at least three discrete types of granules (i.e., primary, secondary and tertiary) have been identified. The azurophilic "primary" granules are the first to appear in the developing neutrophils (i.e., promyelocyte stage) (Klebanoff, 1978; Silber and Moldow, 1983). Mature primary granules contain acid hydrolases, neutral proteases, myeloperoxidase,lysozyme, and acid mucopolysaccharide (Table R.1) (Baggiolini et al., 1985; Bainton and Farquhar, 1968; Pember et al., 1983). Myeloperoxidase is present exclusively in the azurophil granules of the resting neutrophil.

The secondary "specific" granules appear during the myelocyte stage of neutrophil development (Bainton et al., 1971; Capone et al., 1964; Scott and Horn, 1970). These granules are smaller (0.5 µm) and less electron-dense than are the primary granules. Secondary granules contain lysozyme, alkaline phosphatase, the neutral protease collangenase, vitamin B12-binding proteins, and the glycoprotein lactoferrin (Table R.1) (Baggiolinin et al., 1985; Bainton et al., 1971; Masson et al., 1985). Secondary granules contain approximately two thirds of the

Table R.1 Neutrophil Granule Components

Primary (Azurophil) Granules	Secondary (Specific) Granules	Tertiary Granules
Acid Hydrolases		
Acid B-glycerophosphatase		Acid B-glycerophosphatase
B-glucuronidase		B-glucuronidase
N-acetyl-B-glucusaminidase		N-acetyl-B-glucosaminidase
Alpha-mannosidase		alpha-manosidase
Acid protease (Cathepsin B & D)		Acid protease (Cathepsin B&D)
Arylsulfatase		Arylsulfatase
B-galactosidase		
5'-nucleotidase		
α-fucosidase		
Neutral Proteases		
Collagenase*	Collagenase*	Gelatinase
Elastase		Plasminogen Activator
Chymotrypsin-like protease (Cathepsin G)		
Antimicrobial		
Lysozyme (muramidase)	Lysozyme	
Myeloperoxidase		
Defensin		
Other		
Acid mucopolysacharide	Lactoferrin	Proton Pump ATPase
Cationic proteins	Alkaline phosphatase*	
	Vitamin B12-binding proteins	

* There is conflicting data on the location of these components.

neutrophil's total lysozyme. Recently, Cramer and Breton-Gorius (1987), demonstrated ultrastructural localization of lysozyme in both the primary and secondary granules of blood and bone marrow neutrophils utilizing immunoelectron microscopy.

The third type of granules in mature neutrophils are referred to as C-particles or tertiary granules. The tertiary granules are relatively small (approximately 0.3 μm), contain a number of acid hydrolases also found in primary granules (Table R.1), the neutral protease gelatinase and the plasminogen activator (Baggiollini et al., 1985; Murata and Spicer, 1973; Sopata and Dancewicz, 1974; Wetzel et al., 1967). Recent evidence suggests that a proton pump ATPase, responsible in part for the acidification of phagolysosomes following neutrophil phagocytosis, is associated with tertiary granules (Mollinedo and Schneider, 1984; Mollinedo et al., 1986).

B. Cytoskeletal proteins

The cytoskeleton of neutrophil is formed by microtubules and microfilaments (Stossel et al., 1984). Microtubules are composed primarily of tubulin. Tubulin is a complex dimeric protein which can polymerize into microtubular structures both *in vivo* and *in vitro*, in the presence of Ca^{++} and GTP (Borisy et al., 1974). Microtubules form a cytoskeleton which is utilized in the movement of some interacellular organelle. Colchicine is an alkaloid which binds with tubulin and causes the rapid and complete disassembly of cytoplasmic microtubules in all mammalian cells (Oliver, 1978). The contractile microfilaments consist of actin and myosin. Microfilaments appear to function in the maintenance of cell shape, motility and in the intracellular movement of organelles (Howell and Tyhurst, 1982; Neiderman, et al., 1983; Oliver, 1978). Cytochalasin B (CB), a metabolite of the mold Elmintho-Sporium Dematioideum, causes a disruption of microfilaments and an associated loss of the cellular functions dependent on them (Oliver, 1978). In addition, CB inhibits the active transport of sugar across the cell membrane at lower concentration than those required to affect microfilament organization (Enstensen and Plagemann, 1972). In neutrophils, CB inhibits chemotaxis and phagocytosis and enhances secretion to the outside of the cell, (Becker, et al., 1972; Becker, 1976; Malech, et al., 1977). It has been presumed that they do this by preventing the gelation of actin with actin binding proteins, which would allow granules to gain access to the membrane, a process otherwise prevented by subplasmalemmal microfilamentous structures (Hartwig and Stossel, 1976).

C. Plasma membrane receptors

Neutrophils have different receptors on their plasma membrane, including receptors for the Fc portion of IgG, formylated peptides, and the complement components (i.e., C_3b, C_3bi, and C_5) (Fearon, 1980; Fleit, et al., 1982; Goetzl and Hoe, 1979; Sanchez-Madrid et al., 1983). The presence of B-adrenergic receptors and insulin-binding sites have also been demonstrated, even though these receptors have not been purified (Silber and Moldow, 1983).

C.1. LFA-1, p150-95, and MO1 (C_3bi) glycoproteins

The use of monoclonal antibodies to cell surface structures has identified surface glycoproteins (GPs) (i.e., MO1, LFA-1, and p150, 95) which functionally appear to be involved in some cellular adhesiveness reactions (Anderson et al., 1985, 1986; Arnaout et al., 1983; Beller et al., 1982; Dana et al., 1984; Springer et al., 1984, 1986). Structurally these GPs consist of two noncovalently linked glycoproteins of an alpha subunit and a beta subunit (Arnaout et al., 1983; Davignon et al., 1981; Sanchez-Madrid et al., 1983). The beta subunits (94 kilodalton) of these GPs are highly homologous or identical, and the alpha chain contain the antigenic epitopes.

LFA-1, an antigen associated with lymphocyte functions, is a heterodimer (alpha subunit, 177 KD; beta subunit, 94 KD) and is present on T and B cells, monocytes, and neutrophils (Davignon et al., 1981; Sanchez-Madrid et al., 1983). Studies have shown the involvement of LFA-1 in neutrophil-mediated antibody-dependent cellular cytotoxicity (ADCC) and lymphocyte-mediated natural killer cytotoxicity (NKD) (Kohl et al., 1984), homotypic adherence of B lymphocytes and monocytes (Mentzer et al., 1985, 1986), T-cell dependent antibody production (Fisher et al., 1986; Howard et al., 1986) and the interaction between activated macrophages and tumor cells (Strassman et al., 1986).

The p150, 95 GP has been found on neutrophil and monocyte membranes and it appears to be involved in adherence functions of these cells (Anderson et al., 1986; Gallin, 1985; Harlan et al., 1985; Sanchez-Madrid et al., 1983; Springer et al., 1986). Diene et al., (1985) have shown that both Gp-150 deficient and monoclonal antibody-treated normal neutrophils failed to adhere to cultured human umbilical vein endothelial cell monolayers when activated by phorbol myristate acetate (PMA).

MO1, a structure closely associated or identical with the human complement C_3bi receptor, consists of two noncovalently associated alpha chain of approximately 155 KD and beta chain of 94 KD. It is present on human neutrophils, monocytes, natural killer cells, null cells, and erythrocytes (Arnaout et al., 1983; Sanches-Madrid et al., 1983). MO1, is also known as OKM1, Mac-1 and p150, 95 (Berger et al., 1984; Dana et al., 1984; Springer et al., 1986). MO1 recognizes the complement protein fragment C_3bi, the product of C_3b cleavage by the serum proteins factor H and I, and is termed CR_3 (CRIII), or the C_3bi receptor (Ross and Lambris, 1982; Yoon and Fearon, 1985). Wright et al., (1987) have shown that CRIII recognizes a region of C_3 that contains the sequence Arg-Gly-Asp (RGD), and appears to be a member of a larger family of adhesion-promoting receptors (i.e., fibronectin receptor, vitronectin receptor, platelet glycoprotein IIb/III,and CSAT antigen). It is believed that the residues beyond the RGD triplet participate in binding.

The exposure of normal neutrophils to anti-MO1 or anti-LFA-1 monoclonal antibodies produces functional defects of neutrophils which are linked to cell adhesive properties (e.g., adherence to a substratum, aggregation, orientation, motility, phagocytosis and cytotoxicity) (Anderson, et al., 1984; Arnaout et al., 1983; Beller et al., 1982; Kohl et al., 1984). Arnaout et al.,(1983) reported that monoclonal antibodies to MO1 (C_3bi) receptors specifically inhibited rosetting between neutrophils and sheep erythrocytes coated with C_3bi. In addition, anti-MO1 blocked phagocytosis by human neutrophils of C_3-or IgG-opsonized (C_3-coated) particles as well as lysosomal enzyme release and superoxide generation by neutrophils in response to opsonized zymosan (coated with C_3bi). However, the anti-MO1 antibody did not inhibit IgG Fc, C_3b or C_3d receptor-mediated binding of erythrocytes coated with the respective proteins.

Impaired phagocytosis, opsonized zymosan-induced degranulation and superoxide production have previously been noted in neutrophils from a patient with recurrent bacterial infections (Arnaout et al., 1982). Defects of neutrophil functions associated with the deficiency or absence of the surface glycoprotein antigens (e.g., MO1 and LFA-1) have been described in several patients (Anderson et al., 1984; Arnaout et al., 1984; Crowely et al., 1980; Dana et al., 1984; Kohl et al., 1984; Thompson et al., 1984). Their clinical symptoms are characterized by recurrent bacterial or fungal infection, progresssive periodontitis, persistent leukocytosis, and/or delayed umbilical cord separation (Bowen et al., 1982). The patients' neutrophils have shown severely depressed adherence, chemotaxis and phagocytic function.

Depressed neutrophil adherence-dependent functions have also been reported in human neonatal neutrophils (Anderson et al., 1981 and 1984; Boner et al., 1982; Klein et al., 1977). Becker-Freement et al., (1984) have shown that neonatal neutrophils exhibit depressed baseline levels of MO1 receptors on their plasma membranes when compared to adult neutrophils. Neonatal neutrophils demonstrated a dose-dependant increased expression of MO1 receptor following activation, but the degree of expression was significantly less than those expressed by activated adult neutrophils. It has also been reported that the levels of lactoferrin (i.e., secondary granule enzyme) in lysates prepared from neonatal neutrophils were approximately 50% of those found in adult lysate. However, the amounts of lysozyme (i.e., secondary granule enzyme) and myeloperoxidase and beta-glucuronidase (i.e., primary granule enzyme) were comparable to those found in adult neutrophils (Ambruso et al., 1984; Becker-Freement et al., 1984). Upon stimulation, neonatal neutrophils secreted equivalent amounts of myeloperoxidase and lysozyme as adult neutrophils, but the level of lactoferrin was 50% of that secreted by adult cells. Becker-Freeman et al., (1984) have suggested that the low levels of MO1 receptor expression and lactoferrin content in neonatal neutrophils may contribute to the various depressed functions observed in neonates and their increased susceptibility to bacterial infections.

Neutrophil activation promotes an increased expression of MO1 receptors on the cell membrane (Arnaout et al., 1984; Berger et al., 1984). Few studies favor the secondary granules as the interacellular pool for MO1 antigens (Arnaout et al., 1984; Todd et al., 1984; O'Shea et al., 1985). Todd et al., (1984) examined neutrophils and their subcellular components by applying immunological and electrophoretic techniques. They demonstrated that the major intracellular pool for MO1 antigens was located primarily in the specific (secondary) granules. Arnaout et al., (1984) and Todd et al., (1984) have suggested that expression of MO1 antigens on neutrophil membrane is probably due to degranulation and fusion of the specific granule fractions with the plasma membrane. O'Shea et al., (1985) presented evidence showing that neutrophils from a patient with a secondary granule deficiency were unable to exhibit an enhanced expression of MO1 receptor upon activation. Further evidence for the association of the MO1 antigens with the secondary (specific) granules was demonstrated by the myeloid lines KG1 and THP-1. These cells lack secondary granules and do not express MO1 receptors on their plasma membrane (Arnaout et al., 1984).

However; Petrequin and coworkers (1985) have presented evidence suggesting that the MO1 antigens may be more closely associated with tertiary than secondary granules. Under controlled conditions, activated neutrophils expressed a 3-fold increase in cell surface MO1 antigens and concomitant extracellular gelatinase (tertiary granule enzyme) release without significant release of secondary granule vitamin B12-binding protein or primary granule myeloperoxidase.

C.2. C_3b receptor (CRI)

Neutrophils exhibit on their plasma membrane receptor which recognizes C_3b, the major cleavage fragment of C_3, and is termed the C_3b receptor, or CRI (i.e., C_3b/C_4b receptor) (Fearon, 1980, 1984, 1986). C_3b receptor recognizes the C_{3c}

region of C_3, has greatest affinity for C_3b, and also binds iC_3b and C_4b. C_3b (CRI) receptor is composed of a single polypeptide chain that exhibits genetically determined differences in size ranging from 160,000 to 260,000 Daltons that are not the result of differences in carbohydrate (Wong et al., 1983). Recently, Holers and associates (1987), reported that the molecular weight polymorphism is determined at the genomic level and may have been generated by unequal crossing-over. C_3b (CRI) receptor is also present on human erythrocytes, eosinophils, monocytes, macrophages, B lympho-cytes, a subpopulation of T lymphocytes, and glomerular podocytes. C_3b receptor has been purified and specific monoclonal or polyclonal antibodies have been prepared which have provided a useful tool in studying the function and modulation of this receptor in human cells.

Human erythrocytes express an average of 500-600 C_3b receptors per cell, and a wide range of 100-1000 receptor sites per cell has been found among different normal individuals (Arnaout, et al., 1981; Fearon, 1980). The function of C_3b receptors on erythrocytes may be to pro-mote the clearance of immune complexes from the circulation. Several studies have reported a relative deficiency of C_3b receptors on erythrocytes in patients with systemic lupus erythematosus (Dykman, et al., 1984; Fearon, 1986; Minota, et al., 1984; Wilson et al., 1982). Studies from Boston and Japan have implicated the role of genetic factors in the occurrence of the deficiency (although, other reports have suggested that the deficiency may also be secondarily acquired in some individuals).

Circulating neutrophils express only a limited number (i.e., average of 5,000) of C_3b sites per cell, but activation of these cells induces a rapid up-regulation (i.e., increased expression) of these receptors (i.e., as many as 50,000 per cell) available for binding of ligands (Berger et al., 1984; Fearon and Collins, 1983; Torabi et al., 1986). Up-regulation of neutrophil-C_3b receptors has been reported to occur in vivo with patients experiencing intravascular complement activation secondary to hemodialysis or thermal injury (Lee et al., 1984; Moore et al., 1986). Increased expression of C_3b receptors on neutrophil membrane is a temperature dependent process and does not require the presence of extracellular Ca^{++}. Although, the release of intracellular Ca^{++} is necessary and appears to be sufficient for increased C_3b receptor expression in response to different stimuli (Berger et al., 1985). The latent receptors are presumed to be intracellular, although the precise site has not been identified. O'Shea and his associates (1985a), examined membrane- and granule-enriched fractions of neutrophils for the presence of C_3b receptor antigens using RIA and immunofluorescent techniques. They found that the C_3b

receptor was present only in the plasma membrane-enriched fraction.

The primary function of C_3b receptor on neutrophils is to mediate or enhance the endocytosis of soluble complexes and particles to which C_3b or C_4b have attached during activation of complement (Fearon, 1984; Mantovani et al., 1972). Earlier studies indicated that the phagocytosis of particles was mediated through Fc receptor interaction, and could not occur via C_3b receptor alone. It had been suggested that C_3 fragments facilitate recognition of particle-cell attachment without leading to ingestion (Newman, 1979; Scribner and Fahrney, 1976; Taylor et al., 1983). It was considered that C_3b receptor has relatively a passive role in phagocytosis, and is involved only in the adherence to the phagocytes of particles bearing C_3b. However, recent studies indicate an active role of C_3b receptor in cellular reactions. Neutrophils activated with phorbol esters ingested particles bearing only C_3b, and neutrophils internalized C_3b receptors that had been cross-linked with $F(ab')_2$ anti-C_3b receptor or with multimeric C_3b (Chagelian et al., 1985; Fearon et al., 1981; Jack et al., 1986; Wright and Silverstein, 1982). Further evidence for the active role of C_3b receptor is provided by the association of the C_3b receptor with the cytoskeleton of neutrophils (Jack and Fearon, 1983, 1984). A rapid capping of the C_3b receptors on the plasma membrane of neutrophils occurred after binding of F(ab')$_2$ anti-C$_3$b receptor, but not after uptake of Fab' anti-C$_3$b receptor (Jack and Fearon, 1984). Capping on neutrophils did not represent a passive aggregation by the anti-receptor antibody of laterally diffusing receptors. In contrast, the reaction was accompanied by the subplasmalemmal accumulation of actin network at the site of caps. The distribution of the receptor was inhibited by the presence of chlorpromazine, which may interfere with intracellular Ca^{++}-dependent reactions by binding to calmodulin (Salisbury et al., 1981) or of cytochalasin D, which prevents actin elongation (Lin et al., 1980). Jack et al., (1986) studied the nature of C$_3$b receptors in human erythrocytes. They observed no association between cross-linking C$_3$b receptors on erythrocytes and the cytoskeleton, and indicated that erythrocyte C$_3$b receptors may differ structurally from neutrophil C$_3$b receptors in it's cytoplasmic extension. These investigators strengthen their suggestion by the recent finding that activation of protein kinase C with phorbol myristate acetate causes the phosphorylation of C$_3$b receptor in neutrophils but not in erythrocytes (Changelion and Fearong, 1986). O'Shea and his coworkers (1985b), and Changelion and his associates (1985), studied internalization of the C$_3$b receptors by tumor-promoting phorbol myristate acetate (PMA). Phorbol myristate acetate had a biphasic effect on the plasma membrane expression of C$_3$b receptors by neutrophils. Treatment of neutrophils with PMA first led to translocation of latent C_3b receptors to the plasma membrane and this reaction was followed within minutes by the internalization of the C_3b receptors. Internalization of C_3b receptors by PMA, occurred in the absence of ligand, suggesting an effect on the C_3b receptor. This process was temperature-dependent and required functional microfilaments. Despite the decreased expresion of C_3b receptors on neutrophils membrane after treatment with PMA, C_3b-dependent phagocytosis was markedly enhanced. The mechanism by which PMA altered the phagocytic function of C_3b receptor has not been interpreted. O'Shea and his associates (1985b), suggested the association of C_3b receptors with the cytoskeleton may be impor-tant to the process of "activation" of C_3b phagocytosis.

C.3. C_3dg receptor

The existence of a fourth type of C_3 receptor on human neutrophil membranes has been demonstrated by Vik and Fearon (1985). This receptor binds the C_3d region of iC_3b, and C_3dg and is distinct from CRI (C_3b), CRII (C_3d), and CRIII (C_3bi). A 10-fold variation in receptors for C_3dg was observed among normal individuals which was not caused by variable expression of latent receptors, as has been observed for C_3b and C_3bi receptors. Since pretreatment of neutrophils with the chemotactic peptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), did not greatly augment the subsequent uptake of C_3dg. Binding of ^{125}I-labelled C_3dg to neutrophils was saturable, and an average of 12,400 sites/cell among normal individuals was reported. Specific binding of C_3dg was cation independent and was competitively inhibited by iC_3b and C_3d, whereas C_3b was less able to compete with C_3dg for binding to these receptor sites. On the other hand, anti-Mac-1 and OKM10, monoclonal antibodies reported to be directed to the iC_3b binding of CRIII (C_3bi), did not inhibit the cellular uptake of C_3dg. Vick and Fearon (1985), suggested that, two iC_3b-binding proteins are present on neutrophils, with CRIII (C_3bi) perhaps having more critical functions in the adherence of iC_3b-coated particles and the C_3dg-binding protein having a role in the cellular uptake of soluble complexes bearing iC_3b or C_3dg.

C.4. Laminin receptor

Recently, Bryant et al., (1987) and Yoon et al., (1987), demonstrated that neutrophils possess a receptor for laminin. Laminin (MW 800×10^3 - 1000×10^3 daltons) is a large and abundant basement membrane-specific glycoprotein (Foidart et al., 1980; Timple et al., 1979). It contains structurally and functionally distinct domains for attachment to the extracellular matrix components such as type IV collagen, to heparan sulfate proteoglycan, as well as to cell surface receptors (Kennedy et al., 1983; Liotta, 1984; Rao, et al., 1982; Terranova et al., 1980 and 1982; Von der Mark and Kuhl, 1985). Laminin has been shown to interact with a variety of cell types including human breast carcinoma (Hand et al., 1985; Terranova et al., 1983), murine fibrosarcoma (Malinoff and Wicha, 1983), mouse melanoma (Rao et al., 1983), and human melanoma (Turpeenniemi-Hujanen et al., 1986) by binding to specific cell surface receptors (MW 68,000 to 72,000 daltons). In addition, existance of a 50,000 daltons bacterial laminin has been reported (Lopes et al., 1985). Furthermore, laminin promotes haptotactic migration of murine B16 melanoma cells (McCarthy and Furcht, 1984).

Terranova and his associates (1986), studied the effects of laminin on rabbit neutrophil motility and attachment. Laminin was shown to be chemotactic for neutrophils. In the Boyden chamber and human amnion system, laminin was shown to stimulate migration of neutrophils. Pretreatment of neutrophils with antibody to laminin blocked neutrophil migration. In an attachment assay system, laminin was found to increase protease-treated neutrophil attachment to type IV (basement membrane) collagen-coated plastic and to a plastic substrate itself. In addition, laminin caused significant release of the specific granule constituent vitamin B12-binding protein. However, laminin had no detectable effect on the release of lysozyme (found in specific and primary azurophil granules), of beta glucuronidase (a primary granule marker), or of the cytoplasmic enzyme, lactic dehydrogenase. These investigators suggested that neutrophils bind laminin on their plasma membrane, use laminin to attach to basement (type IV) membrane collagen, and migrate toward a gradient of laminin. These properties may be important for the migration of neutrophils from circulation to sites of infection.

Bryant and his coworkers (1987), reported about a single class of saturable high affinity binding sites (kd = 6.15 nM/L) for laminin on rabbit neutrophils and $3.6\text{x}10^4$ sites per cells. Human granulocytes were shown to possess a high affinity laminin receptor, Kd = 1.2 nM with $2.18\text{x}10^4$ sites per cell using ^{125}I-labelled laminin. The number of binding sites on myeloid cells from chronic myelogenous leukemic patients and those from chronic lymphocytic leukemic patients was reported to be at least 10-fold less.

Yoon and his associated (1987), examined the effect of stimulation on the expression of laminin receptors on human neutrophils. Unstimulated cells exhibited very low levels of binding for ^{125}I-laminin. However, upon treatment of neutrophils with PMA, an eighteen-fold to twenty-fold increase of laminin binding to cells was observed. The chemotactic peptide, FMLP, also induced an enhanced expression of laminin receptors. At $10^{-6}M$ of FMLP a five-fold to ten-fold increase was observed. Cytochalasin-B pretreatment enhanced sensitivity to FMLP. In the presence of cytochalasin B(5 ug/ml), $10^{-7}M$ of FMLP, stimulated specific laminin binding, with Kd = 3.9 nM and 64.7×10^4 binding sites/cell. In addition, treatment of neutrophils with A23187 ($10^{-6}M$), an effective secretagogue, resulted in an increased expression of laminin binding sites. Yoon and his coworkers (1987), have implicated the secondary/tertiary granule release with increased surface expression of the laminin receptor. Electroblot transfer and autoradiography of subcellular fractions from unstimulated neutrophils exhibited the presence of a 68,000 dalton laminin-binding component in the secondary/tertiary (beta) fraction, which these investigators presumed to be intracellular laminin receptor pool.

C.5. Fc receptors

Human neutrophils, as well as monocytes, macrophages and lymphocytes possess Fc receptors (FcR) on their membranes that bind the Fc domain of IgG and may, as a consequence, initiate the adherence and destruction of IgG-coated targets (Dickler, 1976; Fleit and Unkeless, 1982). This IgG-dependent, leukocyte-mediated destruction may be important in host defense against infectious microorganisms, in various types of inflammatory diseases, in the destruction of autologous cells in immune hemolytic anemia, and perhaps in other immunologic disorders. These FcR each have a characteristic binding affinity and IgG subclass specificity. For example, evidence indicates the presence of two distinct Fc receptors on mouse macrophages. The trypsin-resistance Fc receptors for binding to IgG2b and IgG1 and the trypsin-sensitive Fc receptors which bind IgG2a (Unkeless, 1977). Mellman and Unkeless (1980) purified a monoclonal rat anti-mouse Fc antibody (i.e., 2.4G2 IgG) that was specifically directed against the trypsin-resistance IgG2b.

Fleit and his associates (1982), analyzed the structure, distribution, and heterogeneity of human neutrophil Fc receptors using monoclonal antibodies (mAb). They prepared two monoclonal antibodies, 3G8 and 4F7, which Fab fragments of both inhibited binding to neutrophils of soluble rabbit IgG complexes and sheep erythrocytes coated with rabbit IgG. Approximately 135,000 Fc receptor sites per neutrophil were shown using the 3G8 antibody. The neutrophil FcR immunoprecipi-tated with 3G8 or 4F7 Fab-Sepharose exhibited a broad band ranging from 51,000 to 73,000 daltons, when examined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. In many cases, this broad band was resolved into two poorly separated components, centered at 66,000 and 53,000 daltons. These investigators found the 3G8 antigen to be present on all neutrophils, mature chronic myelogenous leukemia cells, eosinophils, 10-20% of SIg-bearing lymphocytes, and 6% of erythrocyte-rosetting T lymphocytes. No binding of 3G8 Fab was observed on the FcR-bearing cell lines, the human promyelocyte cell line HL-60 and macrophage-like line U937., Raji, Daudi, and K562 or blood monocytes. On the other hand, these cell lines and monocytes bound human IgG1 with high avidity, while the human neutrophils bound IgG1 dimer with a low avidity (i.e., ka 1/100th of that of monocytes). Neutrophils did not bind monomeric IgG1. Kurlander and Batker (1982), reported that monocytes and neutrophils differ strikingly in their avidity for IgG1 polymers. In contrast to the avid binding of IgG1 to monocytes, IgG1 bound to neutrophils very loosely at 37°C, and even at 4°C, where binding is more stable. IgG1 polymers bound to neutrophils 100-1000-fold less avidly than they bound to monocytes at 37°C. Both neutrophils and monocytes bound IgG1 and IgG3 with roughly comparable avidity, and bound IgG4 and Ig2 much less avidly. Fleit and his coworkers (1982), have suggested that the human neutrophil FcR for IgG is different from that on monocytes. With respect to both antigenic composition and binding of monomeric IgG1.

A second class of IgG Fc receptors on human neutrophils has been identified by using the monoclonal antibody IV3 (mAb-IV3) (Looney et al., 1986). The second class of FcR has a 40,000 daltons M.W. and is distinct from the FcR

(51,000 - 73,000 daltons) on neutrophils recognized by mAb-3G8. This 40,000 daltons FcR is also present on human monocytes, eosinophils, platelets, U937, and K562 cells. Fab fragments of mAb-IV3 or the intact molecule (i.e., IgG mAb) were capable of completely and selectively inhibiting immune complex-mediated generation of superoxide by human neutrophils; superoxide generation by other stimulants (i.e., PMA and FMLP) was not prevented by mAb-IV3. Looney et al., (1986), indicated the 40,000 daltons FcR recognized by mAb-IV3 is the human homologue of a class of murine receptors recognized by monoclonal antibody 2.4G2, and which on macrophages is called FcRII.

Perussia and Trinchieri (1984), have described a mAb-B73.1 which reacts with a discrete population of human lymphocytes, natural killer (NK) and antibody-dependent killer (K) cells. Monoclonal antibody B73.1 and mAb-3G8 reacted with the same human lymphocyte subset containing virtually all NK and K cytotoxic activity. These antibodies recognize two distinct epitopes on the same FcR on the membranes of these cells.

Walsh and Kay (1986), examined the binding of immunoglobulin classes and subclasses to human neutrophils and eosinophils using human IgG-1, IgG-2, IgG-3, IgG-4, IgA-1, IgA-2, IgM, IgD and IgE myeloma proteins. Sheep erythrocytes (E) were coated with different myeloma proteins and the rossette formation by neutrophils and eosinophils was analyzed. The rossette assay in which human myeloma proteins are covalently linked to red cells, allows the identification of human Ig(Fc) receptors using a homologous system. It was shown that human neutrophils bound homologous IgG subclass of myeloma protein, and readily expressed demonstrable receptors for IgA-1 and IgA-2. Neutrophils did not express receptors for IgE, IgM or IgD. On the other hand, eosinophils formed rosettes with E-IgE, but not with E-IgA-1 or E-IgA-2. Similar to neutrophils, eosinophils expressed receptors for IgG, and lack receptors for IgM or IgD myeloma proteins.

II. FUNCTIONS OF NEUTROPHILS: Signal mechanism for neutrophils - Chemotactic factors and opsonins

The primary function of the neutrophil is to protect the host against pyogenic infection. It's function is closely integrated with that of macrophages and lymphocytes, cells also involved in response to infection. Polymorphonuclear neutrophils (PMN) in the peripheral blood are attracted to sites of infection by chemotactic factors, which are produced by the interaction of plasma proteins with antigens or pathogens. The diffusion of these chemotactic factors creates a chemical gradient which influences the direction of neutrophils migration. Plasma, in addition to generating chemical attractants, provides specific proteins (e.g. immunoglobulins and/or complement) that coat foreign materials, rendering them capable of ingestion by neutrophils. This process of immunoglobulin and complement coating is referred to as "opsonization". It can enhance recognition of foreign particles by phagocytic cells through binding to specific receptors on the surface of phagocytes (e.g. Fc receptor, C_3b receptor) (Fanton and Ward, 1982; Mantovani, 1975). Neutrophils ingest the opsonized particles by surrounding them with moving pseudopodia. These pseudopodia fuse to enclose the particle within a vesicle called the phagosome. The cytoplasmic granules of the neutrophil fuse with the phagosome and release their contents into it, a process called "degranulation". The neutrophil reduces oxygen enzymatically to generate reactive metabolites such as superoxide anion (O^-_2), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$). Various oxygen radicals with materials released into the phagosome by the cytoplasmic granules contribute to the anti-bacterial defense mechanism of the neutrophils. Some of the granule contents and oxygen metabolites may leak from the neutrophils into extracellular environment, where they can injure the surrounding tissue elements, as well as the foreign particles. This effect can be counteracted and prevented to some extent by some of the plasma inhibitors. The leakage results from secretion and from incompletely fused phago-somes. In addition, under some circumstances, activation of neutrophils initiates synthesis of arachidonic acid (AA) which is normally stored in biological membrane structures. Leukotreines, metabolites of AA are important mediators in inflammatory reactions, which can modulate neutrophil func tion. This side effect of neutrophil response against antigens or pathogens may be an important cause of tissue inflammation and in certain locations may be harmful to the host.

A. Activating factors for neutrophils

In general the circulating neutrophils may respond to a focus of inflammation (whether on going or potential) by vascular margination, emigration and chemotaxis, adherence to particles or surfaces, phagocytosis, degranulation, and secretion of granule constituents. The first essential step for these processes is a signal mechanism(s) to prompt the neutrophils. Activating factors (i.e., chemotactic factors, opsonins, aggregated IgG, bacterial endotoxin, cellular enzymes, and etc.) play an important role for the signal mechanism(s). Bacterial invasion or tissue damage initiates the elaboration of chemoattractants. The activation of protein cascade systems (e.g. complement system, clotting system) in plasma can generate activating factors (AF). Activating factors also can be derived from lymphocytes (e.g. lymphokines), monocytes, and neutrophils (O'Flaherty and Ward, 1979).

A.1. Complement components

Complement components are important biological substrates for the formation of activating factors. A small molecular weight, heat stable fragment of the fifth component of complement (e.g. C_5a) generated during the activation of complement by either the classical or alternate pathway exerts chemotactic activity (Chenoweth and Hugh, 1980; Jose et al., 1981). The native molecule (C_5a) possesses classical anaphylatoxic properties, i.e., it causes smooth muscle contraction, histamine release from mast cells and enhanced vascular permeability. A carboxypeptidase B-type enzyme present in plasma converts C_5a to C_5a-desArg, leading to a loss of its biological activity. However, the chemotactic activity can be largely restored by the addition of an unidentified "helper factor" which is also present in plasma (Chenoweth and Hugli, 1980). Various components such as aggregated IgG, antigen-antibody complexes, bacterial endotoxin, microbial cell walls, damaged tissue and cellular enzymes can generate complement-related chemotactic factors.

A.2. Bacterial products

Products of bacterial growth are chemotactic, which appear to be related to two groups of compounds; N-Formyl-methionyl-oligopeptides and oxidized lipids (Sahu and Lynn, 1977; Schiffmann and Wahl, 1975; Schiffmann et al., 1975).

The formylated peptides can be synthesized in large quantities in pure form, and can be used as probes for studying neutrophil responses to chemotactic peptides.

A.3. Cellular products

A.3.1 Platelet activating factor (PAF)

Platelet activating factor is a low molecular weight lipid molecule (1-lyso-glycerophosphocholine) which is released from sensitized leukocytes after challenge with specific antigen (O'Flaherty and Wykle, 1983). Studies indicate that basophils, mast cells, macrophages and neutrophils are capable of releasing PAF (Betz et al., 1980; Lotner et al., 1980). *In vitro*, neutrophils have released PAF in response to different stimuli such as PMA, calcium ionophore A23187, serum-opsonized zymosan, aggregated IgG, and surface-bound immune complexes (Betz and Henson, 1980; Lotner et al., 1980; Virella et al., 1982). Platelet activating factor is a potent chemotactic substance for human neutrophils and modulates other leukocyte functions (Goetzl et al., 1980; Shaw et al., 1981). In addition, PAF has aggregating and degranulating effects on rabbit, guinea-pig, dog, and human platelets (Lynch et al., 1979; O'Flaherty and Wykle et al., 1983). Furthermore, it has been suggested that PAF may mediate human anaphylaxis. Platelet activating factor enhances capillary permeability in humans and causes anaphylactic symptoms in subhuman primates (Benveniste, 1974; Pinckard et al., 1980).

A.3.2 Leukotriene-B_4

Leukotriene-B_4 (LTB_4), an arachidonic acid metabolite, can be synthesized through the lipoxygenase pathway by different types of cells, including human neutrophils. Leukotriene-B_4 was first identified as a potent mediator of leukocyte function by Ford-Hutchinson et al., (1980), who indicated that it was a potent chemokinetic and aggregating agent for neutrophils. In addition, it hs been demonstrated that LTB_4 causes degranulation, superoxide generation, receptor expression, and mobilization of membrane-associated calcium (Lew et al., 1984; Naccache et al., 1981; Ringertz et al., 1982; Shaafi et al., 1981; Showell et al., 1982; Volpi et al., 1984).

A.4. Opsonins

The complement mediated reactions which generate chemotactic factors in plasma also elaborate by-products which coat foreign particles and opsonize them. Immunoglobulin (Ig) and fragments of complement (e.g., C_3, C_3b) are considered opsonins (Opsonins; Greek, "to prepare food") (Goldstein et al., 1976). The opsonic fragments (C_3b, Ig) bind with extreme tenacity to the surface of a particle by both hydrophobic and covalent interactions. This can enhance recognition of foreign particles by neutrophils through binding to specific receptors (e.g. C_3b and Fc receptors) on the plasma membranes of cells. It is generally accepted that binding via C_3b receptor serves only to provide more intimate contact between the neutrophils surface and the ligand (e.g. IgG) which serve as a trigger for the internalization phase of phagocytosis (Mantovani, 1975; Scribner and Fahrney; 1976).

A.5. Ionophores; Calcium ionophore A23187

Ionophores are small hydrophobic molecules that dissolve in lipid bilayers of cell membranes and increase the ion permeability of the bilayer (Alberts et al., 1983). Most are synthesized by microorganisms (presumably as biological weapons to weaken their competitors), and some have been used as antibiotics. Ionophores have been widely used to increase membrane permeability to specific ions in studies on synthetic bilayers, cell organelles, and intact cells. The ionophore A23187 is a mobile ion carrier, which transports divalent cations such as Ca^{++} and Mg^{++}. When living cells are exposed to A23187, Ca^{++} rushes into the cytosol down a concentration gradient. A23187 is widely used in cell biology to increase the concentration of free Ca^{++} in the cytosol.

Increased cytoplasmic free Ca^{++} occur rapidly following the binding of ligands to their appropriate receptors on neutrophils (Becker and Stossel, 1980; Korchak et al., 1984). In addition, ligand binding to surface receptor initiates a graded displacement of intracellular bound Ca^{++} from membranous stores into the neutrophil cytoplasm. Further-more, ligand-receptor binding enhances membrane permeability to calcium. It is believed, these events (i.e., increased interacellular Ca^{++} and membrane permeability) may serve as a second messenger to mediate the subsequent physiological responses.

Calcium ionophore A23187, mimics the effect of ligand-receptor occupancy on neutrophils without binding to receptors.

Treatment of neutrophils with the calcium ionophore A23187 have been shown to initiate the responses of $O^{-}{}_2$ generation (Korchak et al., 1984; Smolen et al., 1981), degranulation (Hoffstein and Weissman, 1978; Smolen et al., 1981), up-regulation of the C_3b and C_3b_i receptors (Berger et al., 1985), and synthesis of LTB_4 (Borgeat and Samuelsson, 1979; Seeger et al., 1986; and personal observation).

A.6. Temperature transition

Accumulating data indicates that temperature transition of isolated neutrophils can induce cellular activation.

Temperature mediated enhanced receptor expression have been observed (Berger et al., 1984; Fearon and Collins, 1983; personal observation). Fearon and Collins (1983), reported up-regulation of C_3b on neutrophils and monocytes that had been subjected to mechanical force during centrifugation and resuspension and had been subsequently incubated at temperatures greater than 20°C. Berger et al., (1984), also observed a spontaneous increase in expression of C_3b and C_3bi receptors on isolated neutrophils upon warming to 37°C. Charo et al., (1985), reported that temperature significantly influenced adherence of neutrophils to cultured endothelial cells or albumin-coated plastic. Neutrophils incubated with endothelial cells at 37°C for 10 minutes became tightly adherent, and resisted dislogment forces. In contrast, almost all of the neutrophils incubated with endothelial cells at 4°C attached very loosely and were easily dislodged.

Actin polymerization is an event which occurs following neutrophil activation (Anderson et al., 1982; Oliver and Berlin, 1982). Howard and Oresajo (1985), reported that the temperature transition from 4°C to 37°C caused a four-fold increase in neutrophils actin contents, as compared to actin contents of cells held at 4°C.

Spontaneous temperature-mediated extracellular granule release by neutrophils has been reported. Heerdt (1986), presented evidence suggesting existence of a population of peroxidase negative lactoferrin-containing granules in human neutrophils which are selectively released early in response to cellular activation or in response to a temperature transition from 4°C to 37°C. She detected significant quantities of lactoferrin in supernatant within one minute after neutrophils isolated and maintained at 4°C were warmed to 37°C. The extracellular lactoferrin release was not due to aging of cells or cell death, but required that the cells be isolated in the cold. Dewald et al., (1982), observed that incubation of aged neutrophils (i.e., neutrophils isolated from buffy coat cells held at 4°C for 24 hours) at 37°C for 5 minutes, in the absence of any stimulus was sufficient to induce a rapid and massive release of gelatinase (tertiary granule) which was accompanied by some release of vitamin B12-binding protein (secondary granule). However, incubation of neutrophils isolated from freshly drawn blood did not induce the release of these substances. Similar release to those of aged neutrophils was observed only when freshly isolated neutrophils were incubated with FMLP or ionophore A23187. In the other hand, Corcin et al., (1970), have detected vitamin B12-binding protein in supernatants from freshly isolated neutrophils incubated for five minutes at 37°C. No measurable amount of vitamin B12-binding protein was present in supernatants from neutrophils incubated up to five hours at 4°C. Other investigators have reported presence of lysozyme in the supernatant of neutrophils incubated for 30 minutes at 37°C (Goldstein et al., 1974; Wright and Gallin, 1979).

III. SENSORY MECHANISMS OF NEUTROPHILS - THE MEMBRANE RESPONSE COMPLEX: Morphology of Locomotion, Adherence, Ingestion and Degranulation

The exposure of neutrophils to chemotactic factors initiates a series of coordinated biochemical and biological events including alteration of ion fluxes and membrane permeability, morphological polarization, locomotion, adhesiveness, release of granule contents, superoxide anion generation, and release of arachidonic acid metabolites. These responses are initiated by the generation of signal mechanism (S) as a result of chemotactic factors binding to their specific receptors on neutrophils.

Neutrophils in the peripheral blood are attracted to sites of infection by chemoattractants generation *in vivo* through bacterial invasion, tissue degredation or activation of the complement system. The initial neutrophil response is margination and adherence to endothelial cells of vasculature. The marginated neutrophils then migrate out of the microcirculation into specific tissue compartments, where they phagcytose the infectious agents, and eventually will burst and die. Neutrophils have never been observed re-entering circulation and are true end cells with half lives of minutes to hours. The leukocyte margination and migration through the blood vessels to the inflammed tissue was first noted by Dutrochet in 1824, who studied the vasculature of the tadpole's tail during inflammation.

A. Morphology

Morphologically, freshly isolated, nonactivated, neutrophils are basically rounded, as seen by light microscopy. By scanning electron microscopy, the cells are seen to have relatively smooth surfaces (Figure R.1a). Within seconds of stimulation, cells begin to exhibit small ruffles and other surface irregularities (Figure R.1b), which are randomly extended over all regions of the cell surface. The ruffles become fewer and longer and predominately at one pole of the cells which is referred to as lamellipodium (pseudopodium). The remainder of the cell is rounded with some small irregularities (Figure R.1c). With continued exposure to chemotactic factors, the cell becomes elongated and forms a knoblike tail called the "uropod". The neutrophil has now a bipolar configuration (Figure R.1. d,e,f), with the lamellipodium in the front region, the uropod at the rear region, and a mid-region which is relatively smooth with small surface ridges.

Bipolar formation can also occur rapidly (i.e., within 2 minutes after the exposure to chemotactic factor) in suspension, in the absence of chemotactic gradient or attachment of cells to a surface, indicating that neutrophils have an interinsic polarity (Smith et al.,

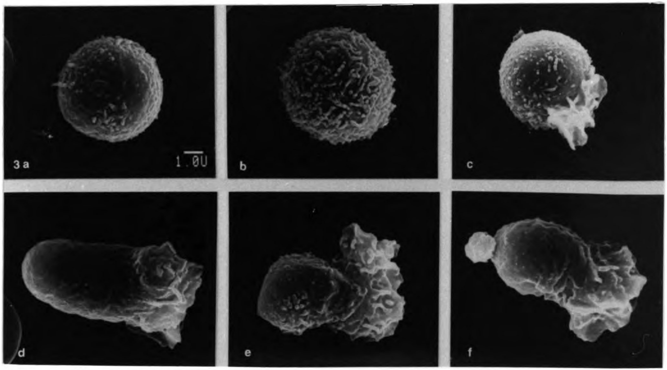
Figure R.1 Change in shape of neutrophils exposed to chemotactic factors

(a) Neutrophils suspended in HBSS (10^6 cells/ml) at room temperature and fixed in suspension in cold (4 C), buffered glutaraldehyde for 1 hour.

(b & c) Neutrophils exposed to 10^{-8}M f-Met-Phe for 10 minutes before fixing in glutaraldehyde.

(d, e, & f) Neutrophils exposed to 10^{-6}M f-Met-Phe for 10 minutes before fixing in glutaraldehyde.

Scanning electron microscopy, 5,000X.



1979). Shape change responses require intact functional microfilaments, glucose metabolism, and are temperature dependent (Heerdt et al., 1984; Smith et al., 1979). Shape change response is reversible and cells become spherical upon removal of the chemotactic factor by washing.

The change in cell shape from spherical to ruffled occurs within seconds, and it has been suggested that membrane is added to the cell surface from an intracellular store (Heerdt, 1986; Hoffstein et al., 1982). It has been demonstrated that the increased cell surface membrane was mediated by specific granule membrane merger, was dose-dependent, and kinetically a transient event (Heerdt, 1986; Hoffstein et al., 1982).

During locomotion toward a chemotactic source, neutrophils acquire a bipolar configuration. The anterior lamellipodium ruffles as the neutrophil moves, at a rate of up to 50 µm/minute. Within the cell, bundles of microfilaments form along the inner surface of the plasma membrane at the advancing front (Oliver et al., 1978). The centromere moves to a position in front of the nucleus and microtubules form around the centromere, radiating peripherally toward, but not contacting, the advancing surface of the cell along with intermediate-sized filaments (Hoffstein et al., 1977; Malech et al., 1977). The mean number of microtubules in stimulated neutrophils significantly increase, implying the involvement of microtubules in stimulated shape change. Microtubule assembly appears to be transient and the number of microtubules returns to baseline levels a few minutes after activation (Hoffstein et al., 1977). Anderson et al., (1982) demonstrated that microtubules lengthen along the axis of cellular polarity and shorten perpendicular to the polar axis. As the cell moves, the cytoplasmic contents behind the anterior lamellipodium streams forward into the front part of the cell, almost obliterating the lamellipodium. The uropod is organelle-free, composed of microfilaments and frequently a core of intermediate filaments (Oliver and Berlin, 1982). At this point, some granules appear to contact the cell periphery, and the release of lysosomal contents occurs at this time (Stossel and Boxer, 1983). It has been observed that neutrophils moving through the vasculature also formed "lamellipoida" between endothelial cells and that the cell contents streamed from the tail to the lamellipodium (Marchesi and Florey, 1960).

B. Adherence

Adherence is a cell property by which non-adhesive circulating neutrophils stick to endothelial cells of the vessel wall and emigrate into the inflammed tissues. A degree of neutrophil adherence appears essential for margination, locomotion, and phagocytosis, all prerequisites for normal neutrophil function in host defense.

Most of the data used to evaluate neutrophil adhesiveness has been obtained using in vitro assays. These techniques allow for neutrophil attachment to an artificial substrate (i.e., plastic or glass coated with different types of substances) or to cultured endothelial cells. Despite the diversity of the assay systems employed, the results obtained for neutrophil function in adherence are generally in agreement. However, the importance of these results derived from in vitro techniques may not apply to neutrophil adhesiveness in vivo since the substrates used, either glass or endothelial monolayers, are foreign to the neutrophil and the testing is done under artificial conditions.

Studies have shown that neutrophil adherence is glucose, temperature and extracellular Mg^{++} dependent, but is independent of microfilaments, cell polarity and extracellular Ca^{++} (Charo et al., 1985; Kvarstein, 1969; Smith et al., 1979). Upon activation, neutrophils become more adhesive which is probably due to an enhanced expression of adhesion sites on the plasma membrane (Berger et al., 1984; Smith et al., 1979). In a non-activating condition, neutrophils express minimal numbers of adhesion sites (receptors) on their plasma membrane, activation of cells with an optimal dose of stimulating factors enhances the availability of the adhesion sites on neutrophil surfaces up to 10 to 12-fold (Berger et al., 1984; Torabi et al., 1986). Studies have documented that the latent adhesion sites (receptors) are stored in specific granules of neutrophils, which will be incorporated into the cell surface, possibly by the fusion of specific granules with the plasma membrane of stimulated neutrophils (Todd et al., 1984). Smith and his coworkers (1979), reported that chemoattractant mediated-enhanced adherence is not reversible by washing the cells free of the chemotactic factor.

Since neutrophil adherence is implicated in cell motility, numerous investigators have focused on studies of the relationship of adherence and motility. Smith and Hollers (1981), studied the attachment and crawling of neutrophils on an albummin-coated glass. The regions of adherence to the substrate were under the lamellipodia, cell body, tail and tips of retraction fibers. It was observed that the detachment of the adherent neutrophils from the substratum occured first at the region of the lamellipodia with the result that cells hung by their uropods, and eventually dropped off the substratum. In another assay system, these investigators studied the attachment and movement of albumin-coated beads on neutrophil surfaces in suspension upon activation with chemotactic factors. It was shown that exposure of neutrophils to low concentrations of chemotactic factor causes the appearance of adhesion sites for albumin coated glass on the neutrophils surface. A second exposure of these neutrophils to a high concentration of chemotactic factor initiates the movement of these adherence sites to the uropod with the appearance of new adhesion sites at the front (lamellipodium) of the cell. It was hypothesized that the continued appearance of adhesion sites and the movement of these adherence sites to the tail upon sensing a concentration gradient of chemotactic factor provides the frictional forces required for locomotion. It has also been suggested that the surface proteins in moving cells continously circulate within the membrane from the front of the cell to its trailing end, then become internalized. After a hypothetical passage through the cytoplasm, these proteins become incorporated again into the front section of the cell (Harris, 1976).

The use of monoclonal antibodies to cell surface structures has identified surface glycoprotein molecules, MO1 (C_3bi) and LFA-1, which appear to be involved in some of the adherence reactions (Arnaout et al., 1983; Beller et al., 1982). MO1 is a structure closely associated or identical with the human complement C_3bi receptor. The adherence associated glycoproteins, MO1 and LFA-1 are required for normal adhesion-dependant neutrophil functions. Defects of neutrophil function associated with the deficiency or absence of the surface glycoprotein antigens (e.g. MO1 and LFA1) have been described in several patients (Anderson et al., 1984; Arnaout et al., 1984; Crowely et al., 1980). Their clinical symptoms are characterized by recurrent bacterial or fungal infection, progressive periodontitis, persistent leukocytosis, and/or delayed unbilical cord separation (Arnaout et al., 1982; Bowen et al., 1982; Thompson et al., 1984). The patients neutrophils have shown severly depressed adherence, chemotaxis and phagocytic function. Depressed neutrophil adherence-dependent functions have also been reported in human neonatal neutrophils (Anderson et al., 1984; Klein et al., 1977). In contrast to the normal adult cells, neonatal neutrophils exhibit only slight elevations in adherence upon stimulation with chemotactic factors. In addition, these cells do not demonstrate normal redistribution of adhesion sites (Anderson et al., 1981; Smith and Hollers, 1980). The abnormal adherence function of neonatal neutrophils may be due to a deficiency of glycoproteins (MO1 and LFA-1) which are present on mature neutrophils. Further evidence supporting the involvement of these glycoproteins in adherence and adherence-associated functions is provided by utilizing monoclonal antibody against the adherence associated glycoproteins. Arnaout and his coworkers (1983), demonstrated that pretreatment of neutrophils with monoclonal anti-MO1 antibody inhibited rosetting between neutrophils and erythrocytes coated with C_3bi. It also inhibited ingestion of IgG or C_3-coated particles by neutrophils.

C. Migration

Chemotaxis, the unidirectional migration of cells in response to a gradient of specific chemical substances is believed to be an important process by which leukocytes migrate from the blood to inflammatory sites in the tissues. One of the processes during neutrophil migration is attachment to the endothelial basement membrane. It has been suggested that the attachment might occur through binding to the basement membrane glycoprotein laminin (Terranova et al., 1986; Yoone et al., 1987). Human neutrophils possess on their plasma membrane receptors to bind laminin (Bryant et al., 1987). Laminin stimulates neutrophil adherence, and migration, as well as the release of granule contents (Terranova et al., 1986). On the other hand, activating agents (i.e., FMLP, PMA, and etc.), enhance the expression of laminin receptors on human neutrophils (Yoon et al., 1987). It has been speculated that neutrophils could respond chemotactically to laminin, and could preferentially use laminin as an attachment factor to type IV collagen during their emigration from the vasculature (Terranova et al., 1986).

Attempts have been made to develop *in vitro* techniques which simulate *in vivo* conditions, to study cellular motility. Since most leukocyte chemotactic factors increase the rate of cell locomotion at moderate concentrations and inhibit the rate at high concentrations, careful consideration of assay systems is essential. Two basic types of systems exist to detect and measure chemotaxis. In the first, one measures the migration and distribution of a sample of a cell population. In the second, one measures the locomotion of individual cells (Wilkonson, 1974; Zigmond and Hirsch, 1973). Widely used techniques evaluate leukocyte migration in response to chemoattractant on plastic (Zigmond, 1977), under agarose, through micropore filters (Gallin and Quie, 1978), and through cultured endothelial cells (Beesley et al., 1979). Few significant in vivo techniques have been developed. The skin window technique which was developed by Rebuck and Crowley (1955) is the most common one. A circular abrasion is made on the human forearm and test agents are added to the abrasion. The site is then covered with a sterile glass coverslip and the accumulation of leukocytes in response to the trauma or test agent is observed. The skin window is still a popular technique which is used by a number of groups in clinical studies.

Studies indicate that neutrophil chemotactic responses require establishment of a chemotactic factor gradient, presence of albumin, extracellular Ca^{++} and Mg^{++}, glucose, microfilament and microtubules function (Wilkinson, 1974; Zigmond, 1978).

D. Phagocytosis and Degranulation

The formation of pseudopodium is also essential for ingestion. As the neutrophil meets a particle, the pseudopodium flows around the particle, its extensions fuse, and it thereby encompasses the particle. The particle thus becomes enclosed within a phagosome into which cytoplasmic granules are rapidly releasing their contents. Locomotion is not prerequisite for ingestion. If neutrophils come in contact with a particle not generating a chemotactic substance, pseudopodia form abruptly at the contact point and envelope the particle. Pseudopodia form whether neutrophils are suspended in a liquid medium or are adhered to a surface. The neutrophil membrane adheres firmly to the particles it ingests, probably to provide the frictional force needed to move pseudopodia around the particles (Hoffstein et al., 1982; Stossel and Boxer, 1983).

Neutrophils become more adhesive during chemotaxis or phagocytosis. It has been suggested that this may be due to secretion of fibronectin by neutrophils (Stossel and Boxer, 1983). In addition, the release of specific granule products and binding to the plasma membrane of "sticky" proteins from these granules, such as acidic proteins and lacatoferrin increase neutrophil adhesiveness (Stossel and

Boxer, 1983). Recently, it has been reported that specific granules might be the major intracellular source for MO1 and laminin glycoproteins which are believed to have an important role in neutrophil adherence. (Todd et al., 1984; Yoon et al., 1987). Furthermore, the granules of neutrophils contain substances which are capable of splitting a chemotactic factor from C_5 directly or by activating complements (Wright and Gallin, 1975, 1977). Moreover, release of platelet activating factor and leukotriene B_4 by neutrophils, which have chemotactic activity for neutrophils may contribute to enhanced neutrophil adhesiveness (Berger et al., 1985; Camussi et al., 1981; Ford-Hutchinson, 1981).

The recognition of signals, the formation of pseudopodia, membrane fusion, and membrane adhesiveness are all characteristics associated with the functional responses of neutrophils.

IV. NEUTROPHILS IN IMMUNITY AND INFLAMMATION

Acute inflammatory reactions may be initiated in tissues by a wide variety of stimuli such as microorganisms, immune complexes, or direct physical and chemical trauma. During acute inflammation, vascularized tissues develop well-known clinical signs of erythema and warmth due to vasodilation and swelling due to the exudation of fluid and protien from the local microvasculature.

Experimental models of acute inflammation have suggested that neutrophils play a central role in the degree of vasodilation and of increase in permeability leading to protein and fluid exudation (Issekutz, 1984; Issekutz et al., 1983). Studies indicate that platelet-activating factor (PAF) may be responsible for the associated protein and fluid exudation in inflammatory reaction. Platelet-activating factor can be produced *in vitro* and *in vivo* by neutrophils following neutrophil activation by a variety of stimuli (Camussi et al., 1981; Lotner et al., 1980; Virella et al., 1982). Platelet-activating factor can induce prolonged vascular permeability and aggregation of platelets. Therefore, PAF, once generated in sufficient quantities by chemotactic factor, could be responsible for the transient platelet accumulation in the microvasculature of accutely inflamed tissues, an event that coincides with and is also dependent on neutrophils margination and migration (Issekutz, 1984; Issekutz et al., 1983). In addition, metabolites of arachidonic acid (i.e., prostaglandins, thromboxanes and leukotrienes) participate in inflammatoy reaction by direct or indirect action (Samuelsson, 1983). Leukotrienes C, D, and E (Cysteinyl-containing leukotrienes), the constituents of the slow reacting substance of anaphylaxis (SRS-A) exert potent effects on smooth muscle, increase vascular permeability in postcapillary venules, and stimulate mucus secreation

(Goetzl et al., 1984; Samuelasson, 1983). Leukotriene-B_4 has potent effects on eosinophils and neutrophils that is related to their adhesion to postcapillary venules and extravasation as well as migration to areas of inflammation and degranulation. Leukotriene-B_4 enhances the proliferation of supressor T lymphocytes and inhibits that of helper T lymphocytes (Goetzl et al., 1984). Studies have shown that stimulation of neutrophils with the activating agents FMLP, Ca A23187, and zymosan-coated serum induced release of LTB_4 by neutrophils (Borgeat et al., 1979; Calesson et al .,1981; Jubiz et al.,1982). Elavated levels of leukotrienes have been detected in tissues or exudates from patients with some spontaneous or elicited hypersensitivity and inflammatory states (Goetzl et al.,1984; Barnes and Costello, 1984). Therefore, the activation of neutrophils may lead to the production of vasopermeability mediators (possibly PAF and leukotrienes). Which account for the protein exudation and swelling. Alternatively, the seperation of endothelial gap by neutrophils during migration may be directly responsible for the protein and fluid exudation in the absence of a specific vasopermeability meditor (Issekutz, 1984).

A. Immune Complexes: Background

It is generally recognized that inflammatory injury in tissues may result from the deposition of immune complexes

(IC) in tissues (Fish et al., 1966). This has been demonstrated not only during the formation of antigen-antibody complexes *in vivo* but also after the injection of immune complexes that have been performed *in vitro* (Johnson et al., 1979 and 1981; Scherzer and Ward, 1978; Von Pirquet and Schick, 1951).

The concept that circulating immune complexes (antigen-antibody) could play a role in human disease was first suggested by Clemens Von Pirquet and Schick in 1905. Their studies on the efficiency of horse anti-diphtheria toxin led them to suggest that the reaction between a foreign protein and the host antibody could have a harmful, as well as a beneficial effect on the host. They postulated that the variation in onset and clinical signs of rash, fever, lymphadenopathy, joint involvement, and, rarely, glomerulonephritis after therapeutic horse serum administration was initiated by the interaction of the foreign antigen and the host antibody. On the basis of these observations Clemens Von Pirquet coined the term allergy in 1906 to define the host's altered immunologic reactivity (hypersensitivity), which develops during initial exposure to antigen or in an accelerated tempo upon reexposure. Despite this early recognition of possible pathogenic role of circulating immune complexes, it was only recently that the role of immune complexes in human disease has been actively studied. In 1953 Germuth made the observation that immune complexes were present in the circulation at the time of the tissue injury. Later, these initial observations were extended and it was possible to correlate the levels of circulating soluble immune complexes with their deposition in glomeruli and vessel walls and the subsequent development of tissue injury. However, in some cases, the detectable immune complexes may not correlate with disease activity (Johnson and Ward, 1982; Jones, 1983).

B. Immune Complexes: Clearance Versus Vascular Localization

Physiochemical characteristics of immune complexes have a role in tissue injury. The most pathogenic complexes are those that are insoluble, either at antigen equivalence or slight antigen excess. These same complexes are also more effective in activation of the complement system (Johnson and Ward, 1982; Scherzer and Ward, 1978). The quality and immunoglobulin class of the antibody involved in immune complexes appears to be important. Immune complexes composed of IgM antibodies are larger and more rapidly cleared than are those formed from IgA or IgG antibodies. One of the most important features of immune complexes is related to their complement fixing activity. Therefore, immune complexes composed of more than one antigen molecule and cross-linked latice-wise by several bivalent IgG molecules are often capable of an effective complement activation. This is also true for immune complexes that consist of IgM antibodies, which in general more effectively activate complememnt. In contrast, IgA immune complex deposits within tissues clinically are not associated with intense complement cascade activation or the other amplifying inflammatory effects associated with such sequelae (Ghebrehiwet, 1983; Williams, 1981).

The clearance of circulating immune complexes by the mononuclear phagocyte system (MPS), formerly called the reticulo-endothelial system (RES) takes place primarily in the liver and spleen. The former mediated predominantly by C_3b receptors, the latter by Fc receptors. Studies have shown that the MPS can be saturated with large doses of immune complexes. With a subsequent persistence of immune complexes in the circulation resulting in increased nonhepatic tissue deposition which may play a role in the pathogenesis of lesions in immune complex diseases (Haakenstad and Mannik, 1974). On the other hand, activation of the system enhances the number and function of Fc and C_3b receptors, increases uptake of circulating immune complexes, and decreases vascular circulating complex localization (Barcelli et al., 1981). The concentration of immune complexes in the circulation depends on the rate of immune complex formation and on the rate of immune complex removal. The rate of immune complex formation in turn depends on the rate of specific antigen availability and on the rate of antibody synthesis. The removal of circulating immune complexes by the RES depends on the physiochemical nature of the antigen, characteristics of the antibody moiety (i.e., affinity and immunoglobulin class), the lattice of immune complexes, and the status of the RES.

In addition to the role of the reticuloendothelial system in the clearance of circulating immune complexes, the presence of Fc receptors on a number of circulating blood cells such as monocytes, macrophages, lymphocytes, polymorphonuclear cells, and platelets is of great importance (Williams, 1981).

C. Neutrophils Interaction with Immune Complexes

The interaction of immune complexes, complement, and neutrophils is thought to be important in the pathogenesis of immune complex diseases (Starkebaum et al., 1982). The interaction between immune complexes (IC) and neutrophils proceeds in several steps; a) adherence, b) phagocytosis, c) cellular metabolic alteration, and d) release of mediators from neutrophils (Goetzl et al., 1984; Lucisano and Mantovani, 1984).

Studies have shown that the phlogestic potential of immune complexes relates to several factors; a) the ability of the immune complexes to activate the complement system resulting in the generation of chemotactic factors which are ultimately responsible for the attraction of neutrophils, b) immune complexes can stimulate neutrophils to release mediators (i.e., lysosomal proteases), which mediate tissue damage directly by hydrolyzing the susceptible substates such as basement membrane, collagen, elastin, etc., or indirectly by enhancing the inflammatory reaction via the generation of chemotactic peptide from C5, c) immune complexes can induce neutrophils to generate superoxide anion and hydrogen peroxide which may play an important role in subsequent tissue damage, d) interaction of immune complex with neutrophils can result in release of platelet-activating factor which can induce neutrophil platelet aggregation, and e) immune complexes can induce the release of leukotrienes from monocytes/macrophages, and neutrophils (personal observation), which leukotrienes, in turn, can modulate cellular functions of neutrophils and other cell types (Camussi et al., 1981; Gimbrone et al., 1984; Johnson and Ward, 1981; Ringertz et al., 1982; Rouzer et al., 1980).

C.1. Extracellular Granule Release

Neutrophils contain a number of different granules which can release their lysosomal constitutents (e.g. acid proteases, cationic proteins and etc.) upon a proper stimulation. The involvement of these mediators in tissue injury in experimental animals has been well established. Several groups of investigators have shown that immune complexes can induce the release of lysosomal constituents from neutrophils (Hawkins and Peeters, 1971; Henson and Oades, 1975). As was mentioned earlier, neutrophils possess surface membrane "receptors" for a fragment of the third component of complement (i.e., C_3b) and for Fc regions of certain immunoglobulin classes which have undergone a conformational change either as a result of union with an antigen or as a result of aggregation. Recognition at the cell surface and binding of such "altered" immunoglobulin mediate adherence and ingestion of immunoglobulin coated-particles or aggregated immunoglobulins by neutrophils (Goldstein et al., 1976; Mantovani, 1975).

In 1971, Hawkins and Peters studied the response of rabbit peripheral blood neutrophils in vitro to immune complexes (IC) in both percipitate and soluble form. They observed that normal rabbit neutrophils, when incubated with immune complexes, released into the external-medium a number of intracellular enzymes (e.g. P-Glucoroniades and Cathepsin E). Homologous antigen-antibody complexes in precipitate form and soluble antigen-antibody complexes made in antigen excess of 20 times equivalence proved equally effective as releasing agents. Highly soluble immune complexes made at 100 times equivalence antigen excess were totally ineffective. However, the greatest release of enzymes was observed from those neutrophils exposed to immune complexes in precipitate form. Release of enzymes appeared to correlate with uptake of immune complexes from the external medium, and the release occurred from intact viable cells.

The effect of immune complexes in both phagocytosable and nonphagocytosable forms on rabbit neutrophils was studied by Peter Henson (1971a, 1971b). Bovin Serum Albumin - rabbit anti BSA was used as the immune complex. In each case, release of constituents from the neutrophil granules was observed. He noted that smaller quantities of immunoglobulins were required to induce release if bound to the nonphagocytosable surface than if phagocytosed by the neutrophils in suspension. Similar observations have been reported using aggregated immunoglobulin as the stimulus. In 1975, Henson and Oades studied the effect of soluble and insoluble aggregated human gamma-globulin on the hexose monophosphate pathway (HMP) and exocytosis of granules on human neutrophils. They reported that insoluble aggregated gamma-globulin in suspension stimulated HMP and secretion of enzymes, while soluble aggregates stimulated HMP only and had no effect upon secretion. However, soluble aggregates bound to a surface readily induced exocytosis of granules from the adherent neutrophils. This process may mimic _in vivo_ situations where neutrophils adhere to immune complexes on surfaces such as the glomerular basement membrane and release their granule enzymes, which may then cause tissue injury.

The Fc receptors on the neutrophil surface have different specificities for particular classes and subclassees of immunoglobulins. Henson and Spiegelberg (1972), studied the effect of different classes and subclasses of immunoglobulin from normal human and myeloma serum. They reported that aggregated human myeloma proteins of subclasses, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2 and normal IgG induce the release of lysosomal enzyme beta-glucuronidase from human neutrophils. In contrast, IgD, IgE and IgM macroglobulins did not stimulate this release. Enzyme secretion occurred with the insoluble form of aggregates. The soluble aggregates did not induce liberation of enzymes when incubated with neutrophils in suspension. Later, Lucisano and Mantovani (1984), investigated the response of rabbit neutrophils to immune complexes (ovalbumin-Rabbit Immunoglobulin) of IgM and of IgG1. They observed that insoluble IgM immune complexes were able to stimulate the release of lysosomal enzymes beta-glucuronidase, alkaline, and acid phosphatase, in contrast to heat-aggregated IgM, which had only a small effect. The reaction required the presence of extracellular Ca^{++}. Insoluble IgG immune complexes from the equivalence region were the most effective, those at antibody excess have a smaller but comparable capacity, whereas the immune complexes at antigen excess were the least effective. They noted that the enzyme release induced by IgM immune complexes was not inhibited by competition with free immunoglobulin in the medium, either IgM or IgG inapproximate physiologic concentrations; contrarily, with IgG IC, free IgG (but not IgM) could completely block the reaction. They suggested that "this difference may have a bearing *in vivo*; IgG present in the plasma or interstitial fluid would competitively block the interaction between the tissue-deposited IgG immune complexes and neutrophils, inhibiting the lysosomal enzyme release, whereas no such interference would exist with the IgM immune complexes. It is possible that for IgG immune complexes the presence of complement could be an important factor in the mechanism of tissue injury, because interaction could be made through C3 receptors,thus bypassing the competitive inhibition".

Johnson and Ward (1981), suggested that the leukocytic neutral proteases released from lysosomal granules have the potential to amplify the acute inflammatory response. With human neutrophils, the major neutral proteases, elastase and chymotrypsin, have the ability, given the proper conditions, to generate leukotactic peptides from C3 and C5. They suggested that perhaps, leukocytic proteases act mainly in the amplification of acute cellular inflammatory reactions rather than as primary effectors of tissue damage.

C.2 Production of Toxic Metabolites of Oxygen (O^-_2,H_2O_2) and Phagocytosis.

In Neutrophils, binding of ligands to their appropriate receptors (Fc, concanavalin A, chemotactic peptide and phorbol myristate acetate receptors) and perturbation of the membrane with the calcium ionophore A23187, have been shown to initiate several responses including the generation of toxic metabolites of oxygen (O^-_2,H_2O_2) (Becker et al., 1979; Korchak et al., 1984; Smolen et al., 1980). A lag period (within seconds) exists between receptor-ligand binding and the onset of oxygen metabolites generation, and the length of this lag period is specific to the particular stimulus.

Korchak et al., have reported that the chemotactic peptide N-formyl-methionyl-leucylphenyl-alanin (f-Met-leu-Phe) exhibited the shortest lag period of approximately 16 seconds, the length of the lag period being independent of the f-Met-Leu-Phe concentration. In the presence of immune complexes (150 ug/ml), a lag period of 45 seconds was observed. The length of this lag period, unlike f-Met-Leu-Phe, was dependent on the concentration of stimulus.

Studies have shown that products of oxygen metabolism are able to inflict damage on isolated cells. Johnson and Ward (1981), using an animal model have shown that production of H_2O_2 and/or it's metabolic products play a key role in the acute lung injury associated with deposition of immune complexes within the tissue of the lung. Ward et al., (1983), have also shown that rat alveolar macrophages can be activated by in vivo exposure to immune complexes resulting in the production of oxygen metabolites. The production of oxygen metabolites was a linear function of cell number, the duration of incubation, and the amount of immune complex employed. In the case of neutrophils, there was a direct relationship between the amounts of immune complex internalized, secretory release of lysosomal enzymes, and production of O^-_2 and H_2O_2. With both neutrophils as well as alveolar macrophages, maximal production of O^-_2 occurred with the largest complexes formed under conditions of antigen equivalence.

A sensitive indicator of increased neutrophils oxidative metabolism, as well as of production of oxygen radicals such as superoxide anion and hydroxyl radical, is the emission of light, termed "chemiluminescence". A portion of these oxidative reactions yield electronically excited products relaxing to ground state by photon emission. The resulting light emission, or chemiluminescence can be measured using a scintillation spectrometer modified for single photon counting. In this manner, CL has been applied to the study of phagocyte physiology and pathophysiology as well as humoral opsonic capacity (Doll and Salvaggio, 1982).

Starkebaum et al., (1981), examined the ability of soluble and insoluble immune complexes, IgG aggregates and anti-neutrophil antibody to stimulate human neutrophils' CL. The stimulation of neutrophils' CL and uptake of immune complexes by neutrophils were both closely correlated with the percent precipitation of immune complexes. Nevertheless, increased levels of neutrophils' CL were observed with soluble immune complexes that were poorly ingested by the neutrophils. A similar dependence of peak CL on immune complex size in guinea-pig peritoneal macrophages was also observed. Soluble aggregated IgG also enhanced the neutrophils' CL as compared to equal amounts of monomeric IgG. In the presence of fresh, normal, human serum (NHS), IgG aggregates induced significantly higher levels of neutrophils' CL than did equal amounts of IgG aggregates in phosphate buffer. Heat-inactivated normal serum didn't have a significant effect on neutrophils' CL, which a role of complement in amplication of this response has been suggested. Rabbit antiserum to neutrophils also was found to stimulate neutrophils' CL.

Doll and Salvaggio (1982), observed that incubating neutrophils with either aggregated immunoglobulin or immune complexes reduced or inhibited the CL response of the cells to subsequent challenge with a particulate stimulant. A direct correlation between percent inhibition in CL and the amount of complex exposed to neutrophils was observed. This observation is consistent with other reports that the microbicidal activity of neutrophils is depressed following immune complex exposure (Matheisz and Allen, 1979). It has been suggested that the inhibition observed in neutrophils CL after preincubation with immune complexes and the subsequent challenge with a secondary phagocytic stimulus may serve as a diagnostic assay for the detection of in vivo immune complexes (Doll et al., 1980).

The interaction of lymphocyte surface-bound immune complexes and neutrophils was studied by Archibald et al., (1983). It was found that CL could be induced by stimulation of neutrophils with surface-bound aggregated human gamma-globulin (AHG) or bovine serum albumin-immune complexes (BSA-IC). Opsonization of the AHG or bovine serum albumin - immune complexes (BSA-IC) with NHS enhanced the CL that was produced. Moreover, B lymphocyte-enriched cell preparations with surface-bound AHG stimulate the production of neutrophils CL to a much greater extent than T lymphocyte-enriched cell preparation.

The production of oxident metabolites, although not enough to cause a significant decrease in lymphocyte viability can cause impaired lymphocyte function (i.e., antibody-dependent and non antibody-mediated cytotoxicity, attachment to sheep red blood cells, concanavalin A cap formation, and etc.). Physiologically, this may relate in the long term to immunologic malfunction observed in patients with high levels of circulating immune complexes (Archibald et al., 1983).

Studies have shown neither phagocytosis nor lysosomal degranulation are prerequisites for enhanced $O^-{}_2$ production (Goldstein et al., 1975). The $O^-{}_2$ generating system is very likely associated with the external plasma membrane of neutrophils. Study employing distrupted neutrophils was shown that $O^-{}_2$ generating activity was associated with membrane fraction.

Weiss and Ward (1982), studied the human neutrophil responses to antigen-antibody complex (Ag-Ab) prepared at different ratios (i.e.; 1:8, 1:4, 1:2, 1:1 and 2:1 ratio of Ag:Ab). The Ag-Ab complexes were made using hyperimmune rabbit IgG rich in antibody to bovine serum albumin (BSA). They found striking differences between; 1) the ability of immune complexes to fix Complement (C'); 2) the ability of immune complexes to be internalized by neutrophils, 3) the ability of the complexes to mediate lysosomal enzyme release, and 4) the ability of complexes to stimulate $O^-{}_2$ and H_2O_2 generation from human neutrophils. There was no correlation between cell uptake of the immune complexes, enzyme release, and production of $O^-{}_2$ and H_2O_2.

At an antigen:antibody weight ratio of 1:8 (molar ratio of 1:2.7), both the ingestion of complexes and lysosomal enzyme release were maximal, whereas $O^-{}_2$ and H_2O_2 production were best achieved by complexes containing an Ag:Ab ratio of 1:2 (molar ratio of 1.5:1). Indeed, at an Ag:Ab ratio of 1:1, the ingestion of complexes was almost undetectable, enzyme release was supressed by more than 50%, and the amount of O^-_2 and H_2O_2 produced were near maximal levels.

The greatest amount of C' fixation resulted when the Ag:Ab ratio was 1:8, somewhat less fixation resulted with the ratio was 1:4, and no significant amount of C' fixation resulted when the ratio of Ag:Ab was increased.

They found that the Fc portion of the IgG molecule interacts with Fc receptors on the neutrophil surface to initiate cell activation, since immune complexes containing pepsin-degraded IgG in the form of F(ab')2 with an antigen:antibody ratio of 1:2 were unable to stimulate O^-_2 or H_2O_2 production by the neutrophils. They suggested that surface activation of the neutrophil with immune complexes via the Fc receptor can lead to quantitatively independent changes in internalization, enzyme release, and oxygen metabolite generation.

These investigators found that small immune complexes that are formed at antigen exess, do not effectively fix C', and are not effectively internalized can maximally stimulate the generation of O^-_2 and H_2O_2 from neutrophils. They suggested that the fact that non-C'-fixing immune complexes are optimal in the stimulation of neutrophils to generate O^-_2 and H_2O_2 could be taken to indicate that soluble complexes in relative antigen excess have considerable phlogistic potential, although it has been believed for some time that complexes of this type do not have a role in the pathogenesis of immune complex-induced tissue damage. Therefore, the larger complexes (containing less antigen) fix C' and initiate the inflammatory reaction via the production of C5a. Even though the same complexes can also stimulate neutrophils, resulting in genereation of O^-_2 and H_2O_2 with the induction of tissue damage, these complexes will be rapidly ingested, terminating the inflammatory reaction. If small immune complexes (containing relative antigen excess) are present, those may lead to a more intense generation of O^-_2 and H_2O_2 as well as a more sustained production of the toxic metabolites, since the complexes are not rapidly ingested by neutrophils.

Starkebaum et al., (1982), examined the effect of immune complexes on human neutrophil phagocytic function. They employed human serum albumin (HSA) as an antigen and rabbit immunoglobulin rich in IgG antibodies to HSA as the antibody. Direct uptake of immune complexes by neutrophils was closely related to the percent of precipitation of the complexes. Less than 1% of soluble complexes prepared at 3-fold antigen excess were internalized by neutrophils compared to 65% internalization of insoluble complexes made at equivalence. Preincubation of neutrophils with immune complexes depressed the subsequent ingestion of insoluble immune complexes which correlated with the percentage of precipitation of the complexes utilized for preincubation. Furthermore, preincubation of neutrophils in normal serum containing soluble aggregates of IgG decreased the internalization of insoluble immune complexes by neutrophils in a dose-dependent fashion. Maximum binding of soluble immune complexes or IgG aggregates to neutrophils occurred after 5 to 15 minutes, however, inhibition of neutrophils phagocytosis was seen only after 90 minutes of preincubation with the complexes or with IgG aggregates. They showed that the decreased phagocytic activity induced by soluble complexes was not due to capping of Fc receptors of neutrophil surfaces. They suggested that prolonged contact of neutrophils with soluble immune complexes or IgG aggregates results in decreased phagocytic activity of the cells via a process that involves metabolic activation of the cells and may be accompanied by the loss or denaturation of surface (IgG) Fc receptors. This reaction may have an important role _in vivo_, where the interaction of soluble immune complexes with circulating neutrophils could lead to abnormalities in phagocyte function.

C.3. Neutrophils Migration

Evidence indicates that the exposure of neutrophils to immune complexes depresses neutrophils migration. Deahlgren and Elwing (1983), examined locomotion of neutrophils on solid surfaces with bound antigen-antibody complexes. Locomotion of neutrophil was inhibited on surfaces coated with bilayers of human serum albumin and the corresponding antibody. Once immobilized on an antigen antibody coated surface, neutrophils did not move chemotactically in response to formylmethionyl-leucyl-phenylalanine (FMLP). However, the receptor for FMLP appeared to be intact since the cells responded metabolically to FMLP as judged from the chemiluminescence response. Furthermore, locomotion inhibition was observed also in the present of super oxide dismutase (SOD) and/or catalase (scavengers of O^{-}_{2} and H_2O_2), thus, autooxidation induced by surface bound Ag-Ab complexes was ruled out as the basis for locomotion inhibition.

C.4. Release of Platelet Activating Factor

It is likely that platelets play a role in inflammatory reactions. These cells contain and can release a wide variety of mediators including vasoactive amines and peptide, proteases, as well as chemotactic factor for neutrophils, and some other mediators. Issekutz et al., (1984), and Lundberg, et al., (1984), observed that during the acute inflammatory reaction induced in the dermis of rabbits by killed or live E. Coli, maximal platelet deposition occurred early, during the first 1 to 2 hours prior to any histologic evidence of hemorrhage or thrombosis. They investigated the relationship between the deposition of platelets and the infiltration of neutrophils into the inflamed tissue since neutrophils infiltration is also an early event. A temporal association between leukocyte accumulation and platelet deposition in the lesions was observed. Platelet deposition in response to inflammatory stimuli did not occur in neutrophil-depleted but platelet-sufficient rabbits. Platelet responses to inflammatory stimuli were normal when neutropenia was prevented. They suggested that platelets selectively deposit in acutely inflamed tissues primarily during neutrophil margination in and emigration across, the microvasculature. It is not clear which mediators may be involved in this neutrophil platelet intereaction. Platelet-activating factor (PAF) is the most likely mediator which has been suggested.

Platelet activating factor has been reportely released from a variety of cell types, in several species, and by different stimuli (O'Flaherty et al., 1983; Pinckard et al., 1979). Evidence has implicated the basophil as the cell of origin. However, recent studies have shown that other cell types such as mast cells, macrophages and neutrophils are capable of releasing PAF (Betz et al., 1980; Lotner et al., 1980).

PAF is a 1-lyso-glycerophosphocholine, which has aggregating and degranulating effects on rabbit, guinea-pig, dog, and human platelets (Lynch et al., 1979; O'Flaherty and Wykie, 1983). In addition, PAF has the ability to aggregate and degranulate rabbit and human neutrophils in nonomolar and lower concentration. With similar potency it stimulates human neutrophils chemotaxis and oxidative metabolism, hnuman monocyte aggregation, infiltration of neutrophils into rabbit skin, and various other actions. Platelet activating factor does not activate rats platelet (Camussi et al., 1982; O'Flaherty and Wykie, 1983; Shaw et al., 1981).

In 1981, Shaw et al., studied the effect of AGEPC on human neutrophil exocytosis, migration, superoxide production and aggregation over a concentration range of 10^{-10} and 10^{-5}M. AGEPC is a class of lipid mediator documented to be functionally and structurally identical to rabbit-derived platelet-activating factor. In this study, AGEPC was prepared by partial chemical synthesis from beef heart choline plasmalogen. AGEPC-induced exocytosis of azurophilic (myeloperoxidase and beta-glucuronidase) and specific (lactoferrin and lysozyme) lysosomal granules was rapid (T1/2 = 20 seconds). Similar to C5a, secretion caused by PAF was minimally affected by the absence of extracellular Ca^{++}, but was significantly inhibited by replacing glucose in the media by 2-deoxyglucose. The enzyme release was markedly temperature dependent, and did not occur at 4^{o}C. The process was dependent on the presence of cytochalasin B, but was unassociated with release of cytoplasmic LDH. PAF caused neutrophil migration into cellulose filters over a concentration range of 10^{-10} to

10^{-5}M. A gradient analysis of this migration showed that AGEPC induced migration was primarily chemotactic in nature, with little stimulation of random migration (chemokinetic stimulation). An unusual characteristic for both enzyme release and migration was a decrease in response between 10^{-6}M and 10^{-5}M AGEPC. The authors suggested that this decreased responsiveness might be due to rapid neutrophil desensitization occurring at high AGEPC concentration, limiting the overall cellular response. The degree of desensitization for lysozyme and MPO secretion was dependent on the concentration of AGEPC during the initial exposure and desensitization was much more complete for the azurophilic granule marker, MPO, than for the specific granule enzyme lysozyme.

The PAF-induced desensitization for secretion appeared to be stimulus specific, in that neutrophils desensitized for subsequent challenge with PAF, responded normally to C5a. Moreover, neutrophils preincubated with C5a prior to the addition of CB, responded normally to AGEPC, whereas they were fully desensitized for lysozyme and MPO release by subsequent challenge with C5a.

Neutrophil aggregation and superoxide production occurred upon exposure to AGEPC. However, in comparison with secretion and chemotaxis, superoxide production and aggregation failed to show a peak or plateau of the response relationship, possibly indicating different control mechanisms for limiting these processes.

Studies have documented that neutrophils activated with different stimuli are able to release PAF (Camussi et al., 1977 & 1980; Virella et al., 1982 & 1983).

In 1977 Camussi et. al. studied the effect of immune complexes, complement and neutrophils on human and rabbit mastocytes and basophils. They showed that: 1) in the presence of immune complexes, with or without the complement, neutrophils released not only lysosomal enzymes but also neutrophil cationic proteins (CP) which were capable of degranulating human basophils and human and rabbit mastocytes; 2) C3a and C5a anaphylatoxins, generated during complement activation by immune complexes, had an effect on basophils/mastocytes similar to that of CP; and 3) histamine and PAF released upon stimulation of basophils and mastocytes by CP and anaphylatoxins. Aggregation of platelets and release of their vasoactive amine content occurred following the exposure of platelets to PAF. The release of PAF by anaphylatoxins and CP was an active process, which was suppressed in the absence of Ca^{++} and at low temperature. The effect of immune complexes on neutrophils was enhanced when neutrophils were incubated with immune complexes in Ab excess in the presence of serum. These investigators suggested that basophils, mastocytes and platelets become involved in inflammatory reaction not only by way of the IgE-mediated mechanism but also as a result of the interaction of immune complexes with complement and immune complexes with neutrophils.

The intravascular release of PAF was demonstrated after the intravenous injection of immune complexes which temporarily correlated with the development of neutropenia. Furthermore, Camussi et. al.(1982) showed that <u>in vivo</u> injection of purified PAF into rabbits lead both to formation of intravascular neutrophil aggregates and to development of acute neutropenia , which had the same features as those observed after challenge with immune complexes, C5a and CP. They suggested that immune complex-induced neutropenia is either due to C5a production or to the interaction between immune complexes and neutrophil surface receptors, resulting in phagocytosis of immune complexes and release of neutrophil CP. Both C5a, CP and phagocytosis are effective stimuli for the release of PAF from neutrophils. Platelet activating factor in turn aggregates neutrophils which <u>in vivo</u> could embolize to microvascular sites, thus possibly playing a pathogenic role in several human immunopathologic states (Issekutz, 1984).

Virella et al. (1982) investigated the interactions of immune complexes and human neutrophils. They found that human neutrophils can be stimulated by large aggregated complexes (heat-aggregated IgG, chemically polymerized IgG, or heavily aggregated human complexes) and by surface-bound immune complexes to release enzymes (lysozyme, beta glucuronidase) and PAF which are able to induce platelet aggregation and ATP release from the platelets. Surface-bound immune complexes were most effective in stimulating the release of PAF. They used several substrates for their preparation: plastic-absorbed antigen, sepharose-coupled antigen and polymerized antigen.

In another study Virella et al. (1983) reported that incubation of human neutrophils with homologous red blood cells (RBC) preincubated with soluble immune complexes resulted in the activation of neutrophils. The corresponding neutrophil supernatants were able to induce platelet aggregation. The stimulation by RBC was also complement-independent and the immune complexes prepared in antigen excess were usually more effective in stimulating neutrophils. They observed that significant immunoglobulin binding only occur when the RBC were incubated with antigen antibody complexes, and not with antibody alone, the incubation of immune complex-coated RBC was an effective stimulus for the release of PAF by neutrophils.

Lotner et al. (1980) reported the release of PAF from both human and rabbit neutrophils after phagocytic stimulus. Human neutrophils from nonatopic individuals were isolated and incubated with opsonized zymosan. They found that the resultant supernatant had the ability to react with washed rabbit platelets and initiate aggregation and release of previously incorporated tritiated serotonin, and with human platelets to induce aggregation.

Betz and Henson (1980) studied the generation of human PAF using neutrophils from normal and Chronic granulomatous disease (CDG) donors. The release of PAF occurred in response to different neutrophils stimuli, indicating PMA and the calcium ionophore A23187, which initiate the relatively selective release of specific granule constituents. The release of PAF was dissociable from neutrophils degranulation and was dependant on the presence of extracellular Ca^{++}. Furthermore, cytochalasin B was required for either enzyme or PAF release by soluble stimuli; however, it had little or no effect on ZC-induced (serum-opsonized zymosan) PAF production at concentrations that consistently enhanced secretion. They suggested that, cytochalasin B may not be required for PAF production, but only for release and that once the signal for production has occured, release will follow as a natural consequence in the presence of cytochalasin B. The release of PAF was also normal in neutrophils from patients with chronic granulomatous disease, indicating that the formation and release of PAF was not dependent upon an intact superoxide generating pathway.

With the use of appropriate inhibitors, it has been documented that the release of PAF is independant of both adenosine-diphosphate (ADP) and arachidonic acid (AA) pathways. Albumin and calcium are required for PAF activity. It appears that albumin binds to PAF and stabilizes its activity (Camussi et al.,1981; Lotner et al. 1980).

C.5. Release of leukotreines

Phospholipids are the major constituent of cell membranes and are subjected to degradation by phospholipases. Phospholipids contain a glyceral backbone which is joined to two fatty acid chains and a phosphate group, which is in turn attached to another small hydrophilic compound such as ethanolamine, choline, inositol, or serine (Figure R.2) (Alberts et al., 1983; Nalbandian and Henry, 1978). Each phospholipid molecule has a hydrophobic tail, composed of two fatty acid chains, and a hydrophilic polar head group, where the phosphate is located.

Phospholipase A_2 cleaves unsaturated fatty acids from the phospholipid, leaving a by-product of lysophospholipid. Other phospholipases such as A_1, C, D or B can cleave bonds at additional sites of the phospholipid. If the fatty acid released by phospholipase A_2 contains 20 carbons and four double bonds it is called an eicosatetraenoic acid. The prefix eicos indicates 20 carbons, and tetra-enoic indicates four double bonds; 20:4 is the symbolic representation of that combination.

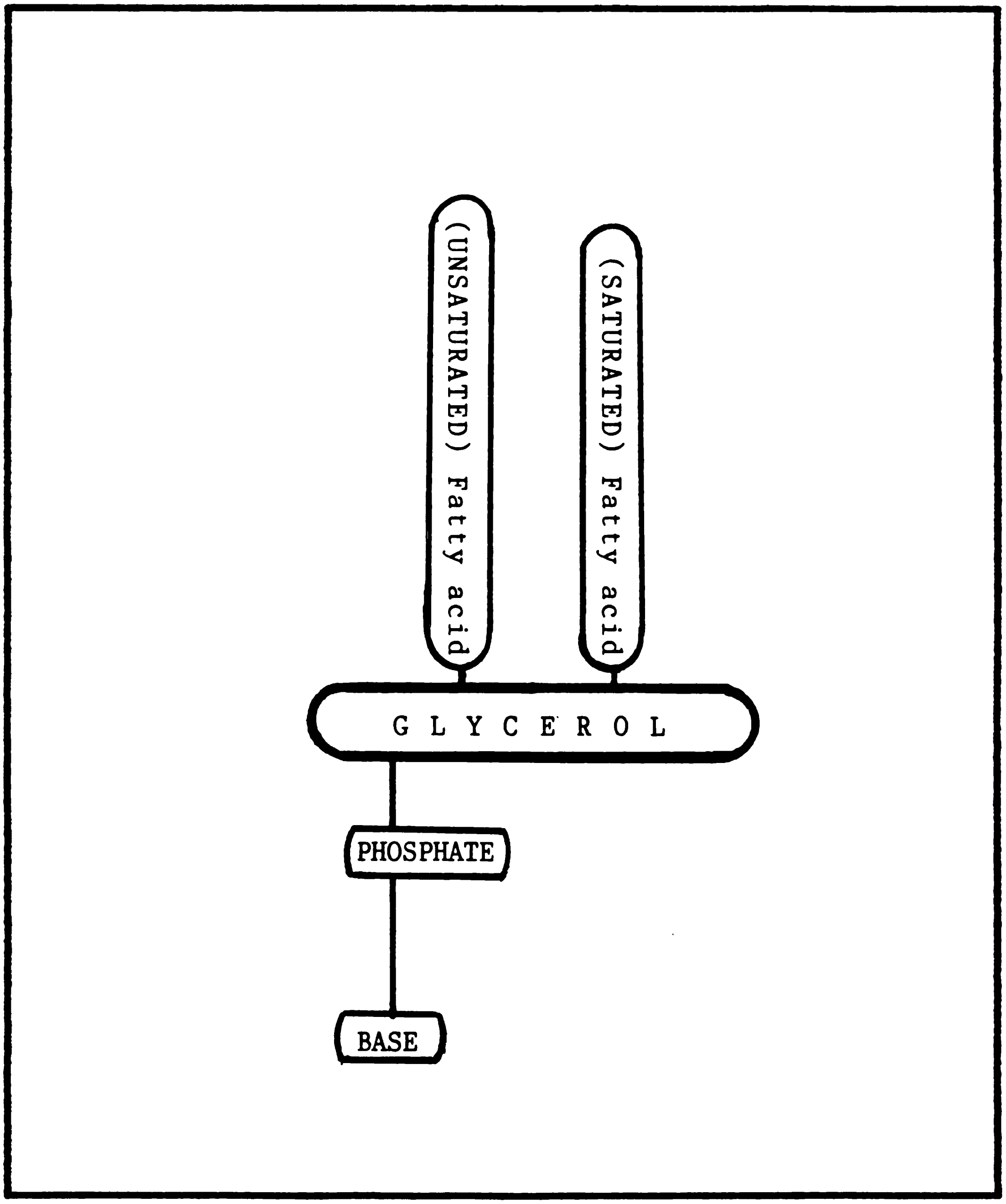


Figure R.2. General Structure of a Phospholipid

Arachidonic acid is 5, 8, 11, 14 eicosatetraenoic acid. The numbers designate the location of it's double bonds. Once freed from the phospholipid, arachidonate can be metabolized by at least two distinct pathways: (1) the lipoxygenase pathway, whose products are the 5-hydroxyeicosatetraenoic acids (5-HETE) and leukotrienes, and (2) the cyclooxygenase pathway, whose products are prostaglandins, prostacyclin (PGI_2), and thromboxane (Figure R.3). Leukotrienes and prostaglandins are formed in many tissues of the body.

C.5.1 Lipoxygenase Pathway

The lipoxygenase pathway has been described in relatively few cell types: platelets, neutrophils, lymphocytes, mast cells, macrophages, testis, a line of rat basophilic leukemia cells (RBL-1), and skin (Borgeat and Sameulsson, 1979; Stenson and Parker, 1979). The products of the lipoxygenase pathway are mono-, di-, and trihydroxy fatty acids. They are formed by the addition of molecular oxygen to arachidonic acid to form unstable hydroperoxy fatty acid intermediates. These peroxide intermediates are then reduced, either enzymatically by a peroxidase or spontaneously, to more stable hydroxy fatty acids. There are a number of different hydroxy fatty acids formed through the lipoxygenase pathway by different cell types. Neutrophils make predominately 5-hydroxy-6, 8, 11, 14-

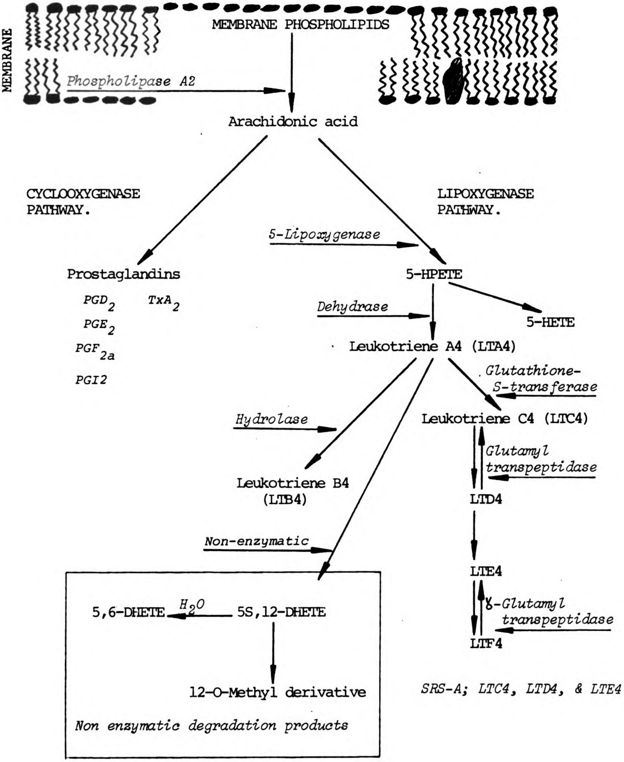


Figure R.3 Metabolism of arachidonic acid (AA).

eicosatetraenoic acid (5-HETE), and 5, 12-dihydroxy-6, 8, 10, 14-eicosatetraenoic acid (5, 12-diHETE, leukotriene B). In addition, neutrophils also make 8-HETE, 9-HETE, and 11-HETE in small quantities. It is not clear if these are all products of the same enzyme or if there is a series of distinct but related lipoxygenases.

In neutrophils arachidonic acid is converted to an unstable peroxide, 5-HPETE (5-hydroperoxyeicosatetraenoic acid) by the 5-lipoxygenase (Figure R.3). 5-HPETE can be reduced to 5-HETE and also can be converted to the unstable expoxide leukotriene A_4 by leukotriene A synthetase. Leukotriene A_4 (LTA_4) is converted by enzymatic hydration (i.e., leukotriene A hydrolase) to leukotriene B_4 (LTB_4) and by the addition of 6-S-gluthathione to leukotriene C_4 (LTC_4). Alternatively, LTA_4 can undergo nonenzymatic degradation. The nonenzymatic degredation products have much less biologic activity than LTB_4 (Figure R.3). Leukotriene C_4 is metabolized to leukotriene D_4 by elimination of glutamyl residue. Further transformation of LTD_4 by elimination of glycine yields leukotriene E_4. Together LTC_4, LTD_4 and LTE_4 form what has been known for many years as the slow-reacting substance of anaphylaxis (SRS-A). LTC_4, LTD_4 and LTE_4 (cysteine-containing leukotrienes) are released from leukocytes and have been found in lung tissue after antigenic challenge. LTC_4, LTD_4 and LTE_4 are bronchoconstrictors and increase the permeability of microvasculature, while LTB_4 is chemotactic and chemokinetic for human leukocytes (Goetzl, 1983; Samuelsson, 1983; Stenson et al., 1984).

In addition, further metabolism of leukotrienes may convert the primary principles to mediators of different activities or to inactive products (Figure R.4). The conversion of LTB_4 to 20-hydroxyl-LTB_4 and 20-carboxyl-LTB_4 depresses the neutrophil chemotactic potency and the smooth muscle contractile activity in several *in vitro* systems (Ford-Hutchinson et al., 1983; Goetzl, 1983; Hansson et al., 1981). The term "leukotriene" was chosen because the compounds were discovered in leukocytes and the common structure feature is a conjugated triene.

C.5.2 Leukotriene B_4 Biosynthesis by Neutrophils and Other Cell Types

Studies have shown that exposure of neutrophils to different stimuli results in the release of the LTB_4. Leukotriene B_4 is produced by other leukocyte preparations including human eosinophils (Goetzl, et al., 1980), monocytes (Ferreri et al., 1986), and both resting and elicited rat peritoneal macrophages (Doig and Ford-Hutchinson, 1980). It has also been detected in perfusates from isolated guinea-pig lungs challenged with antigen (Morris et al., 1979). *In vivo*, LTB_4 has been shown to be present in amniotic fluid embolism (Azegami and Mori, 1986),

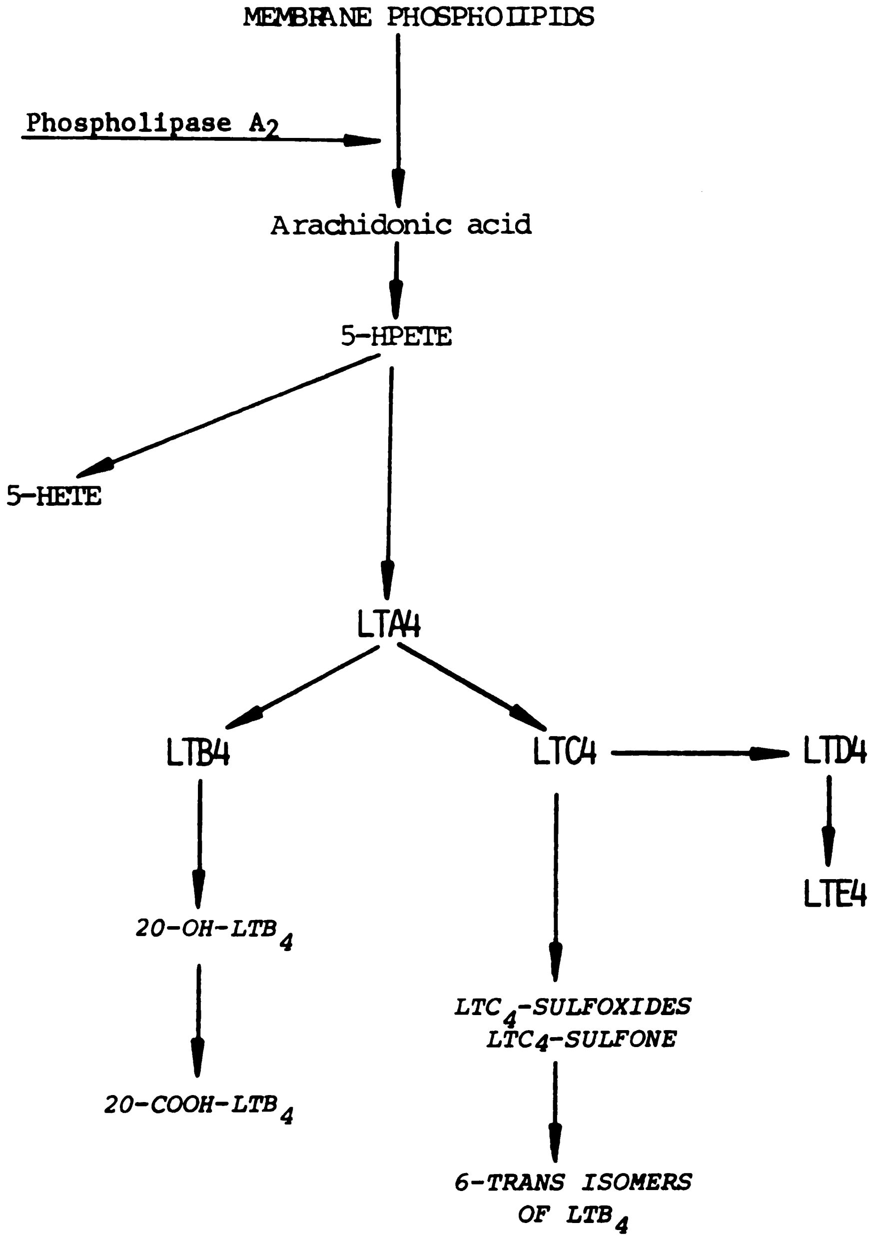


Figure 2.4 Metabolism of leukotrienes.

in synovial fluid from patients with either non-inflammatory arthropathies or rheumatoid arthritis (Klickstein et al., 1980). Leukotriene B_4 has also been found in rat peritoneal fluid five minutes after the anaphylactic challenge (Bray et al., 1981) and is present in the sputum of patients with cystic fibrosis (Cromwell et al., 1981).

In 1979, Borgeat and Samuelsson studied the effects of ionophore A23187 and the exogenous arachidonic acid (AA) on LTB_4 synthesis by human neutrophils. Addition of AA to the suspension of neutrophils led to variable results when cells from several subjects were compared. Some cell preparations showed little activity with respect to the conversion of added AA to LTB_4. However, addition of A23187 together with AA caused a strong conversion of the fatty acid into LTB_4. Ionophore A23187 alone also had strong stimulatory effects on the synthesis of LTB_4 from endogenous substrates. These investigators suggested that, ionophore A23187 not only activates Ca^{++}-dependent phospholipase, it might also directly activate the enzymatic system (or unmask it's activity) involved in the formation of LTB_4.

Jubiz et al., (1982), studied the LTB_4 synthesis by stimulating neutrophils with FMLP. Human neutrophil suspension (10 ml, 100×10^6 cells/ml) was incubated with FMLP (final concentration ranged from 10^{-5} M to 10^{-8}M) for 10 minutes. After extraction and purification, HPLC was performed and the amount of LTB_4 metabolites were estimated from the peak area. The predominant product was 20-carboxyl-LTB_4 (20-COOH-LTB_4), which was derived from LTB_4 by ω-oxidation. A dose-response relationship between FMLP concentration and the amount of 20-carboxyl-LTB_4 released was observed. The amount of 20-carboxyl-LTB_4 recovered was 290 ± 10 ng at 10^{-6}M FMLP, 160 ± 5 ng at 10^{-7}M FMLP, 100 ± 60 at 10^{-8} FMLP and 10 ± 10 ng at zero concentration of FMLP. In addition to the dicarboxylic acid (20-COOH-LTB_4), two other compounds were isolated for the FMLP incubation, including the 20-hydroxyl-LTB_4 (20-OH-LTB_4) compound. The two metabolites of LTB_4 (i.e., 20-carboxyl-LTB_4 and 20-hydroxyl-LTB_4) exhibited chemotactic properties for human neutrophils but were less active in this respect than the parent compount, LTB_4.

Phagocytosis of serum-coated particles (i.e., zymosan and staphylococcus aureus) by human neutrophils have shown to induce the release of LTB_4. Henricks et al., (1985), reported that human neutrophils challenged with serum-coated staphylococcus aureus in the presence of exogenous AA released LTB_4. The release of LTB_4 was dependent on the number of bacteria ingested by the neutrophils. In a similar study, Claesson et al., (1981), demonstrated that ingestion of serum-treated zymosan particles by human neutrophils resulted in the release of LTB_4 from the endogenous substrate. Increased levels of cyclic AMP led to complete inhibition of LTB_4 synthesis. Since cyclic AMP is an important mediator of many processes in leukocytes, these investigators suggested that elevated cyclic AMP levels might decrease chemotaxis by inhibiting the formation and liberation of LTB_4.

Release of LTB_4 and LTC_4 from human monocytes stimulated with aggregated immunoglobulins (Ig) was examined by Ferreri and his coworkers (1986). They found that aggregated IgG, IgA and IgE, but not IgM or monomeric IgG, human myeloma proteins stimulate peripheral blood monocytes to release LTC_4 and LTB_4. Release of leukotrienes was Ca^{++} dependent. Phagocytosis of Ig aggregates was not required for the leukotrienes release, since cytochalasin B did not inhibit the release. In fact, in the case of IgG, release of the leukotrienes was actually potentiated several-fold. Similar effects of cytochalasin B on the release of LTB_4 from monocytes has been reported by Williams et al.,(1986). They reported that FMLP only initiated the generation of LTB_4 from monolayers of human monocytes pretreated with cytochalasin B. Ferreri and his coworkers suggested that cross-linking of Fc receptors by aggregated Ig induced synthesis of LTB_4 and LTC_4 by human monocytes.

The role of functional microtubules for the formation of LTB_4 by neutrophils was investigated by Reibman and her associates (1986). Colchicine or vinblastine (i.e., microtubular-distruptive agents) decreased the formation of LTB_4 and 5-HETE from human neutrophils stimulated with

A23187 in the absence of exogenous AA even more than in its presence. It was pointed out that colchicine did not act by inhibiting the uptake and utilization of exogenous arachidonate, but either decreased the release of arachidonate from membrane phospholipids, or altered the interaction of arachidonate with the lipoxgenases. In addition, colchicine might have modulated the synthesis of lipoxgenase products by an effect on cyclic AMP. Distruption of microtubules by colchicine has been shown to increase the levels of cyclic AMP in the neutrophil (Keller et al., 1984; Rudolph et al., 1977). Increased levels of cyclic AMP has also been shown to inhibit LTB_4 synthesis by neutrophils stimulated with serum-treated zymosan (Claesson et al., 1981). The distruption of microtubules by colchicine might have led to an increase in cyclic AMP levels, which might then have inhibited the formation of the LTB_4 and 5-lipoxygenase products. Therefore, it appears that intact functional microtubules and the presence of Ca^{++} are essential for optimal activity of the lipoxygenase pathway by human neutrophils.

C.5.3 Effects of LTB_4 on Neutrophil Functions

Saturable, sterospecific receptors (a mean of 4400 high-affinity and 270,000 low-affinity) for the potent chemotactic factor of LTB_4, on human neutrophils have been identified (Kreisle and Parker, 1983). The binding of LTB_4 to its receptor initiates activation of the neutrophil.

Leukotriene B_4 enhances the neutrophils adhesiveness which may play an important pathophysiologic role in vivo. Ringertz and his associates (1982), studied the effect of LTB_4 and its metabolites on human neutrophil aggregation. The peak response occurred for LTB_4 at $10^{-7}M$ and for FMLP at $10^{-6}M$, but, while the lowest concentration of FMLP that caused aggregation was $10^{-9}M$, LTB_4 had some effect even at $10^{-10}M$. The 20-hydroxyl-LTB_4 was more active than 20-carboxyl-LTB_4, and almost as active as LTB_4, but did not initiate any aggregation at $10^{-10}M$.

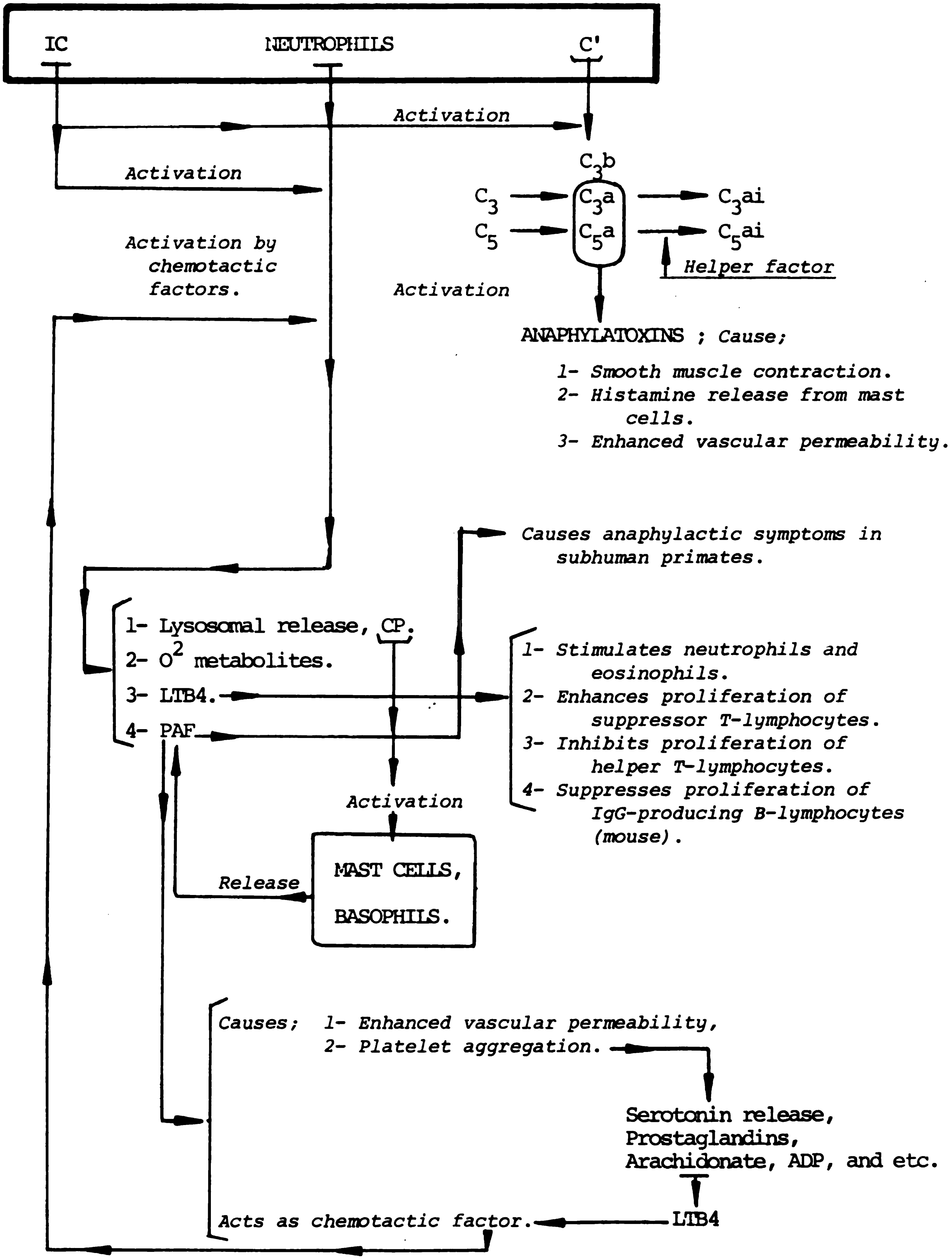
Gimbrone et al., (1984), examined the role of leukotrienes in the regulation of neutrophil adhesion to cultured endothelial cells. They observed that LTB_4 could effectively enhance neutrophil adherence to endothelial cell surfaces in contrast the LTC_4, LTD_4 and LTE_4 that had little or no effect on human neutrophill adhesiveness. The response was dose related and sensitive to the protein (i.e., Albumin) content of the incubation medium. At near physiologic albumin concentration (40 mg/ml), activation was noticeable at $>10^{-8}M$ of LTB_4, whereas at lower albumin concentration, (1 mg/ml), the threshold was reduced to $<10^{-11}M$. At $10^{-6}M$ of LTB_4, the magnitude of adhesiveness was enhanced several fold. These investigators also studied the effect of the 5-HETE (i.e., leukocyte-5-lipoxygenase) and the 12-HETE (i.e., platelet 12-lipoxygenase) that has been reported to have chemoattractant activity. Both 5-HETE and 12-HETE enhanced neutrophil adherence to serum- or fibronectin-coated coverslips, but neither compound had a significant effect on neutrophil adherence to endothelial monolayers. In addition, LTC_4 and LTD_4 that have been shown to increase neutrophil adherence to Sephadex G-25, were ineffective in stimulating neutrophil adherence to endothelial monolayers. These investigators suggested that the mechanism of neutrophil adherence to endothelial cell surfaces may be qualitatively different than that involved in neutrophil interactions with artificial surfaces. Leukotriene B_4 may modulate leukocyte-vessel wall interactions.

A similar study has been done by Palmblad and her associates (1981), who investigated the effects of LTs on neutrophil adherence to nylon fibers and migration under the agarose gel. They found that LTB_4 stimulated the migration of neutrophils under the agarose gel with an optimum concentration at 10^{-6}M. Whereas the 20-carboxyl-LTB_4 induced this response at 10^{-5}M. The LTC_4 and the 5-HETE did not influence neutrophil migration. At the same optimum concentration LTB_4 and 20-carboxyl-LTB_4 enhanced neutrophil adherence to nylon fibers. However, neither LTs nor 5-HETE stimulated the spontaneous or phagocytosis-associated chemiluminescence, nor the bactericidal capacity. Migration of neutrophils through human endothelial monolayers has also been stimulated by LTB_4. Whereas, LTC_4 and LTD_4 were inactive as chemoattractants for neutrophil migration (Hopkins et al., 1984).

The release of lysosomal enzymes from neutrophils by leukotrienes have been studied (Hafstrom et al., 1982; Showel et al., 1982). LTB_4 was found to induce a significant release of lysozyme and beta-glucuronidase from cytochalasin B-treated human neutrophils at 10^{-5}M, but not at lower concentrations. Neutrophils not treated with cytochalasin B also released small amounts of lysozyme but not of beta-glucuronidase once stimulated with 10^{-5}M of LTB_4. However, 20-carboxyl-LTB_4, LTC_4 or 5-HETE did not stimulate secretion of any of the enzymes. The release of both enzymes from cytochalasin B-treated human neutrophils by LTB_4 at 10^{-5}M was approximately half of that detected after stimulation with FMLP at the same concentration (Hafstrom et al., 1981). Showell and his associates (1982), examined the secretory activity of LTB_4 toward rabbit neutrophils. They observed that LTB_4 (10^{-7}M), induced a substantial release of lysozyme into the extracellular medium, which was absolutely dependent on the presence of cytochalasin B, and was enhanced by extracellular calcium. This study indicated that rabbit neutrophils responded to exogenous LTB_4 in a significantly different manner than human cells.

C.5.3.1 Mechanism (S) of LTB_4-Neutrophil Activation

Based on the similarities between the effects of LTB_4 and chemotactic peptide (f-Met-Leu-Phe, FMLP) on neutrophils, it has been suggested that a common mechanism is responsible which leads to neutrophil activation (Molski et al., 1981; Shaafi et al., 1981). The activation of neutrophils by chemotactic factors and other stimuli is believed to be mediated by a rise in the level of intracellular calcium and/or activation of the protein kinase C system (Lew et al., 1984; Shaafi et al., 1981; Volip et al., 1984). Protein kinase C is widely distributed in tissues and organs (Nishizuka, 1984 and 1986). The activation of protein kinase depends on Ca^{++} as well as phospholipid, particularly phosphatidylserine. Chemotactic peptide (i.e., f-Met-Leu-Phe) and LTB_4, activate several of the neutrophil responses by increasing the concentration of intracellular free calcium by mobilization of the same pool (Goldman et al., 1985; Molski et al., 1981; Volpi et al., 1984). Although similar in many ways, the activation of the neutrophil by these two stimuli exhibits significant and important differences. For example, leukotriene B_4, like f-Met-Leu-Phe, mobilizes calcium and is a potent chemotactic agent. On the other hand, LTB_4 is less potent for cell degranulation and a very poor stimulus for the metabolic burst. In addition, contrary to f-Met-Leu-Phe, it does not stimulate the production of leukotrienes, or 5-HETE, from endogenous arachidonate, nor does it stimulate the release of platelet-activating factor (Hafstrom et al., 1981; Palmblad et al., 1981; Prescott et al., 1984).



NEUTROPHIL ACTIVATION. *Overview.*

SECTION I

THE EFFECTS OF N-FORMYL-L-METHIONYL-L-PHENYLALANINE, LEUKOTRIENE-B4, AND TEMPERATURE TRANSITION ON THE EXPRESSION OF C_3b, C_3bi, AND Fc RECEPTORS ON HUMAN NEUTROPHILS IN WHOLE BLOOD AND ISOLATED NEUTROPHILS

MATERIALS AND METHODS

I. PREPARATION OF CHEMOTACTIC SOLUTIONS

A. Chemotactic Peptides

The synthetic chemoattractant, N-formyl-L-methionyl-phenylalanine (f-Met-Phe), was obtained from Sigma Chemical Company, St. Louis, MO., and stored at -70°C as a 10^{-3}M stock solution in 0.1% dimethyl sulfoxide. As needed, the 10^{-3}M f-Met-Phe solution was thawed and diluted to working concentrations.

B. Leukotriene B_4

Leukotriene B_4 (LTB_4) (MW. 336) was obtained from Calbiochem, Behring Diagnostics, La Jolla, CA, 92037. An aqueous stock solution of 10^{-4}M was prepared and stored frozen under liquid nitrogen. As needed, an aliquot was diluted further with Hanks' buffer (HBSS) to the desired concentration.

II. MONOCLONAL ANTIBODIES

Monoclonal antibody to the human neutrophil Fc receptor was kindly provided by Dr. J.C. Unkeless, Mt. Sinai Hospital, School of Medicine, New York. The anti-human Fc receptor was a mouse immunoglobulin of the IgG1 class (3G8). The Fc receptor antibody was diluted ten-fold with PBS containing 5.0% bovine serum albumin (BSA) and stored at-70°C. Monoclonal antibody to the human C_3b receptor was obtained from Dako Corp., Santa-Barbara, CA. The anti-C_3b receptor was a mouse immunoglobulin of the IgG1 class, and stored in the dark at 4-6°C as recommended. Monoclonal anti-human C_3bi receptor (i.e., MO1) was a mouse IgM which was purchased from Coulter Immunology, Hialeah, Florida. A mouse monoclonal antibody against human-T4 antigen which was IgG1 and a purified mouse IgM were also obtained from Coulter Immunology. Fluorescein isothiocyanate (FITC)-labeled goat $F(ab')_2$ anti-mouse immunoglobulins (FITC-GAM) was purchased from Cappel, Cooper Biomedical, West Chester, PA.

III. BLOOD COLLECTION

Peripheral blood was collected in acid citrate dextrose (ACD) solution (Bectin-Dickinson, Rutherford, New Jersey) by the standard venipuncture technique from healthy adult volunteers, who had not taken non-steroidal anti-inflammatory drugs during the preceeding 10 days. All the experiments were immediately performed after the blood was drawn.

IV. ISOLATION OF HUMAN NEUTROPHILS

Five milliliters of citrated blood was overlaid on 4-5 ml of Ficoll-Hypaque (2.16 grams Ficoll, 3.396 grams hypaque; q.s. 34 ml distilled water) (Sigma Chemical Company, St. Louis, MO), and centrifuged at 800 G for 30 minutes at 4°C. Ficoll-Hypaque solution provided a density gradient to separate granulocytes and erythrocytes from platelets and other leukocytes in the peripheral blood. After centri- fugation, the supernatant was removed by suction and the cell button, consisting of granulocytes and erythrocytes, was resuspended in 9.0 ml of CA^{++}-free HBSS. A 1.0 ml of a 6% (W/V) solution of dextran (70,000 MW; Sigma Chemical Company) in Ca^{++}-free HBSS was mixed with the 9.0 ml cell suspension to enhance erythrocyte sedimentation. The mixture was incubated for 45-60 minutes at 20°C. The granulocyte rich supernatant was removed and washed with an equal volume of HBSS. The cells were then resuspended in HBSS and counted by an electronic cell counter (Coulter Counter, Model Z_{BI}). This cell suspension contained greater than 90% granulocytes, of which approximately 95% were neutrophils. Rare platelets or mononuclear cells were observed in the suspension and the neutrophil-to-erythrocyte ratio was consistently greater than 5:1. The neutrophil viability was greater than 98% as determined by trypan blue (0.04% W/V) exclusion. The neutrophils were resuspended at 10^7/ml in HBSS and were used within one hour of isolation.

V. NEUTROPHIL PRETREATMENT

Neutrophil suspensions (10^6 cells/0.1 ml HBSS) or whole blood (0.1 ml) were incubated with 0.1 ml of a specified concentration of f-Met-Phe, LTB_4, or HBSS for a selected time interval and temperature. The treated cells while in the pretreatment solution, were then brought to 4°C and immunofluorescence studies were performed on the cells. All cell pretreatments were carried out in polystyrene microcentrifuge tubes (1.2 ml).

VI. IMMUNOFLUORESCENCE STUDIES

An indirect immunofluorescent procedure was employed using the different monoclonal antibodies and an FITC-conjugated goat anti-mouse IgG. The experiments were carried out at 4°C. For the procedure, 0.1 ml of whole blood was added to a 1 ml polystyrene microcentrifuge tube containing 0.1 ml PBS, LTB_4, or f-Met-Phe as described in Neutrophil Pretreatment. The contents of the tubes were gently mixed using a Vortex mixer and incubated at the desired temperature for 10 minutes. After incubation, the different monoclonal antibodies, Fc, MO1, C_3b, T4 and mouse IgM were added in excess, as determined in preliminary saturation experiments, and the mixtures were incubated at 4°C for 10 minutes. The blood was washed by centrifugation three times using HBSS or 10^{-6}M f-Met-Phe and then the fluorescein-conjugated goat anti-mouse IgG solution was added and incubated with the cells for an additional 10 minutes at 4^{o}C. The blood was washed an additional three times and the erythrocytes were removed using 1 ml of lysing reagent prepared by adding Coulter Immunolyse concentrate to HBSS at a 1:25 dilution. After incubation in the lysing reagent for 20 seconds, 250 µl of Coulter fixative was added to each tube and the cells were washed 2-3 times until the residual erythrocytes were removed. All washes were performed by centrifugation at 600 g for one minute in a microcentrifuge (Model 59A, Fisher) using 1 ml HBSS or 10^{-6}M f-Met-Phe. When isolated neutrophils in suspension were used, the lysing step for erythrocytes was omitted.

The cell-surface fluorescence was analyzed using an EPICS-V flow cytofluorograph (Coulter Electronics, Inc., Hialeah, FL.) set at 488 nm. Forward light scatter, right angle light scatter (i.e., 90^{o}LS), and green fluorescence were measured as each cell passed through the argon laser beam. Fluorescence emission was logarithmically amplified and displayed as a fluorescence profile histogram. Fluorescence intensity of neutrophils was reported as the mean fluorescent channel, average intensity of fluorescence emitted by at least 10,000 cells measured. The T4 monoclonal antibody and a Mouse IgM were used to determine the non-specific binding to neutrophil surface.

VII. STATISTICAL EVALUATION

The data are expressed in terms of a mean ± standard error of the mean; n representing the number of separate experiments and in all cases, the number of separate donors. The analysis of variance (ANOVA) and the least significant difference (lsd) tests were used to assess significance (Steel & Torrie, 1985).

RESULTS

I. C_3b, C_3bi, AND Fc RECEPTOR EXPRESSION BY HUMAN NEUTROPHILS IN WHOLE BLOOD

The assay for assessing the expression of C_3b, C_3bi and Fc receptor antigens on neutrophils was performed by staining the cells in whole blood with different monoclonal antibodies at 4°C. After staining the cells were analyzed using a flow cytometer that simultaneously measured the forward angle light scatter (FALS), right angle light scatter (90°LS), and fluorescence of each cell as it passed through an argon laser beam. Polymorphonuclear leukocytes, monocytes, and lymphocytes have different light-scattering properties, and are represented by three distinct clusters when 90°LS and FALS signals are displayed as a cytogram. Each cluster can be gated and the fluorescence of cells comprising the cluster can be measured. Figure 1.1 shows the cytograms of peripheral blood leukocytes. Immunofluorescent analysis showed a distinct difference in neutrophil expression of C_3b, C_3bi and Fc receptors. C_3b and C_3bi receptors were minimally expressed on the plasma membrane of resting neutrophils, while Fc receptors were expressed in greater amounts as indicated by the difference in the mean fluorescent channel distribution (Figure 1.2).

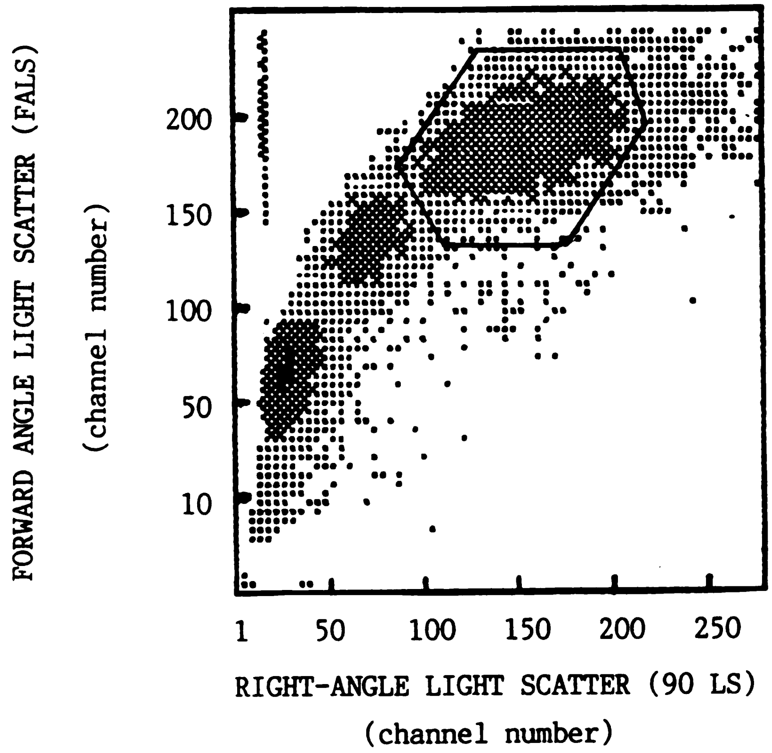


Figure 1.1 Cytogram produced by the forward light scatter (Y axis) and right-angle light scatter (X axis) of cells within the whole blood preparations after the erythrocytes being lysed. Signals for neutrophils, monocytes, lymphocytes, erythrocytes and cell debris have been recorded at different regions of the cytogram according to their light-scattering properties. The dotted lines that form a box in the cytogram delineate the neutrophil clusters and select these cells for analysis of immunofluorescence.

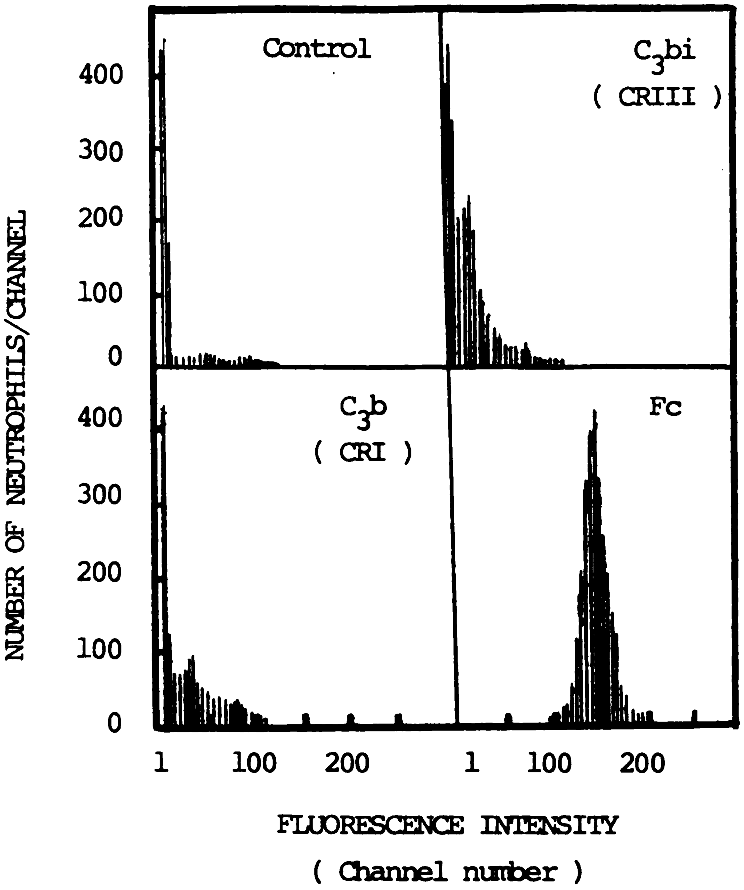


Figure 1.2 Quantitation by flow cytofluorography of C_3b, C_3bi and Fc receptors on resting neutrophils in whole blood. Samples (0.1 ml) of freshly drawn anticoagulated peripheral blood were immediately placed at 4°C. Neutrophils were stained for the antigens using monoclonal antibodies and FITC-conjugated $F(ab')_2$ fragments of goat anti mouse immunoglobulins as described in Methods. A mouse IgG1 anti-T_4 and a purified mouse IgM were used to determine the non-specific binding to neutrophil surface and served as controls. Relative immunofluorescence of neutrophils was determined using the Coulter EPICS-V. Ten thousand cells were analyzed. The cytograms depict the number of neutrophils (Y axis) having variable amounts of fluorescence (X axis).

II. EFFECTS OF TEMPERATURE TRANSITION ON MEMBRANE EXPRESSION OF C_3b,C_3bi, AND Fc RECEPTORS

To determine the possible effect of temperature transition on C_3b, C_3bi and Fc receptor expression, freshly drawn blood was anticoagulated, then immediately divided into four samples designated as A, B, C, and D. All samples were incubated at different temperatures as following. A and B samples were placed at 4°C, and C and D samples were held at 37°C. To D sample, the chemotactic factor f-Met-Phe (final concentration 10^{-6}M), was added. All the samples (A, B, C, and D) were incubated for 10 minutes. After the first incubation, sample B that was held at 4°C was transferred into 37° C. All blood samples were then incubated for an additional 10 minutes. At the end of the second incubation, all the samples (A, B, C, and D) were brought to 4°C and the neutrophils were stained for the receptor antigens and assessed for fluorescence. Neutrophils in the whole blood samples (i.e., A and B) maintained at 4°C or 37°C had a low mean fluorescence. However, neutrophils in whole blood samples (i.e., C) that had been incubated at 4°C for the first incubation and then transferred into 37°C for the second incubation had significantly increased the expression of their receptors relative to the cells held at 4°C (Table 1.1). Addition of f-Met-Phe to the whole blood also caused a significant increase in the expression of C_3b, C_3bi and Fc receptors on neutrophil surfaces (Figure 1.3).

Table 1.1 Samples of citrated blood were incubated at different temperatures as indicated below. At the end of the 2nd incubation, the blood samples were brought to 4$_{O}$C and cells stained with monoclonal antibodies. The mean fluorescent channels of neutrophils were determined by flow cytofluorography.

(*) Results are expressed as the mean relative fluorescence ± SEM for 10,000 neutrophils.

(**) The statistical analysis was done by applying the analysis of variance (Anova) test, then a multiple comparison was performed using the least significant difference (lsd) test at the P-value of 0.05.

(a) Compared to values for PMNs incubated at 4°C ---> 4°C.

(b) Compared to values for PMNs incubated at 37°C ---> 37°C.

(c) Compared to values for PMNs incubated at 4°C ---> 37°C.

(N) represents the number of experiments.

(SD) = significant difference.
(NS) = no significant difference.

Table 1.1 Expression of receptors on neutrophils induced by F-Met-Phe and by temperature transition.

Receptors on Neutrophils	N	Incubations (1st incubation ---> 2nd incubation) 10 min. 10 min.									
		A 4°C ---> 4°C	B 37°C ---> 37°C		C 4°C ---> 37°C			D f-Met-phe (10^{-6}M)			
		Relative Fluoresence *	Relative Fluoresence *	P**	Relative Fluoresence *	P**		Relative Fluoresence *	P**		
				(a)		(a)	(b)		(a)	(b)	(c)
T4	4	2 ± 1	2 ± 1	NS	2 ± 1	NS	NS	2 ± 1	NS	NS	NS
C_3b	4	3 ± 1	7 ± 1	NS	15 ± 4	SD	SD	43 ± 11	SD	SD	SD
C_3bi(M01)	4	18 ± 4	21 ± 3	NS	60 ± 8	SD	SD	98 ± 5	SD	SD	SD
Fc	4	109 ± 4	114 ± 3	NS	121 ± 5	SD	NS	135 ± 6	SD	SD	SD

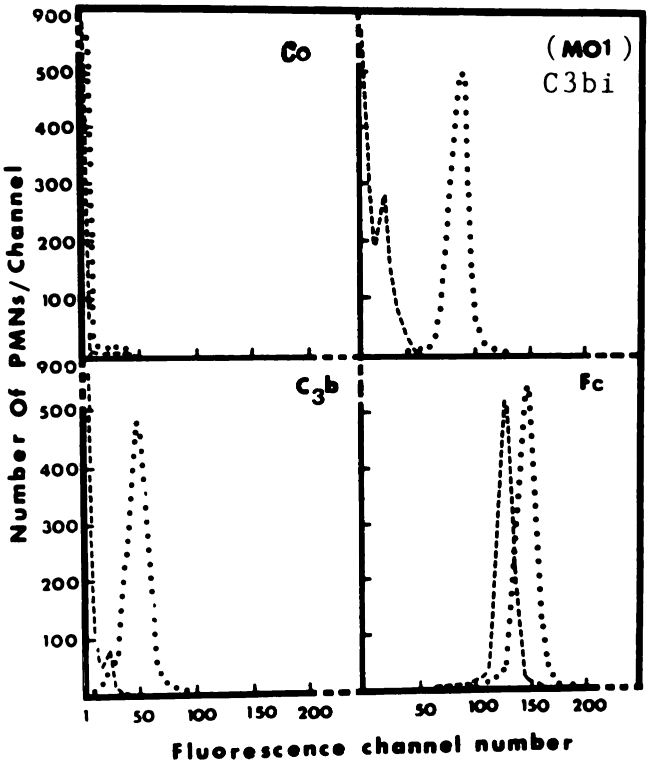


Figure 1.3 Expression of receptors on neutrophil in whole blood. The broken lines represent neutrophils in whole blood stained for the receptors, and incubated at 4°C for 10 minutes, as described in Methods. The dotted lines represent neutrophils in whole blood incubated in 10^{-6}M f-Met-Phe for 10 minutes at 37°C, and stained for the antigens using monoclonal antibodies as described. T4 monoclonal antibody represents the nonspecific binding to neutrophil surface. Relative immunofluorescence of cells was determined using the Coulter EPICS-V. The number of neutrophils (10,000 analyzed) is plotted against log green fluorescence (Channels 1-255).

The percentage of positive neutrophils labeled with C_3b, C_3bi and Fc receptor antibodies were analyzed. The results showed a significant effect of f-Met-Phe stimulation on the percentage of neutrophils expressing C_3b and C_3bi receptors, while no effect was observed on the percentage of neutrophils expressing Fc receptors (Table 1.2). More than 94% of neutrophils expressed Fc receptor on their surfaces before and after incubation with f-Met-Phe. Less than 10% of neutrophils exhibited the C_3b receptor before incubation with f-Met-Phe; while 94% expressed this receptor following a short incubation with chemotactic peptide. For the C_3bi receptor, 45% of neutrophils expressed this receptor before incubation with f-Met-Phe and 98% did after incubation.

III. EFFECTS OF LTB_4 ON MEMBRANE EXPRESSION OF C_3b, C_3bi, AND Fc RECEPTORS

To assess the influence of LTB_4 on neutrophil receptor expression, 0.1 ml of fresh blood was incubated with different concentrations of LTB_4 (10×10^{-12}M to 5×10^{-8}M) at 37^{o}C. After 10 minutes, the different monoclonal antibodies and FITC-F(ab')$_2$ goat anti-mouse immunoglobulins were added as described in methods. The expression of C_3b, C_3bi and Fc receptors on neutrophils were all increased significantly after incubation with LTB_4 at 10^{-10}M to 5×10^{-8}M concentrations. When compared to neutrophils in whole blood incubated with HBSS (Figure 1.4).

Table 1.2 Percentage of positive neutrophils with various antibodies.

Receptors on Neutrophils	N	% Fluorescence*		P**
		PBS	f-Met-Phe (10^{-6}M	
C_3b	6	6	94	SD
C_3bi (M01)	6	45	98	SD
Fc	6	94	95	NS

Stimulated and non-stimulated neutrophils in whole blood were stained for C_3b, C_3bi and Fc receptors at 4°C. Relative fluoresence of cells was measured using the Coulter EPICS V.

(*) Results are expressed as the percentage of positive neutrophils expressing various receptors on their plasma membranes. These were calculated by subtraction of background fluoresence after labeling with control antibody. A Coulter MDADS using the IMMUNO software program was applied for calculation of data. Ten thousand cells were analyzed.

(**) Statistical analysis was done using the T-test variance by transformation of the percentage data into arcsine value at the P-value of 0.05.

(N) Represents the number of experiments.

Figure 1.4 Effect of LTB_4 on the expression of C_3b, C_3bi and Fc receptors on neutrophils in whole blood. Whole blood anticoagulated with ACD was incubated with LTB_4 and f-Met-Phe at the designated concentration for 15 minutes at 37°C. The blood samples were then brought to 4°C and reacted with monoclonal antibody to C_3b, C_3bi and Fc as described in Methods. Relative immunofluorescence of neutrophils was determined using the Coulter EPICS-V and is expressed as the mean fluorescent channel (Channels 1-255) which is the average fluorescence of each population. 10,000 cells were analyzed.

(*) The statistical analysis was done by applying the analysis of variance (Anova) test, then a multiple comparison was performed using the least significant difference (lsd) test at the P-value of 0.05. Comparison was made between the stimulated neutrophils (i.e., LTB_4 and f-Met-Phe) and the neutrophils in a non-stimulatory medium (i.e., PBS).

(SD) = significant difference.

(NS) = no significant difference.

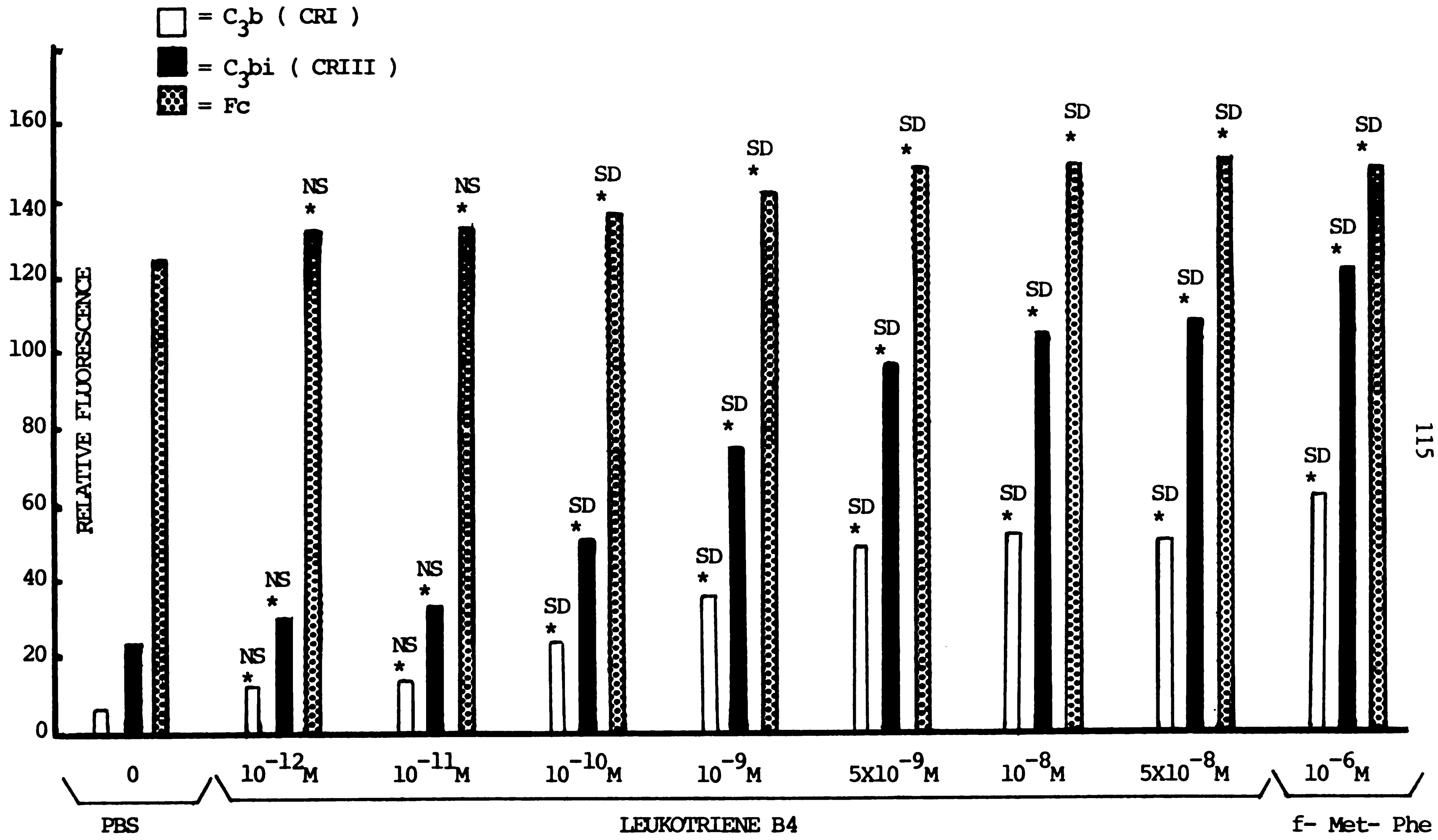


Figure 1.4 Effect of LTB4 on the expression of C_3b, C_3bi, and Fc receptors on neutrophils in whole blood.

Under this condition, LTB_4 at $10^{-11}M$ and $10^{-12}M$ did not significantly effect neutrophil receptor expression for C_3b, C_3bi or Fc.

IV. EFFECTS OF THE ISOLATION PROCEDURE ON RECEPTOR EXPRESSION

To determine the possible effects of the isolation procedure on receptor expression, anticoagulated blood from three donors was divided into paired aliquots and neutrophils were isolated from one aliquot by the standard Ficoll-Hypaque centrifugation as described in Methods. The isolated neutrophils were then used for the experiment and stained with monoclonal antibodies for different antigens. The other blood aliquot was used as whole blood and immediately stained for the receptors. Neutrophils in whole blood had low mean fluorescence for C_3b and C_3bi receptors (i.e., 7 and 25) compared with the fluorescence levels of these receptors on the isolated neutrophils (i.e., 46 and 82) (Table 1.3). Addition of f-Met-Phe or LTB_4 to PMNs in whole blood or neutrophils in suspension induced a significant increase in C_3b and C_3bi receptor expression on neutrophils in whole blood and on the isolated neutrophils. However, there was no significant effect of stimulation of Fc receptor expression on isolated neutrophils. The increased Fc receptor expression was only significant on neutrophils in whole blood.

Table 1.3 Expression of C_3b, C_3bi and Fc receptors on neutrophils in whole blood or after isolation. Whole blood anticoagulated with ACD or isolated neutrophils were incubated with chemotactic factor (i.e., f-Met-Phe or LTB_4) or PBS at 37°C for 10 minutes, then reacted with monoclonal antibodies to C_3b, C_3bi or Fc as described in Methods. A T4 monoclonal antibody and a mouse IgM were used to determine the non-specific binding to neutrophil surfaces and served as the control. Relative fluoresence of cells was measured using the Coulter EPICS V.

(+) Results are expressed as the mean fluorescent channel (Channels 1-255) which is the average fluorescence of each population. 10,000 cells were analyzed.

(*) The statistical analysis was done by applying the analysis of variance (Anova) test, then a multiple comparison was performed using the least significant difference (lsd) test at the P-value of 0.05.

(a) Compared to the value of neutrophils in whole blood incubated with PBS.

(b) Compared to the value of isolated neutrophils incubated with PBS.

(N) Represents the number of experiments.

(SD) = significant difference.
(NS) = no significant difference.

Table 1.3 **Expression** of C_3b, C_3bi and Fc receptors on neutrophils in whole blood and isolated neutrophils.

Receptors on Neutrophils	N	Neutrophils in whole blood					Isolated Neutrophils					
		Relative Fluorescence$^+$					Relative Fluorescence$^+$					
		PBS	f-Met-Phe (10^{-6}M)	P* a	LTB_4 (10^{-9}M)	P* a	PBS	P* a	f-Met-Phe (10^{-6}M)	P* b	LTB_4 (10^{-9}M)	P* b
C_3b	3	7 ± 2	64 ± 3	SD	57 ± 7	SD	46 ± 9	SD	64 ± 2	SD	62 ± 2	SD
C_3bi	3	25 ± 4	126 ± 6	SD	101 ± 4	SD	82 ± 4	SD	121 ± 6	SD	115 ± 3	SD
Fc	3	125 ± 5	154 ± 2	SD	151 ± 5	SD	129 ± 11	NS	132 ± 10	NS	136 ± 2	NS
Control	3	2 ± 1	2 ± 1	NS	1 ± 1	NS	2 ± 1	NS	2 ± 1	NS	2 ± 1	NS

DISCUSSION

The purposes of the study in this section (I), are to 1) investigate the expression of C_3b, C_3bi and Fc receptors on human neutrophils in whole blood, 2) evaluate the effect of chemotactic factors (i.e., f-Met-Phe and LTB_4) and temperature transition on the expression of these receptors in neutrophils, and 3) to examine the differences, which might exist, between the isolated neutrophil and the neutrophil in whole blood as they relate to surface receptor expressions.

In this study, monoclonal antibodies and cytofluorography were used to assess the expression of receptors for C_3b, C_3bi and Fc on the surface of human neutrophils.

The results indicate that resting neutrophil in whole blood expressed relatively few receptors for C_3b and C_3bi, but expressed greater numbers of surface receptors for Fc (Figure 1.2). With neutrophils isolated using Ficoll-Hypaque gradients and maintained at $4^{o}C$, the results were different for C_3b and C_3bi receptor expressions than those of neutrophils in whole blood. As the data demonstrate in Table 1.3, the isolation procedure significantly increased the expression of C_3b and C_3bi, but not the Fc receptor. A

6.5-fold increase for C_3bi and a 3.2-fold increase in C_3bi receptor expression was observed upon isolation. Part of these results are consistent with the observations of Fearon and Collins (1983) who reported a spontaneous increase in the expression of C_3b receptors on human neutrophils due to the isolation procedure. However, contrary to the results presented here, Berger et al., (1984), reported the existence of no significant difference in the expression of C_3b and C_3bi receptors between the isolated neutrophils and the neutrophils in whole blood. This difference could be due to the assay system. In the whole blood assay used by Berger et al., (1984), after addition of monoclonal antibodies, the whole blood was subjected to a rotator for 30 minutes at 4°C. The buffy coat cells were then obtained by centrifugation, and erythrocytes were lysed by a hypotonic procedure. The leukocytes were washed again and then stained with FITC-conjugate. Therefore, it is possible that the rotatory action and the isolation of the buffy coat cells before the completion of immunofluorescent staining have activated neutrophils. As a result, a higher baseline level for C_3b and C_3bi receptor expression on neutrophils might have been obtained. In the whole blood assay applied in this study, neither a rotator was used nor were the buffy coat cells isolated prior to the completion of the staining.

Activation of the isolated neutrophils or neutrophils in whole blood with either of the chemotactic factors (i.e., f-Met-Phe or LTB_4) induced a significant increase in C_3b and C_3bi receptor expression on neutrophils (Table 1.3). The magnitude of stimulation for both the isolated neutrophils and the neutrophils in whole blood was similar. However, f-Met-Phe or LTB_4 stimulation did not significantly enhance the up-regulation of Fc receptors on the isolated neutrophils when compared with isolated cells in the buffer. Stimulation with chemotactic factors induced only a significant increase in the expression of the Fc receptors on neutrophils in whole blood. Of particular interest is the presence of a significant difference between the stimulated-isolated neutrophils and the stimulated neutrophils in whole blood in regards to their Fc receptor expressions. As the data demonstrate in Table 1.3, isolated neutrophils stimulated with chemotactic factors expressed significantly less Fc receptors than the stimulated neutrophils in whole blood. Further studies are necessary to clarify the possible mechanism(S) involved in the alteration of Fc receptor expression apparent in the isolated neutrophils.

The results in this study clearly indicate that LTB_4, a major product of 5-lipoxygenase pathway in activated neutrophils, can stimulate the expression of C_3b, C_3bi and Fc receptors on the neutrophil surface. A noticeable enhanced expression of these receptors (i.e., C_3b, C_3bi and Fc) was observed at 10^{-10}M concentration of LTB_4. The up-regulation of surface receptors reached its maximum at approximately 5 x 10^{-9} to 10^{-8}M concentration of LTB_4 (5 x 10^{-8}M was the highest LTB_4 concentration used in this study). The results may suggest that modulation of these receptors by LTB_4 could partly account for the activation of neutrophils seen _in vitro_ and _in vivo_. Previous _in vitro_ experiments have shown that LTB_4 stimulated the adhesion of neutrophils to endothelial cell surfaces, and enhanced the migration of neutrophils through endothelial monolayers (Hopkins et al., 1984; Gimbron et al., 1984). _In vivo_ studies have demonstrated neutrophil accumulations following the injection of LTB_4 into the rabbit eye and guinea-pig peritoneum. Neutrophil accumulation in humans has also been observed following application of skin chambers containing a solution of LTB_4 over skin abrasions on the forearm. Additionally, in the skin of the rabbit, in the presence of vasodilator (i.e., PGE_2) and neutrophils, the leukotrienes produced a marked increase in vascular permeability, which appeared to result from the interaction of neutrophils with vascular endothelial cells (Wedmore and Williams, 1981). Therefore, LTB_4 may have an intracellular role as a modulator of neutrophil functions partly through its action upon these receptors.

Neutrophils in whole blood exposed to a temperature transition also exhibited an enhanced expression of C_3b, C_3bi and Fc receptors on their surfaces. The purpose of this study was to find out if a temperature transition could contribute to the up-regulation of receptors. Several studies have reported that a temperature transition (i.e., 4°C --> 37°C) can stimulate neutrophils adhesiveness and the release of granule contents from these cells (Charo et al., 1985; Goldstein et al., 1974; Heerdt, 1986). Additionally, different laboratories have reported data regarding the expression of surface receptors which contradict each other. As a result, uninterpretable information has been reported, and in some cases, technical procedures have been suggested to be implicated in these discrepancies.

In the most common standard technique for isolation of neutrophils, whole blood (with 37°C body temperature) is subjected to erythrocyte sedimentation at room temperature (RT) (i.e., approximately 18-22°C) or at 37°C for 45-90 minutes. Then the leukocyte rich plasma will be washed, and leukocytes will be fractionated by passage through a specific gradient using centrifugal force. This step will usually take place at 4°C or RT. After isolating the neutrophils, the cell suspension will be refrigerated until the performance of the experiments which generally are carried out at 37°C. Therefore, it is obvious that neutrophils will be exposed to different temperatures during the isolation.

Berkow and associates (1983), reported that, compared to neutrophils isolated with ficoll/hypaque gradients, cells obtained by the elutriation technique generated significantly more superoxide anion and released significantly more vitamin B12 binding protein, lysozyme and beta-glucuronidase when stimulated with FMLP in the presence of cytochalasin B. In contrast to this report is the study by Heerdt (1986) who studied the effect of temperature transition and technical procedures on the release of lactoferrin (specific granules) from neutrophils. She applied two different techniques, the ficoll/hypaque gradient and the elutriation technniques for the isolation of neutrophils. She reported that the technique played no apparent role on the extracellular granule release. Temperature transition (i.e., 37°C, body temperature --> 4°C, isolation temperature --> 37°C, experimental temperature) played the key role in the release of granule contents.

In this study, in order to control the temperature transitions and prevent the effect of other factors (e.g., centrifugal force, washing, and etc.) during neutrophil preparations, the experiment was designed so that the isolation procedure was omitted. A whole blood assay was developed and the effect of temperature on the expression of neutrophils was studied. All the necessary reagents and tubes were prepared and left at the desired temperature prior to the blood drawing. The experiment was carried out immediately after the blood was drawn. Blood samples were incubated at the desired temperature for 10 minutes, then subjected to a temperature transition and incubated for another 10 minutes. The expression of the receptors was evaluated immediately by staining the neutrophil receptors in the whole blood at 4°C as described in Methods. As the data demonstrates in Table 1.1, when blood was kept at 4°C for ten minutes and then warmed to 37°C, neutrophils expressed a significantly increased up-regulation of C_3b, C_3bi and Fc receptors. In contrast, neutrophils in whole blood kept at either 4°C or 37°C for the entire incubation period and not subjected to a temperature transition exhibited no significant differences in their receptor expression. The data suggests that the up-regulation of these receptors is through a metabolic process(es) which can be initiated by a temperature transition or a chemotactic stimuli. This metabolic activity appears to occur at a much slower rate by temperature manipulation, since the up-regulation of these receptors did not reach maximal expression, similar to those induced by chemotactic factors. However, it should be considered that, a longer incubation time might be essential for the maximum receptor expression to occur by temperature transition. Further investigation is required to clarify this matter.

In considering if the C_3bi receptor is partially related to neutrophils adhesion to albumin-coated surfaces or cultured endothelial cells, the results presented here for the expression of C_3bi are consistent with the observations of Charo et al., (1985). These investigators reported that a temperature increase from 4°C to 37°C enhanced neutrophil adhesion to albumin coated plastic and cultured endothelial cells.

The data presented above appear to suggest that temperature transition has a significant role in the discrepancy among data reported by different laboratories. However, centrifugal force, the action of breaking the cell pellet, and other cell manipulation during the isolation procedure should not be neglected. The whole blood assay developed in this study appears to eliminate many of the problems (i.e., cell loss, damage, activation, etc.) which are associated with neutrophil isolation. It provides an assay system with less uncertainty about different factors which might interfere with the overall interpretations. In addition, the expression of receptors is examined in whole blood milieu, which represents a more relevant physiologic condition.

In conclusion, the results reported here indicate that 1) unactivated neutrophils in whole blood exhibit a minimal number of C_3b and C_3bi receptors, while expressing a much greater number of receptors for Fc on their surfaces, 2) activation of neutrophils in whole blood by f-Met-Phe, LTB_4, or temperature transition (i.e., 4°C --> 37°C) induced a significant up-regulation of C_3b, C_3bi, and Fc receptors on their plasma membranes, 3) although the up-regulation of

C_3b, C_3bi and Fc receptors by temperature transition was significantly enhanced on neutrophils in whole blood, it was not near the maximal expression of these receptors when induced by the chemotactic peptide, f-Met-Phe, 4) isolated neutrophils in a non-stimulatory medium had significantly more receptors for C_3b and C_3bi on their surfaces than neutrophils in whole blood, isolation had only a moderate effect on the up-regulation of the Fc receptor, and 5) activation of the isolated neutrophils with f-Met-Phe or LTB_4 induced only a significant expression of C_3b and C_3bi receptors on their surfaces.

The results reported here raise the possibility that; 1) LTB_4 which has been considered a potent mediator of hypersensitivity and inflammation, may modulate neutrophil functions by it's effect on the expression of C_3b, C_3bi and Fc receptors, becoming involved in the regulation of leukocyte-endothelial cell adhesion. 2) The discrepancy existing among different reports regarding the up-regulation of neutrophil receptors might be due to the temperature transitions which occur during the isolation of neutrophils. One particular issue is Fc receptor expression, where up-regulation may be a physiologic process obscured by cell isolation procudures. 3) The whole blood assay developed in this study may facilitate assessment of neutrophil responsiveness in clinical and research laboratories, by eliminating many of the difficulties associated with neutrophil isolation.

SECTION II

THE EFFECTS OF SOLUBLE AND INSOLUBLE IMMUNE COMPLEXES ON THE EXPRESSION OF C_3b, C_3bi, AND Fc RECEPTORS ON HUMAN NEUTROPHILS IN WHOLE BLOOD

MATERIALS AND METHODS

I. PREPARATION OF CHEMACTIC SOLUTION

A. Chemotactic Peptides

The synthetic chemoattractant, N-formyl-L-methionyl-L-phenylalanine (f-Met-Phe), was prepared as described in Section I.

II. MONOCLONAL ANTIBODIES

Monoclonal antibody to human Fc, C_3b, C_3bi and T4 antigens were all prepared as described in Methods of Section I.

III. IMMUNE COMPLEXES

Immune Complexes (ICs) were prepared with normal human serum albumin (HSA) (Sigma Chemical Company) and rabbit IgG antibodies (Cappel, Cooper Biomedical Inc., Malvern, PA) to HSA.

A. Preparation of Monomer Albumin by High-Performance Liquid Chromatography

Human Albumin was gel filtered on Waters PROTEIN-PAK_{300SW} column (7.5 mm (ID) X 300. mm) (Waters, Millipore Corporation, Milford, Mass) using high-performance liquid chromatography (HPLC). PROTEIN-PAK_{300SW} column is packed with a rigid, hydrophilic porous silica gel, separates proteins ranging in molecular weight from 10,000 to 500,000 Daltons (native globular). The column provides rapid separation, purification and characterization of proteins. PROTEIN-PAK column accomadates flow rates of up to two ml/min., providing high efficiency separations in minutes compared to hours or days required to accomplish separations with conventional soft gel techniques. This column can be used to isolate up to 50 mg of protein per separation. The Perklin-Elmer TriDet^{TM} Detector/3D^{TM} HPLC (Norwalk, Conn.) support was used. The 3D HPLC system consists of three units, TriDet Detector, Series 100 Pump, and the R50 Recorder. The series 100 Pump is for the accurate delivery of mobile phase. The sample to be analyzed is loaded into the injector. The sample flows from the injector through a packed column (in this case, Waters PROTEIN-PAK$_{300SW}$) and into the unique trifunctional detector flowcell. The flowcell is capable of simultaneously monitoring three physical properties of the sample (ultraviolet absorbance, fluorescence and conductivity). The R50 recorder receives the detector output from only one channel and displays it in chart form (chromatogram).

The column was equilibrated with 0.1 M Potassium Phosphate dibasic, pH 7.0, at a flow rate of 1. ml per minute. The column was calibrated with the following substances: Blue dextram (2000 X 10^3 M.W.), catalase (210 X 10^3 M.W.), aldolase (158 X 10^3 M.W.), human albumin

(66×10^3 M.W.) ovalbumin (43×10^3 M.W.), chymotrypsinogen A (25×10^3 M.W.) and ribonuclease A (13.7×10^3 M.W.). Concentrations of the standard solutions were at 10 mg/0.1 ml buffer. A 0.05 ml of each standard solution was separately loaded into the injector, and the protein molecules were scanned using an ultraviolet (UV) mode. The retention time for each protein standard was recorded and a calibration (standard) curve was prepared (Table 2.1 and Figure 2.5). A four-protein mixture of blue dextran, catalase, human albumin and ribonuclease A was used to observe the resolution, and each protein fraction was collected at their main peak. Each protein fraction was identified by it's specific retention time using the standard curve, and was verified by electrophoresis (Figure 2.1 and 2.2). A solution of human serum albumin (HSA) was prepared at 250 ug/ml buffer, clarified by centrifugation if necessary. 0.1 ml of HSA solution was loaded into the injector, the monomer albumin molecules were identified by their specific retention time using the standard curve, and were collected (Figure 2.3). The protein content of the monomer albumin solution was determined using a modified Folin method (Appendix A).

B. Preparation of Immune Complexes

Immune complexes were prepared by adding a constant amount of anti-HSA antibody (i.e., 0.2 ml antibody which contained 1000 µg antibody protein) to various amounts of antigen HSA (i.e., 5 to 1000 µg). After incubation for one hour at 37°C and overnight at 5°C, the complexes were centrifuged at 2000Xg for 15 minutes at 5°C. The complexes were then washed twice with cold PBS and resuspended in 1 ml of PBS. The total protein in the precipitates was measured by a modified Folin method (Appendix A) with human IgG (Accurate Chemical and Scientific Corp., Westbury, New York, 11590) used as the standard. By quantitative precipitin analysis equivalence was found to be 10 µg HSA per 100 µg antibody (Figure 2.4). For these studies, insoluble immune complexes were prepared at equivalence, and soluble complexes were made in antigen excess as indicated in Results. For some experiments, labeled immune complexes were used in which a fluorescein isothiocyanate-(FITC) labeled rabbit (IgG) anti-human albumin (Cappel, Cooper Biomedical) was used as the antibody. Also, immune complexes which lack the Fc portion of their antibody were prepared. These complexes were made of rabbit $F(ab')_2$ anti-human albumin and monomer HSA. In addition, a different type of immune complex was made. In this complex, a goat anti-human albumin (Cappel, Cooper Biomedical) was used as the antibody source.

All the products were sterilized as provided by the companies and the reagents were examined for endotoxin contamination using a Limulus Amebocyte Lysate (LAL) test (Appendix B).

Protein	Molecular Weight	Retention Time (Minutes)
Blue Dextran 2000		4.2
Catalase	210×10^3	6.6
Aldolase	158×10^3	6.9
Albumin (Human)	66×10^3	7.3
Ovalbumin	43×10^3	8.1
Chymotrypsinogen A	25×10^3	9.2
Ribonuclease A	13.7×10^3	9.4

Table 2.1 Retention time for different protein molecules using a Waters PROTEIN-PAK$_{300SW}$ Column and a 3D HPLC support. 2-5 mg of different protein molecules loaded into a 3D HPLC support. Column: 7.5 mm X 300 mm Waters PROTEIN-PAK$_{300SW}$; mobile phase; 0.1 M potassium phosphate dibasic, pH 7.0; flow rate: 1 ml/minute.

Peak #	Protein	MW
1)	Blue Dextran	2000 X 10^3
2)	Catalase	210 X 10^3
3)	Human Albumin	66 X 10^3
4)	Ribonuclease A	13.7 X 10^3

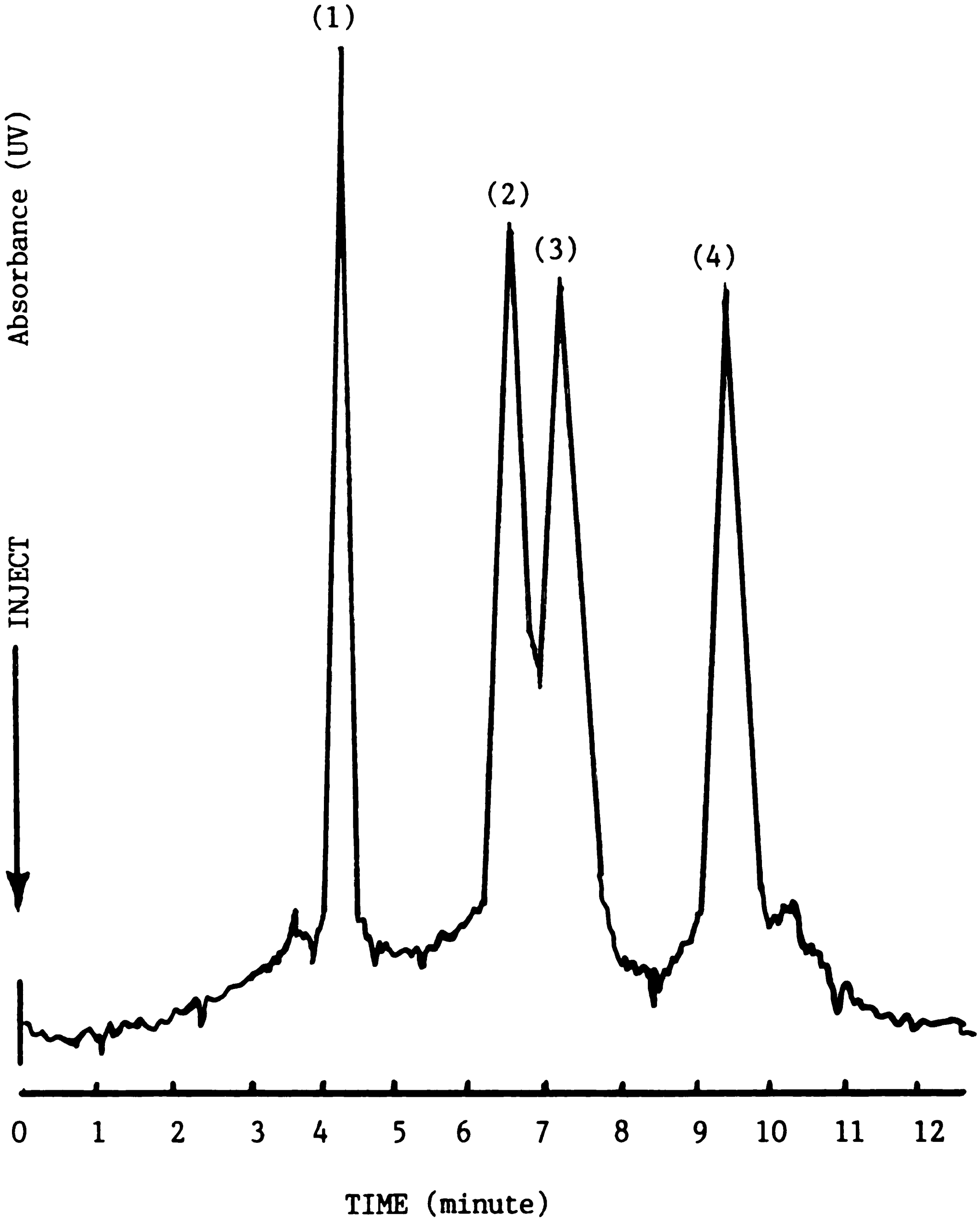
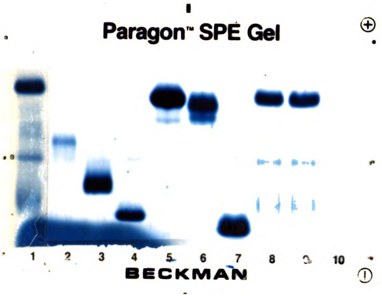


Figure 2.1 Analysis of a protein mixture using a 3D HPLC support. Column: 7.5 mm X 300 mm Waters PROTEIN-PAK$_{300SW}$; Mobile phase: 0.1 M phosphate dibasic, pH 7.0; Flow rate: 1 ml/minute.

Figure 2.2 Electrophoresis of protein molecules in agarose gel. Different protein fractions were collected and identified by their specific retention time. The protein fractions were then verified by electrophoresis. **A:** demonstrate the known standard proteins migrated in an electrical field. **B:** demonstrates the migrational pattern of protein fractions collected using a Waters PROTEIN-PAK$_{300SW}$ Colunm in an electrical field.

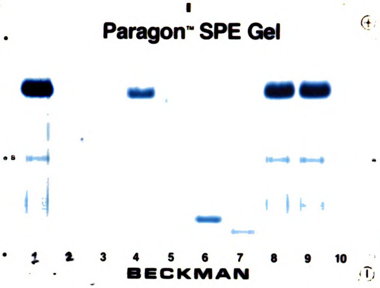
Figure 2.2



The Standard Proteins:

#2: Catalase
#3: Aldolase
#4: Chymotrypsinogen A
#5: Human Albumin
#6: Ovalbumin
#7: Ribonuclease A

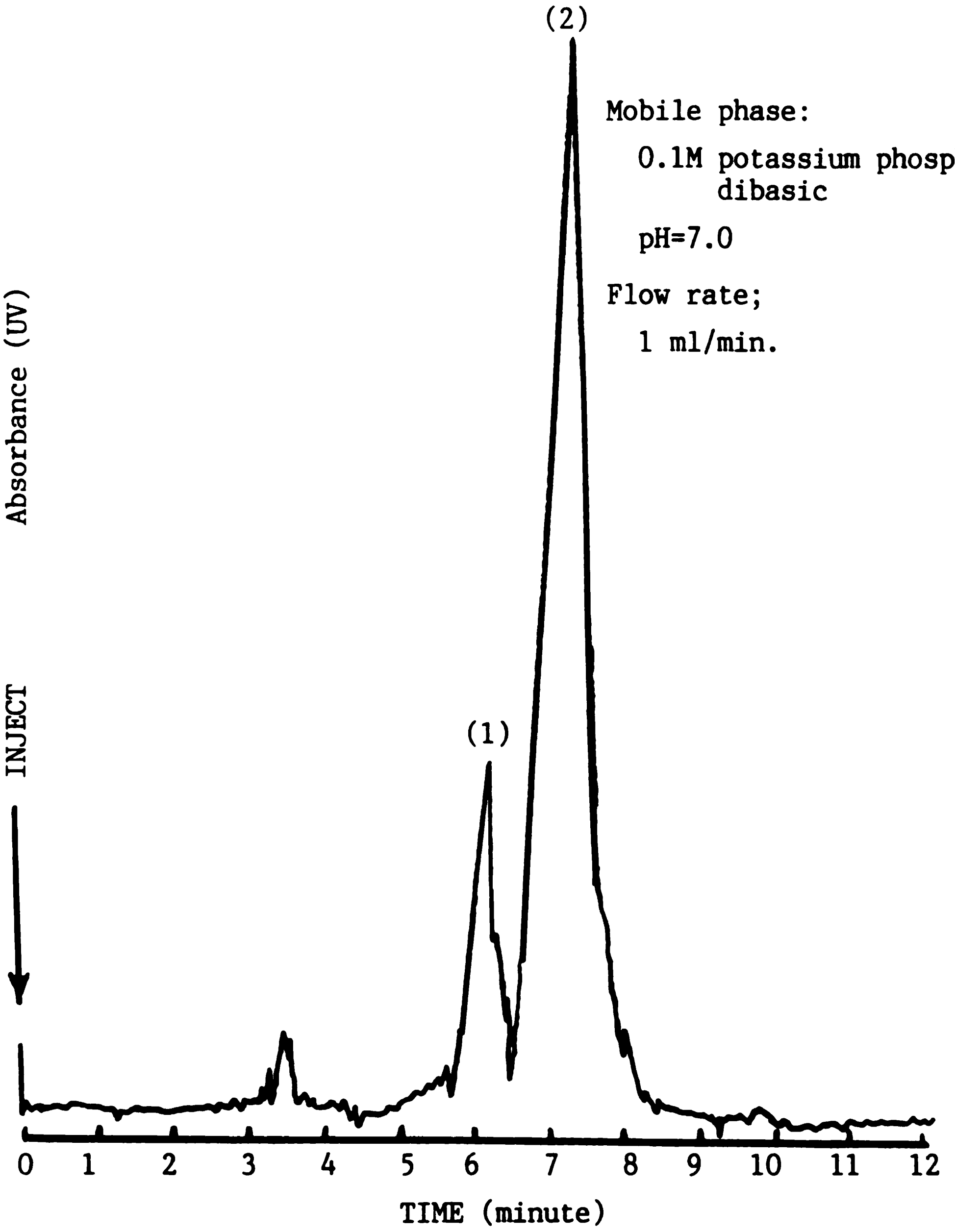
#1, 8 and 9 are the control proteins for the paragon SPE gel.



Protein Fractions Analyzed by 3D HPLC:

#2: fraction C (Catalase)
#3: fraction A (Aldolase)
#4: fraction HAL (Human Albumin)
#5: fraction OAL (Ovalbumin)
#6: fraction CtA (Chymotrypsinogen A)
#7: fraction RA (Ribonuclease A)

#1, 8 and 9 are the control proteins for the paragon SPE gel.



Mobile phase:
0.1M potassium phosphate dibasic
pH=7.0
Flow rate;
1 ml/min.

Figure 2.3 Chromatogram of human serum albumin using a Waters PROTEIN-PAK$_{300SW}$ Column and a 3D HPLC support. 0.1 ml of HSA solution (250 µg/ml) loaded into the injector, and ultraviolet (UV) mode was selected to scan the albumin molecules, and the chromatogram was recorded. By applying the calibration curve and the retention time, it is apparent that peak #1 is correspondent to polymers (retention time = 6.2 minutes; MW > 200 X 10^3) and peak #2 is correspondent to monomers (retention time = 7.3 minutes; MW = 66 X 10^3).

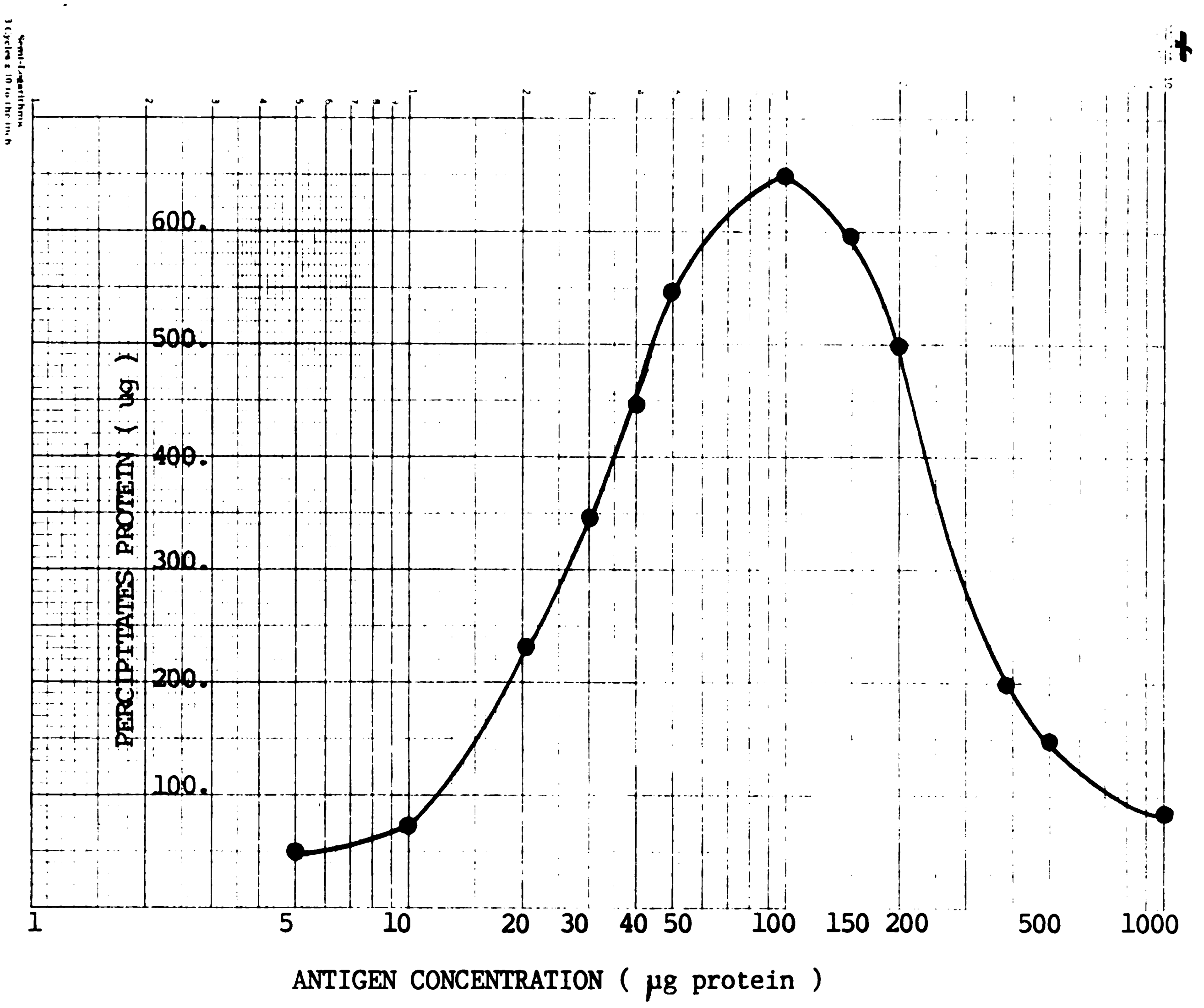


Figure 2.4 Histogram of the immunoprecipitin occuring between anti-HSA (Rabbit) and monomer HSA. Varying concentrations of monomer HSA (5 to 1000 µg protein) were added to a constant concentration of antibody (i.e., 1000 µg antibody protein). After incubation for 1 hour at 37°C and overnight at 5°C, the complexes were centrifuged and washed 2 times with cold PBS. The total protein in the precipitates was measured. The various concentrations of antigen (X-axis) is plotted against the total protein in the precipitates. The data demonstrates the point of equivalence to be 100. µg of HSA per 1000 µg of antibody protein (i.e., 1:10 Ag/Ab ratio).

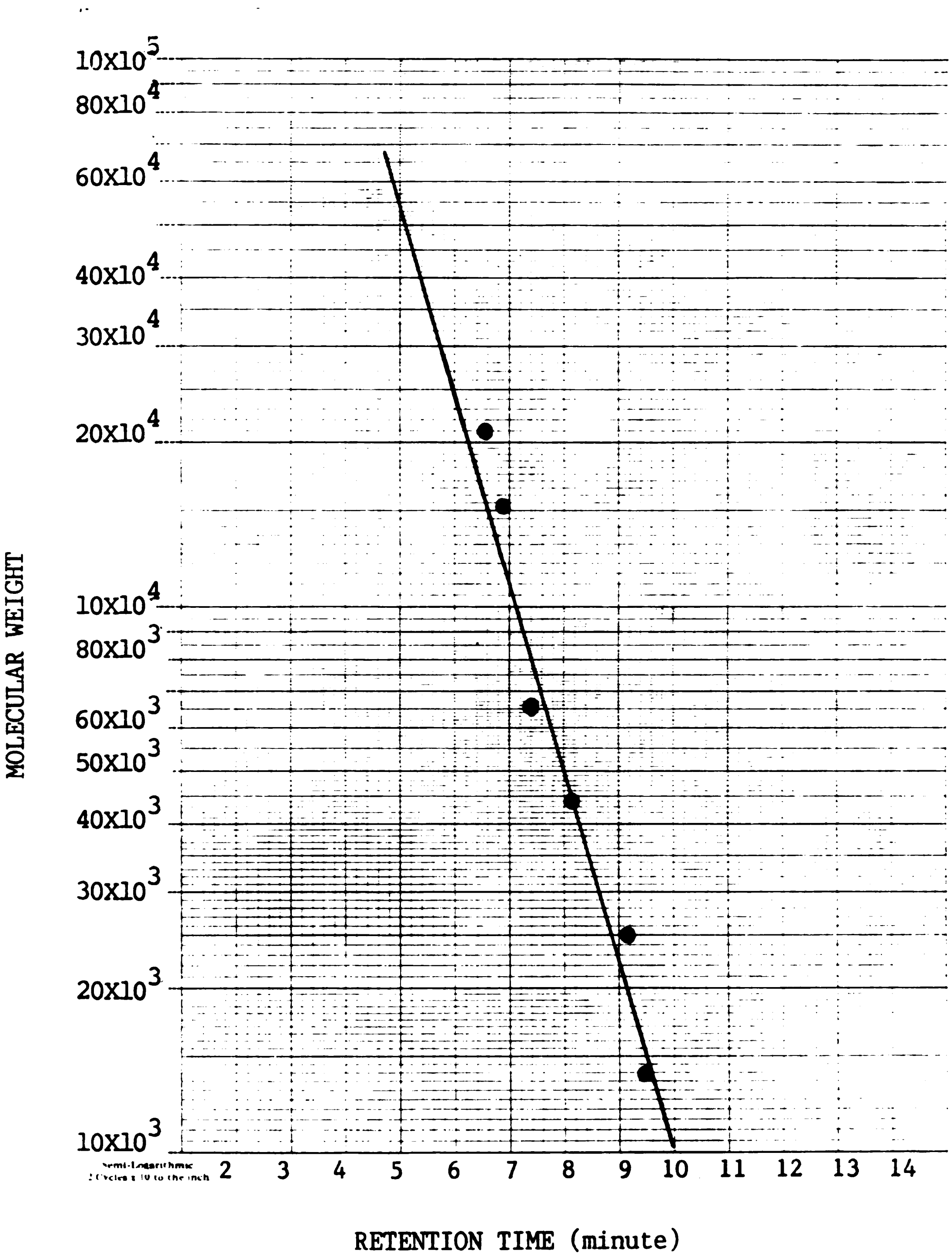


Figure 2.5 The standard curve of different molecular weight proteins using a 3D-HPLC system.

IV. BLOOD COLLECTION

Peripheral blood was collected from healthy adult volunteers as described in Methods, Section I.

V. UPTAKE OF IMMUNE COMPLEXES BY HUMAN NEUTROPHILS IN WHOLE BLOOD

0.1 ml of whole blood was added to a 1 ml polystyrene microcentrifuge tube containing 0.1 ml PBS or f-Met-Phe (final concentration = 10^{-6}M). The contents of the tubes were gently mixed using a Vortex mixer and incubated for 10 minutes at 37°C. The treated cells while in the pretreatment solution, were then incubated with different types of FITC-immune complexes for 15 minutes at 37°C. The samples were then washed three times with magnesium-free HBSS and the erythrocytes were removed using 1 ml of a lysing reagent prepared by adding Coulter Immunolyse concentrate to Mg^{++}-free HBSS at a 1:25 dilution. The contents of the tubes were immediately mixed, then after 20 seconds 0.25 ml of Coulter fixative was added to each tube and mixed well using a pipet. The cells were then washed 2-3 times with HBSS until the residual erythrocytes were removed. All washes were performed by centrifugation at 600 g for one minute in a microcentrifuge (Model 59A, Fisher) using 1 ml Mg^{++}-free HBSS. Florescence of neutrophils was analyzed using an EPICS-V flow cytofluorograph (Coulter Electronics, Inc., Hialeah, FL) set at 488 nm. Forward angle light scatter

(FALS), right angle light scatter (i.e., 90^{o}LS), and green fluorescence were measured as each cell passed through the argon laser beam. Fluorescence emission was logarithmically amplified and displayed as a fluorescence profile histogram. Fluorescence intensity of neutrophils was reported as the mean fluorescent channel, average intensity of fluorescence emitted by at least 10,000 cells measured. In addition, neutrophils were mounted on glass slides, coverslipped and examined for immunofluorescence using a Zeiss microscope with UV illumination.

In a few experiments, neutrophils in whole blood were exposed to immune complexes which were not conjugated with FITC. The presence of the immune complexes on the surface of neutrophil was demonstrated by staining the complexes with the FITC-antibody against them. In duplicate samples, neutrophils in whole blood (0.1 ml) were pretreated with cytochalasin-B (10 µM) for five minutes at 37^{o}C. The treated cells were then incubated with immunce complexes while in the pretreatment solution as described at the above.

VI. UPTAKE OF SOLUBLE IMMUNE COMPLEXES BY HUMAN ERYTHROCYTES

1 ml of citrated blood was placed into a FALCON polystyrene conical tube (17 X 120 mm, Becton Dickinson, Lincoln Park, New Jersey), and washed three times with

saline. Then, a 20% solution of erythrocytes was prepared by resuspending the cell pellet in Mg^{++}-free HBSS. 0.1 ml of erythrocytes was added to a 1 ml polytyrene microcentrifuge tube containing different concentrations of FITC-labeled soluble immune complexes or HBSS. The contents of the tubes were gently mixed and incubated at 37°C. After 15 minutes, erythrocytes were washed two times with Mg^{++}-free HBSS, and the cells were then fixed with 1% paraformaldehyde. To examine the presence of FITC-labeled soluble immune complexes on erythroc tes, the cells were mounted on glass slides, coverslipped, and examined using a Zeiss fluorescence microscope.

VII. NEUTROPHIL PRETREATMENT

Neutrophils in whole blood (0.1 ml) were incubated with 0.1 ml of a specific concentration of f-Met-Phe, soluble immune complexes, insoluble immune complexes, or Mg^{++}-free HBSS for 15 minutes at 37°C. The treated cells in whole blood were washed twice with Mg^{++}-free HBSS and divided into two groups for different experiments. For the first group, the treated cells were brought to 4°C and stained for C_3b, C_3bi and Fc antigens by an indirect immunofluorescent technique using monoclonal antibodies as described in Methods, Section I. For the second group, the treated cells were exposed to f-Met-Phe (final concentration 10^{-6}M) for ten minutes at 37°C. The cells were then stained for C_3b,

C_3bi and Fc antigens at 4°C by an indirect immunofluorescent technique.

VIII. STATISTICAL EVALUATION

The data are expressed in terms of a mean ± standard error of the mean; n representing the number of separate experiments and in all cases, the number of separate donors. The analysis of variance (ANOVA) and the least significant difference (lsd) tests were used to assess significance.

RESULTS

I. UPTAKE OF IMMUNE COMPLEXES BY HUMAN NEUTROPHILS IN WHOLE BLOOD

A. Uptake Of Insoluble Immune Complexes By Neutrophils

These experiments were performed with insoluble (precipitated) immune complexes composed of human monomer albumin and fluorescein conjucated IgG fraction of anti-human albumin at equivalence as described in Methods. Samples (0.1 ml) of freshly drawn anticoagulated whole blood were exposed to different concentrations of insoluble fluoresceinated-immune complexes (FL-ICs). After 15 minutes incubation at 37°C, the blood samples were washed twice with magnesium-free HBSS, and the leukocytes fixed after lysing the erythrocytes. The uptake of immune complexes by neutrophils was quantitated by measuring the relative fluorescence of neutrophils using the Coulter EPICS-V. The results showed that the uptake of immune complexes by neutrophils was in a dose-dependent manner (Figure 2.6). Saturation of uptake by neutrophils was achieved at a dose of 60-100 ug of antibody protein in the immune complexes used (Table 2.2).

An immunofluorescence microscopic examination was also performed, and neutrophils exhibited diffusely distributed

Figure 2.6 Dose-response curves for the uptake of immune complexes by stimulated and non-stimulated human neutrophils. Stimulated and non-stimulated neutrophils in whole blood (0.1 ml) were incubated with FITC-immune complexes (ICs) containing different concentrations of antibodies to human serum albumin for 15 minutes at 37°C. Relative fluorescence of cells was measured using the Coulter EPICS-V. Solid lines represent neutrophils which were exposed to insoluble ICs. The broken lines represent neutrophils which were exposed to soluble ICs prepared at 10 times antigen excess (IC-10XAg). (●), non-stimulated neutrophils in whole blood incubated with ICs. (+), neutrophils in whole blood were preincubated with f-Met-Phe (10^{-6}M) for 15 minutes at 37°C, and then were exposed to ICs.

Results are expressed as the mean fluorescent channel (Channels 1-255) which is the average fluorescence of each population. 10,000 cells were analyzed. The concentration of ICs is plotted against the log green fluorescence (Channel 1-255). Representative figure from three experiments.

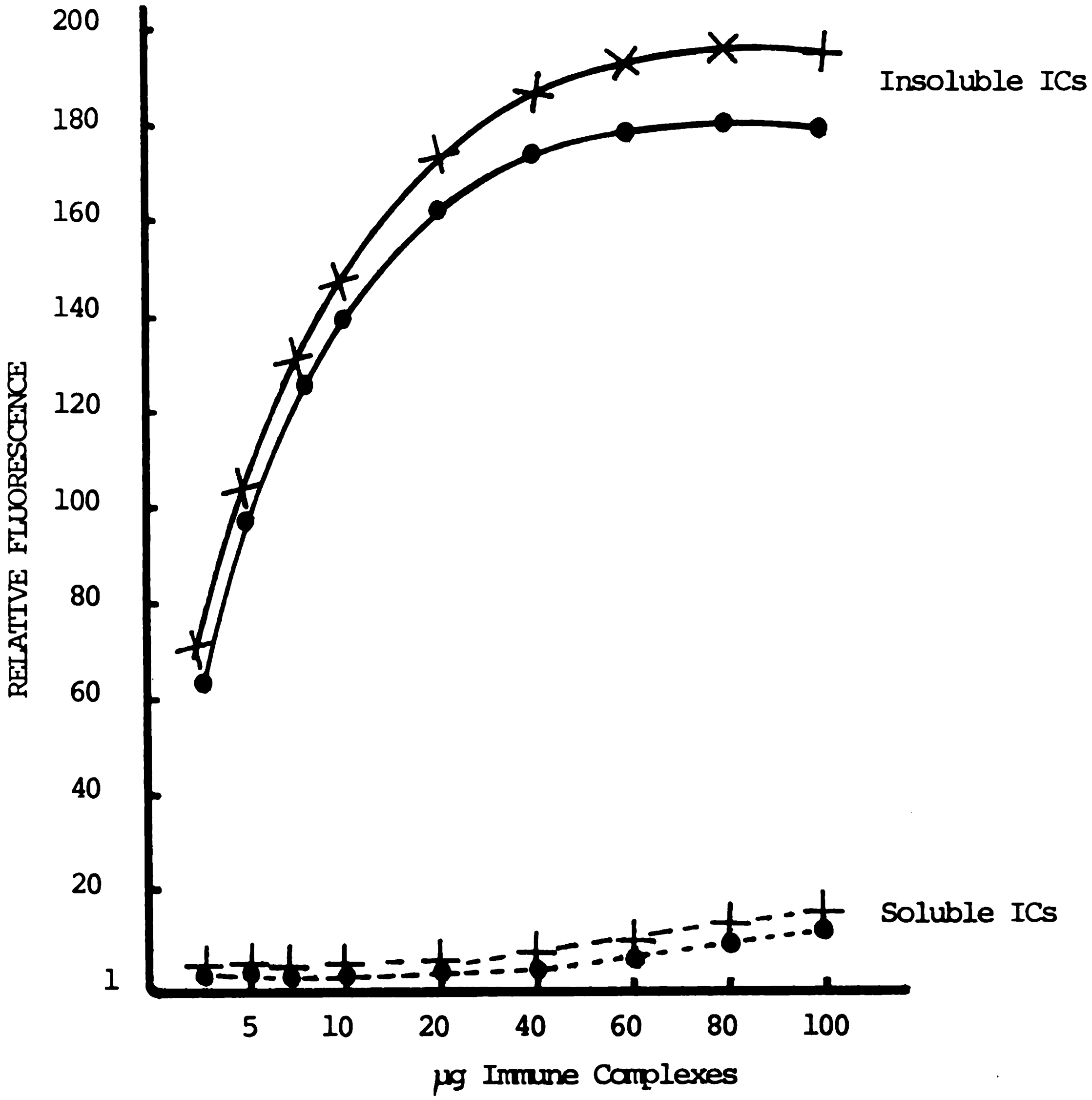


Figure 2.6 Dose-response curves for the uptake of immune complexes by stimulated and non-stimulated human neturophils in whole blood.

(●) = Non-stimulated neutrophils

(+) = f-Met-Phe stimulated neutrophils

Table 2.2 Neutrophils in whole blood incubated with different forms of fluorescien conjugated immune complexes as indicated for 15 minutes at 37°C. Relative fluorescence of neutrophils was measured using the Coulter EPICS-V. Results are expressed as the mean fluorescent channel (Channels 1-255) which is the average fluorescence of each population. 10,000 cells were analyzed.

(*) Immune complexes of human serum albumin and the IgG fraction of anti-human albumin.

(**) These Complexes lack the Fc portion on antibody, composed of human serum albumin and $F(ab')_2$ fragments of anti-human albumin.

(N) represents the number of experiments.

Table 2.2. Uptake of immune complexes by human neutrophils in whole blood.

Immune Complexes*	N	Immune Complexes Concentration (μg antibody Protein)								
		2.5μg	5μg	7.5μg	10μg	20μg	40μg	60μg	80μg	100μg
Insoluble ICs	3	63±12	96±14	125±9	138±16	164±11	173±12	175±12	176±8	176±11
Soluble ICs (10XAg excess)	3	1	1	1	1	2	4±0.5	7±3	9±2	10±1
Insoluble ICs** (F(ab')$_2$	3	4±0.5	3±1	-	-	-	4±0.5	-	-	-

clusters of immune complexes (Figure 2.7.a, and 2.7.b). On rare occasions, small aggregates of neutrophils (2-3 cells) were noticed when cells were exposed to a lower concentration of immune complexes (i.e., 2.5 to 20 µg antibodies protein) (Figure 2.7.c). As the concentration of immune complexes immune complexes increased, over 20 µg antibody protein, a greater aggregation of neutrophils were observed. There was a moderate aggregation of neutrophils when the cells were incubated with 80-100 µg of immune complexes (Figure 2.7.d). All the experiments were performed in a magnesium (Mg^{++}) free system, since the presence of the Mg^{++} caused a strong aggregation of cells after exposure to immune complexes.

The effects of the chemotactic factor upon the uptake of immune complexes by human neutrophils were examined. Neutrophils in whole blood were pre-incubated with f-Met-Phe ($10^{-6}M$) for 10 minutes at 37°C prior to incubation with immune complexes. The stimulated neutrophils in whole blood were then exposed to different concentrations of immune complexes, and the uptake was measured by flow cytometry. Stimulated neutrophils had only a slight increase in the uptake of the immune complexes compared to those neutrophils which had not been stimulated with f-Met-Phe (Figure 2.6).

Insoluble immune complexes which lack the Fc portion of the antibody were formed at equivalence. The complexes were

Figure 2.7 Fluorescence photomicrographs of neutrophils bearing FITC-immune complexes. Neutrophils in whole blood (0.1ml) were incubated with insoluble Immune Complexes (5 µg) at 37°C. After a 15 minute incubation, cells were washed twice with Mg^{++}-free HBSS and the erythrocytes were lysed. The leukocytes were then fixed with 1% paraformaldehyde. (a and b) Neutrophils having diffusly distributed clusters of ICs. (c) Neutrophils were exposed to a low concentration of ICs (i.e., 5 µg antibody protein). (d) Neutrophils were exposed to a high concentration of ICs (i.e., 80 µg antibody protein). All photomicrographs were taken using a Zeiss microscopewith a UV light source (i.e., Mercury Lamps HBO). Magnification approximately 1000 X for figure 2.7.b, and 250 X for figures 2.7.c and 2.7.d.

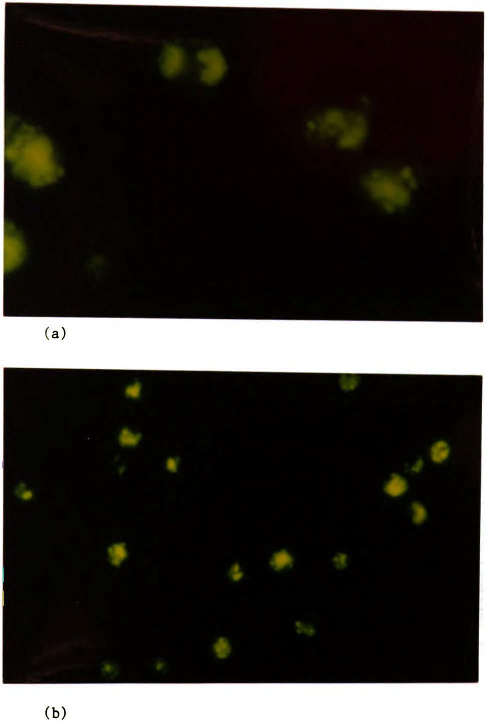
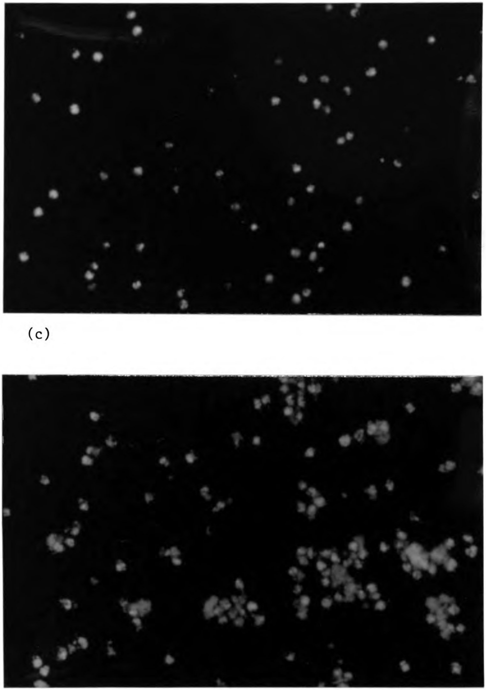


Figure 2.7 Fluorescence photomicrographs of neutrophils bearing FITC-immune complexes.



(c)

(d)

Figure 2.7 Fluorescence photomicrographs of neutrophils bearing FITC-immune complexes.

composed of human serum albumin (HSA) and fluorescein conjugated $F(ab')_2$ fragments of anti-human albumin. These insoluble immune complexes served as negative controls. There was only a minor uptake of these complexes by neutrophils in whole blood.

To assess whether insoluble immune complexes were internalized by the neutrophils in whole blood, cells were pretreated with cytochalasin B (CB) before exposure to immune complexes. Pretreatment of neutrophils with CB inhibited internalization of immune complexes. Insoluble immune complexes were rapidly ingested by the untreated neutrophils, and were not present on the surfaces of the cells to bind to the FITC-antibody against them. However, when phagocytosis of immune complexes was prevented by CB treatment, complexes were left on the cell surfaces and bound to the FITC-antibody against them (Table 2.3).

B. Uptake Of Soluble Immune Complexes By Human Neutrophils In Whole Blood

These experiments were similar to those which were performed with insoluble immune complexes except that soluble immune complexes were used instead. The soluble immune complexes were made at 10 times antigen excess as described in Methods. The results showed that the uptake of soluble immune complexes by neutrophils was also in a dose-dependent manner; however, the uptake was very poor compared to those of insoluble immune complexes (Table 2.2). In immunofluorescence microscopy, neutrophils exhibited only a very weak fluorescence with high concentrations of insoluble immune complexes (i.e., 80 - 100 µg), and there was no apparent clumping of cells (Figure 2.8).

Table 2.3: Uptake of Insoluble Immune Complexes* by Neutrophils

Source	Relative Fluorescence	N
HBSS-treated neutrophils	12 ± 7	2
CB-treated neutrophils	85 ± 12	2

Neutrophils in whole blood were pre-incubated with Hank's buffer (HBSS) or with cytochalasin B (CB) for five minutes at 37°C. The treated cells while in the pretreatement solution, were then exposed to insoluble immune complexes (10 µg) for 15 minutes at 37°C. After two washes, the cells were exposed to $F(ab')_2$ fragments of the antibody against the immune complexes. Results are expressed as the mean fluorescence ± SEM for 10,000 neutrophils.

(*) Immune complexes composed of human serum albumin and IgG fraction of anti-human albumin.

(N) Represents the number of experiments

II. UPTAKE OF SOLUBLE IMMUNE COMPLEXES BY HUMAN ERYTHROCYTES

Since all the experiments for the uptake of soluble immune complexes were performed on human neutrophils in whole blood, the possible uptake of these complexes by erthyrocytes was examined. A 20% suspension of human erythrocytes was prepared as described in Methods. Samples (i.e., 0.1 ml) of the erythrocyte suspension were exposed to different concentrations of soluble immune complexes (i.e., 5, 7.5, 10, 20, 40, 80 and 100 µg) for 15 minutes at 37°C. Under these conditions, immunofluorescent microscopic examination showed no apparent FITC-soluble immune complexes on erythrocytes. Occasional fluoresceinated leukocytes were observed (Figure 2.9).

III. MODULATION OF C_3b, C_3bi, AND Fc RECEPTORS ON HUMAN NEUTROPHILS IN WHOLE BLOOD BY IMMUNE COMPLEXES

These studies were performed to clarify the mechanism whereby preincubating neutrophils with immune complexes led to reduced phagocytic activity and decreased migration of the cells. Neutrophils in whole blood were pre-incubated with 5 µg of insoluble or soluble immune complexes at 37°C. After 15 minutes, the cells were washed twice with Mg^{++}-free HBSS, and the cells were then stained for different antigen receptors. In order to stimulate the maximum receptor

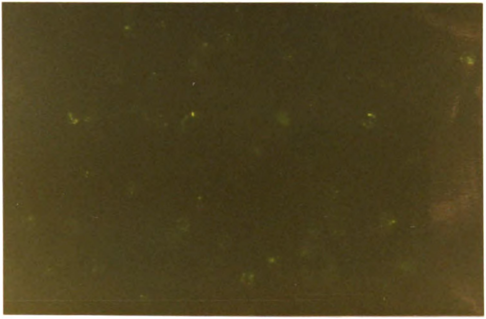


Figure 2.8 Fluorescence photomicrograph of neutrophils bearing soluble FITC-immune complexes. Neutrophils in whole blood (0.1 ml) were incubated with 80 μg of soluble-Immune Complexes for 15 minutes at 37°C. Cells were washed twice with Mg^{++}-free HBSS and the erythrocytes were lysed, then the leukocytes were fixed. Neutrophils exhibited only a very weak fluorescence. Photomicrograph was obtained using a Zeiss microscope with a UV source. Magnification approximately 250 times.

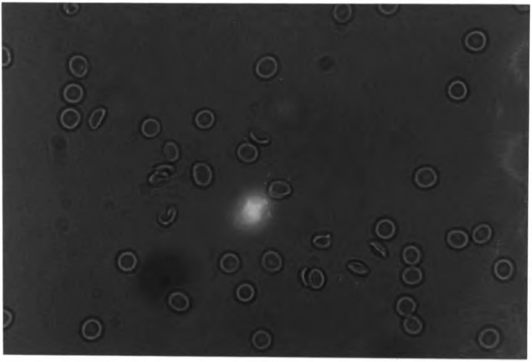


Figure 2.9 Uptake of soluble FITC-immune complexes (FITC-ICs) by human erythrocytes. 0.1 ml of a 20% suspension of human erythrocytes incubated with 80 µg of soluble immune complexes prepared at 10 X antigen excess. After a 15 minute incubation at 37°C, erythrocytes were washed twice with HBSS, and the cells then fixed with 10% paraformaldehyde. Erythrocytes were examined using a Zeiss microscope with a UV and white light sources. Note that there is no evidence of soluble FITC-ICs binding to erythrocytes. The arrow shows a leukocyte bearing soluble FITC-ICs. Magnification approximately 400 times.

expression after the exposute of the cells to the immune complexes, duplicate samples of whole blood were treated as follows: As the neutrophils in whole blood (0.1 ml) were pre-incubated with 5 µg of soluble or insoluble immune complexes for 15 minutes at 37°C and washed twice, the cells were then exposed to f-Met-Phe (10^{-6}M) for 10 minutes at 37°C. Neutrophils were then stained for C_3b, C_3bi and Fc antigens at 4°C.

The results showed that 5 µg of insoluble or soluble immune complexes caused a significant increase in C_3bi receptor expression on the human neutrophil plasma membrane. The results also indicated that this increase was near the maximum expression of C_3bi receptors as caused by f-Met-Phe (10^{-6}M) alone. However, when insoluble immune complexes which lack the Fc portion on their antibodies were used, no increase in the expression of C_3bi receptors were observed. This C_3bi receptor expression was similar to that expressed by neutrophils which were incubated in HBSS alone (Table 2.4).

Immune complexes had different effects on Fc receptor expression on the plasma membrane of neutrophils. Pre-incubation of neutrophils in whole blood with 5 µg of insoluble immune complexes resulted in a significant decrease of the expression of Fc receptors when compared to

Table 2.4 Neutrophils in whole blood were pre-incubated in various reagents as indicated. After two washes, the cells were resuspended in HBSS or f-Met-Phe for 10 minutes at 37^{o}C, and then reacted with monoclonal antibody to Fc as described in Methods. Relative fluorescence was determined using the Coulter EPICS-V. Abbreviations: Hank's buffer (HBSS), and immune complexes (IC).

(*) Results are expressed as the mean fluorescent channel ± SEM (Channels 1-255) which is the average fluorescence of each population. Ten thousand cells were analyzed.

(**) Compared with the values for neutrophils incubated in HBSS alone. The statistical analysis was done by applying the analysis of variance (Anova) test, then a multiple comparison was performed using the least significant difference (lsd) test at the P-value of 0.05.

(N) Represents the number of experiments.

(SD) = significant difference.
(NS) = no significant difference.

Table 2.4 Effect of immune complexes on C_3bi receptor expression on human neutrophils in whole blood

First Incubation (15 min. at 37°C) Additions	Second Incubation (10 min. at 37°C) Additions	N	Relative* Fluorescence	P**
HBSS	HBSS	3	63 ± 2	
f-Met-Phe (10^{-6}M)	f-Met-Phe (10^{-6}M)	3	120 ± 4	SD
Insoluble IC (5 µg) [F(ab')$_2$ fragment]	HBSS	3	67 ± 3	NS
Insoluble IC (5 µg)	HBSS	3	114 ± 3	SD
Insoluble IC (5 µg)	f-Met-Phe (10^{-6}M)	3	126 ± 1	SD
Soluble IC (5 µg)	HBSS	3	110 ± 5	SD
Soluble IC (5 µg)	f-Met-Phe (10^{-6}M)	3	130 ± 3	SD

those of neutrophils which were pre-incubated in HBSS alone (Table 2.5). In parallel, replicate samples of blood were restimulated with f-Met-Phe (10^{-6}M), after cells were pre-incubated with 5 µg insoluble immune complexes. This experiment was performed to see if f-Met-Phe stimulation would up-regulate more Fc receptors on the plasma membrane of neutrophils. No significant up-regulation of Fc receptors was observed on the neutrophil surface. The relative fluorescence of Fc receptors on neutrophils incubated with insoluble immune complexes was 99, and for neutrophils pre-incubated with insoluble immune complexes and then exposed to f-Met-Phe being 100 (Table 2.5).

Soluble immune complexes at 5 µg antibody protein did not reduce the number of Fc receptors on human neutrophils surface (Table 2.5). However, soluble immune complexes at higher concentrations decreased the expression of Fc receptors on plasma membranes, the effect being dose-dependent. Figure 2.10 shows the dose-response curves for soluble and insoluble immune complexes. Statistical analysis showed that there was an inverse relationship between the expression of Fc receptors on neutrophil surfaces and the concentration of immune complexes to which cells were exposed to.

Under these circumstances, the effect of immune complexes on C_3b receptor expression of human neutrophils was similar to those of Fc receptors (Table 2.6). A decreased expression of C_3b receptors was observed and the effect was dose-dependent (Figure 2.11). Soluble complexes at low concentrations did not reduce the expression of C_3b on neutrophil surfaces. However, an inhibitory effect was observed when higher concentrations of soluble Complexes were used (Figure 2.11).

Table 2.5 Neutrophils in whole blood were pre-incubated in various reagents as indicated. After two washes, the cells were resuspended in HBSS or f-Met-Phe for 10 minutes at 37^{o}C, and then reacted with monoclonal antibody to C_3b as described in Methods. Relative fluorescence was determined using the Coulter EPICS-V.

(*) Results are expressed as the mean fluorescent channel ± SEM (Channels 1-255) which is the average fluorescence of each population. 10,000 cells were analyzed.

(**) Compared with the values for the neutrophils incubated in HBSS alone.

(***) Compared with the values for the neutrophils incubated in f-Met-Phe alone.

(P) The statistical analysis was done by applying the analysis of variance (Anova) test, then a multiple comparison was performed using the least significant difference (1sd) test at the P-value of 0.05.

(N) Represents the number of experiments.

(SD) = significant difference.
(NS) = no significant difference.

Table 2.5 Effect of immune complexes on Fc receptor expression on human neutrophils in whole blood

First Incubation (15 min. at 37^{o}C) Additions	Second Incubation (10 min. at 37^{o}C) Additions	N	Relative* Fluorescence	P**
HBSS	HBSS	3	130 ± 4	
f-Met-Phe (10^{-6}M)	f-Met-Phe (10^{-6}M)	3	144 ± 5	SD
Insoluble IC (5 µg) [F(ab')$_2$ fragment]	HBSS	3	138 ± 3	NS
Insoluble IC (5 µg)	HBSS	3	99 ± 3	SD
Insoluble IC (5 µg)	f-Met-Phe (10^{-6}M)	3	100 ± 5	SD
Soluble IC (5 µg)	HBSS	3	136 ± 3	NS
Soluble IC (5 µg)	f-Met-Phe (10^{-6}M)	3	135 ± 4	NS

Table 2.6 Neutrophils in whole blood were pre-incubated in various reagents as indicated. After two washes, the cells were resuspended in HBSS or f-Met-Phe for 10 minutes at 37°C, and then reacted with monoclonal antibody to C_3b as described in Methods. Relative fluorescence was determined using the Coulter EPICS-V.

(*) Results are expressed as the mean fluorescent channel ± SEM (Channels 1-255) which is the average fluorescence of each population. 10,000 cells were analyzed.

(**) Compared with the values for the neutrophils incubated in HBSS alone.

(***) Compared with the values for the neutrophils incubated in f-Met-Phe alone.

(P) The statistical analysis was done by applying the analysis of variance (Anova) test, then a multiple comparison was performed using the least significant difference (lsd) test at the P-value of 0.05.

(N) Represents the number of experiments.

(SD) = significant difference.
(NS) = no significant difference.

Table 2.6 Effect of immune complexes on C_3b receptor expression on human neutrophils in whole blood

First Incubation (15 min. at 37^oC) Additions	Second Incubation (10 min. at 37^oC) Additions	N	Relative* Fluorescence	P**	
HBSS	HBSS	3	25 $\pm$ 1		
f-Met-Phe (10^{-6}M)	f-Met-Phe (10^{-6}M)	3	57 $\pm$ 2	SD	--
Insoluble IC (5 μg) [F(ab')$_2$ fragment]	HBSS	3	28 $\pm$ 1	NS	--
Insoluble IC (5 μg)	HBSS	3	39 $\pm$ 1	SD	--
Insoluble IC (5 μg)	f-Met-Phe (10^{-6}M)	3	39 $\pm$ 2	--	SD
Soluble IC (5 μg)	HBSS	3	48 $\pm$ 2	SD	--
Soluble IC (5 μg)	f-Met-Phe (10^{-6}M)	3	55 $\pm$ 1	--	NS

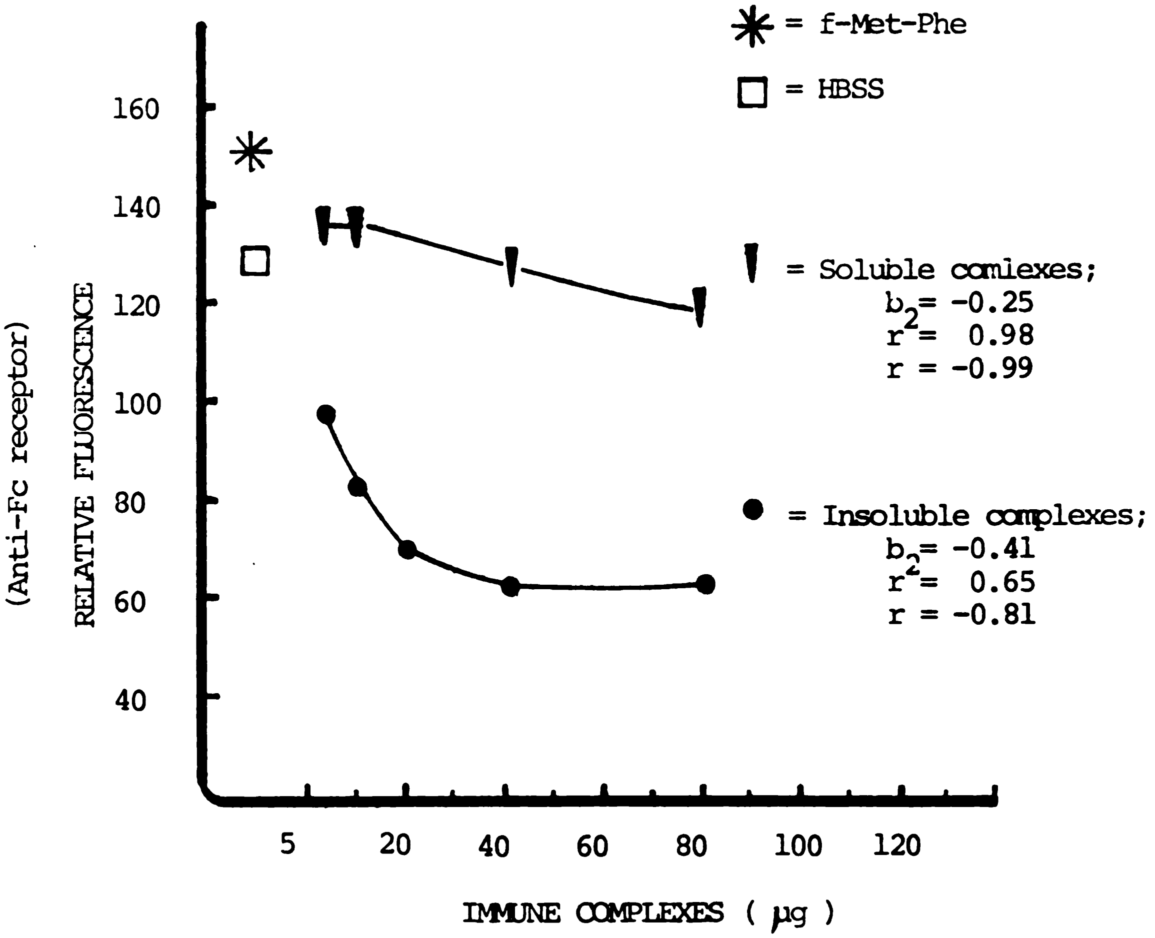


Figure 2.10 Dose-response curves for soluble and insoluble complexes - decreased Fc receptor expression on human neutrophils in whole blood. Neutrophils in whole blood preincubated with various concentrations of immune complexes as indicated for 15 minutes at 37°C. After two washes, cells were stained with a monoclonal antibody to Fc antigens. Fluorescence was determined using the Coulter EPICS V. Results are expressed as the mean relative fluorescence for 10,000 cells. Mean fluorescence for neutrophils stimulated with f-Met-Phe is indicated by an asterisk (*). Each point represents the mean for 3 determinations.

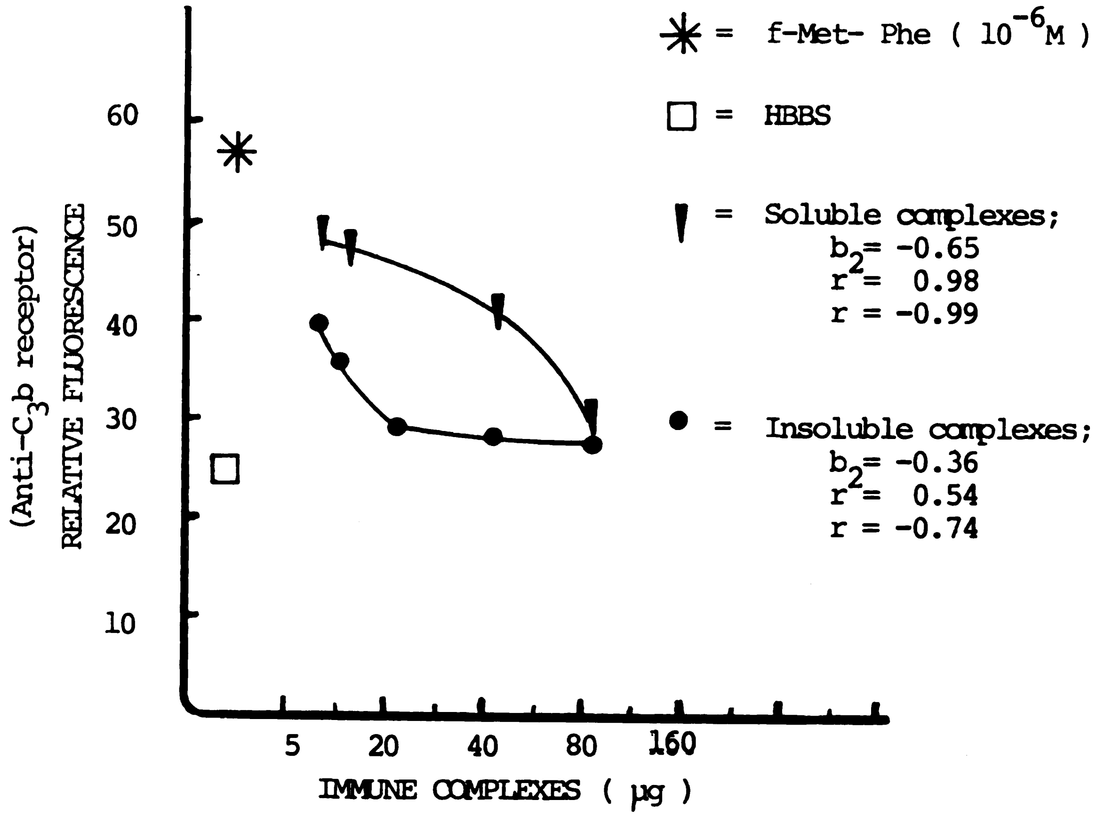


Figure 2.11 Dose-response curves for insoluble and soluble complexes - decreased C3b receptor expression on human neutrophils in whole blood. Neutrophils in whole blood preincubated with various concentrations of immune complexes as indicated, for 15 minutes at 37°C. After two washes, cells were stained with a monoclonal antibody to C3b antigens. Fluorescence was determined using the Coulter EPICS V. Results are expressed as the mean relative fluorescence for 10,000 cells. Mean fluorescence for neutrophils stimulated with f-Met-Phe is indicated by an asterisk (*). Each point represents the mean for 3 determinations.

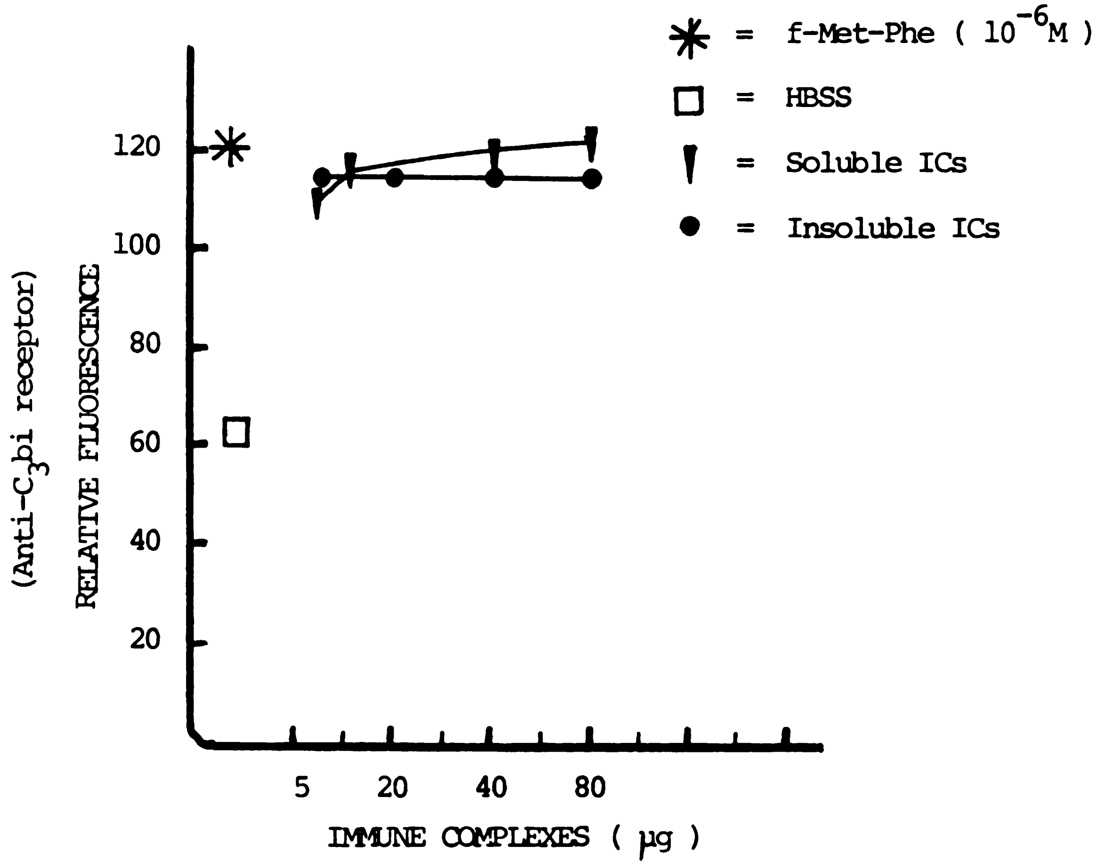


Figure 2.12 Dose-response curves for insoluble and soluble complexes-increased C_3bi receptor expression on human neutrophils in whole blood. Neutrophils in whole blood pre-incubated with various concentrations of immune complexes as indicated for 15 minutes at 37°C. After two washes, cells were stained with a monoclonal antibody (i.e., MO1) to C_3bi antigens. Fluorescence was determined using the Coulter EPICS V. Results are expressed as the mean relative fluorescence for 10,000 cells. Mean fluorescence for neutrophils stimulated with f-Met-Phe is indicated by an asterisk (*). Each point represents the mean for 3 determinations.

DISCUSSION

The purpose of the study in section II was to examine the effects of soluble and insoluble immune complexes (ICs) on the expression of C_3b, C_3bi and Fc receptors on human neutrophils. Previous studies have documented that exposure of neutrophils to immune complexes depresses the locamotion and phagocytic activity of these cells. C_3b, C_3bi and Fc receptors are implicated in immune adherence and adhesion-associated functions (i.e., locomotion, phagocytosis, adhesion, etc.) of neutrophils. Thus, it was of interest to evaluate the fate of these receptors on neutrophils which have been exposed to immune complexes. The experiment was designed so that the interaction between the neutrophil and immune complexes occured in a whole blood milieu, which represents a more relevant physiological condition.

Insoluble-immune complexes were avidly bound by human neutrophils in whole blood. Neutrophils rapidly ingested the insoluble-immune complexes and this process was inhibited by the pretreatment of neutrophils in whole blood with 5 µM cytochalasin B. However, neutrophils poorly bound the soluble-immune complexes. The uptake of soluble-immune complexes was approximately 1-5% of the insoluble-immune complexes uptake by neutrophils depending on the concentration of the complexes used. These results are consistent with those of Starkebaum et al., (1982), who studied the uptake of immune complexes labeled with ^{125}I by isolated neutrophils. These investigators reported that less than 1% of the soluble complexes prepared at a 3-fold antigen excess were taken up by the neutrophils compared to a 65% uptake of insoluble complexes made at equivalence.

The presence of a Mg^{++} ion in buffer solutions caused a strong aggregation of leukocytes which occurred during washing of the cells. Therefore a Mg^{++}-free buffer was used for the assay. Under the condition used in this study (i.e., absence of Mg^{++} and presence of Ca^{++} in the buffer) no aggregation of neutrophils was observed upon the interaction of immune complexes and neutrophils, except when a higher concentration of complexes (i.e., 40-100 µg) was used in which slight aggregations were observed (Figure 2.7.d).

The uptake of immune complexes by neutrophils in whole blood was dependent of the concentration of immune complexes present in the media. Increasing the dose of insoluble-immune complexes (i.e., 2.5, 5, 7.5, 10, 20, 40, 60, 80, and 100 µg) in the incubation media with neutrophils increased the uptake of the complexes by neutrophils which reached a plateau at concentrations higher than 20 µg immune complexes (Table 2.2 and Figure 2.6). When insoluble-complexes which lack the Fc portions on the antibody molecules were used, only a minor uptake was observed. These results suggest that the uptake of immune complexes by neutrophils in whole blood possibly occured via Fc receptors which are saturable.

The binding of soluble-complexes (formed by complement) to human erythrocytes (i.e., RBC) via C_3b (CRI) receptors have been previously reported (Schifferli et al., 1986., Schifferli and Peters, 1983; Sherwood and Virella, 1986). It was of concern in this study to find out if the soluble-complexes bound to RBC and thus were not available for binding to the neutrophils. Under the condition of the assay system used in this study no apparent binding of the insoluble-complexes to RBC was observed (Figure 2.9). Explanation for this discrepancy is out of the scope of this study. Since the purpose of this experiment was to find out if under the experimental condition used in this study the soluble-complexes will bind to RBC, not to provide an assay system for the evaluation of immune complex binding to RBC. However, a few points have to be mentioned. A different assay system was used in this study. The sensitivity of the assay used here is for neutrophils which express approximately 135,000 Fc receptors per cell. Erythrocytes express an average of 500-600 C_3b receptors per cell. In addition, the characteristics of antigen and antibody, the ratio of antigen to antibody, and the physical properties of the reaction medium (i.e., plasma, extracellular fluid, lymph, and so forth) are very important factors which influence immune adherence.

Neutrophils preincubated with insoluble-complexes exhibited significantly reduced Fc receptors on their surfaces. The expression of Fc receptors by neutrophils correlated inversely with the concentrations of complexes present during the incubation. At lower concentrations of insoluble-complexes (i.e., 5, 10 µg) a 30-40% reduction at the higher concentrations of insoluble-complexes (i.e., 20, 40, 80 µg) a 50-55% reduction in Fc receptors on the neutrophil surface were observed. Soluble-complexes at lower concentration (i.e., 5, 10 µg) slightly decreased the expression of Fc receptors, but at higher concentrations (i.e., 100 µg) the reduction was more noticable. A 20% reduction in Fc receptor expression on neutrophils was observed when cells were preincubated with 80 µg of soluble-complexes. These results are in agreement with those of Starkebaum et al., (1982) who utilized a different experimental assay system to examine the effect of immune complexes on human neutrophils phagocytic function. They observed that preincubating isolated neutrophils with insoluble complexes caused significantly greater inhibition of the uptake of additional insoluble complexes than did preincubating neutrophils with an equal amount of soluble complexes. They reported that maximum inhibition of uptake of insoluble complexes occured only after 90 minutes of preincubation of the neutrophils with immune complexes or aggregated IgG.

Down-regulation of Fc receptors on the neutrophil surface reached its maximum plateau at 40 µg concentration of insoluble-complexes in the incubating media (Figure 2.10). It is interesting to notice that the maximum uptake of insoluble-immune complexes by neutrophils also reached its plateau at approximately this concentration, indicating saturation of the receptors associated with insoluble-complexes binding at this concentration (Figure 2.6). In the presence of saturating levels of insoluble-complexes approximately forty percent of the total Fc receptors on the neutrophil were free of ligands and were available for binding to anti-Fc antibody (i.e., 3G8). On the other hand, pretreatment of neutrophils with anti-Fc antibody completely inhibited the uptake of insoluble-complexes, suggesting that Fc receptors play the main role in the binding of these complexes to the cells. A question might be raised of, how the anti-Fc antibody could completely inhibit the uptake of insoluble-complexes, while the exposure of neutrophils to saturating levels of insoluble-complexes did not block all the available Fc receptors on the cell surface. This question could be explained by the assumption that some Fc receptors are present on the surfaces of neutrophils which do not bind to the Fc portion of IgG molecules in immune complexes, but will bind to the anti-Fc antibody. Further study is required to find an appropriate answer to the above question.

Immune complexes also decreased the expression of C_3b (CRI) receptors on human neutrophils in whole blood. Like the Fc receptor, the down-regulation of C_3b receptors on neutrophils is inversely correlated with the concentration of complexes present during incubation (Figure 2.11). Insoluble-immune complexes at lower concentration (i.e., 5, 10 µg) induced a 30-40% reduction in the expression of C_3b receptors, while the soluble-complexes used 15-20% reduction. A maximum reduction (i.e., 50%) of the C_3b receptor expression was observed when neutrophils were incubated with 20, 40, or 80 µg of insoluble-immune complexes. With soluble-immune complexes a 50% reduction was observed when 80 µg of complexes were present in the incubating media. It is interesting to notice that soluble-immune complexes at 80 µg depressed the expression of C_3b receptors as much as the insoluble-complexes did at the same concentration (Figure 2.11). A higher concentration of soluble-immune complexes was not tested in this study to see if this was the maximum reduction or not. These results clearly indicate that C_3b receptors are also associated with the uptake of immune complexes by neutrophils.

Results of the effect of soluble-immune complexes on C_3b and Fc receptors show that the soluble-complexes induced a greater inhibition of C_3b receptors (i.e., at 80 µg IC --> 50% reduction) than of Fc receptors (i.e., at 80 µg IC --> 20% reduction). These results may suggest that C_3b

receptors are associated with the uptake of soluble-immune complexes more than the Fc receptors are. It is suggested that the primary function of the C_3b (CRI) receptor on neutrophils and monocytes is to mediate or enhance the endocytosis of soluble complexes and particles to which C_3b has bound covalently (Abrahamon and Fearon, 1983; Fearon, 1986). In addition, <u>in vitro</u> study has shown that binding of soluble antibody/ds DNA-ICs to neutrophils occurs principally via the CRI receptor (Taylor, et. al., 1983).

It was of interest to see if the reduction of the C_3b receptors on the neutrophil was due to the fact that they had not been fully up-regulated on the cell surface. Therefore, after the neutrophils were incubated with immune complexes and washed twice, they were exposed to the chemotactic factor, f-Met-Phe. No up-regulation of the C_3b receptors on neutrophils preincubated with 5 µg of insoluble-immune complexes was observed upon restimulation with f-Met-Phe (Table 2.5). In fact, there was a significant down-regulation of C_3b receptors on these neutrophils (i.e., preincubated with 5 µg of insoluble complexes) restimulated with f-Met-Phe when compared with the untreated neutrophils (i.e., preincubated in buffer only) stimulated with f-Met-Phe. On the other hand, soluble-immune complexes at a low concentration (i.e., 5 µg) which did not induce a great inhibition of C_3b receptors, significantly enhanced the up-regulation of C_3b receptors on neutrophil surfaces (Table 2.6). The results indicate that a low concentration of soluble-immune complexes enhances the up-regulation of C_3b receptors and a higher concentration of soluble complexes enhances the down-regulation of these receptors.

When neutrophils were treated with soluble-immune complexes lacking the Fc portions on their antibody molecules, no changes were observed in the expression of C_3b receptors. The level of C_3b receptor expression was equal to that of cells in the control buffer solutions. These results may indicate that the attachment of immune complexes to neutrophils is initiated via the interaction between the Fc portion of IgG and the Fc receptors of the cell.

So far, the results demonstrated that interaction of immune complexes with neutrophils depressed the expression of C_3b and Fc receptors on the cell surface. The decreased neutrophil phagocytic activity which have been shown *in vivo* upon exposure to immune complexes could be due to down-regulation of C_3b and Fc receptors. In addition the data may suggest that the decreased expression of these receptors could be responsible for the impaired phagocytic functions found with neutrophils from some patients with rheumatoid arthritis and systemic lupus erythematosus.

The effect of immune complexes on the expression of C_3bi (CRIII or M01) was different from those of C_3b and Fc receptors on neutrophils in whole blood. Soluble and insoluble-immune complexes induced a significant up-regulation of C_3bi receptors at different concentrations of immune complexes (i.e., 5,20, 40, and 80 µg) (Figure 2.12). In fact, there was no apparent difference in the magnitude of the enhanced receptor expression of 5 µg of soluble and insoluble-immune complexes (Table 2.4). Insoluble-immune complexes lacking Fc portions on their antibody molecules did not enhance the expression of C_3bi (CRIII) on neutrophils. Restimulation of immune complexes-pretreated neutrophils with f-Met-Phe slightly increased the expression of C_3bi receptors above the level of expression observed with untreated neutrophils activated with f-Met-Phe. The data presented here may suggest that the inhibition of neutrophil migration induced by immune complexes _in_ _vitro_, might be due to a significant increase in the number of C_3bi receptors on neutrophil surfaces. The C_3bi (CRIII) receptor is associated with the adherence property of the neutrophil. Upon exposure of neutrophils to immune complexes, they become very adherent to the substratum, resulting in their depressed locomotion.

In conclusion, the results presented in this section (II) indicated that: 1) neutrophils in whole blood avidly bound and ingested the insoluble-immune complexes, the uptake of complexes was directly correlated with the concentration of complexes present in the blood, and the uptake was saturable, 2) the uptake of soluble-immune complexes by neutrophils in whole blood was 1-5% of the insoluble-immune complexes. Similiar to those of insoluble immune complexes, the uptake of soluble-immune complexes was directly related to the concentration of complexes present in the blood. 3) Insoluble-immune complexes induced a significant down-regulation of Fc receptors on the neutrophil surface. Soluble-immune complexes at higher concentrations had a similar effect on Fc receptors though of a lesser magnitude. 4) Insoluble-immune complexes depressed the C_3b receptor expression on the neutrophil surface. Although, the soluble-immune complexes at very low concentrations (i.e., 5 µg), enhanced the expression of C_3bi (CRIII), at a higher concentration (i.e., 80 µg) depressed the expression of C_3b receptors to the same magnitude as the insoluble-immune complexes did. 5) Insoluble and soluble-immune complexes at low or high concentrations, significantly enhanced the up-regulation of C_3bi (CRIII) receptors on neutrophils in whole blood. 6) None of the results summarized so far, were observed when immune complexes lacking the Fc portion on their antibody molecules were applied in the experiment.

This study suggests that uptake of immune complexes is primarily initiated by the interaction of the Fc portion of the antibody molecule in immune complexes and the Fc receptor on the neutrophil surface. The presence of the Fc receptor is required for this interaction. Once the interaction occurs, C_3b receptors become involved in the attachment of immune complexes with the neutrophil. Upon this attachment on the neutrophil surface a decrease in the number of C_3b and Fc receptors occurs concomitant with an increase in the C_3bi (CRIII) receptors, which together impair the normal functions of the neutrophil (i.e., adherence, locomotion, phagocytosis, etc.). The abnormal migratory and phagocytic activities of neutrophils in response to foreign particles and infectious microorganisms results in a prolonged infection and inflammatory reactions.

SECTION III

THE EFFECTS OF SOLUBLE AND INSOLUBLE IMMUNE COMPLEXES ON THE SYNTHESIS OF LEUKOTRIENE-B4 BY HUMAN NEUTROPHILS

MATERIALS AND METHODS

I. PREPARATION OF REAGENTS

A. Calcium Ionophore A23187

Calcium ionophor (M.W. 523.6), was obtained from Calbiochem, Behring Diagnostic, La Jolla, CA, and stored at -70°C as a 10^{-3} stock solution in 0.1% dimethylsulfoxide. As needed, the 1 mM calcium ionophore solution was diluted to working solution.

B. Arachidonic Acid (AA)

The arachidonic acid (M.W. 304.) procine liver, (cis, cis, cis, - 5, 8, 11, 14-eicosatetraenoic, 20:4), (Calbiochem, La Jolla, CA) was provided in glass ampule as a liquid. The purity of this substance was greater than 99% by GC and TLC. A stock solution of 0.1 M was prepared and stored at -70°C. The stock solution was diluted further with Ca^{++} and Mg^{++}-free HBSS to the desired concentrations.

C. Cytochalasin B

Cytochalasin B (CB) (M.W. 479.6) was purchased from Sigma Chem. Co., St. Louis, MO. A stock solution of 10^{-3} M was stored at -70°C, and diluted further with Ca^{++} and Mg^{++}-free HBSS to the desired concentrations.

D. Nordihydroguaiaretic Acid

Nordihydroguaiaretic acid (NDGA), (M.W. 302.4)., (4, 4'-(2, 3-Dimethyl-1, 4-butanediyl) - bis [1,2-benzenediol]), was obtained from Sigma Chem. Co., St. Louis, MO, and stored at 4°C as a 10^{-3} stock solution in 0.1% ethanol. As needed, the 1 mM NDGA was diluted to working concentrations. Fresh stock solution was prepared weekly.

II. MONOCLONAL ANTIBODIES

Monoclonal antibody to human Fc, C_3b, C_3bi and T4 receptors were all prepared as described in Methods of Section I.

III. PREPARATION OF IMMUNE COMPLEXES

Immune complexes were prepared as described in Methods of Section II.

IV. BLOOD COLLECTION AND ISOLATION OF HUMAN NEUTROPHILS

Human venous blood from healthy donors was collected, and neutrophils were isolated as described in Methods of Section I.

V. INCUBATION OF HUMAN NEUTROPHILS WITH IMMUNE COMPLEXES

Neutrophils (5×10^6 cells/0.1 ml HBSS) were incubated with 0.9 ml of a specified concentration of calcium ionophore and immune complexes (ICs) in polystyrene tubes, for 15 minutes at 37°C. At the end of the incubation, tubes were placed in ice water for 5 minutes to stop the reaction, and were then centrifuged for 10 minutes at 2000 G at 4°C. The supernatants were immediately analyzed for their LTB_4 contents as described below. In a few experiments, neutrophils were pretreated prior to their incubation with immune complexes or calcium ionophore. Briefly, neutrophils (5×10^6 cells) were preincubated with specified concentration of arachidonic acid, CB, NDGA, or HBSS for a selected time interval and temperature. The treated cells were then incubated with immune complexes or calcium ionophore while in the pretreatment solutions.

For the experiments in which human serum (HS) was used, a desired concentration of HS was added to immune complexes prior to their incubation with neutrophils. Heat inactivated HS was prepared by incubating the sera for 30 minutes at 56°C.

VI. PRETREATMENT OF HUMAN NEUTROPHILS WITH C_3b, C_3bi AND Fc MONOCLONAL ANTIBODIES

5×10^6 neutrophils in 0.1 ml HBSS were preincubated for 15 minutes at 37°C with saturating amounts of monoclonal antibodies against C_3b, C_3bi or Fc receptors. The treated cells were then incubated with the desired concentrations of immune complexes or HBSS for 15 minutes further at 37°C, in the presence of monoclonal antibodies. At the end of the incubation, the cell mixtures were chilled in ice water and centrifuged (2000 G, 10 minutes) at 4°C. The supernatants were then assayed for their LTB_4 contents.

VII. ANALYSIS OF LTB_4 BY RADIOIMMUNOASSAY (RIA)

Immunoreactive LTB_4 was measured by RIA, using the Amersham (Arlington Heights, IL) TRK.840 kit. Briefly, 0.1 ml of cells supernatant with fixed amounts of antibody and tritiated leukotrien B_4 was added into glass tubes (12 mm X 75 mm). After a 15 to 18 hour incubation at 4°C, tubes were transferred into an ice water bath and allowed to equilibrate for 10 minutes. Then a 0.2 ml of dextran-coated charcoal suspension was added to the tubes, mixed well, and incubated in ice water for 10 minutes. Charcoal separates the bound LTB_4 from the unbound LTB_4. At the end of the incubations, the tubes were centrifuged at 2000 G for 15 minutes at 4°C. Immediately after centrifugation, the supernatants were decanted gently into the scintillation vials containing 15-18 ml of scintillation cocktail. The amount of ^3H-LTB_4 in the supernatant was determined by counting for four minutes in a beta scintillation counter (Searle Analytic Inc., ISOCAp/300, Model 6868). A standard curve was prepared by plotting bound cpm against pg of LTB_4 per assay tube on a semi-log graph paper. The concentration of LTB_4 in each sample was then calculatd from the standard curve. This assay was sensitive for the determination of

LTB_4 in vitro over the range 12.5 to 400 pg per assay tube. According to the manufacturer, the anti-sera to LTB_4 cross-reacted 0.03% with LTC_4 and LTD_4, <0.03% with PGE_2, and other prostaglandins and arachidonic acid, and 0.14% with 5(S), 12(S)-di HETE.

RESULTS

I. STIMULATION OF LTB_4 RELEASE BY CALCIUM IONOPHORE A23187.

An initial assessment of the enzymatic capacity of human neutrophils to metabolize endogenous arachidonic ascid via the C_5-lipoxygenase pathway was accomplished by using the calcium ionophore A23187. Isolated neutrophils (5 X 10^6/ml) were incubated with 5 µM of calcium ionophore for 15 minutes at 37°C, after which their supernatants were collected and subjected to LTB_4 analysis by RIA. Ionophore induced release of LTB_4 by human neutrophils (Table 3.1).

II. RELEASE OF LTB_4 FROM HUMAN NEUTROPHILS INDUCED BY IMMUNE COMPLEX

Human neutrophils (5 X 10^6/1 ml) were incubated with 15 ug of insoluble immune complexes for 15 minutes at 37°C, and the release of LTB_4 was determined by RIA. Under this condition, insoluble complexes did not induce release of any measurable amount of LTB_4 by neutrophils using the RIA assay.

In a few experiments, the release of LTB_4 by neutrophils was measured in the presence of arachidonic acid (AA). In a preliminary series of experiments, an AA concentration of 50 µM was the lowest concentration yielding

Table 3.1 Release of LTB_4 from human neutrophils stimulated with Calcium ionophore A23187.

Stimulus	Nanograms per 10^6 cells* LTB_4	N
None (HBSS)	<0.01**	7
A23187	6.4 ± 2	7

Neutrophils (5 x 10^6/1 ml) were incubated with A23187 (5 µM) or HBSS for 15 minutes at 37°C, after which the supernatants were collected and analyzed by RIA.

(*) Nanograms of LTB_4 released from neutrophils are expressed per 10^6 cells. Values represent mean ± SEM.

(**) Minimum sensitivity level of the RIA assay for LTB_4 was 0.012. Any value smaller than the sensitivity level (i.e., >0<0.012) is shown as <0.01.

(N) represents the number of experiments.

optimal release of LTB_4 by neutrophils. Therefore, thisconcentration was applied in this part of the study. Human neutrophils (5 X 10^6) were pre-incubated with 50 µM AA for 2 minutes, and then exposed to insoluble immune complexes for 15 minutes at 37°C. The results showed that insoluble ICs induced the release of LTB_4 by neutrophils in the presence of exogenous AA (Table 3.2).

The effects of human serum (HS) or bovine serum albumin (BSA) on the release of LTB_4 by the human neutrophils upon the stimulation with insoluble complexes was assessed. In a preliminary series of experiments, a 10% HS and a 10% BSA solution were used. The insoluble-immune complexes (15 µg) were exposed to a 10% HS or a 10% BS solution and then the neutrophils (5 X 10^6) were incubated with these complexes for 15 minutes at 37C. The LTB_4 released by neutrophils into the supernatants were measured by RIA. The results showed that the presence of 10% HSA with the insoluble-complexes induced the release of LTB_4 by neutrophils. However, when the serum was inactivated by incubating it for 30 minutes at 56°C, the effect of insoluble-complexes on the release of LTB_4 was abolished. In addition, no measurable amount of LTB_4 was detected in the supernatant when a 10% BSA solution was added to the insoluble-complexes (Table 3.3).

Since the presence of serum was required for the release of LTB_4 by neutrophils, different concentrations of

Table 3.2 Neutrophils (5 x 10^6/1 ml) in the presence or absence of 50 μM arachidonic acid were exposed to immune complexes and various reagents as designated, for 15 minutes at 37°C. The supernatants were then collected and analyzed for LTB_4. Abbreviations: Hank's buffer (HBSS), immune complexes (IC), and antibody (AB).

(*) Nanograms of LTB_4 released from neutrophils are expressed per 10^6 cells. Values represent mean ± SEM.

(**) Minimum sensitivity level of the RIA assay for LTB_4 was 0.012. Any value smaller than the minimum sensitivity level (i.e., >0<0.012) is shown as <0.01.

(***) Antibody was the IgG fraction of rabbit anti-human albumin which was used for the formation of Immune Complexes.

(N) represents the number of experiments.

Table 3.2 Effect of insoluble immune complexes on the metabolism of arachidonic acid in human neutrophils.

First incubation (2 min. at RT)	Second incubation (15 min. at 37^{o}C)	Nanograms per 10^6 cells*	N
		LTB_4	
HBSS	HBSS	<0.01**	3
Arachidonic acid	HBSS	0.14 ± 0.06	3
HBSS	AB***(15µg)	<0.01	3
Arachidonic acid	AB (15µg)	0.12 ± 0.07	3
HBSS	Insoluble ICs (15µg)	<0.01	3
Arachidonic acid	Insoluble ICs (15µg)	0.21 ± 0.14	3
HBSS	f-Met-Phe (10^{-6}M)	0.01 ± .002	3
Arachidonic acid	f-Met-Phe (10^{-6}M)	0.32 ± 0.2	3

Table 3.3 Neutrophils (5 x 10^6) were incubated with various reagents as indicated, in the presence or absence of HS and BSA for 15 minutes at 37°C. (AA+), neutrophils were exposed to 50 μM arachidonic acid before incubation with different reagents. (AA-), neutrophils were not exposed to arachidonic acid before incubation.

Abbreviations of reagents used are bovine serum albumin (BSA), heat inactivated human serum (I-HS), Human serum (HS), Hank's buffer (HBSS), calcium ionophore A23187 (A23187), antibody (Ab), and immune complexes (ICs).

(*) Nanograms of LTB_4 released from neutrophils are expressed per $1X10^6$ cells. Values represent mean ± SEM.

(**) Minimum sensitivity level of this RIA assay for LTB_4 was 0.012. Any value smaller than the minimum sensitivity level (i.e., >0<0.012) is shown as <0.01.

(***) Maximum sensitivity level of the RIA assay was 0.5 ng/tube. The values higher than this level (i.e., >0.5ng) is shown as >0.5, since no dilution was made at the time the experiments were performed.

(****) Antibody was the IgG fraction of rabbit anti-human albumin which was used for the formation of Immune Complex.

(N) represents the number of experiments.

Table 3.3 Release of LTB_4 from human neutrophils incubated with various reagents in the presence or absence of human serum or bovine serum albumin.

Stimulus	LTB_4 Nanograms per 10^6 Cells*		N
	AA-	AA+	
None (HBSS)	<0.01**	0.14 ± 0.06	2
None (HBSS) + 10% HS	0.02 ± 0.008	0.03 ± 0.006	2
None (HBSS) + 10% I-HS	<0.01	0.01 ± 0.006	2
None (HBSS) + 10% BSA	0.01 ± 0.003	0.01 ± 0.002	2
Ab****(15 µg)	<0.01	0.12 ± 0.07	2
Ab****(15 µg) + 10% HS	0.02 ± 0.007	0.03 ± 0.009	2
Ab****(15 µg) + 10% I-HS	0.01 ± 0.006	0.01 ± 0.002	2
Ab****(15 µg) + 10% BSA	0.02 ± 0.002	0.01 ± 0.002	2
Insoluble-ICs (15 µg)	<0.01	0.21 ± 0.14	2
Insoluble-ICs (15 µg) + 10% HS	0.10 ± 0.01	0.10 ± 0.003	2
Insoluble-ICs (15 µg) + 10% I-HS	<0.01	0.01 ± 0.002	2
Insoluble-ICs (15 µg) + 10% BSA	0.02 ± 0.002	0.01 ± 0.001	2
A23187	>0.5***	> 0.5	2

serum solutions were used. The LTB_4 in the supernatant of the stimulated neutrophils and in the serum solutions were determined. Small amounts of LTB_4 were detectable in a 10% solution of the serum from some individual subjects. The variation among individual donors was great. However, in lower concentrations of serum (i.e., 2.5% and 5.%) no detectable levels of LTB_4 were observed (Table 3.4). The release of LTB_4 by insoluble-immune complexes was higher when a lower concentration of serum was applied (Table 3.4).

III. DOSE-RELATED RELEASE OF LTB_4 FROM NEUTROPHILS INDUCED BY IMMUNE COMPLEXES

Neutrophils were incubated with various concentrations of soluble and insoluble-immune complexes for 15 minutes at 37°C, and the release of LTB_4 was determined by RIA. The threshhold dose for activation of lipoxygenase enzyme system by insoluble-immune complexes was 5 µg for 5 X 10^6 cells/1 ml (Figure 3.1). Soluble-immune complexes with an excess of antigen concentration at 10 times that of equivalence were also able to stimulate the LTB_4 release, but this capacity was two to fourfold less than those of insoluble-immune complexes. Saturation for immune complexes-induced release was achieved at a dose of 30-50 µg for both complexes. The variation among individual donors was great, which is typical of arachidonic acid metabolism. In a few cases, when 50 µg insoluble-complexes were used, no detectable level of LTB_4 was measured in the supernatant of neutrophils.

Table 3.4 Human neutrophils (5 x 10^6/1 ml) were incubated with insoluble-immune complexes (10 µg) and calcium ionophor A23187 (5 µM) in the presence of serum for 15 minutes at 37°C. The LTB_4 present in the serum solutions and supernatants were measured by RIA.

Abbreviations of reagents used are Hank's buffer (HBSS), human serum (HS), and insoluble immune complexes (ICs).

(*) Nanogram of LTB_4 present in 1 ml solution of serum, or released into 1 ml of supernatant by 5 x 10^6 neutrophils. Values represent mean ± SEM.

(**) Minimum sensitivity level of the RIA assay for LTB_4 was 0.012. Any value smaller than this value (i.e., >0<0.012) is shown as <0.01.

(N) represents the number of experiments.

Table 3.4 Level of LTB_4 in human serum and in the supernatants of stimulated neutrophils.

Source	LTB_4*	N
HBSS	0	5
HS (2.5%)	<0.01** ng/ml	2
HS (5.%)	<0.01 ng/ml	5
HS (7.5%)	<0.01 ng/ml	2
HS (10%)	0.01 ng/ml	5
Cells + ICs + HS (2.5%)	0.62 ± 0.14 ng/5x10^6 cells	2
Cells + ICs + HS (5.%)	0.56 ± 0.10 ng/5x10^6 cells	2
Cells + ICs + HS (7.5%)	0.46 ± 0.07 ng/5x10^6 cells	2
Cells + ICs + HS (10%)	0.41 ± 0.12 ng/5x10^6 cells	2
Cells + A23187	30.0 ± 4.9 ng/5x10^6 cells	2

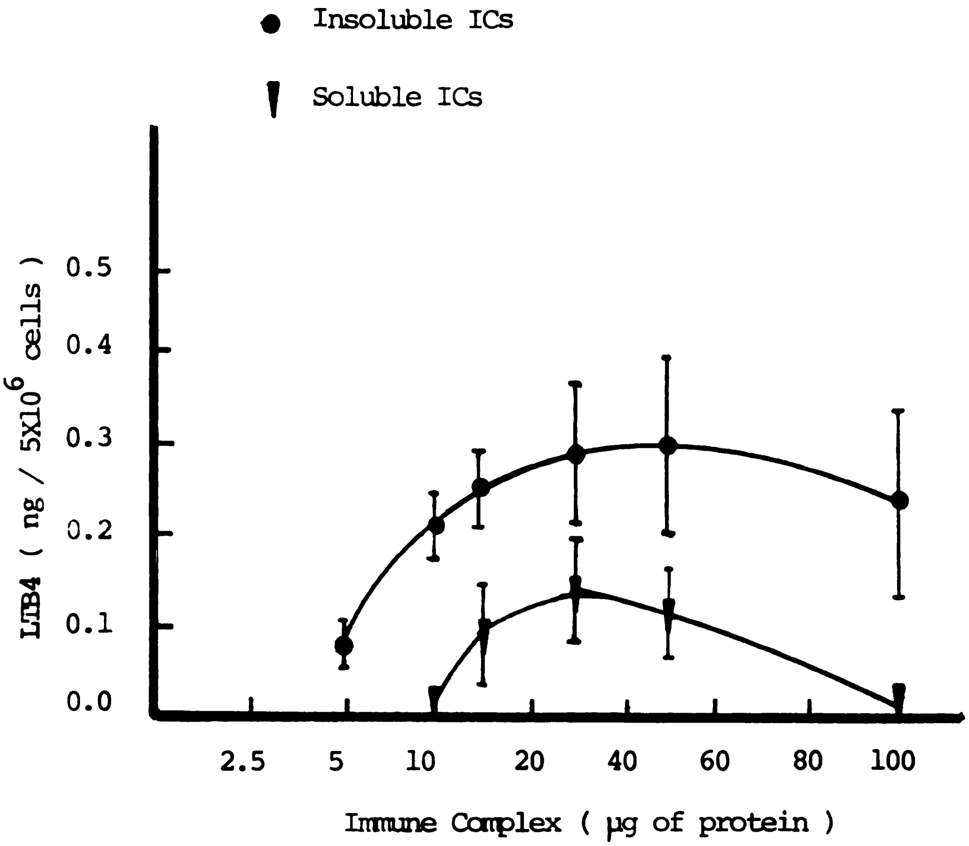


Figure 3.1 Dose-response curves for immune complexes-induced release of LTB_4 from human neutrophils. Neutrophils were incubated for 15 minutes at 37°C with soluble or insoluble immune complexes, and the amount of LTB_4 was determined by RIA. Representative figure from four experiments. Values represent mean ± SD.

IV. EFFECTS OF CYTOCHALASIN-B AND NORDIHYDROGUAIARETIC ACID (NDGA) ON IMMUNE COMPLEXES-INDUCED LTB_4 RELEASE

To assess whether phagocytosis of immune complexes by neutrophils was required for initiation of LTB4 synthesis, cells were pretreated with cytochalasin B (CB) (10 µM) before exposure to immune complexes. Pretreatment of neutrophils with CB did not inhibit immune complexes-induced LTB_4 release. In fact, CB enhanced immune complexes-induced LTB_4 synthesis by 1.5-fold to 3-fold (Figure 3.2). Cytochalasin B alone did not have any effect on the release of LTB_4 from neutrophils.

When neutrophils were pre-incubated with the lipoxygenase inhibitor NDGA (10 µM), LTB_4 release induced by immune complexes was completely inhibited (Figure 3.2).

V. ROLE OF C_3b, C_3bi AND Fc RECEPTORS ON LTB_4 RELEASE FROM HUMAN NEUTROPHILS STIMULATED WITH INSOLUBLE-IMMUNE COMPLEXES

The role of these receptors in initiation of LTB_4 synthesis by neutrophils, upon stimulation with immune complexes was assessed using monoclonal antibodies. Neutrophils (5×10^6) were preincubated with monoclonal antibodies against C_3b, C_3bi or Fc receptors for 15 minutes at 37°C. The cells were then exposed to insoluble-immune complexes (10 µg) and incubated for 15 minutes further at 37°C. The supernatants were collected and analyzed for the LTB_4 by RIA. The results showed that anti-Fc completely

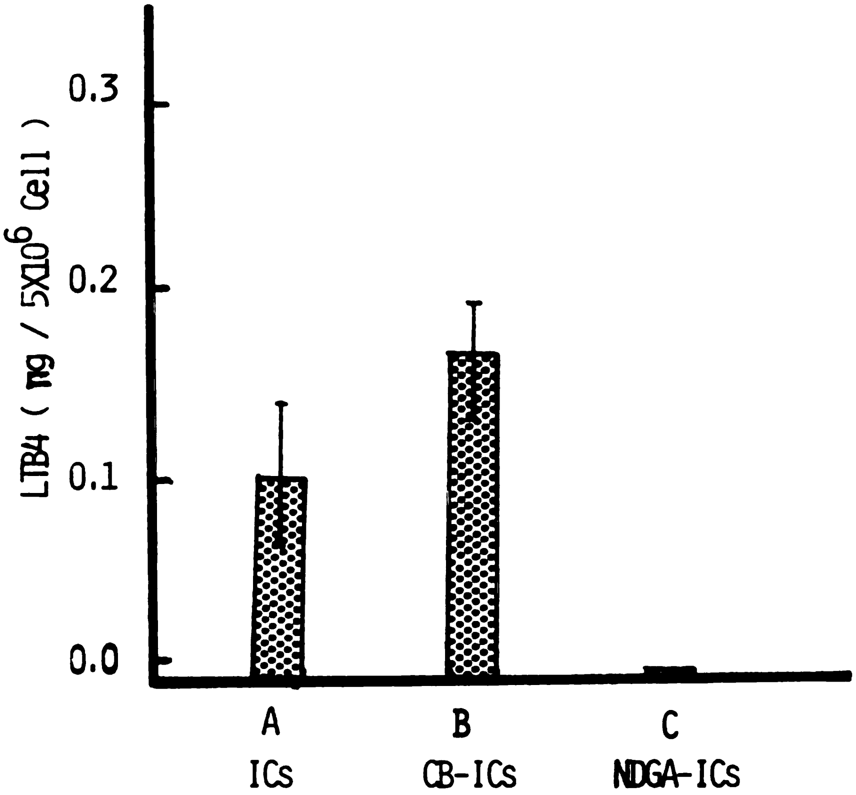


Figure 3.2 Release of LTB_4 from human neutrophils induced with insoluble immune complexes (ICs): Effects of CB and NDGA. The results of three experimental conditions are given; (A), neutrophils (5×10^6) suspended in HBSS exposed to insoluble-ICs (10 µg) for 15 minutes at 37°C; (B), neutrophils (5×10^6) preincubated with CB (10 µM) and then exposed to insoluble-ICs (10 µg) for 15 minutes at 37°C; (C), neutrophils (5×10^6) preincubated with NDGA (10 µM) and then exposed to insoluble-ICs (10 µg) for 15 minutes at 37°C. The amounts of LTB_4 were then determined by RIA in the supernatants.

Abbreviations: Hank's buffer (HBSS), Cytochalasin B (CB), nordihydroguaiaretic acid (NDGA), and immune complexes (ICs). Each point represents the mean ± SD for three determinations.

inhibited the release of LTB_4 by neutrophils (Table 3.5). Human neutrophils (5 X 10^6) incubated with insoluble complexes in the presence of 5% serum released 0.106 ± 0.037 ng of LTB_4, while, cells preincubated with anti-Fc and then exposed to insoluble complexes released <0.001 ng of LTB_4. However, pre-incubation of neutrophils with antibodies to C_3b, C_3bi receptors, or with both antibodies simultaneously, did not inhibit immune complexes-induced LTB_4 release. In fact, C_3b, or C_3bi receptor blocked with the monoclonal antibodies potentiated immune complexes-induced LTB_4 release (Table 3.5). No effect was observed with a control mouse monoclonal IgG directed against human T_4 receptor or with a purified mouse IgM.

Table 3.5 Neutrophils (5 x 10^6) preincubated with Hank's buffer (HBSS) or different monoclonal antibodies for 15 minutes at 37°C. The cells were then exposed to Hank's buffer or insoluble immune complexes (ICs) (10 µg) in the presence of 5% serum for an additional 15 minutes at 37°C. The supernatants were collected and analyzed for LTB_4 by RIA.

(*) Nanograms of LTB_4 released from neutrophils are expressed per 5 x 10^6 cells. Values represent mean ± SEM.

(**) Minimum sensitivity level of the RIA assay for LTB_4 was 0.012 ng. Any value smaller than this level (i.e., >0<0.012) is shown as <0.001.

(***) Insoluble immune complexes composed of human serum albumin and IgG fraction of Goat anti-human albumin.

(****) Insoluble immune complexes composed of human serum albumin and IgG fraction of Rabbit anti-human albumin.

Table 3.5 Effect of C_3b, C_3bi and Fc receptors blocked by monoclonal antibodies on immune complexes induced LTB_4 release from human neutrophils.

First Incubation (15 min. @ 37°C) Additions	Second Incubation (15 min. @ 37°C) Additions	Nanograms per $5X10^6$ Cells LTB_4	N
HBSS	HBSS	<0.001**	3
HBSS	A23187	32. ± 4.9	3
HBSS	Insoluble-ICs (G)***	<0.001	3
HBSS	Insoluble-ICs (R)****	0.106 ± 0.03	3
Anti-Fc	Insoluble-ICs (R)	<0.001	3
Anti-Fc	HBSS	<0.001	3
Anti-C_3bi	Insoluble-ICs (R)	0.185 ± 0.06	3
Anti-C_3bi	HBSS	<0.001	3
Anti-C_3b	Insoluble-ICs (R)	0.192 ± 0.06	3
Anti-C_3b	HBSS	<0.001	3
Anti-C_3b + Anti-C_3bi	Insoluble-ICs (R)	0.320 ± 0.07	3
Anti-C_3b + Anti-C_3bi	HBSS	<0.001	3
Anti-T4	Insoluble-ICs (R)	0.109 ± 0.03	3
Anti-T4	HBSS	<0.001	3

DISCUSSION

The central role of arachidonic acid (AA) metabolites in hypersensitivity reactions and inflammation has led to an explosive growth of interest and research activity regarding these substances. Leukotriene B_4, one of the AA metabolites, is chemotactic for granulocytes and promotes neutrophil aggregation as well as the expression of receptors (i.e., C_3b, C_3bi, and Fc) (discussed in Section I). Human neutrophils can metabolize AA to several important types of eicosanoids, including LTB_4. On the other hand, neutrophils are implicated in inflammatory reactions. Of particular interest was whether the interaction of immune complexes and neutrophils can initiate the synthesis of LTB_4 by these cells.

Human neutrophils were isolated as described in Methods. Immune complexes were prepared from human serum albumin (HSA) and the IgG fraction of rabbit anti-human albumin. The amounts of LTB_4 released by neutrophils into the extracellular media was measured by RIA.

The data presented in this study demonstrate that immune complexes stimulate peripheral blood neutrophils to release LTB_4 from the endogenous AA. Insoluble-immune complexes were the most potent stimulators when compared with soluble complexes. Immune complexes stimulated neutrophils via interaction with Fc receptors, since the release of LTB_4 was completely inhibited by the exposure of neutrophils to anti-Fc antibody (i.e., 3G8). In the absence of exogenous AA, the release of LTB_4 was dependent on the presence of a small fraction (i.e., 5%) of human serum. The role of serum in the synthesis of LTB_4 by human neutrophils is not clear and further studies are necessary. Cytochalasin B (CB), a potent inhibitor of phagocytosis, did not prevent the synthesis of LTB_4, indicating that cross-linking of Fc receptors on neutrophils in the absence of phagocytosis activated the C5-lipoxygenase pathway of AA metabolism. To my knowledge, this is the first observation describing release of LTB_4 by soluble and insoluble immune complexes in the absence of exogenous AA from human neutrophils. This study indicates that not only insoluble-immune complexes but also soluble-complexes, which has been believed for some time to be a "dead end" and not have a role in the pathogenesis of immune complexes induced tissue damage, can contribute to inflammatory reactions by stimulating neutrophils to produce LTB_4.

The result obtained for the abscence of LTB_4 released from neutrophils stimulated with immune complexes in the absence of human serum (i.e., 5%) is in agreement with the observations of Smith et al., (1986). These investigators reported that aggregated IgG (i.e., 400 µg/ml) failed to stimulate neutrophils (5 x 10^6) to release LTB_4. However, they showed that in the presence of exogenous AA (i.e., 30μM), neutrophils stimulated with aggregated IgG released LTB_4. Smith et al., (1986) did not examine the effect of aggregated IgG on LTB_4 synthesis by neutrophils in the presence of human serum. In the study presented here, immune complexes, in the presence of AA also stimulated the release of LTB_4 by neutrophils (Table 3.2). However, the addition of AA to suspensions of human neutrophils by itself also led to the release of LTB_4 as has previously been reported by Borgeat and Samuelsson (1979). The level of the LTB_4 release was two to three-fold higher from neutrophils activated with the stimuli (i.e., ICs or f-Met-Phe) in the presence of AA than the level of LTB_4 released by unstimulated neutrophils in the presence of AA alone (Table 3.2). Never the less, this study concentrated only on endogenous AA metabolism, since the use of an exogenous substrate may not reflect the fate of AA released from endogenous cellular phospholipids after receptor stimulation.

In the absence of exogenous AA, the presence of human serum (HS) was required for the production of LTB_4 by neutrophils stimulated with immune complexes. Substitution of serum with a bovine serum albumin solution or heat-inactivated serum did not promote LTB_4 release. The presence of serum in the incubating media also enhanced the production of LTB_4 by neutrophils stimulated with ionophore A23187. The data suggest that the presence of serum was not required for the opsonization of immune complexes, and the heat labile factor present in human serum might somehow have enhanced the reaction which is involved in LTB_4 synthesis by neutrophils, since it also increased the production of LTB_4 by the soluble stimulus A23187.

Inhibition of immune complexes-induced LTB_4 release with the lipoxygenase inhibitor NDGA suggests that LTB_4 was formed by enzymatic transformation of fatty acid in the plasma membranes of neutrophils.

Both insoluble and soluble-complexes were capable of stimulating the release of LTB_4 by neutrophils. However, the quantities of LTB_4 released by soluble-complexes were two to four-fold less than those of insoluble-complexes. Although the amounts of LTB_4 released after immune complexes stimulation are relatively low compared with A23187 stimulation, they are significant. As it is presented in section I of this study, LTB_4 at 10^{-10}M (i.e., approximately equals to 0.03 µg/ml) could induce a significant up-regulation of C_3b, C_3bi and F_C receptors on the neutrophil surface. The amount of immune complexes tested in this study were within the probable concentration range found in the circulation of some patients with rheumatoid arthritis or systemic lupus erythematosus. Therefore, it is likely that interaction of neutrophils with immune complexes will result in the production of quantities of LTB_4 that can influence local inflammatory reactions. In addition, peripheral monocytes can synthesize LTB_4 as well as LTC_4 and PGE_2 upon interaction with aggregated IgG, IgA and IgE (Ferreri et al., 1986). Therefore leukocytes in peripheral blood upon stimulation with immune complexes can release a more substantial amount of leukotrienes. This suggestion could be supported further by a recent report by Gresele and associates (1986) who reported that higher amounts of LTB_4 are produced by whole blood than those produced by isolated neutrophils.

Phagocytosis of insoluble particulate substances such as opsonized zymosan (Claesson et al., 1981) and Staphylococcus aureus (Henricks et al., 1985) have been shown to initiate the release of LTB_4 from human neutrophils. However, the data presented in this study show that human neutrophils can also release LTB_4 in the absence of a phagocytic stimulus. Inhibition of immune complexes phagocytosis by CB treatment did not inhibit LTB_4 production. In fact, the release of LTB_4 was actually potentiated. An enhanced production of LTB_4 by CB-treated monocytes after stimulation with aggregated immunoglobulin has also been reported (Ferreri et al., 1986). This enhancement is not understood. Cytochalasin B also increases the magnitude of the Quin-2 fluorescence response elicited by fMLP (Korchak et al., 1984) and LTB_4 (Goldman, unpublished data),(Quin-2 measures the cytoplasmic concentration of interacellular calcium). It is believed that an increase in intracellular Ca^{++} may serve to enhance the physiological responses of the cell. Perhaps the increments in cytosolic free calcium induced by CB, somehow contributes to the enhancement of LTB_4 production. In addition, CB stimulates the release of neutrophil granules contents. The fusion of granule membranes with the plasma membrane may provide additional phospholipids and AA on the cell surface, which upon stimulation could be metabolized further to LTB_4. Furthermore, the release of granule contents into the extracellular medium may somehow contribute to the release of LTB_4 by neutrophils. Extensive research and study is required to clarify these speculations.

It was of interest to discover how the metabolism of AA by immune complexes is related to Fc, C_3b (CRI), and/or the C_3bi (CRIII) receptors on human neutrophils. To fulfill this part of the study, neutrophils were preincubated with different monoclonal antibodies against Fc, C_3b, or C_3bi receptors. Cells in the presence of the antibody were then exposed to immune complexes and the amount of LTB_4 released into the extracellular medium was measured. When Fc receptors on the neutrophil surface were blocked with anti-Fc antibody (i.e., 3G8), the release of LTB_4 was completely inhibited. On the other hand, pretreatment of neutrophils with either C_3b or C_3bi antibodies potentiated the release of LTB_4 upon exposure to insoluble complexes. A much higher enhancement of LTB_4 release was observed when both receptors, C_3b and C_3bi on the neutrophil surface were simultaneously blocked by their respective antibodies. The treatment of neutrophils by antibodies alone did not induce the release of LTB_4. Complete inhibition of LTB_4 release by anti-Fc antibody indicates that the interaction between the neutrophil Fc receptor and IgG molecules of immune complexes is necessary for the initiation of AA metabolism by these cells. This suggestion is further supported by the finding that insoluble-immune complexes containing an IgG fraction of goat anti-human albumin did not induce the release of LTB_4 by neutrophils (Table 3.5). As previously reported, human neutrophils failed to bind particles coated with goat immunoglobulins, apparently because of a difference between the Fc component of goat immunoglobulins and other animal immunoglobulins (Newman and Johnston, 1979; York, 1983).

The enhancement of LTB_4 release from immune complexes-stimulated neutrophils by blocking the C_3b and C_3bi receptors with their respective antibodies is not understood at present. To my knowledge, this is the first observation regarding this issue, and since the study of the mechanism(s) involved in this reaction(s) was not within the scope of this research investigation, it will remain unknown until some future study can clarify it.

The results may suggest that the release of LTB_4 by neutrophils upon exposure to soluble-complexes is initiated, somewhat, through the F_C receptor interaction and is further enhanced by binding to C_3b receptors. Since, it appears that soluble-complexes bind more to C_3b receptors (as discussed in Section II). If this hypothesis is true, then higher quantities of LTB_4 are expected to be released upon interaction of neutrophils with higher concentrations of complexes. However, in this study a decline of the LTB_4 released by neutrophils was observed when the cells were exposed to higher concentrations of both soluble and insoluble-complexes (Figure 3.1). The following assumptions may offer a solution to this puzzle. The occupancy of C_3b receptors by immune complexes may be different from that of the anti-C_3b antibody therefore causing different physiological responses to occur, as can be observed from the previous study. Abrahamson and Fearon (1983) reported that $F(ab')_2$ antibodies (but not Fab') to the C_3b receptor were rapidly internalized by neutrophils at 37°C. They suggested that soluble-immune complexes bearing C_3b may be similarly internalized by neutrophils. Contrary to this suggestion, Taylor et al., (1983) observed no significant internalization of soluble antibodies/dsDNA immune complexes after they were bound by neutrophils. Another assumption is that, the decline in LTB_4 might not be due to the presence of a high concentration of immune complexes, resulting instead as a further metabolism of LTB_4 to ω-oxidation products, as previously reported in neutrophils stimulated with FMLP (Jubiz et al., 1982; Goetzl, 1983).

In conclusion, the results reported here indicate that: 1) soluble and insoluble immune complexes induced the synthesis of LTB_4 from endogenous arachidonic acid through the 5-lipoxygenase pathway in human neutrophils, 2) the transformation of arachidonic acid to LTB_4 initiated by the interaction of Fc receptors with IgG molecules of immune complexes, 3) the synthesis of LTB_4 was not only due to the Fc receptor occupancy by the ligand, since anti-Fc antibody alone did not induce the release of LTB_4, and 4) the release of LTB_4 was potentiated upon preincubation of neutrophils with cytochalasin B (CB), and antibodies against C_3b (CRI) and C_3bi (CRIII) receptors.

This study suggests that the interaction of neutrophils with immobilized immune complexes (such as those deposited in blood vessel walls or glomerular basement membranes), and with soluble circulating immune complexes (which have been believed to be a "dead end"), could initiate metabolism of arachidonic acid. Such a mechanism could contribute to inflammatory reactions characterized by the infiltration of leukocytes.

SUMMARY

In this study it was shown that unactivated neutrophils in whole blood exhibit a minimal number of C_3b (CRI), C_3bi (CRIII) receptors, while expressing a large number of receptors for Fc on their surface. Each of the chemotactic factors, f-Met-Phe, LTB_4, and a temperature transition (i.e., 4^oC --> 37^oC) significantly enhanced the up-regulation of C_3b, C_3bi and Fc receptors on the plasma membranes of neutrophils in whole blood. When neutrophils were isolated by the standard isolation procedure (i.e., dextran sedimentation and ficoll/hypaque gradients), the expression of C_3b and C_3bi receptors were only enhanced significantly on cell surfaces upon stimulation with f-Met-Phe or LTB_4. No significant increased expression of Fc receptors was observed on the isolated neutrophil surface.

The interaction of immune complexes with neutrophils was studied. Neutrophils in whole blood avidly bound and ingested the insoluble immune complexes. However, the uptake of soluble immune complexes by neutrophils was 1-5% compared to the insoluble immune complex uptake. The interaction of immune complexes with neutrophils depressed the expression of C_3b and Fc receptors, in contrast to the expression of the C_3bi receptor which was significantly

enhanced. In addition, soluble and insoluble immune complexes induced the synthesis of leukotriene B_4 from the endogenous arachidonic acid via the 5-lipoxygenase pathway. The interaction between the Fc receptor and the Fc portion of the antibody molecule in immune complex was required but not sufficient for the release of LTB_4 by neutrophils (the mechanism is unknown, however, the presence of a small fraction of human serum was required). Pre-incubation of neutrophils with antibodies against C_3b, C_3bi receptors or cytochalasin B prior to the interaction with immune complexes potentiated the release of LTB_4 from these cells.

The results suggest that immune complexes modulate the surface receptors associated with the immune adherence, and may be responsible for the depressed locomotion and phagocytic activity of neutrophils observed in some patients with inflammatory diseases (Brandt and Hedberg, 1969; Corberand et al., 1977). The leukotriene B_4 released by neutrophils upon the interaction with immune complexes could potentiate the inflammatory reaction by increasing leukocytic infiltration.

APPENDICES

APPENDIX A

This modified "FOLIN" assay was used for the measurement of protein in Immune Complexes. The assay is sensitive for the determination of protein *in vitro* over the range 5-100 ug per assay tube.

REAGENTS

1) 2% Na_2Co_3 in 0.1 N NaOH
   20. gm Na_2Co_3 (Sodium Carbonate)
   5.56 ml of 18 N NaOh or 4 gm of NaOH pellets
   Up to 1000. ml with distilled water

2) 1% $CuSo_4$ (W/V)

3) 2% Natartrate ($Na_2C_4H_4O_6$,$2H_2O$)
   Fresh solution was prepared, since it precipitates with standing.

4) Phenol reagent of Folin-Ciocalteau
   Diluted 1:2 with distilled H_2O.
   (the light sensitive phenol reagent was stored in the dark).

PROCEDURE

1) Measured out an aliquot 0.05 ml of sample.

2) Added 2 ml of Na_2Co_3 mixture.
   Mixed in the following order:
      1 ml of 2% Natartrate
      1 ml of 1% $CuSo_4$
      100 ml of 2% Na_2Co_3 in 0.1 N NaOH.

3) Added 0.2 ml of phenol reagent and mixed immediately.

4) Read at OD 700 after 30 minutes incubation at RT.

APPENDIX B

LIMULUS AMEBOCYTE LYSATE

The Limulus Amebocyte Lysate (LAL) test is a quantitative test for gram negative bacterial endotoxin. The test kit was purchased from Whittaker M.A. Bioproducts, Walkersville, Maryland. The use of LAL for the detection of endotoxin evolved from the observation by Bang that a gram-negative infection of Limulus polyphemus, the horseshoe crab, resulted in fatal intravascular coagulation. Levin and Bang later demonstrated that this clotting was a result of the reaction between endoxtin and a clottable protein in the circulating amebocytes of Limulus. Levin and Bang prepared a lysate from washed amebocytes which was an extremely sensitive indicator of the presence of endotoxin. They have purified and characterized the clottable protein from LAL and have shown the reaction with endotoxin to be enzymatic. The lysate prepared from the circulating amebocytes of the horse-shoe crab Limulus polyphemus standardized to detect at least 1.25 EU/ml of FDA Reference endotoxin.

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