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Intracellular processing, recycling and membrane anchoring of human polymorphonuclear leukocyte N-formyl-Met-Leu-Phe receptors

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Intracellular Processing, Recycling and Membrane Anchoring of Human Polymorphonuclear Leukocyte N-formyl-Met-Leu-Phe Receptors.

by

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DISSERTATION

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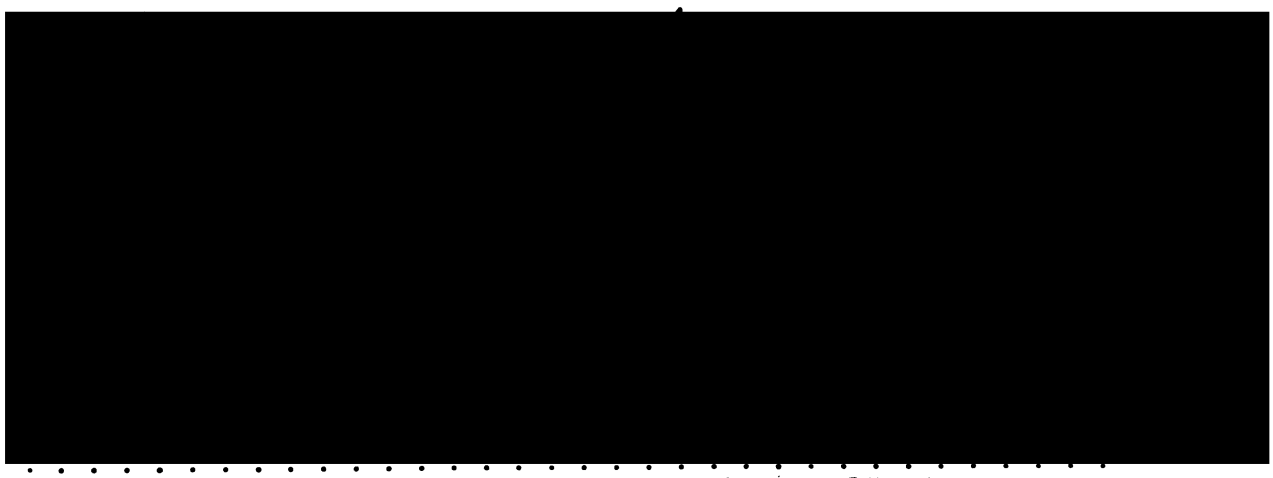

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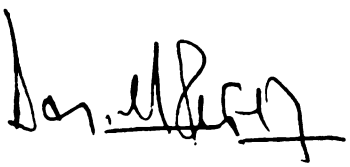
Abstract.

Human polymorphonuclear leukocytes (PMN) possess a heterogeneous population of receptors for the chemotactic peptide N-formyl-met-leu-phe (FMLP). When exposed to FMLP at 37°C, PMN internalize FMLP and its receptor, segregate the ligand-receptor complex to compartment of similar density and enzymatic characteristic as the light Golgi apparatus. Receptors are subsequently recycled back to the plasma membrane. This recycling (or reexpression) of receptors is essential for chemotaxis. When terminal sugar residues are manipulated by either, treatment of PMN with a derivative of the plant lectin wheat germ agglutinin (binds N-acetyl glucosamine), or by treatment of PMN with neuramindase (removes sialic acid residues), FMLP-induced PMN chemotaxis is inhibited, (but not FMLP-induced enzyme release). Cell fractionation studies show that in the presence of these chemotactic inhibitors, there is an accumulation of FMLP receptors within the Golgi, thereby preventing the recycling of receptors. A subpopulation of receptors which modulate FMLP-induced PMN chemotaxis appear to be anchored to the membrane by phosphatidyl inositol. Such receptors may be cleaved by phosphatidyl inositol specific phospolipase C. These latter studies strongly suggest receptor heterogeneity.



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Chairperson Dissertation Committee

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CHAPTER I

INTRODUCTION AND HISTORICAL PERSPECTIVES.

Chemotaxis.

The directed migration of cells and organisms along a concentration gradient of chemical substances is a well recognized biologic phenomenon referred to as chemotaxis. The term chemotaxis was first used by Pfeffer in 1884 to describe the attraction of spermatoziods of certain ferns towards malic acid. (1,2). Many lower organisms including bacteria, protozoa and slime moulds are capable of exhibiting chemotactic responses (3,4). These organisms use chemotaxis for their survival. Indeed chemotaxis plays a major role in the ability of these organisms to obtain their nutrients, avoid noxious stimuli and aggregate at critical times of their development. In higher organisms various cell systems have been reported to exhibit chemotaxis.

The principal vertebrate cells that possess the ability to migrate in a directed fashion are the leukocytes, although chemotaxis has been shown in primordial germ cells, neurons, tumor cells and fibroblasts (5,6,7,8). Leukocyte chemotaxis was first recognized in 1888 by Leber (9). He noted that when guinea pig corneas were injected with moulds or with putrefied rabbit muscle, the corneas were infiltrated with leukocytes. This observation, made under light microscopy, opened the door for the study of chemotaxis and its role during the process of acute inflammation.

In higher animal forms, the chemotaxis of leukocytes, in particular polymorphonuclear neutrophils (PMN), is an essential part of the body's response to infection and tissue destruction, (i.e. acute inflammation). The acute inflammatory response serves to maintain the integrity of the host by eliminating dead tissue,microbes , toxins and inert foreign substances.

Inflammation.

The concept of inflammation can be traced to around 1650 BC when a word translated as inflammation is used several times in an Egyptian scroll (1). Over a thousand years later, the Greek Philosopher Hippocrates wrote about inflammation, calling it "phlegmon" or burning thing. It was believed at this time that pus represented the "badness"coming out the wound and it was not unusual for soldiers injured in battle to have blood removed from them to "remove more of badness". The first recognition of the major signs of inflammation came during the time of the Romans when Celsus wrote of the first signs of inflammation: RUBOR ET TUMOR CUM CALORE ET DOLORE, "redness and swelling with heat and pain"(1).

In more recent times, Julius Cohnheim wrote the first major paper on the subject of inflammation in 1867 (10). While Cohnheim was not privy to the use of the tools of modern pathology, especially processed and embedded tissue, he made his astute observations in living animals using the mesenteric tissue and the tongue of the frog. The thin membrane-like configuration of these tissues allowed him to observe the events of inflammation. While observing the mesentery of a frog under a microscope, Cohnheim noted a series of changes

in the vasculature of the mesenteric tissue. Initially there was dilatation of the arterioles and the acceleration of blood flow, followed by the slowing of blood flow and the lining of the leukocytes on the walls of the venules. Eventually there was an "escape of the leukocytes from the blood vessels into the extravascular spaces", a process now known as diapedesis. Our understanding of the the events of inflammation have been significantly advanced since the time of Cohnheim, nevertheless his description of the major events of the inflammatory response,i.e. changes in vasculature tone, increased vascular permeability and accumulation of leukocytes in tissues, are immortalized.

The inflammatory response is initiated by tissue injury, invasion of tissue or simply the presence of noxious stimuli. All of the latter mentioned stimuli trigger the release of several chemical mediators, which account for the initial changes seen in inflamed tissue. Following injury, there is an alteration in the vasculature, particularly in the post-capillary venules, initially manifested as a transient vasoconstriction followed by vasodilation and increased vascular permeability (2,11). The vascular phenomenon of the acute inflammatory response is mediated by several chemical mediators, including the vaso-active amines histamine and serotonin. Other major chemical mediators are derived from the coagulation system, the fibrinolytic system, the Kallikren-kinin system and the complement system. These mediators act in concert to orchestrate the acute inflammatory response. It is difficult to determine the relative importance of each chemical mediator since a great deal of overlap exists (11,12). A brief list of chemical mediators and their functions is summarized in Table 1.

Polymorphonuclear Leukocytes - Acute Inflammatory Cells.

The principal cell of the acute inflammatory response and the major focus of this dissertation is the or PMN. Polymorphonuclear neutrophils are specifically equipped to migrate to sites of invasion and injury, to seek out, ingest and destroy most invading organisms. The participation of PMN in the inflammatory response involves a series of coordinated events, which are initiated by the chemical mediators mentioned above. Stimulated PMN adhere to the vascular endothelium and then migrate into the extravascular space to sites of injury. This is followed by the engulfment of invading organisms (or phagocytosis), and destruction of organisms by fusion of lysosomal granules with endosomes containing ingested material. PMN contain two types of granules , the specific and the azurophilic granules , which contain a myriad of enzymes and bactericidal agents which aid in the killing and digestion of ingested material (13,14,15). PMN granule contents are summarized in Table 2. The anti-bacterial activity of PMN is further enhanced by a burst of oxidative metabolism, and generation of superoxide anion and other reactive oxygen species. Release of the superoxide anions occurs directly into phagosomes, (i.e.,vessicles which contain material ingested during phagocytosis), and also at the cell surface.

The average life span of the mature human PMN in the peripheral circulation is approximately 10 hours (16). Aging PMN are actively replaced by mature PMN from the bone marrow, where active proliferation and maturation of these cells takes place. Egression of PMN from the bone marrow to peripheral circulation,in the absence of infection, is limited to mature cells (16). In the peripheral circulation PMN are found in the midstream of blood vessels or

sequested on the endothelial cells lining the blood vessels. These latter pool of marginated PMN consist of about half to a third of the peripheral PMN (17). It is these PMN that are the initial responders to injury (18).

Chemotactic Factors.

Before PMN are able to participate in phagocytosis they must first be directed to inflamed or infected tissue. The directed migration and the adherence of PMN to endothelial cells is mediated by chemotactic stimuli with the aid of certain specific cell surface proteins known as adherence proteins, (i.e. integrins). Several chemotactic stimuli (chemotractants) are released from injured or infected tissue. These include bacterial products including formyl peptides (19), C5a produced by the activation of either the classical or the alternative pathway of the complement system, and leukotriene B4 (LTB4) which is released by lipoxygenation of arachidonic acid by cells adjacent to sites of inflammation (20,21).[In this dissertation much of the studies utilize the synthetic formyl peptide N-formyl -met -leu-phe (FMLP).]

Most PMN chemotractants that have been studied bind to specific receptor sites on the membrane of PMN. It is the recognition and binding of the chemotractant to its specific receptor site on the PMN that triggers the process of adherence, chemotaxis, degranulation (i.e.selective release of lysosomal contents) and oxidative metabolism. (22,23). The interaction of chemotractants with PMN initiates a series of complex but coordinated biochemical responses. These include ion fluxes (Ca^{++}, Na^{+}) resulting in changes in the membrane potential (24,25,26), and changes in the cell shape from round to elongated with specific polarity at each end of the cell.

Chemotractants stimulate the adherence of PMN to endothelial cells thereby enabling PMN to migrate between the endothelial cells and into extracellular spaces (i.e., diapedesis), (28, 29). Specific binding of chemotractants to their membrane receptor sites on the PMN triggers an array of events leading to phagocytosis and killing of invading organisms (2).

This dissertation will examine the process of chemotaxis by human PMN stimulated with the synthetic formyl peptide FMLP. The major focus of the thesis is to examine the processing of the chemotactic peptide FMLP, and its specific receptor by PMN, and how this process is altered in the presence of specific inhibitors of FMLP-induced chemotaxis. Moreover the ability of PMN to continuously express a population of unoccupied FMLP receptors during chemotaxis, and the hypothesis that PMN re-express (or re-cycle) a population of FMLP receptors will be examined. Furthermore, this thesis will also delineate if there there are subpopulations of FMLP receptors which may modulate induction of chemotaxis without receptor recycling based on their structural differences. While such receptors may contain a common fundamental structure, the anchoring of these receptors within the plasma membrane and possibly glycosylation of the receptors may play a role in the ability of these receptors to modulate these different different functions. Results of experiments designed to test these hypotheses are presented.

In the second chapter of the dissertation, experiments showing the processing of [^{3}H]-FMLP and its receptor by PMN will be reported. The results of the experiments performed, show that [^{3}H]-FMLP and its receptor co- migrate with organelles of similar density as the light Golgi apparatus.

In the third chapter the inhibition of chemotaxis by a derivative of the plant lectin wheat germ agglutinin (termed as WGA-D) will be discussed. Experiments will show that in the presence of WGA-D, FMLP induced chemotaxis is inhibited and more importantly there is an accumulation of [^{3}H]-FMLP that co-sediments with the light Golgi fraction. The mechanism of inhibition of FMLP induced-PMN chemotaxis by cells exposed to the enzyme neuraminidase is examined in chapter four. Removal of terminal sialic acid residues for surface proteins by the enzyme neuraminidase, hence altering the surface glycosalation , also resulted in a similar accumulation of [^{3}H]-FMLP within the Golgi containing fractions of fractionated cells previously incubated with FMLP . The association between accumulation of FMLP and its receptor within the Golgi component and inhibition of FMLP -induced PMN chemotactic responses, (together with the binding studies performed with whole cells in the presence and absence of inhibitors), strongly suggests the occurrence of receptor recycling.

Evidence of receptor recycling in PMN chemotaxis is again presented in chapter five. Formyl peptide is covalently linked to its receptor and cells are then incubated at 37^{o}C for various time periods. Cells are then exposed to an ecto-enzyme which cleaves any formyl peptide-receptor complex present on the surface of the PMN. Recycling in this system was dependent on stimulation by phorbol myristate acetate (PMA), suggesting a role for protein kinase c in formyl peptide receptor recycling.

In the chapter six of the dissertation the postulate that different subpopulations may modulate FMLP-induced functions is examined. This chapter will demonstrate that there is a subset of FMLP receptors that are anchored to the

plasma membrane via phosphatidylinositol. Such receptors can be released by the action of phosphatidylinositol specific phospholipase C. Data will will be presented to show that removal of receptors anchored in the plasma membrane by phosphatidylinositol results in a decreased FMLP-induced chemotactic activity and enzyme release thereby providing evidence for the hypothesis that subsets of FMLP receptors that may control different functions exist.

CHEMICAL MEDIATORS OF INFLAMMATION*

SYSTEM	MEDIATOR	EFFECT
THE COAGULATION CASCADE	FIBRINOPEPTIDES	VASCULAR LEAKS
	FIBRIN	PMN CHEMOTAXIS
	FACTOR XII	TRIGGERS CLOTTING CASCADE, AND KALLIKREN SYSTEMS
THE FIBRINOLYTIC SYSTEM	PLASMIIN	DEGRADATION OF FIBRIN COMPLEMENT ACTIVATION
THE KININ-KALLIKREIN SYSTEM	BRADYKININ	SMOOTH MUSCLE CONTRACTION, VASODILITATION , PAIN, VASCULAR PERMEABILITY
THE COMPLEMENT SYSTEM	C5a	PMN CHEMOTAXIS
THE EICOSANOIDS	LEUKOTRIENE B4	PMN CHEMOTAXIS
OTHER	HISTAMINE	VASODILITATION PAIN AND VASCULAR PERMEABILITY
	SEROTONIN	VASOCONSTRICTION

Table 1

CHEMICAL MEDIATORS AND THEIR EFFECTS (ROLES) IN THE INFLAMMATORY RESPONSE. * MODIFIED FROM REFERENCE 1.

NEUTROPHIL GRANULE CONTENTS

AZUROPHILIC OR PRIMARY GRANULES.

LYSOZYME.
ACID HYDROLASES
NEUTRAL PROTASES (ELASTASE)
CATIONIC PROTEINS (BACTERICIDAL AND PERMEABILITY INCREASING FACTOR)
MYELOPEROXIDASE
ACID MUCOPOLYSACCHARIDE
BETA- GLUCURONIDASE
DEFENSINS

SPECIFIC OR SECONDARY GRANULES.

LYSOZYME
LACTOFERRIN
VIT. B-12 BINDING PROTEIN.
NEUTRAL PROTEASES (COLLAGENASES)

Table 2.

Granule contents of PMN.. (From ref. 13 and 14)

CHAPTER II

PROCESSING OF FORMYL PEPTIDES AND THEIR RECEPTORS BY PMN.

Introduction.

In the introductory chapter of this thesis, the process of chemotaxis was defined. While the primary result of PMN chemotaxis is the directed migration of PMN to the site of tissue injury or invasion, modulation of chemotaxis involves the interaction of chemotactic factors (chemotractants) and PMN. In this thesis a detailed study of some of the possible mechanisms by which a chemotractant might modulate PMN chemotactic responses is undertaken. All of the studies in this thesis were performed using the synthetic N-formylated peptide N-formyl-methionyl-leucyl-phenylalanine or FMLP.

In this chapter the interaction of FMLP and PMN will be examined. The interaction of the chemotractant (FMLP) and PMN sets off a series of molecular events that culminate in the directed locomotion of PMN (1). More specifically this chapter will trace the processing of FMLP by PMN from the initial binding of the chemotractant (FMLP) to its specific PMN receptor, to the subsequent intracellular migration of bound FMLP over time.

Chemotractants

The ability to quantify PMN responsiveness (chemotaxis) *in vitro* has not only led to the characterization of chemotractants but has also contributed to the understanding of the complex process of chemotaxis (1,2,3).The first chemotractant to be identified was the complement (C5)-derived component C5a, a peptide that is produced during the activation of the complement cascade (4,5,6). Most of the current and prior studies on PMN chemotaxis however, have been done using the tripeptide FMLP. It should be also noted that not all PMN chemotractants are peptides, in fact some the more potent PMN chemotactic factors are non- protein, most notably the derivatives of arachidonic acid lipoxygenation such as leukotriene B4 or LTB4 (7).

FMLP.

Induction of PMN chemotaxis by the oligopeptide FMLP was first demonstrated by Schiffman *et. al.* (8). The identification of a potent synthetic peptide provided a vital tool for the study of chemotaxis. The well-defined structure of the peptide, and the studies that showed that manipulation of the structure of the oligopeptide changed it's chemotactic potency led to the realization of another important concept : that FMLP-induced PMN chemotaxis may be initiated by specific binding of the ligand (FMLP) to a specific receptor on the PMN surface (9). Initial binding studies, (using [^{3}H]-FMLP) reported rapid (t1/2=<2min) and reversible binding of the ligand to the PMN membrane, with an equilibrium dissociation constant (K_D) of 12-14 nM at 37^{o}C, and the number of binding sites approximated to 20,000 per cell (10). Subsequent

studies have demonstrated that PMN exhibit high and low affinity receptors for FMLP (11,12).

Scatchard analysis of specific binding of [^{3}H]FMLP binding to intact human PMN showed the presence of two classes of receptor - a high affinity receptor with a K_D of 2.1 nM and approximately 15,000 binding sites, and a low affinity receptor with a K_D of 22.2 nM and approximately 66,000 binding sites (11). Similar results were reported with on the binding of [^{3}H]FMLP to PMN membranes (12).

Structural Requirements of Formylated Peptides.

In studies of the structural requirements of formylated peptides, it has been shown that manipulations of the structure of the peptide alters its biological activity. There are however, certain properties of the structural features that are imperative. The most important of these being the N-formyl group, which confers activity to the peptide (13). Other manipulations of the amino acids contained within the formylated peptides has shown that removal of the terminal amino acid does not significantly reduce chemotactic responsiveness, provided that the second (and now terminal) amino acid is neutral and non-polar (13). What these studies have done is allowed the creation of formylated peptides with variations in structure, allowing for the use of different radiolabels and hence, increasing the availability of tools for the study of PMN chemotaxis.

Physiologic correlates of synthetic formyl peptides.

Initial studies of FMLP-induced PMN chemotaxis were performed utilizing synthetic peptides. Marasco et al. however, were able to isolate oligopeptides resembling the synthetic peptides in both structure and function, from supernates of bacterial cultures (14). This finding was instrumental in implicating oligopeptide-induced PMN chemotaxis as a possible mechanism in the host defense.

FMLP Receptor.

While significant progress on the studies of oligopeptide kinetics has been made, the complete isolation, characterization and sequencing of the receptor has yet to achieved. Initial attempts to isolate the receptor using covalent binding of a radiolabelled oligopeptide demonstrated the receptor to be a highly glycosylated protein exhibiting a molecular weight of between 50-60k daltons (15,16).

Peptide Receptor Modulation.

Endocytosis of receptor ligand complexes has been demonstrated in several systems, including the low density lipoprotein (LDL) (17), mannose-6-phosphate (18), insulin (28), and the transferrin systems (19,20).It has been shown that in the process of endocytosis plasma membrane components are internalized, segregated to intracellular compartments and subsequently recycled back to the surface membrane (17,18,19,20). When PMN are first incubated with FMLP there is a down regulation of receptors (11,21,22). Over

time however, the number of cell surface receptors increases reaching a plateau (11,21). This continued availability of receptor can be explained by three different mechanisms. First, by the continued synthesis of peptide receptor. Second, by the presence of intracellular (i.e. stored) receptor within the cell that is brought to the cell surface. Finally as has been established in other systems, by the recycling of receptors. Since PMN are not noted to be 'synthetic' cells and because the receptor number on the surface increases even in the presence of cycloheximide (22), the first explanation is not plausible. The concept of spare receptors (stored receptors) was based on the findings that PMN can contain a population of chemotactic receptor within their specific granules, (23). Such granules are believed to fuse with the PMN membrane when cells are stimulated by FMLP (22,23). Finally, there has been speculation that re-expression of the receptor occurs (11). This latter hypothesis will be discussed in subsequent chapters of this thesis. This chapter deals with the processing of FMLP by PMN and is the background to some of the subsequent chapters.

PROCESSING OF FMLP AND ITS RECEPTOR BY PMN. EVIDENCE THAT INTRACELLULAR PROCESSING OF FMLP MAY INVOLVE THE LIGHT GOLGI COMPARTMENT.

The subcellular pathway for ligand-receptor processing is an important starting point for investigation of chemotactic peptide receptor modulation in the process of chemotaxis. It is evident that when ligand receptor interaction takes place a complex network of events involving many different cellular compartments occurs. These events are triggered by the binding of the ligand (i.e. FMLP) to its receptor on the PMN surface membrane. It is important to note

however, that these events are temperature dependent and cellular stimulation by FMLP (beyond the binding of FMLP to its receptor) does not occur at temperatures below 15^{o}C (11,21). At 37^{o}C, FMLP and its receptor are rapidly internalized. The internalized peptide and its receptor appear initially within fractions of similar enzyme characteristics as the light Golgi. Indeed as will be elaborated in the following chapters, segregation to the light Golgi-like fraction may be of paramount importance to the intracellular processing of the FMLP receptor.Detailed below, are the results of studies in which we examined the internalization and compartmentalization of bound [^{3}H]-FMLP.

Methods and Materials.

Preparation of platelet poor leukocyte suspensions.

Platelet-poor leukocyte suspensions containing 97-99% PMN were prepared from venous blood (50 ml), obtained from healthy adult volunteers. Blood was collected in acid-citrate dextrose (24). After platelet removal by low speed centrifugation, cells were resuspended in phosphate buffered saline (PBS), pH 7.4. PMN were separated on Ficoll/Hypaque gradients. Centrifugation of the Ficoll/Hypaque gradients allowed for the removal of mononuclear cells. Red blood cells were removed by dextran sedimentation and hypotonic lysis (11,24). PMN preparations were >97% pure and exhibited a viability > 98%.

Chemotactic Stimuli.

FMLP was purchased from Peninsula Laboratories, Inc., San Carlos Ca. Radiolabelled [^{3}H]-FMLP was purchased from New England Nuclear, Boston Ma.

Preparation of [^{125}I]-WGA-D.

WGA-D was prepared, purified and radioiodinated as described previously (11).Approximately 90 +/- 2% of the radioactivity was protein bound.The specific activity of [^{125}I]-WGA-D was 47.4 uCi/mg protein. In some experiments, PMN were centrifuged through silicone oil in order to separate free from cell bound radioactivity, and the radioactivity associated with the cell pellets determined as described (11).

Subcellular Fractionation of PMN.

Cells (7-9 x10^8PMN) were suspended in cold PBS (14 ml) containing 0.34 M sucrose, 0.1 mM $MgCl_2$, 1.0 mM ethylenediamine tetracetic acid (EDTA, Sigma), 10 mM Hepes, and 1.0 mM diisopropylfluorophosphate (DFP, Sigma) (cavitation buffer), placed in a 50 ml polyethylene tube, and subjected to nitrogen cavitation in a precooled (4^{o}C) Parr cell disruption bomb (Parr Instruments, Moline, IL) with constant stirring (25). After 20 min equilibration at 400 psi N_2, cells were lysed. Lysates were centrifuged at 1000 x g for 10 min to remove intact cells, nuclei and cell fragments.The efficiency of lysis was 80-90%, as assessed by phase microscopy and light microscopy of smears stained with Wright-Giemsa.The 1000 x g supernatant, containing between 75-85% of all markers, was layered onto sucrose density gradients in Beckman SW 27 tubes (Beckman Instruments, Palo Alto, CA). The gradients consisted of 3.0 ml of 60% sucrose as cushion and 22 ml of a linear (55 to 20%) sucrose gradient . Layered samples were centrifuged in a Beckman SW 27 rotor at 82,500 x g for 3 hours. Fractions (1.0 ml) were collected by puncturing the bottom of each tube using a Beckman fractionator. Recovery of markers ranged from 85-98% of the amounts present in the 1000 x g supernatant.

[^{3}H]-FMLP was detected after treatment of samples for 18 h with 1.0 ml NCS tissue solubilizer (Amersham Corp.,Arlington Heights, IL), followed by the addition of 10 ml OCS counting solution (11). Several enzymatic and non-enzymatic markers were measured: lactic dehydrogenase (LDH) (11), (cytoplasmic marker), beta-glucuronidase (11), (azurophilic granule marker), lysozyme , gelatinase and, vitamin B_{12}-binding protein (11), (specific granule marker), alkaline phosphatase (25), (membrane marker), and UDP-galactose galactosyl transferase (25) (golgi marker). Protein content was determined, after treating samples with NaOH (25), by the Bradford method (19) using the BioRad protein assay kit (BioRad Laboratories, Richmond, CA). Bovine serum albumin was used as a standard.

Results.

Intracellular processing in FMLP

When PMN are exposed to FMLP at 37^{o}C there is rapid internalization of the the ligand (25,26). Previously, it has been reported that PMN incubated with 50 nM [^{3}H]FMLP, initially internalized the peptide to a compartment containing galactosyl transferase activity (25,26). Electronmicroscopic analysis of this compartment was highly suggestive of the presence of light and intermediate type Golgi apparatus (25).In the study reported in this chapter, PMN were incubated with 50 nM [^{3}H]FMLP for various periods of time at 37^{o}C, following which the reaction was stopped by adding cold buffer and the PMN were subsequently disrupted by nitrogen cavitation and fractionated on a sucrose density gradient. Enzyme markers were used to localize PMN organelles contained within the various fractions. In most experiments over 98% of the radioactivity was accounted for, with approximately 55% of the radioactivity

recovered from the sucrose fractions. The remaining 43-45% radioactivity was discarded in the pellet-foam residue obtained after nitrogen cavitation of PMN. Analysis of radioactivity obtained from sucrose fractions was performed by ultracentrifugation and radioactivity was divided into sedimentable (organelle associated) and soluble (cytosolic) fractions (Table 1).

All fractions underwent enzyme and non-enzyme analysis to determine location of organelles on the sucrose gradient as described in *Methods and Materials.*

Processing of Bound [^{3}H]-FMLP by PMN at 4^{o}C.

When PMN were incubated with [^{3}H]-FMLP at 4^{o}C for 5 minutes and subsequently disrupted and fractionated, a peak of radioactivity was noted to co-migrate with the fractions containing the membrane marker alkaline phosphatase as well as ^{125}I-labelled WGA-D (surface marker) (Fig.1). Thus internalization of [^{3}H]-FMLP was not observed at 4^{o}C (Fig.2).

Processing of Bound [^{3}H]-FMLP by PMN at 37^{o}C .

As previous studies have shown, FMLP is rapidly internalized at higher temperatures. In experiments performed whereby PMN were incubated with 50 nM [^{3}H]-FMLP for 5 minutes at 37^{o}C, the [^{3}H]-FMLP is observed to co-migrate with three different organelle markers. There is a peak of [^{3}H]-FMLP which migrates with surface membrane marker alkaline phosphatase. There is also a peak of radioactivity noted to migrate with the Golgi marker galactosyl transferase. A significant proportion of [^{3}H]-FMLP however, is found at the top of the sucrose density gradients representing free [^{3}H]-FMLP or, most likely degraded FMLP (Fig. 3). Thus it appears that after binding to its receptor on the

plasma membrane, FMLP is internalized to a compartment with high galactosyl transferase activity.

With increasing periods of incubation at 37°C (10 min.), as more ligand is internalized, there is an increased amount of radioactivity that co-migrates with the Golgi marker galactosyl transferase, with a concomitant decrease in the amount of radioactivity co-migrating with the plasma membrane marker alkaline phosphatase (Fig. 4).

Upon incubation of PMN with 50 nM [^{3}H]-FMLP at 37°C for 20 minutes a diminution of radioactivity was observed in the fractions rich in alkaline phosphatase and galactosyl transferase. A peak of radioactivity is now observed within the fractions containing the enzyme markers for the specific granules. The majority of the radioactivity was detected in the fraction at the top of the gradient and probably represented degraded peptide (Fig. 5).

Analysis of radioactivity contained within the sucrose gradient fractions.

Fractions containing the golgi maker, plasma membrane marker, and cytosol marker, were ruptured by sonication, resuspended in PBS and subjected to high speed ultracentrifugation. The post-centrifugation supernates and pellets were then assayed for radioactivity.

In fractions containing the cytosol marker, 95% of the radioactivity was detected in the supernate indicating free or degraded [^{3}H]-FMLP. In fractions containing the Golgi marker and the membrane marker, 68% and 74% respectively of the radioactivity was contained within the pellets (Table1). This indicated that the majority of radioactivity in the Golgi fraction and plasma

membrane were sedimentable and hence, represented ligand-receptor complexes.

Discussion

In the experiments described above the processing and redistribution of radiolabelled chemotactic peptide was examined. Results obtained from these experiments and from studies performed by other investigators indicate that [^{3}H]-FMLP, is internalized rapidly by PMN at 37^{o}C (11,25,26). Following internalization the ligand is noted to migrate to a Golgi-enriched fraction, although a large amount of the radioactivity is noted in the cytosolic fractions of the disrupted cells. The radioactivity contained within the cytosolic fractions is thought to represent both degraded peptide from hydrolysis of the peptide by membrane hydrolases (27), as well as internally degraded peptided (25).

In the initial studies performed at 4^{o}C, a peak of tritiated peptide is noted to co-migrate with the plasma membrane fractions of the disrupted PMN. Plasma membrane enriched fractions were identified by alkaline phosphatase activity and by [^{125}I]-wheat germ agglutinin (WGA) surface labelling . Even at a temperature at which internalization of intact ligand receptor complexes does not occur, a significant percentage of the radiolabel was observed in the cytosolic fractions. (Binding analysis of [^{3}H]-FMLP is further elaborated in chapter 3).

On exposure of PMN to [^{3}H]-FMLP at 37^{o}C, fast internalization of the ligand occurs.The internalization is receptor-mediated and besides being temperature dependent, can be competed by non-labelled excess peptide, (not shown). At 5

minutes of incubation at 37°C, there is an accumulation of [^{3}H]-FMLP that comigrates with the Golgi-enriched fraction, thus indicating that the internalized ligand migrates initially to the Golgi complex or a compartment of similar density and fractionation characteristics of Golgi. This trafficking of ligand to the golgi or para-golgi compartiments is not a unique property of PMN. Indeed, several other ligand systems have been shown to migrate to the Golgi following internalization (18,28). The precise role of the golgi in the processing of FMLP has yet to be elucidated, although several possibilities exist. These include, the Golgi serving as pre-processing organelle for lysosomes, where degradation of the ligand-receptor complex occur (18); the Golgi as the main processing organelle where the degradation of the ligand occur, followed by transport of the receptor to lysomes (specific granules) or to the plasma membrane. Alternatively, there may be no association between the Golgi and the internalized ligand-receptor complexes, instead this may represent vesicles containing receptorsomes also known as sorting endosomes of similar density to the Golgi. Other studies with chemotactic formyl peptides present convincing evidence that the ligand migrates initially to the golgi and then perhaps to the lysosomes.

Over time, with exposure of PMN to FMLP at 37°C for greater than 15 minutes, a significant amount of radioactivity is noted in the lysomal fractions, specifically fractions rich in markers for the specific granules. This radioactivity probably represents degraded peptide as well as 'neutrally-charged' peptide from the cytosol which may diffuse into lysosome. A significant amount of the radioactivy present in the lysosomal fraction was non-sedimentable, indicating that ligand-receptor complexes were not present.

In summary, formyl peptides are rapidly internalized by PMN when exposed to the cells at 37°C. The ligand migrates to the Golgi enriched fractions, are sedimentable and are probably still contained in a receptor-ligand complex. Dissociation of the ligand-receptor complex (and perhaps subsequent degradation of the ligand) most likely occurs in the Golgi-enriched fractions, with transport of the ligand to the cytosolic and perhaps the lysosomal fractions. It is highly suggestive from these studies that the Golgi organelle plays a major role in the intracellular processing of chemotactic formyl peptides

Table 1.

ORGANELLE MARKER	PERCENT CPM [^{3}H]-FMLP	
	SEDIMENTABLE	NON-SEDIMENTABLE
CYTOSOL.		
LDH--rich fractions	5%	95%
PLASMA MEMBRANE.		
ALKALINE PHOSPHATASE-rich fractions	74%	26%
LIGHT GOLGI.		
GALACTOSYL-TRANSFERASE-rich fractions	68%	32%.

Table 1.

Comparison of sedimentable and non-sedimentable cpm from sucrose gradient fractions with peak enzyme activity for markers for cytosol, plasma membrane and light golgi. Fractions underwent ultracentrifugation at 4^{o}C (50,000 x g), following which cpm from supernate and pellet were quantitated as described in *Methods and Materials*. Data represents results from 6 experiments.

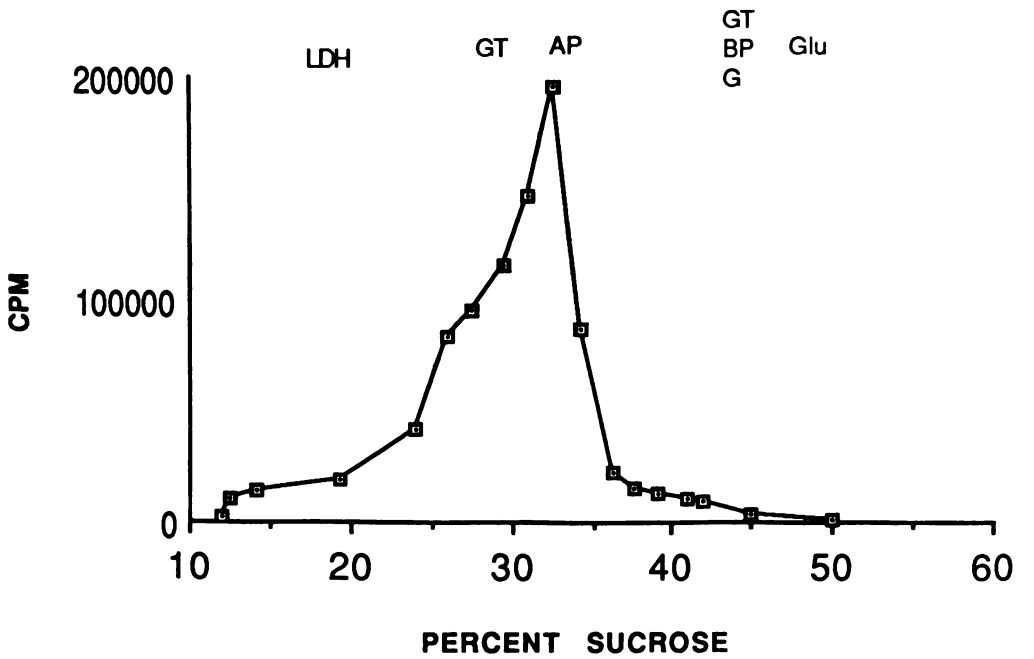

Figure 1

Subcellular distribution of [^{125}I]-WGA-D within PMN. Cells (7 x 10^8 PMN) were suspended in 14 ml of Hanks' buffer containing the appropriate reagents and incubated at 4°C for 5 min with continuous agitation. After incubation, reactions were stopped by adding equal volumes of cold PBS. PMN were washed twice with cold PBS, resuspended in 14 ml of cavitation buffer, and subjected to N_2 cavitation, followed by fractionation on sucrose density gradients as described in Materials and Methods. Subcellular markers: GT, galactosyl transferase; AP, alkaline phosphatase; BP, vitamin B_{12}-binding protein; G, gelatinase; Glu, beta-glucuronidase; LDH, lactate dehydrogenase.

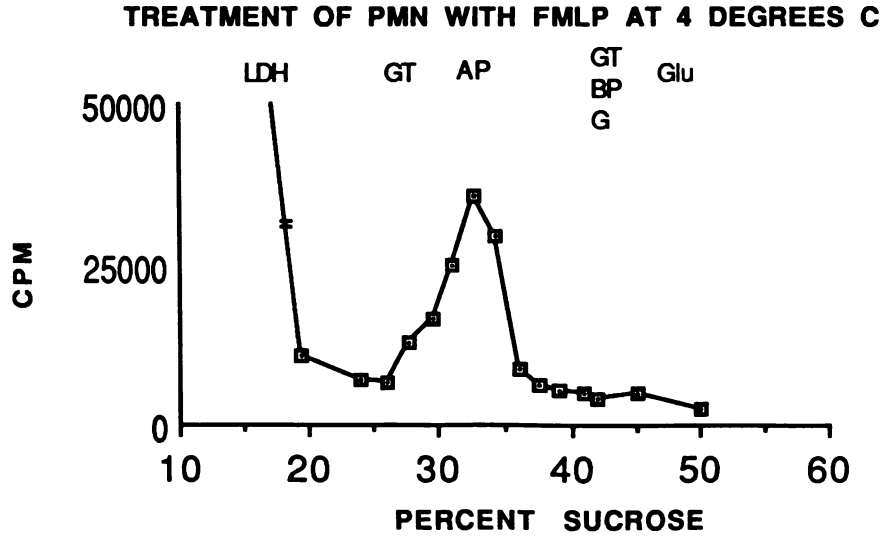

Figure 2

Subcellular distribution of [^{3}H]-FMLP within PMN. Cells (7 x 10^8 PMN) were suspended in 14 ml of Hanks' buffer containing the appropriate reagents and incubated at 4^{o}C for 5 min with continuous agitation. After incubation, reactions were stopped by adding equal volumes of cold PBS. PMN were washed twice with cold PBS, resuspended in 14 ml of cavitation buffer, and subjected to N_2 cavitation, followed by fractionation on sucrose density gradients as described in Materials and Methods. Subcellular markers: GT, galactosyl transferase; AP, alkaline phosphatase; BP, vitamin B_{12}-binding protein; G, gelatinase; Glu, beta-glucuronidase; LDH, lactate dehydrogenase.

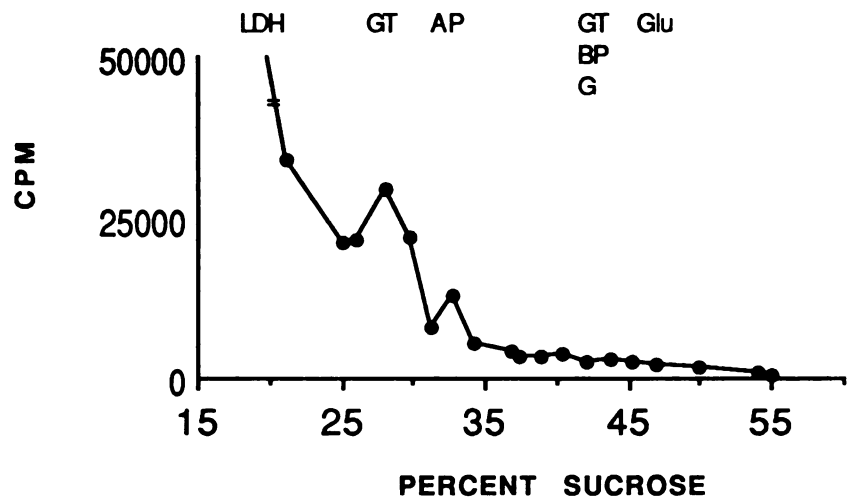

Figure 3

Subcellular distribution of [^{3}H]-FMLP within PMN. Cells (7 x 10^8 PMN) were suspended in 14 ml of Hanks' buffer containing the appropriate reagents and incubated at 37^{o}C for 5 min with continuous agitation. Cells were processed as described in legends for figures 2 and 3. Organelle markers appear at top of graph.

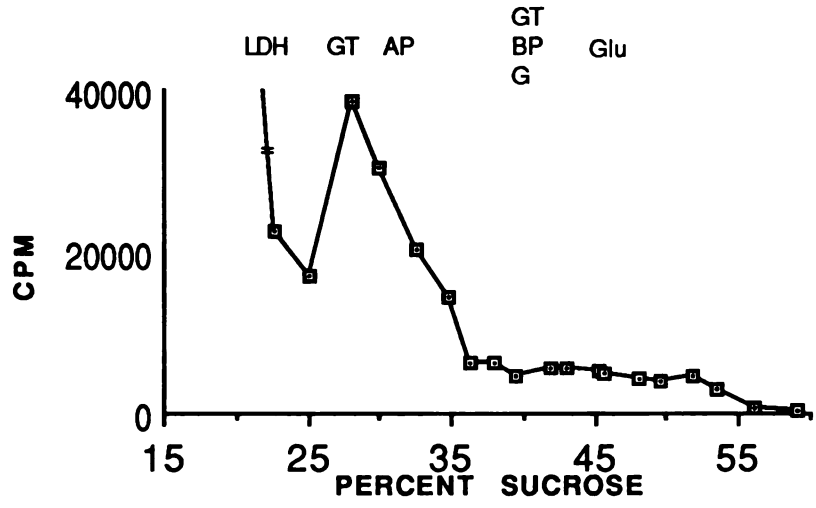

Figure 4

Subcellular distribution of [^{3}H]-FMLP within PMN. Cells (7 x 10^8 PMN) were suspended in 14 ml of Hanks' buffer containing the appropriate reagents and incubated at 37^{o}C for 10 min with continuous agitation. Cells were then processed as described in legends for figures 1 and 2.

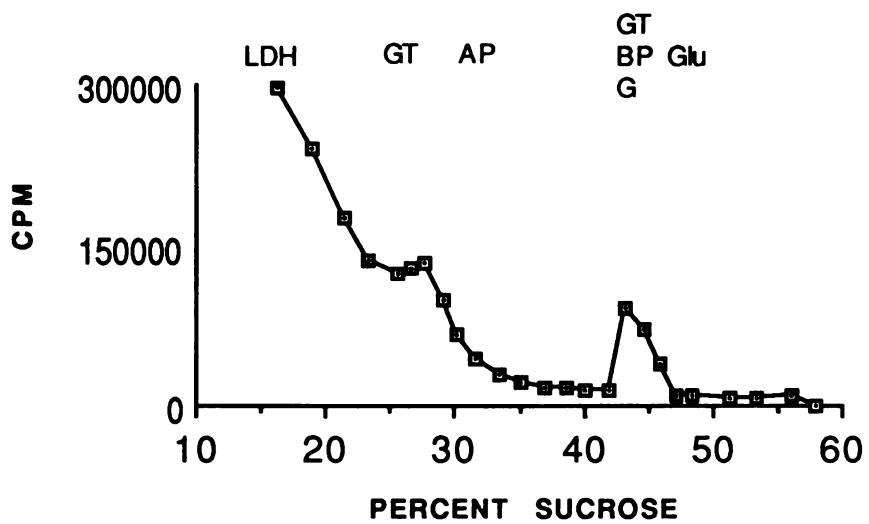

Figure 5

Subcellular distribution of [^{3}H]-FMLP within PMN. Cells (7 x 10^8 PMN) were suspended in 14 ml of Hanks' buffer containing the appropriate reagents and incubated at 37^{o}C for 20 min with continuous agitation. Cells were then processed as described in legends for figures 1 and 2.

Acknowledgement.

The text of this chapter is a reprint of material as it appears in the Journal of Immunology. Errol Lobo is listed as a co-author in this paper and while he contributed in part to all phases of the study, his major contribution came in the cell fractionation studies which are an essential part of the study.

CHAPTER III

INHIBITION OF PMN FORMYL-PEPTIDE RECEPTOR RECYCLING AND CHEMOTAXIS

A DERIVATIVE OF WHEAT GERM AGGLUTININ SPECIFICALLY INHIBITS FORMYL PEPTIDE-INDUCED POLYMORPHONUCLEAR LEUKOCYTE CHEMOTAXIS BY BLOCKING REEXPRESSION (OR RECYCLING) OF RECEPTORS.

Introduction.

Bound [^{3}H]-FMLP is rapidly internalized by PMN and can be localized, on sucrose density gradients, to a compartment exhibiting a density lower than that of plasma membranes and containing Golgi markers (1). Over time, free, undegraded [^{3}H]-FMLP appears to accumulate in a cytosolic compartment (1). The fate of the receptor after internalization is unknown.

Endocytosis of receptor-ligand complexes has been demonstrated to occur in various cell types (2-4). Following internalization, the ligand-receptor complex can be processed in a variety of ways (5,6). First, the ligand may remain associated with the receptor and the ligand-receptor complex delivered to lysosomes where it is degraded. Second, the ligand receptor complex may be stored intracellularly. Finally, an alternative mechanism involves the segregation of ligand and receptors to different compartments. In the latter case, receptors escape the fate of the ligand, can be recycled to the cell surface, and used in multiple cycles of internalization.

The preparation and characterization of a derivative of the plant lectin, wheat germ agglutinin (WGA), that specifically inhibited chemotactic responses of

PMN to FMLP has been reported in previous studies, (7).The derivative (termed WGA-D) had no effect on either FMLP-induced PMN polarization, superoxide anion generation, or degranulation. WGA-D also had no effect on the ability of [^{3}H]-FMLP to bind to its specific receptors on the PMN plasma membrane and did not affect the ability of unlabelled FMLP to displace [^{3}H]-FMLP bound to PMN. Presumably, WGA-D exerted its effects by attaching to N-acetyl-D-glucosamine residues on the PMN plasma membrane (7).

The mechanism(s) whereby WGA-D exerted its inhibitory effect was not elucidated. In this report, we have examined its mechanism of action and present evidence indicating that WGA-D inhibits FMLP-induced PMN chemotaxis by blocking the reexpression (or recycling) of a population of FMLP receptors required for continuous migration.

Materials and Methods

Preparation of Leukocyte Suspensions.

Platelet-poor leukocyte suspensions containing 97-99% PMN were prepared as described in chapter 2.

Assays of PMN Functions.

PMN stimulated random migration and directed migration (chemotaxis) were assayed using a minor modification (7-10) of the leading front method of Zigmond and Hirsch (10). Chemotaxis (net migration) was calculated by subtracting random motility from total migration (7,8). Results are expressed as the distance (um) that the leading front of cells migrated during 35 min of incubation (7,8). Duplicate chambers were used in each experiment and 10 fields were examined in each filter.

Extracellular release of the granule markers, beta-glucuronidase, lysozyme and vitamin B_{12}-binding protein, was measured as described previously (7,9) in reaction mixtures containing 3 x 10^6 PMN, which were preincubated with cytochalasin B (5.0 ug/ml) (Aldrich Chemical CO., Milwakee, WI) for 60 sec prior to the addition of the stimulus.

Superoxide anion generation was measured as described previously in duplicate reaction mixtures containing 3 x 10^6 PMN and 75 uM horse heart ferricytochrome *c* (Type III, SigmaChemical Co., St Louis, MO) (7,9). PMN were preincubated with cytochalasin B as described above.

Chemotactic Stimuli.

FMLP was purchased from Peninsula Laboratories, Inc.,San Carlos, CA. Human C5a was isolated from yeast-activated human serum containing the carboxypeptidase inhibitor, 2-mercaptomethyl-5-guanidinoethyl-thiopropanoic acid (2.0 mM), and was purified as described previously (9,11).

Preparation of [^{125}\]-WGA-D.

WGA-D was prepared, purified and radioiodinated as described in chapter II.

Assays Using [^{3}H]-FMLP.

Binding of [^{3}H]-FMLP to PMN was measured as described, and analyzed by the method of Scatchard (7,9).Data points were fit by a weighted non-linear method of least-squares to one and two receptor models with a technique modified for the LIGAND program (9,12),using an HP-86A computer (Hewlett Packard, Inc., Mountainview, CA).

Uptake of [^{3}H]-FMLP by PMN was measured as follows. PMN (4 x 10^6 cells) and [^{3}H]-FMLP(1.0 nM) (specific activity, 48.5 Ci/mmol) (New England Nuclear

Corp., Boston, MA) were incubated at 37°C for varying durations in 0.4 ml of PBS, in the presence or absence of WGA-D (3.0 ug). This concentration of WGA-D was chosen because it completely inhibited FMLP-induced PMN chemotaxis (7).Following incubation, reactions were stopped by adding an equal volume of cold (4°C) PBS containing 1.0 uM unlabelled FMLP. A collection method involving centrifugation through silicone oil was used to separate free from cell-associated peptide (9). After centrifugation for 30 sec (15,600 x g), the tips of the tubes were excised and processed as described (9).

We also measured the release of [^{3}H]-FMLP-associated radioactivity that had been taken up by PMN. Cells, 4 x 10^6 PMN in 0.4 ml PBS, were preincubated with 1.0 nM [^{3}H]-FMLP in the presence and absence of WGA-D (3.0 ug) for 20 min as described above. After preincubation, the reaction was stopped by adding an equal volume of cold PBS containing 1.0 uM unlabelled FMLP, and PMN were washed twice with 4°C PBS. PMN were finally resuspended to original volume (0.4 ml) in 37°C Hanks' buffer and incubated at 37°C for varying durations. Following incubation, cells were centrifuged through oil and pellets processed as described (9).

In some experiments, we examined the ability of PMN to recover their capacity to bind [^{3}H]-FMLP. Briefly, cells (4 x 10^6 PMN) suspended in 0.5 ml PBS were preincubated at 4°C for 10 min with 1.0 uM unlabelled FMLP in the presence and absence of WGA-D (3.0 ug).This preincubation time was chosen in order to attain equilibrium binding of FMLP (9). After preincubation, cells were washed once with cold PBS and resuspended in 0.25 ml Hanks' buffer (that had been prewarmed to 37°C) and incubated at 37°C for varying durations. Reactions were stopped by adding an equal volume of cold PBS containing 20 nM [^{3}H]-

FMLP. PMN were incubated with [^{3}H]-FMLP for 10 min on ice to achieve equilibrium binding (9). After incubation, PMN were pelleted through oil and processed as described (9). Non-specific binding, that is, uptake by the cells of radioactivity that was not inhibited by 1000-fold excess unlabelled FMLP, represented between 7-11% of total binding.

Binding and Internalization of a Fluoresceinated Analog of FMLP.

Binding of the fluoresceinated hexapeptide, N-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys (N-FNLPNTL-Fl) was monitored as described previously (13). PMN suspensions (2 x 10^6cells/ ml) were exposed to N-FNLPNTL-Fl (3.0 nM)in the presence and absence of WGA-D (3.0 ug), and monitored by flow cytometry using a Becton-Dickinson FACS IV (Sunnyvale, CA). Binding is expressed as mean fluorescence channel number as a function of time. Internalization of the bound peptide was examined by reducing the pH of the cell suspension after 3 min of binding, as described previously (13). Extracellular fluorescein or its derivatives are quenched instantaneously by the change in pH, while intracellular fluorescein derived from fluorescein diacetate is quenched only slowly following the pH change.

Subcellular Fractionation of PMN.

Subcellular fractionation was performed as described in chapter 2. [^{125}I]-WGA-D radioactivity was determined directly using a Beckman 5500 gamma radiation counter. [^{3}H]-FMLP was detected after treatment of samples for 18 h with 1.0 ml NCS tissue solubilizer (Amersham Corp.,Arlington Heights, IL), followed by the addition of 10 ml OCS counting solution (9). Several enzymatic and non-enzymatic markers were measured: lactic dehydrogenase (LDH) (9), beta-glucuronidase (9), lysozyme (9), gelatinase (14), vitamin B_{12}-binding

protein (8), alkaline phosphatase (15), and UDP-galactose galactosyl transferase (1). Protein content was determined, after treating samples with NaOH (1), by the Bradford method (15) using the BioRad protein assay kit (BioRad Laboratories, Richmond, CA). Bovine serum albumin was used as a standard.

In some instances, peak fractions obtained after subcellular fractionation were subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions using 5-15% gradient slab gels (16). Gels were either stained with Coomassie blue (BioRad) or proteins were recovered by transfer to nitrocellulose paper as described by Towbin et al (17). Immediately following transfer, proteins were reacted with WGA-D (2.0 ug/ml) for 90 min. Nitrocellulose sheets were rinsed and and were incubated with a 1/100 dilution of rabbit antiserum to WGA-D, followed by exposure to ^{125}I-labelled Staphylococcus protein A (Pharmacia Fine Chemical, Piscataway, NJ). After 90 min, the nitrocellulose sheet was rinsed, wrapped in Glad-wrap and exposed (for 18 hrs at -70^{o}C) to Kodak XR-1 film using a Cronex intensifying screen (Dupont, Wilmington, DE).

Results

Effect of WGA-D on Uptake and Release of [^{3}H]-FMLP by PMN.

In previous studies it was demonstrated, that, when at 4^{o}C, WGA-D had no effect on the binding of [^{3}H]-FMLP to its receptors on the PMN membrane (7). These experiments represented only true binding, since internalization does not occur at 4^{o}C. Consequently experiments were performed at 37^{o}C to determine the effect of WGA-D on the uptake of [^{3}H]-FMLP by PMN. Uptake represents both receptor-mediated binding to, as well as endocytosis of receptor-peptide

complexes by PMN. Cells (4 x 10^6 PMN) were incubated with 1.0 nM [^{3}H]-FMLP, in the presence or absence of WGA-D (3.0 ug), at 37^{o}C for varying durations and uptake determined as described in Methods. As can be seen in Fig. 1, uptake of [^{3}H]-FMLP by PMN was linear between 2 and 10 min of incubation, reaching saturation at 20 min. A more rapid increase in uptake was observed during the first 2 min, most likely representing rapid binding (8) and some degree of endocytosis. A linear uptake (up to 10 min) followed, probably representing equilibrium between rates of binding and endocytosis. Saturation occurred at 20 min, at a level equivalent to 6.7 x 10^3 molecules/PMN. The kinetics of uptake of [^{3}H]-FMLP by PMN in the presence of WGA-D were identical to those observed using untreated PMN (Fig. 1).Thus, it appeared that WGA-D had no effect on the ability of PMN to bind and take up [^{3}H]-FMLP.

Next the possibility that WGA-D exerted its inhibitory effect on FMLP-induced PMN chemotaxis by interfering with the dissociation of FMLP-receptor complexes at 37^{o}C was examined. PMN (4 x 10^6 cells) were incubated with 1.0 nM [^{3}H]-FMLP, in the presence or absence of WGA-D (3.0 ug), for 20 min at 37^{o}C, washed twice with cold (4^{o}C) PBS, and resuspended at 37^{o}C in Hanks' buffer. PMN were incubated at 37^{o}C for varying durations, after which cells were pelleted through oil and radioactivity in pellets and supernatants determined (Fig. 2). Only a portion (approximately 65-70%) of the cell-associated radioactivity (Fig. 2A) was released into supernatants after 30 min of incubation (Fig. 2B). Longer incubation periods did not result in further release (not shown). There was an initial rapid loss of cell-associated radioactivity (460 molecules/min/PMN)during the first 2 min (Fig. 2A), followed by a slow diminution (100 molecules/min/PMN) between 2 and 15 min. No significant release of [^{3}H]-FMLP was observed between 15 and 30 min of incubation (Figs.

2A and 2B). Again, WGA-D had no effect on the release of [^{3}H]-FMLP that had been taken up by PMN (Figs. 2A and 2B), indicating that it did not interfere with the ability of either surface bound and/or internalized FMLP to dissociate from its receptors.

Effect of WGA-D on Binding and Internalization of N-FNLPNTL-Fl.

Uptake of [^{3}H]-FMLP represented both tight binding to, and internalization of the radiolabeled peptide by PMN. Thus, it was possible that WGA-D affected internalization of the peptide by PMN, but that this was not readily detected. Impairment of FMLP internalization could provide an explanation for the inhibitory effect of WGA-D on FMLP-induced PMN chemotaxis. Consequently, we examined the effect of WGA-D on the kinetics of binding to, and internalization by, PMN of the fluoresceinated hexapeptide, N-FNLPNTL. As shown in Fig. 3, WGA-D (3.0 ug) did not affect the rate of either short-term binding of N-FNLPNTL-Fl to PMN or short-term internalization of bound N-FNLPNTL-Fl by the cells. Approximately 50% of the bound ligand was internalized after 3 min by both normal (Fig. 3A) and WGA-D-treated PMN (Fig. 3B).

From the experiments described above, it appeared that WGA-D had no effect on either binding of FMLP to its receptors, internalization of FMLP-receptor complexes, or release of bound and internalized FMLP by PMN. Consequently, we performed experiments to determine the effect of WGA-D on the reexpression (or recycling) of FMLP receptors by PMN.

Effect of WGA-D on Receptor Reexpression by PMN.

Preincubation of PMN (4 x 10^6 cells) with unlabelled FMLP (1.0 uM) for 10 min at 4^{o}C significantly diminished their subsequent ability to bind [^{3}H]-FMLP (Fig.

4A). While untreated PMN specifically bound an average of 3,450 +/- 280 molecules of [^{3}H]-FMLP/PMN, cells that had been preincubated with unlabeled FMLP bound 1,100 +/- 120 molecules of [^{3}H]-FMLP/PMN, a diminution of 70% in their capacity to specifically bind [^{3}H]-FMLP (Fig. 4A). As reported previously (7), WGA-D (3.0 ug/ml) had no effect on the ability of untreated PMN to bind [^{3}H]-FMLP (Fig. 4A).

After incubation at 37^{o}C, PMN that had been preincubated with unlabelled FMLP alone rapidly recovered their ability to bind [^{3}H]-FMLP (Fig. 4A).Recovery appeared to occur in two phases; a rapid one, taking place within the first 3 min of incubation, and a slow phase occurring between 3 and 8 min of incubation. After 3 min of incubation, PMN that had been preincubated with FMLP alone bound 3,010 +/- 175 molecules of [^{3}H]-FMLP/PMN, a value close to that of untreated PMN (i.e., 3,450 +/- 280 molecules/PMN). After 8 min of incubation, the same cells bound 4,214 + 230 molecules of [^{3}H]-FMLP/PMN. Longer incubation times did not result in further increments of their binding capacity. Thus, it appeared that PMN that had been preincubated with unlabelled FMLP alone recovered 87% of their initial binding capacity within the first 3 min of incubation. At 8 min (i.e., plateau of recovery), PMN that had been preincubated with unlabelled FMLP alone bound approximately 20% more [^{3}H]-FMLP than untreated PMN (Fig. 4A), a phenomenon known as super recovery (18).

Initially, binding of [^{3}H]-FMLP to PMN that had been preincubated with both unlabelled FMLP (1.0 uM) and WGA-D (3.0 ug) was identical to that of PMN that had been preincubated with unlabelled FMLP alone (Fig. 4A).However, PMN that had been preincubated with unlabelled FMLP and WGA-D failed to completely recover their ability to bind [^{3}H]-FMLP(Fig. 4A). Even after 10 min,

these cells bound significantly less [^{3}H]-FMLP (2,150 +/- 134 molecules/PMN) than PMN that had been preincubated with unlabelled FMLP alone (4,214 +/- 230 molecules/PMN) ($p < 0.01$, Student's t test) (Fig. 4A).Identical results were observed after 20 min of incubation (not shown).

The effect of WGA-D on PMN receptor reexpression was specific. Identical experiments were performed using the plant lectin, concanavalin A (Con A) . Con A (4.0 ug) had no effect on the ability of PMN that had been preincubated with 1.0 uM FMLP to recover their capacity to bind [^{3}H]-FMLP (Fig. 4A).

It has been reported previously that PMN specific granules possess a pool of putative receptors for FMLP that are expressed upon fusion of granules with the plasma membrane (19,20). Thus, it was of interest to determine whether WGA-D inhibited spontaneous PMN degranulation during the recovery period. Inhibition of degranulation could account for the differences observed in binding. Consequently, in the same set of experiments we determined release by PMN of the specific granule markers, lysozyme and B_{12}-binding protein (as well as the azurophil granule marker, beta-glucuronidase). As shown in Fig. 4B, PMN that had been preincubated with unlabelled FMLP in the presence of WGA-D released identical amounts of lysozyme and vitamin B_{12}-binding protein into supernatants as did PMN that had been preincubated with unlabelled FMLP alone. Similar results were observed when beta-glucuronidase activity was determined (not shown). Thus, the differences observed in the binding of [^{3}H]-FMLP between the two populations of PMN could not be explained by differences in degranulation.

In the same set of experiments, the measured specific binding of [^{3}H]-FMLP to

PMN that had been preincubated with unlabelled FMLP, with and without WGA-D, and allowed to recover for 8 min (plateau of recovery) was measured,(Fig. 5). Following recovery, the reaction was stopped by adding an equal volume of cold (4°C) PBS. PMN were then washed twice with cold buffer, and incubated with varying concentrations of [^{3}H]-FMLP at 4°C. Binding was measured at equilibrium (8).

PMN that had been preincubated with unlabelled FMLP plus WGA-D prior to recovery bound significantly less [^{3}H]-FMLP than PMN that had been preincubated with unlabelled FMLP alone (Fig. 5). Both populations of cells exhibited similar dissociation constants (K_D) of binding for their high and low affinity sites (Table I). Interestingly, PMN that had been preincubated with unlabelled FMLP plus WGA-D had fewer binding sites than PMN that had been preincubated with unlabelled FMLP alone (Table I). The difference observed in the number of high affinity sites between the two populations of PMN was significant ($p < 0.01$, Student's t test).

Effect of WGA-D on the Recovery of PMN Responses to FMLP.

Experiments were performed to determine the ability of PMN that had been preincubated with FMLP, in the presence and absence of WGA-D, and allowed to recover for varying periods of time, to respond chemotactically, to degranulate and to generate superoxide anion radicals upon subsequent challenge with FMLP. At designated time points during recovery, reactions were stopped by adding equal volumes of 4°C PBS. PMN were washed once with cold PBS, resuspended in Hanks' buffer, and used for the functional assays. These experiments were performed simultaneously with the receptor reexpression assays described in Fig. 4.

As shown in Fig. 6, PMN that had been preincubated with 1.0 uM FMLP at 4°C and washed were unable, initially, to migrate chemotactically in response to 10^{-8}M FMLP. When washed cells were allowed to recover for 5 min at 37°C prior to the chemotaxis assay, PMN that had been preincubated with FMLP alone exhibited similar chemotactic responsiveness to FMLP as untreated PMN (Fig. 6). After 10 min of incubation, PMN that had been preincubated with FMLP alone migrated approximately 20% more than untreated PMN (Fig. 6).Longer incubations (up to 20 min) did not result in further enhancement of their chemotactic responsiveness (not shown).

In contrast, PMN that had been preincubated with 1.0 uM FMLP plus WGA-D (3.0 ug) failed to recover their ability to migrate chemotactically in response to FMLP, even after 10 min of incubation at 37°C prior to the chemotaxis assay (Fig. 6).Their chemotactic responsiveness was similar to that of PMN that had been preincubated with WGA-D in the absence of FMLP (Fig. 6).Interestingly, PMN that had been preincubated with 1.0 uM FMLP, in the presence and absence of WGA-D (3.0 ug), and allowed to recover for 10 min at 37°C exhibited similar chemotactic responses as untreated PMN when challenged with suboptimal (2.0 ng/ml) concentrations of the complement-derived peptide, C5a (not shown).

In the same set of experiments, the ability of PMN that had been preincubated with FMLP, in the presence and absence of WGA-D, to generate superoxide anion radicals and to release lysosomal constituents when exposed to FMLP (10^{-7}M), at various times, was examined . As shown in Fig. 7 (panel A), PMN that had been preincubated with FMLP (1.0 uM) alone and PMN that had been

preincubated with FMLP plus WGA-D (3.0 ug) generated identical amounts of superoxide anion when subsequently challenged with FMLP. Interestingly, the amounts of superoxide anion generated were the same at the beginning as at the end of the recovery period (Fig. 7A). Identical results were obtained when the assays were performed in the absence of cytochalasin B (not shown). Similar results were observed when FMLP-induced PMN degranulation was measured (Fig. 7B). Thus, in contrast to chemotaxis, preincubation of PMN with FMLP in the presence and absence of WGA-D had no effect on the ability of these cells to either exhibit a burst of oxidative metabolism or degranulate upon subsequent exposure to FMLP.

Because WGA-D had no effect on internalization of FMLP by PMN, but did appear to interfere with FMLP receptor reexpression, it was of interest to determine wether bound WGA-D was internalized by PMN and whether FMLP and WGA-D were internalized simultaneously.

Internalization of WGA-D by PMN.

To determine whether WGA-D was internalized by PMN, cells (1 x 10^6/ml) were incubated with 3.0 ug of ^{125}I-labelled WGA-D for 5 min at 4^{o}C, washed twice with PBS, and resuspended at 37^{o}C in Hanks' buffer (0.3 ml). Five min was ample time to allow equilibrium binding of ^{125}I-WGA-D to PMN (7). At varying times, reactions were stopped by adding equal volumes of 4^{o}C PBS containing 0.1 M N-acetyl-D-glucosamine, and PMN were pelleted by centrifugation through silicone oil. This treatment results in the displacement of 85-90% of surface bound ^{125}I-WGA-D. Non-displaceable radioactivity, that is, radioactivity that remained associated with the cell pellet, was determined and used as a measurement of internalization. When exposed to FMLP (10^{-7}M), PMN rapidly

internalized bound ^{125}I-WGA-D (Fig. 8). Maximal internalization was observed after 60 sec, after which time the amount of PMN-associated radioactivity remained constant. Approximately 25% of bound ^{125}I-WGA-D was internalized. In the absence of FMLP, PMN failed to internalize bound ^{125}I-WGA-D (Fig. 8). This was true even after 10 min of incubation at 37°C (not shown).

Internalization of bound ^{125}I-WGA-D by PMN was dependent upon the concentration of FMLP and was specific (Fig. 9). FMLP-induced internalization of bound ^{125}I-WGA-D was maximal when 10 nM FMLP was used (Fig. 9).Higher concentrations of FMLP did not increase the amount of ^{125}I-WGA-D internalized by PMN (Fig. 9). In contrast to FMLP, purified human C5a, at concentrations as high as 1.0 nM, failed to induce internalization of bound ^{125}I-WGA-D by PMN.

Experiments were performed to confirm that WGA-D was indeed internalized by FMLP-stimulated PMN and to determine if, after internalization, ^{125}I-WGA-D and [^{3}H]-FMLP were segregated to similar compartments within PMN. Cells (7-9 x 10^{8} PMN) were incubated with either 50 nM [^{3}H]-FMLP, 0.7 mg ^{125}I-WGA-D, or both, at 37°C for 5 min. Reactions were stopped by adding 2 volumes 4°C PBS. PMN were washed twice with cold PBS, resuspended in cavitation buffer, and subjected to nitrogen cavitation followed by fractionation on sucrose density gradients, as described in *Materials and Methods.*

As reported previously (1), PMN incubated with 50 nM [^{3}H]-FMLP internalized the peptide to a compartment with a density lower than the plasma membrane marker alkaline phosphatase and comigrating with fractions containing galactosyl transferase activity (Fig. 10A). Internalization of [^{3}H]-FMLP did not occur at 4°C, and was blocked completely by the presence of 100-fold excess

unlabelled FMLP (not shown) (1). Incubation of PMN with ^{125}I-WGA-D alone revealed a peak of radioactivity that comigrated with the plasma membrane marker, alkaline phosphatase (Fig. 10A). Interestingly, when PMN were incubated with both [^{3}H]-FMLP (50 nM) and ^{125}I-WGA-D (0.7 mg), both molecules comigrated into galactosyl transferase-rich fractions (Fig. 10B). These experiments indicate that WGA-D and FMLP are internalized together and appear to migrate to the same compartment within PMN.

Finally, experiments were performed to detect the glycoprotein(s) on PMN membranes to which WGA-D binds.PMN (9 x 10^8 cells) were subjected to nitrogen cavitation and fractionated on sucrose density gradients, as described in *Materials and Methods*. Membranes present in fractions containing peak activity of either alkaline phosphatase (plasma membrane) or vitamin B_{12}-binding protein (specific granules) were concentrated by centrifugation at 100,000 x g for 3 hours. Pellets were dissolved in buffer (0.05 ml) containing 1.0% (w/v) SDS and 0.5% (v/v) beta-mercaptoethanol, and subjected to SDS-PAGE. After electrophoresis, parallel lanes were sliced and either stained with Coomassie blue or blotted onto nitrocellulose paper. After blotting, nitrocellulose strips were reacted with WGA-D, followed by rabbit antiserum to WGA-D and ^{125}I-labelled protein A. As expected, gels obtained after SDS-PAGE of PMN plasma membranes, when stained with Coomassie blue, revealed the presence of a variety of proteins with molecular weights ranging from 10,000 to over 200,000 Daltons (Fig. 11A).Similar results were observed when membranes from specific granules were used (Fig. 11B). The 200,000 dalton band (comigrating with myosin standard) was enriched in the plasma membrane fractions (Fig. 11A) as compared with the granular fraction (Fig 11B). Nitrocellulose strips stained with Coomassie blue after protein transfer revealed

similar patterns as those present in the stained gels (not shown). When nitrocellulose strips containing plasma membrane proteins were reacted with WGA-D, antibody and radiolabelled protein A, a single band was detected by autoradiography exhibiting an approximate molecular weight of 62,000 Daltons (Fig. 11C). When nitrocellulose strips containing specific granule membrane proteins were processed in the same fashion, five bands were detected by autoradiography that exhibited molecular weights ranging between 17,000-90,000 Daltons (Fig. 11D). A faint band that comigrated with the 62,000 dalton plasma membrane band was present in blots of specific granule membrane (Fig. 11D). It should be noted that membranes were prepared in the absence of exogenous albumin, and that WGA-D did not react with the human serum albumin standard (Fig. 11E). The only protein standard with which WGA-D reacted was ovalbumin (Fig. 11E), but no bands were observed comigrating with ovalbumin when blotted proteins from either plasma membranes or specific granules were used. When nitrocellulose strips containing blotted proteins were reacted with WGA-D and preimmune rabbit serum, no reaction was observed. Similar results were observed when unrelated rabbit antisera were used.

Discussion

The results of experiments presented here indicate that WGA-D specifically inhibits FMLP-induced PMN chemotaxis by blocking the reexpression (or recycling) of a population of receptors required for continuous migration. WGA-D had no effect on the uptake of [^{3}H]-FMLP by PMN (Fig. 1). Specific uptake was measured by subtracting nonspecific uptake (i.e., uptake of [^{3}H]-FMLP in the presence of a 100-fold excess of unlabelled peptide) from total uptake. By doing so, pinocytotic uptake of [^{3}H]-FMLP can be excluded. PMN have been shown to be capable of pinocytosis (21). Saturation of [^{3}H]-FMLP uptake

indicated that it was a receptor-mediated process (Fig. 1).It should be noted that the uptake data were not corrected for release of cell-associated peptide that may have occurred during the incubation period (18). Thus, the exact rate constant of uptake of [^{3}H]-FMLP by control PMN could not be determined. Nevertheless, it was clear that WGA-D (3.0 ug) had no effect on the uptake of [^{3}H]-FMLP by PMN. This concentration of WGA-D was chosen since it completely inhibits FMLP-induced PMN chemotaxis (7).

In some instances, displacement experiments (using 100-fold excess unlabelled FMLP) were performed at various times during uptake, after stopping the reaction by adding 4^{o}C PBS. WGA-D did not interfere with the ability of unlabelled FMLP to displace surface bound [^{3}H]-FMLP (not shown).

It has been shown recently that, when binding to PMN at 37^{o}C, FMLP forms high affinity receptor complexes that are in transient association with the cell cytoskeleton (22). Peptide within these complexes dissociates at a very slow rate. Thus, it was possible that WGA-D interfered with the internalization of [^{3}H]-FMLP but that this was not readily detected because the uptake experiments determined both the presence of tight complexes and internalized peptide. Consequently, experiments were performed in which we examined short-term binding and internalization by PMN of the fluoresceinated FMLP analog, N-FNLPNTL (Fig. 3). It has been shown previously that N-FNLPNTL-Fl interacts with the FMLP receptor (23). Moreover, this method allows for the discrimination between surface bound peptide and peptide that has been internalized via a receptor-mediated process (12).As observed in the uptake experiments, WGA-D had no effect on the short-term binding and internalization of N-FNLPNTL-Fl by PMN (Fig. 1B). Similarly, WGA-D had no effect on the release of radioactivity

from PMN that had been preincubated with [^{3}H]-FMLP and WGA-D for 20 min at 37°C and then washed (Fig. 2). These results indicated that WGA-D had no effect on the spontaneous dissociation of FMLP from either surface receptors or internalized FMLP-receptor complexes. As has been shown previously using rabbit PMN (18), only a portion of the cell-associated radioactivity (approximately 70%) was released into supernatants during the incubation period (Fig. 2). The amount of [^{3}H]-FMLP that remained associated with the cells may represent peptide that is present within a nonreleasable compartment and/or high affinity FMLP-receptor complexes (22).

Since it appeared that WGA-D had no effect on either binding of FMLP to its receptors, internalization of bound FMLP, or release of bound and internalized peptide, we examined the effect of WGA-D on FMLP-receptor reexpression (or recycling).Receptor recycling has been demonstrated to occur in a variety of cells (5,6). For example, using rat hepatocytes it has been shown that binding of low density lipoprotein (LDL) to its membrane receptor results in rapid internalization of the LDL-receptor complex within coated vesicles. Coated vesicles rapidly shed their clathrin coats and fuse with one another to form larger vesicles called endosomes. Within endosomes, receptor and LDL dissociate and the receptor recycles to the cell membrane, where it can bind another LDL particle and initiate a new cycle of endocytosis (5).It has been estimated that each LDL receptor makes one round trip every 12 min. Similar results have been reported for the macrophage Fc receptor and K562 cell transferrin receptor (24, 25).

Results presented in this chapter support the assumption that WGA-D may inhibit reexpression (or recycling) of a population of FMLP receptors that

mediate chemotaxis. To test this hypothesis, PMN were preincubated with 1.0 uM FMLP at 4°C for 10 min, in the presence and absence of WGA-D, and washed before measuring receptor reexpression (Fig. 4). This concentration of FMLP was used because it completely deactivated PMN chemotactic responses to a subsequent challenge with FMLP (Fig. 6). Preincubation with lower concentrations of peptide produced variable results. Preincubations were carried out at 4°C to prevent internalization of bound FMLP, thus allowing examination only of occupied surface receptors. WGA-D inhibited the reexpression of a population of receptors by FMLP-treated PMN (Fig. 4A). Inhibition became apparent after 2 minutes of incubation. Receptor reexpression by PMN that had been preincubated with FMLP alone could not be explained solely by degranulation (19). After 10 min, both sets of cells had released identical amounts of lysozyme and vitamin B_{12}-binding protein into supernatants, but had significant differences in their ability to bind [^{3}H]-FMLP (Fig. 4B) . Degranulation can explain the partial recovery of binding observed in PMN that had been preincubated with FMLP and WGA-D and allowed to recover for 10 min. The differences observed between cells preincubated with FMLP in the presence and absence of WGA-D most likely represent WGA-D-mediated inhibition of FMLP receptor recycling. It would be unlikely that surface bound WGA-D inhibits expression of an intracellular, non-lysosomal receptor pool. Scatchard analysis of [^{3}H]-FMLP binding to PMN that had been preincubated with FMLP in the presence and absence of WGA-D, and allowed to recover for 8 min, demonstrated significant differences in receptor number (Fig. 5). PMN that had been preincubated with FMLP and WGA had approximately 45% fewer high affinity and 10% fewer low affinity receptors than PMN that had been preincubated with FMLP alone. The K_D's of binding were identical for both populations of PMN indicating that the differences observed in

binding were due to a true diminution of receptor number.

Functional studies demonstrated that WGA-D-mediated inhibition of receptor reexpression was associated with inhibition of recovery of chemotactic responsiveness to FMLP (Fig. 6). In contrast, PMN that were incubated with FMLP alone fully recovered their chemotactic responsiveness to FMLP (Fig. 6). It is difficult to establish a direct correlation between kinetics of receptor recovery and chemotaxis because of the inherent differences between the two assays. It is apparent, however, that blocking of FMLP-receptor reexpression by WGA-D results in inhibition of PMN chemotactic responsiveness. Furthermore, inhibition of chemotaxis was specific for FMLP since both sets of cells responded equally well to C5a (not shown). We have shown previously that WGA-D does not inhibit C5a-induced PMN chemotaxis (7).

WGA-D mediated inhibition of FMLP-receptor reexpression had no effect on the ability of PMN to either generate superoxide anion radicals (Fig. 7A) or degranulate (Fig. 7B) when challenged with 10^{-7}M FMLP. These results suggest that, under the conditions used in these studies, WGA-D interfered only with the reexpression of receptors that mediate chemotactic responses to FMLP. Is possible that high affinity receptors mediate PMN chemotactic responsiveness, while low affinity receptors mediate superoxide anion generation (26). Indeed, results of Scatchard plots obtained at plateau of reexpression suggest that this may be the case. Also, it is possible that while high affinity receptors recycle, low affinity receptors do not.

Bound FMLP is internalized rapidly by PMN (12). Using a fluoresceinated FMLP analog, Sklar et al. (27) have demonstrated that PMN internalize bound peptide at a rate of 24% of occupied receptors/min. After 3 min, approximately

60% of receptor-bound ligand is internalized by PMN (13, 27).

FMLP-stimulated PMN rapidly internalized bound ^{125}I-WGA-D (Fig. 8). Internalization was complete after 60 sec of incubation and required the presence of FMLP since it did not occur when PMN were incubated with buffer alone (Fig. 8). Furthermore, internalization of bound ^{125}I-WGA-D was specific for FMLP and did not occur when highly purified C5a was used (Fig. 9). It should be noted that the concentrations of C5a used in these experiments are comparable, functionally, to the concentrations of FMLP used (8). Results of these experiments indicate that WGA-D binds to a site close to, or within, a population of FMLP receptors that are rapidly internalized by PMN. While FMLP receptors continue to be internalized after 60 sec (27), ^{125}I-WGA-D is not. Moreover, the fact that ^{125}I-WGA-D is not internalized by C5a-stimulated PMN is compatible with previous findings that WGA-D-treated PMN respond chemotactically to C5a (7). Interestingly, while C5a does not mediate ^{125}I-WGA-D internalization by PMN, it induces capping of fluoresceinated WGA-D to the same extent as FMLP (28). Leukotriene B_4, however does not induce internalization or capping of WGA-D. There are two possible explanations for these findings. One is that WGA-D binds to a site on the PMN membrane that is close to the FMLP and C5a, but not leukotriene B_4, receptors. Alternatively, WGA-D may bind to the FMLP receptor and C5a induces capping of WGA-D because both receptors are closely associated on the PMN membrane. Internalization of WGA-D by PMN, however, is stimulus specific.

Experiments using subcellular fractionation demonstrated that FMLP and ^{125}I-WGA-D were internalized to the same compartment within PMN (Fig. 10B). As observed in the internalization studies using intact PMN (Fig. 8), it appeared

that only a fraction of bound ^{125}I-WGA-D was internalized into galactosyl transferase-rich fractions by FMLP-stimulated PMN. A significant amount remained associated with alkaline phosphatase-containing fractions, as evidenced by the shoulder of ^{125}I-radioactivity detected within these fractions (Fig. 10B). Also, as observed using intact PMN (Fig. 8), in the absence of [^{3}H]-FMLP, ^{125}I-WGA-D comigrated with the alkaline phosphatase-containing fractions, indicating lack of internalization (Fig. 10A).

Finally, blotting experiments were performed using peak fractions of plasma membrane and specific granules. Only peak fractions were used to increase purity and specificity. It should be noted that PMN were purified after platelet depletion. This step was essential to avoid detection, by WGA-D, of platelet-associated glycoproteins. Interestingly, blots of PMN plasma membrane, when reacted with WGA-D and specific anti WGA-D rabbit antiserum, demonstrated the presence of a single band with a molecular weight of 62,000 (Fig. 11C). This molecular weight is similar to that reported for either affinity-labelled or purified FMLP receptors (14, 29, 30). Binding studies using ^{125}I-WGA-D and intact PMN have demonstrated that, at saturation, WGA-D bound to many more sites on PMN than the estimated number of FMLP receptors (11). It is likely, however, that WGA-D interacts with a variety of glycolipids present on the cell membrane that would not be detected by SDS-PAGE. Alternatively, WGA-D may not react with glycoproteins on the PMN membrane that are denatured by treatment with SDS and mercaptoethanol. This possibility seems unlikely because N-acetyl-D-glucosamine residues should still be available for binding by WGA-D. WGA has been shown to bind to purified, affinity-labelled PMN FMLP receptors (30). Thus, it is possible that WGA-D also binds to the FMLP receptor.

Summary

In this chapter, the mechanism of action of a derivative of wheat germ agglutinin (WGA-D) that specifically and irreversibly inhibits N-formyl-methionyl-leucyl-phenylalanine or (FMLP)-induced polymorphonuclear leukocyte (PMN) chemotaxis is examined. At a concentration that completely inhibited PMN chemotaxis, WGA-D had no effect on either the uptake or release of [^{3}H]-FMLP by PMN. Similarly, WGA-D did not affect either the short-term binding to, or internalization did not affect either the short-term binding to, or internalization by, PMN of a fluoresceinated FMLP analog. WGA-D did interfere, however, with the reexpression (or recycling) of FMLP receptors by PMN that had been preincubated with 1.0 uM FMLP for 10 min at 4^{o}C. This effect was specific for WGA-D, since it was not observed when concanavalin A was used. Scatchard plot analysis of FMLP binding to PMN after receptor reexpression demonstrated that WGA-D- treated PMN had a significant diminution in the number of high affinity receptors. WGA-D-mediated inhibition of FMLP receptor reexpression was associated with inhibition of FMLP-induced PMN chemotaxis, but had no effect on either FMLP-induced PMN superoxide anion generation or degranulation. Studies using [^{125}I]-WGA-D demonstrated that PMN did not internalize WGA-D spontaneously. PMN did internalize [^{125}I]-WGA-D, however, when stimulated with FMLP.Internalization of WGA-D by FMLP-stimulated PMN was rapid, dependent on the concentration of FMLP, and specific. Internalization of [^{125}I]-WGA-D by PMN did not occur when highly purified human C5a, instead of FMLP, was used as a stimulus. Subcellular fractionation studies demonstrated that [^{125}I]-WGA-D and [^{3}H]-FMLP were cointernalized by PMN, and segregated to a compartment comigrating with Golgi markers. Western blot analysis, using PMN plasma membranes, demonstrated that WGA-

D bound to a single membrane glycoprotein that migrated with an apparent molecular weight of 62,000 daltons. The data indicate that WGA-D, perhaps by binding to the FMLP receptor, inhibits FMLP-induced PMN chemotaxis by blocking the reexpression (or recycling) of a population of receptors required for continuous migration.

TABLE I.

Binding of [^{3}H]-FMLP to PMN that had been Preincubated with Either Unlabelled FMLP alone or Unlabelled FMLP plus WGA-D and Allowed to Recover

	PMN preincubated with Unlabelled FMLP alone	PMN preincubated with Unlabelled FMLP + WGA-D
K_D		
High affinity	2.1 +/- 0.2 nM	1.7 +/- 0.3 nM
Low affinity	22.2 +/- 0.5 nM	23.0 +/- 0.6 nM
Sites		
High affinity	15,000 +/- 1,250	8,490 +/- 875
Low affinity	66,000 +/- 2,275	59,510 +/- 3,420

Results represent mean values (+/- SE) obtained in 3 experiments.

P vs unlabelled FMLP < 0.01 (Student's t test).

P vs unlabelled FMLP < 0.2 (Student's t test).

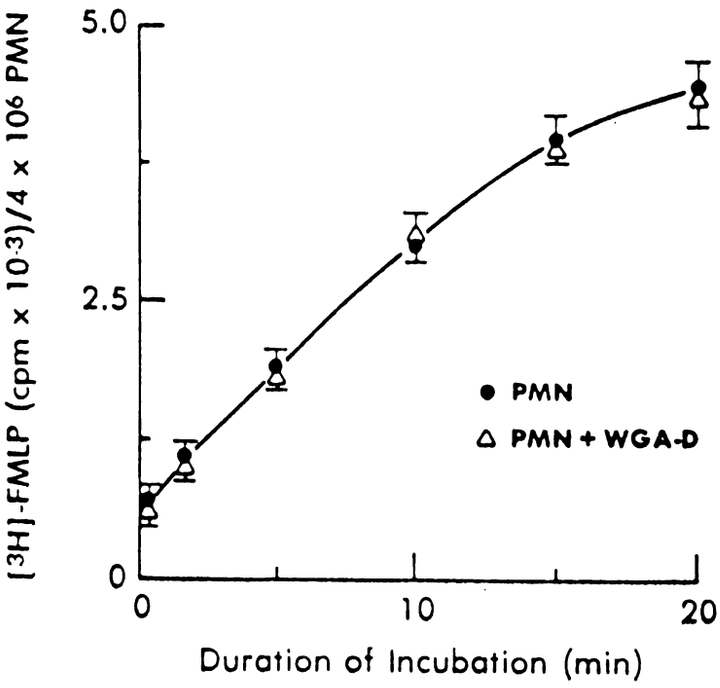

Figure 1

Effect of WGA-D on the specific uptake of [^{3}H]-FMLP by PMN. Cells, (4 x 10^6 PMN) in 0.4 ml PBS, were incubated with 1.0 nM [^{3}H]-FMLP in the presence (triangles) and absence (dots) of WGA-D (3.0 ug) for varying durations. Following incubation, cells were centrifuged through silicone oil and pellets processed as described in Materials and Methods. Results represent mean values (+/- SE) obtained in 4 experiments.

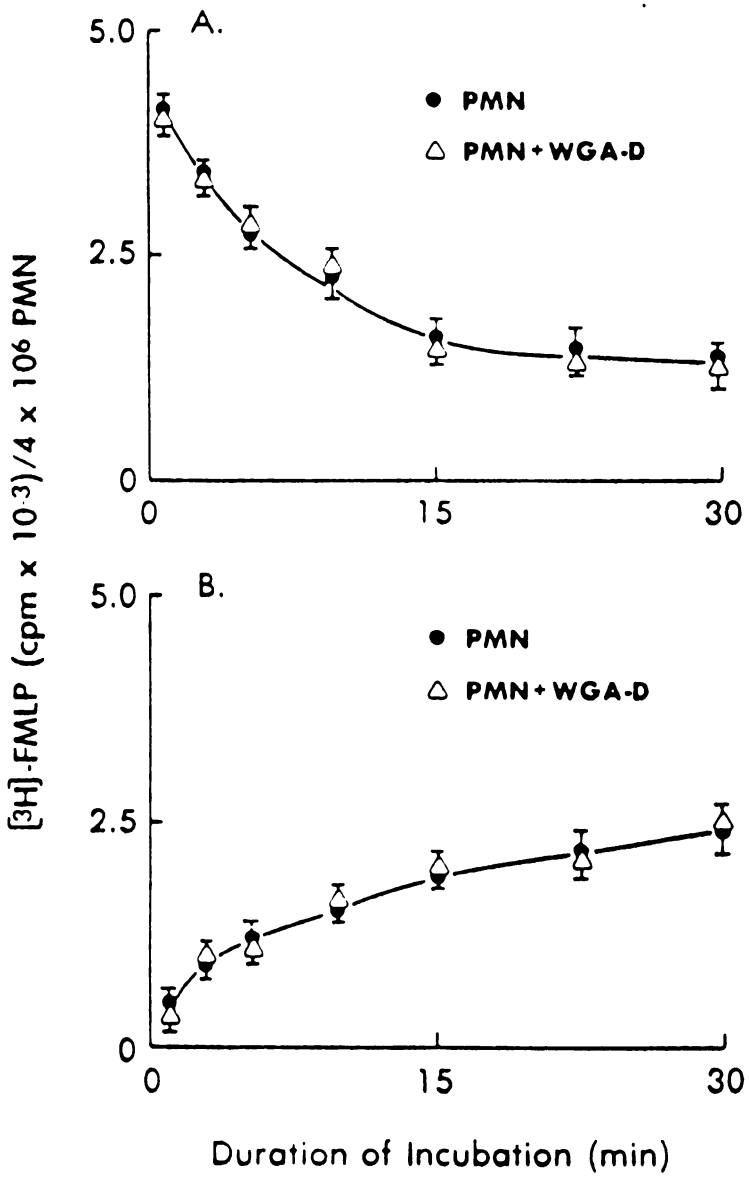

Figure 2

Effect of WGA-D on the ability of PMN to release [^{3}H]-FMLP that had been taken up by the cells. PMN, (4 x 10^6 cells) in 0.4 ml PBS were incubated with 1.0 nM [^{3}H]-FMLP in the presence (triangles) and absence (dots) of WGA-D (3.0 ug) at 37°C for 20 min. Reactions were stopped by adding excess cold PBS. PMN were washed twice and resuspended in 0.4 ml of Hanks' buffer at 37°C for varying durations. Incubations were terminated by centrifugation through oil and pellets processed as described in Materials and Methods. (A).Cell-associated radioactivity; (B). Radioactivity released into supernatants. Results represent mean values (+/- SE) obtained in 3 experiments.

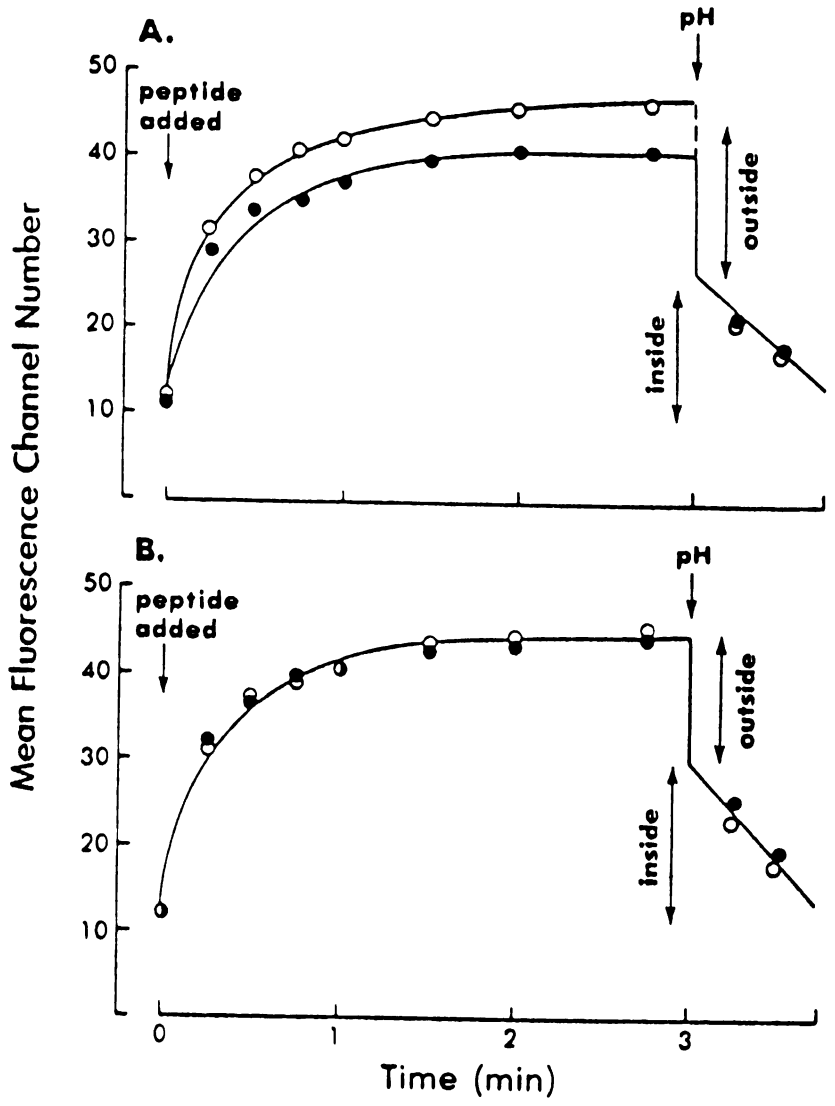

Figure 3

Effect of WGA-D on short-term binding and internalization of N-FNLPNTL-Fl by PMN. Cells (2×10^6 ml) were allowed to bind N-FNLPNTL-Fl (3.0 nM), in the presence and absence of WGA-D (3.0 ug), for 3 min at 37°C. After this time, the external pH was dropped to 4.0 and the amount of internalized peptide determined. A, Normal PMN. B, WGA-D-treated PMN. Duplicate tracings are presented for each condition. Shown is one of two experiments that yielded essentially identical results.

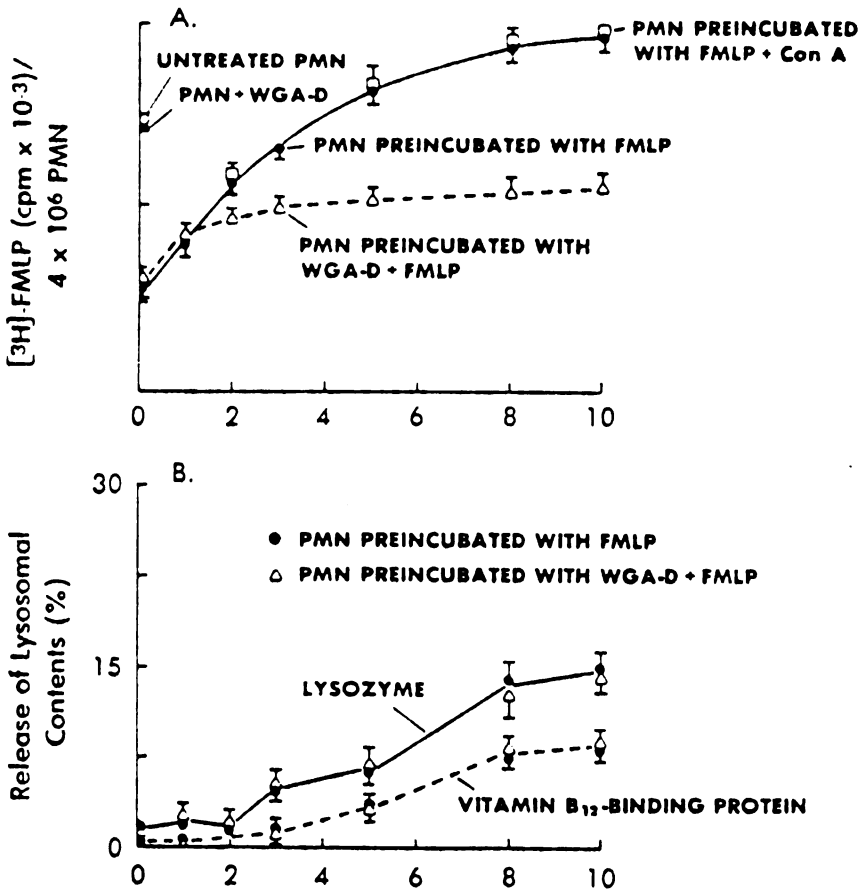

Figure 4

Effect of WGA-D on FMLP-receptor reexpression by PMN.Cells, (4×10^6 PMN) in 0.4 ml PBS were preincubated with the reagents at 4°C for 10 min, washed once, and resuspended in 0.25 ml of Hanks' buffer at 37°C for varying durations. At each time point, reactions were stopped by adding equal volumes of cold PBS containing 20 nM [^{3}H]-FMLP, and processed as described in *Materials and Methods*. A, Kinetics of reexpression of FMLP receptors by PMN that had been preincubated with either 1.0 uM FMLP alone (closed circles), 1.0 uM FMLP plus WGA-D (3.0 ug) (open triangles), or 1.0 uM FMLP plus Con A (4.0 ug) (open squares). Binding of [^{3}H]-FMLP by untreated PMN (open circles), binding of [^{3}H]-FMLP by PMN preincubated with 3.0 ug WGA-D alone (closed triangles). (B). Spontaneous release of lysosomal contents by PMN during reexpression. Lysozyme, solid line; vitamin B_{12}-binding protein, dashed line. PMN preincubated with 1.0 um FMLP alone (closed circles), PMN preincubated with 1.0 uM FMLP plus WGA-D (3.0 ug) (open triangles). Results represent mean values (+/- SE) obtained in 5 experiments.

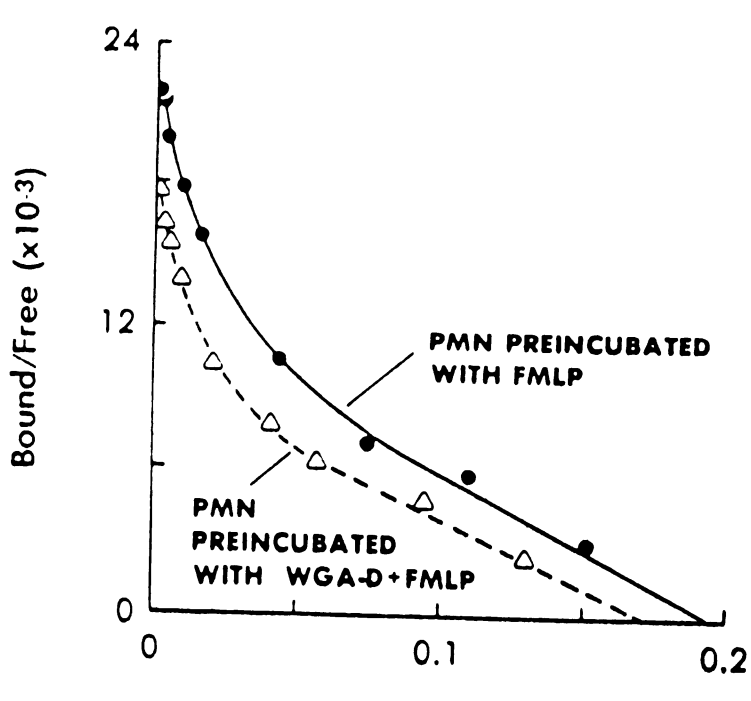

Figure 5

Scatchard plot analysis of specific binding of [^{3}H]-FMLP to PMN after 8 min reexpression. PMN were treated as described in Materials and Methods and in legend to Figure 4. After 8 min reexpression, reactions were stopped by adding equal volumes of cold PBS, and tubes placed on ice for 10 min. Binding was measured at equilibrium and data subjected to computer analysis using the LIGAND program (12, 15). PMN preincubated with 1.0 uM FMLP alone (closed circles), PMN preincubated with 1.0 uM FMLP plus WGA-D (3.0 ug) (open triangles). Results represent mean values obtained in 3 experiments performed in duplicate.

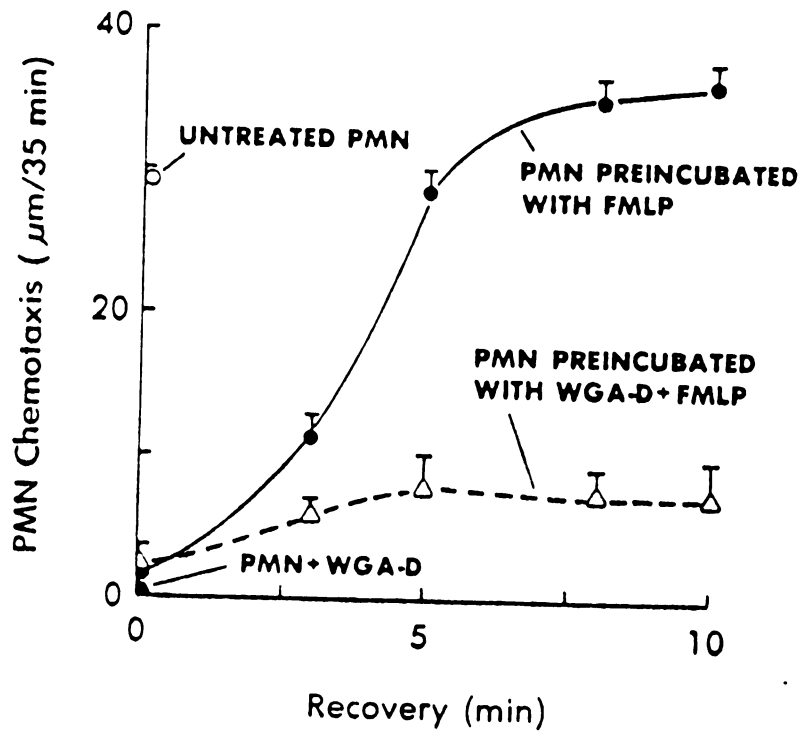

Figure 6

Effect of WGA-D on FMLP-induced PMN chemotaxis during receptor reexpression. Cells, (4 x 10^6 PMN) in 0.4 ml PBS were preincubated with 1.0 uM FMLP in the presence (open triangles) and absence (closed circles) of WGA-D (3.0 ug) at 4°C for 10 min. After preincubation, PMN were washed and resuspended in 0.25 ml of Hanks' buffer at 37°C. At varying durations during receptor reexpression, reactions were stopped by adding equal volumes of cold PBS. PMN were washed once and used in the chemotaxis assays. FMLP was used as a chemoattractant at a final concentration of 10^{-8}M. Results represent mean values (+/-SE), obtained in 3 experiments performed in duplicate.

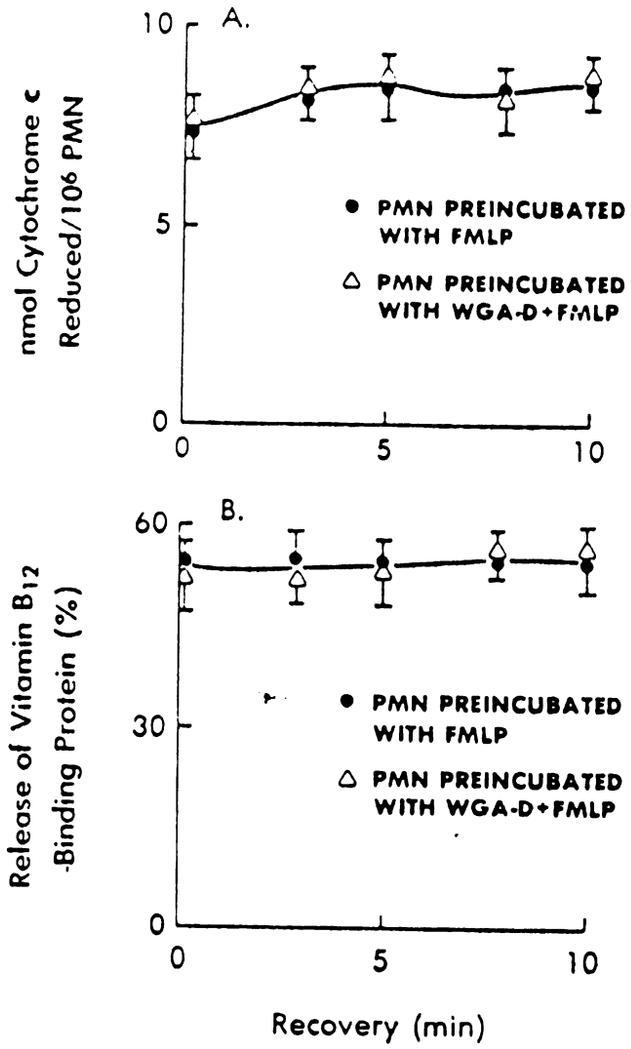

Figure 7

Effect of WGA-D on FMLP-induced superoxide anion generation and degranulation by PMN during receptor reexpression. Cells were treated as described in legend to Figure 6. PMN were preincubated with cytochalasin B (5.0 ug/ml) for 60 sec prior to exposure to 10^{-7}M FMLP for an additional 60 sec. (A). Superoxide anion generation; (B). Degranulation. PMN preincubated with 1.0 uM FMLP alone (closed circles); PMN preincubated with 1.0 uM FMLP plus WGA-D (3.0 ug) (open triangles). Results represent mean values (+/- SE) obtained in 4 experiments

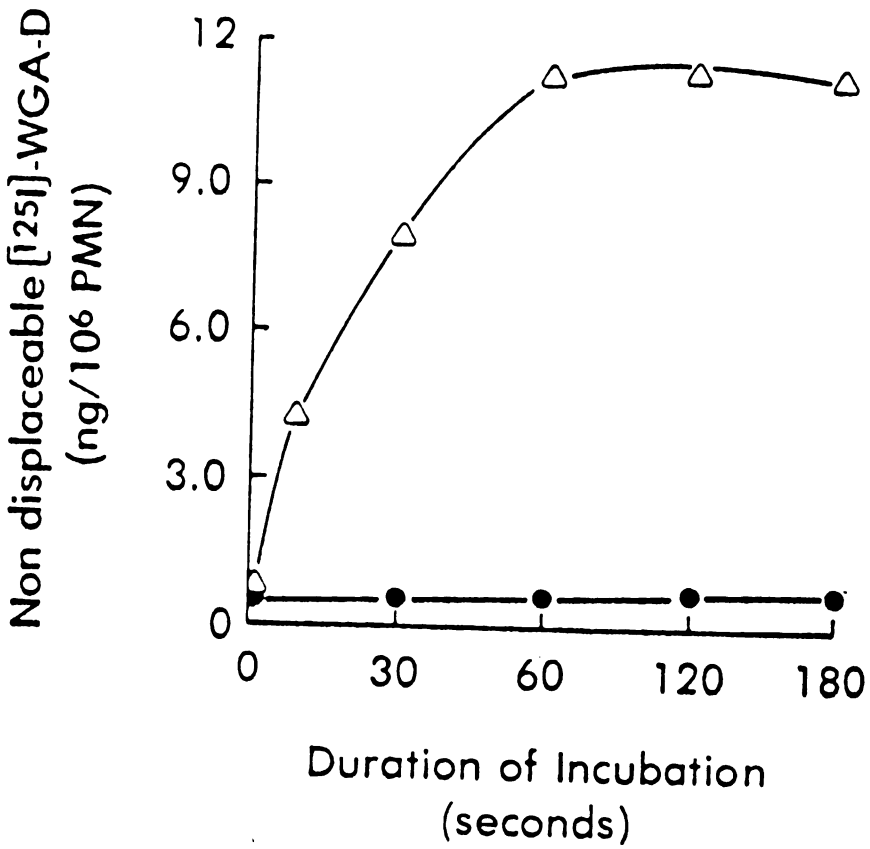

Figure 8

Kinetics of internalization of bound [^{125}I]-WGA-D by PMN. Cells, (1 x 10^6 PMN/ml), were incubated with 3.0 ug [^{125}I]-WGA-D at 4°C for 5 min, washed twice and resuspended in 0.5 ml of Hanks' buffer. At time zero, FMLP was added at a final concentration of 10^{-7}M. After varying durations (10-180 sec), reactions were stopped by adding equal volumes of 4°C PBS containing 0.1 M N-acetyl-D-glucosamine, followed by centrifugation through silicone oil. PMN plus [^{125}I]-WGA-D alone (closed circles); PMN plus [^{125}I]-WGA-D and FMLP (open triangles). Results represent the average of two experiments performed in duplicate.

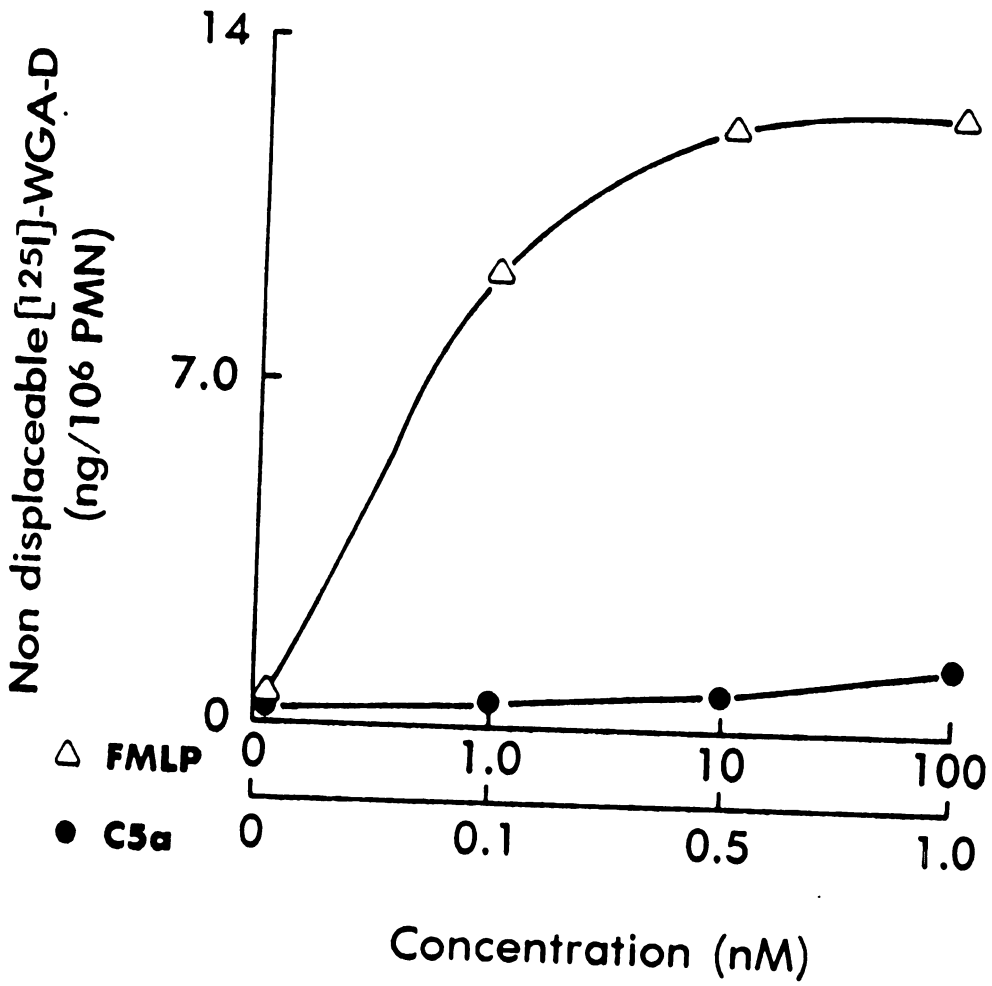

Figure 9

Internalization of bound [^{125}I]-WGA-D by PMN that had been stimulated for 60 sec with varying concentrations of either FMLP (open triangles); or C5a (closed circles). Cells were treated as described in legend to Figure 8. Results are the average of two experiments performed in duplicate

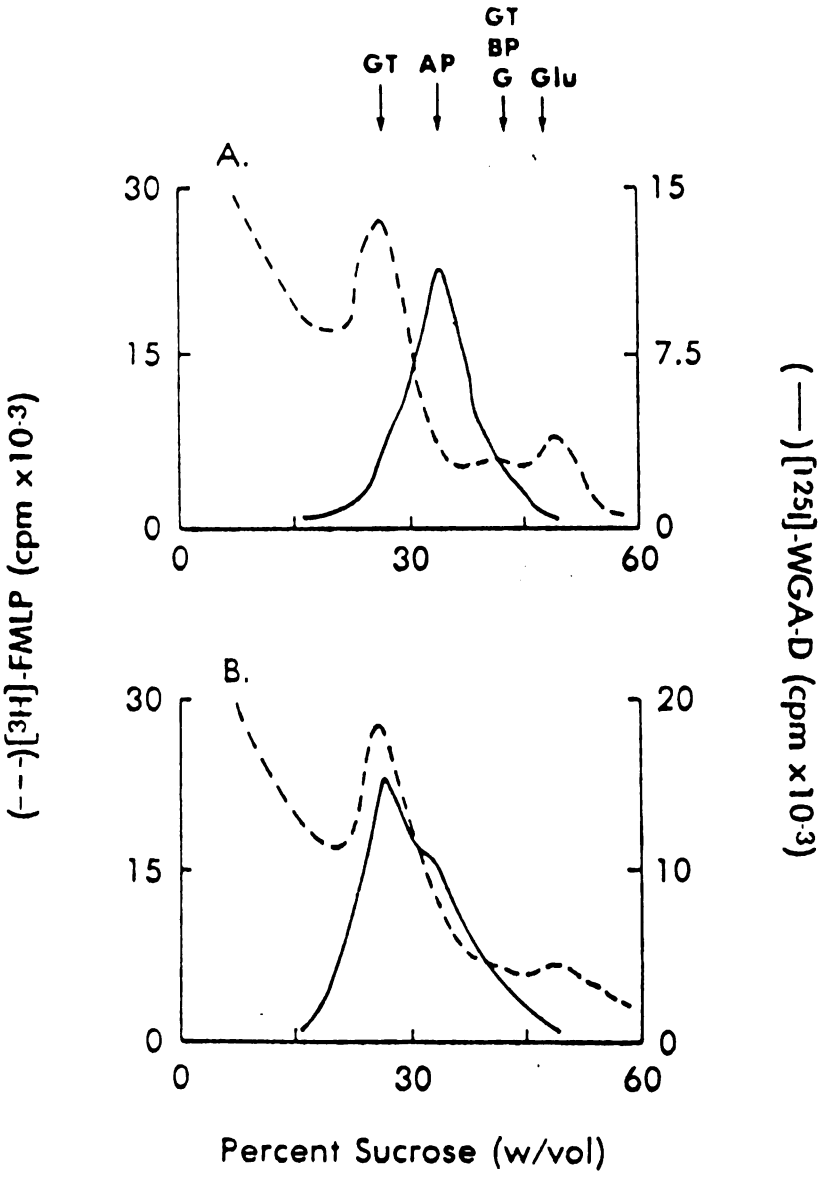

Figure 10

Subcellular distribution of [^{3}H]-FMLP and [^{125}I]-WGA-D within PMN. Cells (7 x 10^{8} PMN) were suspended in 14 ml of Hanks' buffer containing the appropriate reagents and incubated at 37°C for 5 min with continuous agitation. After incubation, reactions were stopped by adding equal volumes of cold PBS. PMN were washed twice with cold PBS, resuspended in 14 ml of cavitation buffer, and subjected to N_2 cavitation, followed by fractionation on sucrose density gradients as described in Materials and Methods. (A). Dashed line, PMN incubated with 50 nM [^{3}H]-FMLP alone; solid line, PMN incubated with 0.7 mg of [^{125}I]-WGA-D alone. (B). PMN incubated with both, 50 nM [^{3}H]-FMLP (dashed line) and 0.7 mg [^{125}I]-WGA-D (solid line). Subcellular markers: GT, galactosyl transferase; AP, alkaline phosphatase; BP, vitamin B_{12}-binding protein; G, gelatinase; Glu, beta-glucuronidase.

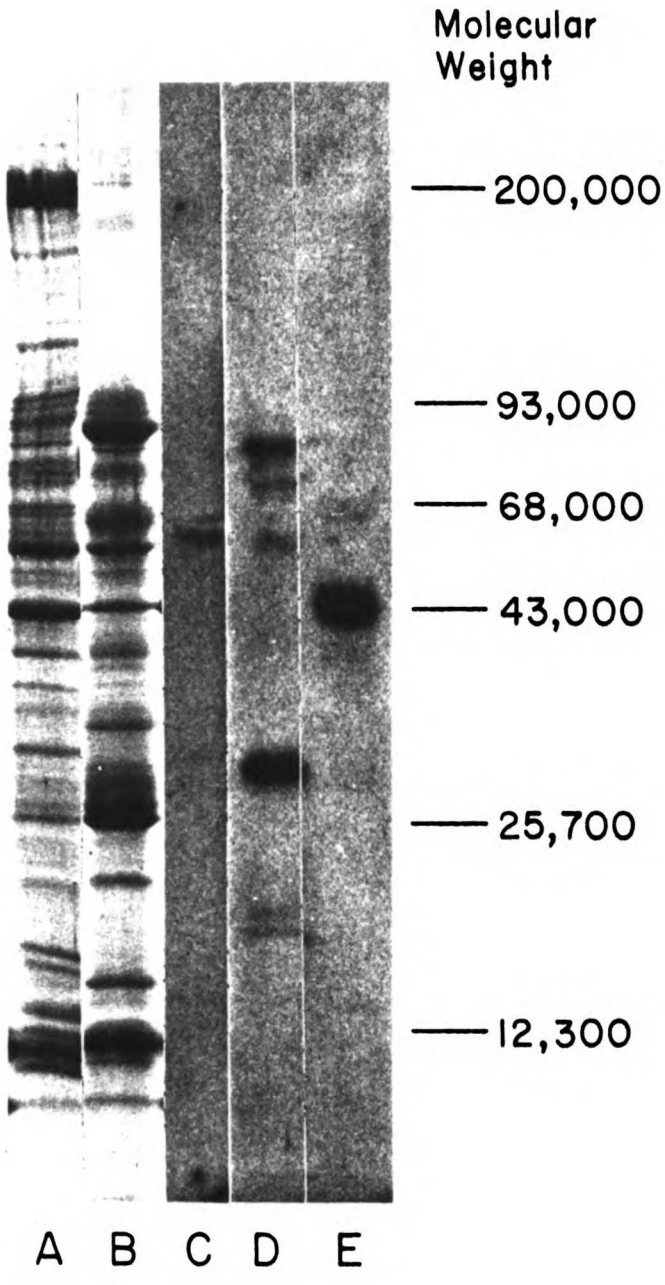

Figure 11

SDS-PAGE (7-15% gradient gels) and Western blots of PMN plasma membrane and specific granule membrane proteins under reducing conditions. Approximately 30 ug protein was applied to each lane. A. SDS-PAGE of plasma membrane proteins stained with Coomassie blue. B. SDS-PAGE of specific granule membrane proteins stained with Coomassie blue. C. Western blot of plasma membrane proteins reacted with WGA-D, anti-WGA-D, and ^{125}I-protein A. D. Western blot of specific granule membrane proteins reacted with WGA-D, anti-WGA-D, and ^{125}I-protein A. E. Molecular weight standards reacted with WGA-D, anti-WGA-D, and ^{125}I-protein A.

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CHAPTER IV

REMOVAL OF HUMAN POLYMORPHONUCLEAR LEUKOCYTE SURFACE SIALIC ACID INHIBITS REEXPRESSION (OR RECYCLING) OF FORMYL PEPTIDE RECEPTORS. A POSSIBLE EXPLANATION FOR ITS EFFECT ON FORMYL PEPTIDE-INDUCED POLYMORPHONUCLEAR LEUKOCYTE CHEMOTAXIS.

Introduction

Cell surface carbohydrates are known to play a role in receptor processing and function by mammalian cells (1, 2). In previous studies, it was reported that enzymatic (i.e., neuraminidase) removal or oxidation of surface sialic acid from PMN, inhibited their ability to respond chemotactically to the synthetic peptide FMLP (3). Removal of PMN surface sialic acid had no effect on the ability of FMLP to induce these cells to selectively release lysosomal constituents (i.e., degranulation) or to generate superoxide anion radicals (3). Furthermore, removal of PMN surface sialic acid had no effect on either the kinetics, reversibility or specificity of [^{3}H]-FMLP binding to its cell surface receptor (3). Inhibition of PMN chemotactic responsiveness to FMLP was specific and not due to a change in cell surface charge, since removal of PMN surface sialic acid had no effect on the chemotactic responsiveness of these cells to either the complement-derived peptide C5a or the arachidonic acid metabolite leukotriene B_4 (3). The mechanism(s) whereby removal of PMN surface sialic acid inhibited FMLP-induced PMN chemotaxis was not elucidated.

In the previous chapter, it was reported that FMLP-induced PMN chemotaxis appears to require reexpression (or recycling) of a population of FMLP

receptors (4). Upon binding to its specific receptor on the PMN membrane, [^{3}H]-FMLP is rapidly internalized by these cells and segregates to a compartment containing Golgi markers (4, 5). Over time, [^{3}H]-FMLP-associated radioactivity accumulates in a cytosolic compartment (5) and free receptors reappear on the PMN membrane (4). Inhibition of FMLP receptor reexpression is not associated with inhibition of other FMLP-induced PMN functions (i.e., polarization, superoxide anion generation and degranulation) (4, 6).

In this chapter, the possibility that removal of PMN surface sialic acid results in abnormal processing of the FMLP-receptor by these cells is examined. Using [^{3}H]-FMLP as a receptor marker the ability of normal and neuraminidase-treated (NT)-PMN to internalize, process and reexpress FMLP receptors was studied. NT-PMN exhibit a defect in the intracellular processing of the FMLP receptor resulting in accumulation of FMLP-receptor complexes within the cell, a process that results in abnormal FMLP receptor re-expression (or recycling) and is associated with inhibition of FMLP-induced PMN chemotaxis.

Materials and Methods

Preparation of Leukocyte Suspensions and Assay of PMN Chemotaxis.

Platelet-poor leukocyte suspensions containing 97-99% PMN were prepared as described in chapter 2. For chemotaxis experiments, HBSS was supplemented with 20 mg/ml bovine serum albumin (BSA) (Grand Island Biological Co., Grand Island, NY)(6). PMN were more than 95% viable, as assessed by trypan blue exclusion.

PMN stimulated random migration and directed migration (chemotaxis) was assayed as described in chapter III.

Neuraminidase Treatment of PMN.

Neuraminidase from *Clostridium perfringens* (chromatographically purified, Type IV) was from Sigma Chemical Co., St Louis, MO. One unit is defined as the amount of enzyme that liberates 1.0 umol. of sialic acid per minute at pH 5.5 and 37°C. Enzyme activity was determined using bovine submaxillary mucin as substrate, and liberated sialic acid determined by the thiobarbituric acid procedure (3, 9). This preparation of neuraminidase showed no detectable contamination with beta-galactosidase, beta-glucosaminidase or alpha-mannosidase. Also, it was devoid of protease activity under the conditions used for PMN digestion (3).

PMN (2×10^6/ml) were incubated with neuraminidase (20 milliunits/ml) in PBS, pH 7.0, containing 0.5% BSA and 10 ug/ml each of superoxide dismutase and catalase (Sigma), for 5 min at 37°C. Under the conditions used in these studies the enzymatic activity of the neuraminidase preparation was 30% of that observed under optimal conditions (3). This treatment resulted in the release of approximately 6 nmol sialic acid/10^8 PMN, and represented 30% of total PMN sialic acid (3). After incubation, cells were washed twice at 4°C, and resuspended in Hanks' buffer. It should be noted that in all experimental conditions normal PMN were subjected to the same manipulations as NT-PMN, except for the absence of neuraminidase.

Assays using [^{3}H]-FMLP.

Uptake of [^{3}H]-FMLP by normal and NT-PMN was measured as reported previously (4) using 4×10^6 PMN (in 0.4 ml HBSS) and 20 nM [^{3}H]-FMLP (specific activity 48.5 Ci/mmol) (New England Nuclear Corp., Boston, MA) per condition. At each time point reactions were stopped by the addition of equal volume of 4°C buffer containing 20 uM unlabeled FMLP. A collection method

involving centrifugation through oil was used to separate free from cell-associated peptide (3, 4). After centrifugation for 30 sec (15,600 x g), the tips of the tubes were excised and processed as described (3). We also measured release of [^{3}H]-FMLP-associated radioactivity that had been taken up by normal and NT-PMN (4). Cells (4 x 10^6 PMN in 0.4 ml HBSS) were preincubated with 20 nM [^{3}H]-FMLP for 16 min at 37^{o}C. After preincubation, the reaction was stopped by adding equal volumes of 4^{o}C PBS containing 20 uM unlabeled FMLP, and PMN washed three times at 4^{o}C. Cells were resuspended to original volume in 37^{o}C HBSS and incubated at 37^{o}C for varying times. Following incubation, PMN were spun through oil and radioactivity present in supernatants and pellets determined (4).

In some experiments, the ability of normal and NT-PMN to recover their capacity to bind [^{3}H]-FMLP, using a minor modification of the method described previously (4), was investigated. Briefly, PMN (5 x 10^6 cells/ml) suspended in HBSS were incubated at 37^{o}C for 5 min with 50 nM unlabeled FMLP (Peninsula Laboratories, San Carlos, CA). After preincubation cells were washed once at 4^{o}C, resuspended in prewarmed HBSS (16 x 10^6 PMN/ml) and aliquots (0.25 ml) incubated at 37^{o}C for varying durations. Reactions were stopped by the addition of equal volumes of 4^{o}C HBSS containing 20 nM [^{3}H]-FMLP. PMN were incubated with [^{3}H]-FMLP for 10 min on ice to achieve equilibrium binding (3, 4), pelleted through oil and pellets processed as described (3). Non-specific binding, that is, uptake by PMN of radioactivity that was not inhibited by 1000-fold excess unlabeled FMLP, represented 8-10% of total binding.

Subcellular distribution of [^{3}H]-FMLP within normal and NT-PMN was

assessed as described previously (see chapter 2 and 3). PMN (6 x 10^8 cells) were suspended in HBSS (15 ml) containing 50 nM [^{3}H]-FMLP and 10 ug/ml each of superoxide dismutase and catalase, and incubated at 37^{o}C for either 5 or 15 min. After incubation, PMN were washed 3 times with 4^{o}C PBS pH 7.4, resuspended in 14 ml of 4^{o}C PBS, pH 7.4, containing 0.34 M sucrose, 1.0 mM ethylendiaminen tetraacetic acid (EDTA), 10 mM Hepes and 1.0 mM diisopropylfluorophosphate (DFP) (cavitation buffer). Suspended PMN were subjected to nitrogen cavitation at 4^{o}C, and supernatants containing 75-85% of all markers were fractionated on linear (20-55%) sucrose density gradients, as described (4, 5). Recovery of markers was between 85-95% of the amount present in starting supernatants (4). Lactic dehydrogenase (LDH), beta-glucuronidase (Glu), vitamin B_{12}-binding protein (BP), alkaline phosphate (AP), UDP-galactose galactosyl transferase (GT), protein content and [^{3}H]-FMLP-associated radioactivity were determined as described previously (4). In some instances, aliquots of peak light golgi-containing fractions, obtained from sucrose (27-28%) density gradients, were fractionated further using iso-osmolar stractan gradients (10). Stractan (arabinogalactan, Sigma) was prepared as described (11). Tubes containing discontinuous stractan gradients (1.0 ml each, 18 to 36%) were centrifuged at 48,000 x g for 30 min at 4^{o}C, using a Beckman SW-40 rotor (Beckman Instruments, Palo Alto, CA). Fractions (0.5 ml) were collected by puncturing the bottom of each tube, using a Beckman fractionator.

Iodination of Formyl Peptide.

N-formyl-norleucyl-leucyl-phenylalanyl-tyrosine (FP) (Sigma, St. Louis) was radioiodinated using carrier-free Na^{125}I (Amersham, Arlington Heights, IL), by the chloramine-T method, as described previously (12). The specific activity of ^{125}I-FP was approximately 500 Ci/mmol.

Affinity Labeling of the Formyl Peptide Receptor.

Affinity labeling of PMN formyl peptide receptor was performed with minor modifications of previously described methods (13, 14). Purified PMN plasma membranes (approximately 0.3 mg, obtained by nitrogen cavitation) were suspended in 1.0 ml of PBS, pH 6.7, containing 10 nM ^{125}I-FP, and incubated at 4^{o}C for 20 min. Controls contained 1000-fold excess unlabeled FP. Ethylene glycolbis succinimidylsuccinate (EGS) (Pierce Chemical Co., Rockford, IL) (0.3 mg/ml) was subsequently added, and mixtures incubated for an additional 40 min at 4^{o}C. Reactions were stopped by the addition of equal volumes of 2.0 mM glycine in PBS. Membrane suspensions were layered on 10% (w/v) sucrose and subjected to centrifugation (100,000 x g, 90 min). Membrane pellets were resuspended in buffer (0.1 ml) containing 15 mM Tris, 3.0 mM glycine (pH 8.0), 1.0 mM beta-mercaptoethanol, 1.0% sodium dodecyl sulfate (SDS, Sigma) and 2.0 mM DFP, for 10 min at 4^{o}C. After centrifugation at 15,600 x g for 2 min, supernatants were removed, boiled for 20 sec, and used for electrophoretic analysis

Polyacrylamide Gel Electrophoresis.

Solubilized supernatants containing affinity-labeled formyl peptide receptor were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, using 7-17% gradient slab gels (4, 15). After SDS-PAGE, gels were dried and exposed (48 hr at -70^{o}C) to Kodak XR-1 film, using a Cronex intensifying screen (DuPont, Wilmington,DE).

Results

Previously, studies demonstrated that NT-PMN specifically bind [^{3}H]-FMLP

(4°C), equilibrium conditions) as well as normal PMN (3). These experiments represented true binding, since internalization does not occur at 4°C. Consequently, we examined the ability of normal and NT-PMN to take up [^{3}H]-FMLP when incubated with peptide at 37°C for varying durations. At each time point, reactions were stopped by the addition of equal volumes 4°C PBS containing 1000-fold excess unlabeled FMLP. Under these conditions, uptake represents endocytosis of receptor-peptide complexes by, PMN. Uptake of [^{3}H]-FMLP by normal PMN was linear between 4-16 min of incubation (Fig. 1), reaching saturation at approximately 18 min (Fig. 1). Normal cells exhibited a rapid uptake within the first 3 min of incubation (rate 1.9 x 10^3 molecules/min/PMN), followed by a linear uptake (4-16 min) at a rate of 1.4 x 10^3 molecules/min/PMN. Saturation occurred at approximately 18-20 min, at a level of 35.5 2.8 x 10^3 molecules/PMN (mean+/-SE, n=3). Longer incubation times did not result in further increments in the uptake of [^{3}H]-FMLP by PMN.

Uptake of [^{3}H]-FMLP by NT-PMN was similar to that observed using normal PMN (Fig. 1). After 20 min, NT-PMN exhibited an uptake of [^{3}H]-FMLP equivalent to 34.7 +/- 1.8 x 10^3 molecules/PMN (mean+/-SE, n=3). Thus, it appeared that removal of surface sialic acid had no effect on the ability of PMN to bind and take up [^{3}H]-FMLP.

Consequently, experiments were performed to determine the ability of normal and NT-PMN to release [^{3}H]-FMLP that had been taken up by the cells. Normal and NT-PMN were suspended in Hanks' buffer and incubated with 20 nM [^{3}H]-FMLP for 16 min at 37°C and washed three times with cold buffer. After washing, PMN were suspended to original volume and incubated in Hanks' buffer at 37°C for varying durations, as described in *Materials and Methods.* At

each time point PMN suspensions were pelleted through oil and radioactivity in pellets (Fig. 2) and supernatants determined. Less and supernatants determined. Less than 2.0% of cell associated radioactivity could be displaced from PMN, after washings, by a new addition of 20 uM unlabeled FMLP indicating that radioactivity corresponded to either [^{3}H]-FMLP bound in a non-dissociable fashion (16) or, most likely, internalized peptide (17). Normal PMN released only a portion (approximately 60%) of cell-associated radioactivity into supernatants after 20 min of incubation (Fig. 2) (4). There was a linear loss (equivalent to 0.48 x 10^3 molecules/PMN/min) during the first 18 min of incubation. After 18 min, PMN released small amounts of radioactivity (rate of 0.04 x 10^3 molecules/PMN/min) (Fig. 2). NT-PMN released 42.6% of cell associated radioactivity after 20 min of incubation (Fig. 2). Two rates of release were observed. A initial (first 6 min) slow rate of loss (0.22 x 10^3 molecules/PMN/min) followed by a more rapid loss (6-18 min) at a rate of 0.36 x 10^3 molecules/PMN/min. These rates (first 6 min and 6-18 min) represented 45% and 75% of the linear rate observed using normal PMN.

Subcellular Distribution of [^{3}H]-FMLP within Normal and NT-PMN.

The experiments described above suggested that NT-PMN had a defect in the intracellular processing of [^{3}H]-FMLP-associated radioactivity (some of which may represent peptide-receptor complexes) (5, 16). Consequently, we performed experiments to examine the subcellular distribution of [^{3}H]-FMLP-associated radioactivity within normal and NT-PMN. PMN (6 x 10^8 cells) were suspended in 5 ml of PBS, pH 7.0, in the presence or absence of neuraminidase (20 milliunits/2 x 10^6 PMN) and preincubated for 5 min at 37^{o}C. After preincubation, 10 ml of 37^{o}C HBSS containing 50 nM [^{3}H]-FMLP were added and cell suspensions incubated for an additional 5 or 15 min at 37^{o}C.

Reactions were stopped by the addition of equal volumes 4°C HBSS, chilled on ice, and washed 3 times with cold buffer. PMN were resuspended in 14 ml of 4°C cavitation buffer, disrupted by N_2 cavitation and fractionated on linear sucrose density gradients, as described in *Materials and Methods.* As reported previously (4, 5), normal PMN when incubated with 50 nM [^{3}H]-FMLP for 5 min at 37°C internalized a fraction of the peptide to a compartment with a density lower than the plasma membrane marker alkaline phosphatase (AP) and comigrating with fractions containing the Golgi marker, galactosyl-transferase (GT) (Fig. 3,A). A significant amount of radioactivity comigrated with the cytoplasmic marker lactate dehydrogenase (LDH) (4, 5) and probably represents either free peptide (5) and/or degradation products (18). It should be noted that internalization of [^{3}H]-FMLP did not occur at 4°C and could be prevented completely by the presence of 100-fold excess unlabeled FMLP (not shown) (4, 5).

After 5 min of incubation with [^{3}H]-FMLP, NT-PMN retained approximately 50% more radioactivity within light golgi-containing fractions than normal PMN, with concomitant diminution of radioactivity within cytosol (Fig. 3,A and Table I). After 15 min of incubation with [^{3}H]-FMLP, normal PMN continued to accumulate radioactivity within the light Golgi compartment (5), but counts had segregated also to the lysosomal (BP) and cytoplasmic (LDH) compartments (Fig. 3,B and Table I). In contrast, NT-PMN continued to accumulate more [^{3}H]-FMLP-associated radioactivity within the light Golgi compartment with concomitant diminution of counts within the lysosomal and cytoplasmic compartments (Fig. 3,B and Table I). After 15 min of incubation, NT-PMN accumulated 2.1-fold more sedimentable (100,000 x g for 180 min) radioactivity within light golgi fractions than normal cells with approximately 80% of the counts remaining

sedimentable (100,000 x g for 180 min) after sonication of the samples at 4°C for 2 min (5).

To characterize further the light golgi-associated radioactivity, aliquots of peak light golgi-containing fractions (obtained from normal and NT-PMN that had been incubated with [^{3}H]-FMLP for 15 min at 37°C; Fig 3,B) were applied to discontinuous stractan gradients (11). When light Golgi-containing fractions from normal PMN were fractionated on stractan, most of the radioactivity comigrated with the Golgi marker galactosyl transferase (GT) (Fig 4). A small peak of radioactivity was detected migrating at a higher density probably representing sequestration of [^{3}H]-FMLP within sealed vesicles (perhaps trapped within endocytic vesicles), since radioactivity became non-sedimentable (100,000 x g for 180 min) after sonication. When identical experiments were performed using light golgi-containing fractions from NT-PMN, there was an increase in the amount of radioactivity comigrating with the golgi marker (Fig. 4) but also there were more counts migrating in the high density fractions (Fig. 4). Thus, it appears that NT-PMN retained more radioactivity than normal PMN both in the light golgi-containing fractions and in the high density fractions.

Retention of radioactivity by NT-PMN in sedimentable, light Golgi-containing fractions most likely represents [^{3}H]-FMLP-receptor complexes from which the peptide did not dissociate in a normal fashion. This process will lead to retention of receptors intracellularly, a phenomenon that could manifest itself by diminished reexpression of FMLP receptors on the PMN membrane (i.e., diminished recycling). Consequently, we examined the ability of normal and NT-PMN to reexpress FMLP receptors.

Receptor Reexpression and Recovery of Chemotactic Responsiveness by Normal and NT-PMN.

Preincubation of either normal or NT-PMN (4 x 10^6 cells/ml) with unlabeled FMLP (50 nM) for 5 min at 37°C resulted in a 70% diminution in their subsequent ability to bind [3H]-FMLP (Fig. 5), a phenomenon known as down-regulation (4, 19). Whereas untreated (i.e., not preincubated with unlabeled FMLP) PMN specifically bound 7.8 0.25 x 10^3 molecules of [^{3}H]-FMLP/PMN, cells that had been incubated with unlabeled FMLP bound 2.5 0.17 x 10^3\molecules of [^{3}H]-FMLP/PMN (mean +/-SE, n=3; $p < 0.01$, Student's t test) (Fig. 5). Furthermore, before preincubation with unlabeled FMLP normal and NT-PMN bound identical amounts of [^{3}H]-FMLP (Fig. 5).

After incubation at 37°C, normal PMN that had been preincubated with unlabeled FMLP rapidly recovered their ability to bind [^{3}H]-FMLP (Fig. 5) (4). After 12 min of incubation, normal PMN that had been preincubated with unlabeled FMLP bound 8.0 +/- 0.43 x 10^3 molecules of [^{3}H]-FMLP/PMN, a value similar to that observed prior to incubation with unlabeled FMLP (7.8 x 10^3 molecules/PMN) (Fig. 5). After 16 min of incubation at 37°C, normal PMN bound of [^{3}H]-FMLP/PMN. Longer incubation times did not result in further increments in their binding capacity. Thus, it appeared that normal PMN that had been preincubated with unlabeled FMLP recovered and after 16 min bound approximately 10% more [^{3}H]-FMLP than untreated normal PMN (i.e., super-recovery) (4, 19).

Initially, binding of [^{3}H]-FMLP to NT-PMN that had been preincubated with unlabeled FMLP was identical to that of normal PMN preincubated with unlabeled FMLP (Fig. 5). After incubation at 37°C however, NT-PMN that had

been preincubated with unlabeled FMLP failed to completely recover their ability to bind [^{3}H]-FMLP (Fig. 5). Even after 16 min of incubation at 37^{o}C, these cells bound less [^{3}H]-FMLP (7.0 +/- 0.17 x 10^3 molecules/PMN) than normal PMN that had been preincubated with unlabeled FMLP (8.6 +/- 0.32 x 10^3 molecules/PMN) (mean +/-SE, n=3; $p < 0.01$, Student's t test). Identical results were obtained when receptor reexpression by normal and NT-PMN was assessed in the presence of the glycosylation inhibitor, tunicamycin (10^{-6}M) (Fig. 5). It should be noted that, during the reexpression experiments, all cell types released similar amounts of the granular constituent lysozyme into supernatants (not shown). Thus, it appears that NT-PMN exhibited a defect in the reexpression of a population of FMLP receptors.

Experiments were performed to determine the ability of normal and NT-PMN, that had been preincubated with unlabeled FMLP and allowed to recover for varying periods of time, to respond chemotactically on subsequent challenge with FMLP. At designated time points during recovery, reactions were stopped by adding equal volumes of 4^{o}C PBS. Cells were washed once with cold PBS, resuspended in HBSS containing BSA and used for the chemotactic assays. These experiments were performed simultaneously with the receptor reexpression assays.

Normal PMN that had been preincubated with unlabeled FMLP and washed were unable, initially, to migrate chemotactically in response to 10^{-8}M FMLP (Fig. 6). When allowed to recover for 12 min at 37^{o}C before the chemotactic assay, normal PMN that had been preincubated with unlabeled FMLP exhibited chemotactic responsiveness to FMLP similar to that of normal PMN that had not been preincubated with unlabeled FMLP (Fig. 6, legend). After 16 min of

recovery, normal PMN that had been preincubated with FMLP migrated approximately 15% more than control normal PMN (Fig. 6).

In contrast, NT-PMN that had been preincubated with unlabeled FMLP failed to recover their ability to migrate chemotactically in response to FMLP (Fig. 6). Even after 16 min of incubation at 37°C, NT-PMN that had been preincubated with unlabeled FMLP migrated NT-PMN that had been preincubated with unlabeled FMLP migrated approximately 75% less that normal PMN that had been subjected to the same manipulations (Fig. 6). These cells, however, were as capable as normal PMN to respond chemotactically when challenged with suboptimal concentrations of C5a (legend to Fig. 6).

Affinity Labeling of the Formyl Peptide Receptor of Normal and NT-PMN.

It was of interest to determine if removal of PMN surface sialic acid altered the structure, and hence the electrophoretic mobility, of the formyl peptide receptor. The formyl peptide receptor from normal and NT-PMN was covalently labeled with ^{125}I-FP using EGS (13, 14) and solubilized receptors subjected to SDS-PAGE under reducing conditions (Fig. 7). Plasma membranes from normal and NT-PMN bound ^{125}I-FP specifically to a protein exhibiting an approximate molecular weight of 60-62,000 (Fig. 7, A and C).

Discussion

The data presented here indicates that removal of PMN surface sialic acid results in the intracellular trapping of [^{3}H]-FMLP associated radioactivity (some of which may represent FMLP-receptor complexes). Also, NT-PMN exhibited a defect in the reexpression (or recycling) of a population of formyl peptide receptors. As reported previously (4), inhibition of recycling is associated with inhibition of FMLP-induced PMN chemotaxis.

In previous studies from this laboratory, it was reported that NT-PMN bind [^{3}H]-FMLP (4^{o}C) as well as normal PMN (3). Scatchard analysis of [^{3}H]-FMLP binding to normal and NT-PMN failed to detect differences between the two cell types. Similarly, NT-PMN were as able as normal PMN to take up (37^{o}C) [^{3}H]-FMLP (Fig. 1). Specific uptake was measured by subtracting nonspecific uptake (i.e., uptake of [^{3}H]-FMLP in the presence of 100-fold excess unlabeled ligand) from total uptake. Results represent true uptake since pinocytotic uptake was excluded (4, 18). Furthermore, saturation of [^{3}H]-FMLP uptake indicated that it was a receptor-mediated process (Fig. 1).

One would assume that if NT-PMN reexpress less FMLP receptors than normal PMN, their late uptake of [^{3}H]-FMLP would be less than normal PMN (i.e., less receptors being available). It should be noted, however, that uptake experiments represent binding, internalization and release of [^{3}H]-FMLP by PMN during the incubation period. One possible explanation for the lack of differences in uptake between normal and NT-PMN is that NT-PMN would release less [^{3}H]-FMLP associated radioactivity than normal PMN during the uptake experiments. Thus, while the total radioactivity associated with the cells would be identical, the mechanisms involved would be quite different.

Indeed, NT-PMN released less cell-associated radioactivity than normal PMN (Fig. 2). Normal and NT-PMN were incubated with [^{3}H]-FMLP for 16 min (plateau of uptake) at 37^{o}C and washed three times with cold buffer. Displacement experiments (using 1000-fold excess unlabeled FMLP) revealed that normal and NT-PMN had less than 2% displaceable radioactivity at the beginning of the release experiments (i.e., after washings following uptake). Thus, [^{3}H]-FMLP was either bound in a non-dissociable fashion on the cell

membrane or radioactivity represented internalized peptide. Digestion of normal and NT-PMN (performed at the beginning of the release experiments), with trypsin (100 ug/ml) (4°C for 15 min) failed to release radioactivity into supernatants (not shown) (12). Thus, it is likely that the cell-associated radioactivity that was released into supernatants over time represented [^{3}H]-FMLP that had been internalized. It should be noted that we do not have any evidence indicating that released radioactivity represents only intact peptide. Diminution in the ability of NT-PMN to release [^{3}H]-FMLP suggested that these cells retained some of the radioactivity perhaps in association with the receptor (i.e., ligand-receptor complexes). This suggestion was confirmed by the subcellular fractionation studies (Fig. 3). While normal and NT-PMN incorporated similar amounts of radioactivity after 5 and 15 min of incubation with [^{3}H]-FMLP, NT-PMN retained more counts within light Golgi-containing fractions than normal PMN (Fig. 3). After 15 min of incubation with [^{3}H]-FMLP, NT-PMN had approximately 2.1-fold more radioactivity within light golgi-containing fractions than normal PMN (Fig. 3,B and Table I). The fact that most of the radioactivity in these fractions remained sedimentable after sonication suggested that it was tightly associated with protein (i.e., most likely [^{3}H]-FMLP-receptor complexes) (5, 16). Supporting evidence was obtained when aliquots of peak light golgi-containing fractions (from normal and NT-PMN), that appeared homogeneous on hyperosmolar sucrose density gradients, were fractionated further on iso-osmolar stractan gradients (Fig. 4). These experiments revealed, also, that NT-PMN retained radioactivity within sealed vesicles (Fig. 4). It is possible that inability of NT-PMN to process ligand-receptor complexes, perhaps via a light Golgi pathway, results in the accumulation of radioactivity within endocytic vesicles (i.e., "back-up phenomenon").

Based on these observations, we postulated that NT-PMN may have a defect in the recycling of FMLP receptors. To address this possibility, normal and NT-PMN were incubated with 50 nM unlabeled FMLP for 5 min, washed twice at $4^{o}C$, and their ability to bind [^{3}H]-FMLP overtime determined (Fig. 5). This concentration of unlabeled FMLP was chosen because it completely deactivated PMN chemotactic responses to a subsequent challenge with FMLP (Fig. 6). Removal of PMN surface sialic acid inhibited the reexpression (or recycling) of a population of receptors by FMLP-treated cells (Fig. 5). Inhibition was apparent after 6 min of incubation at $37^{o}C$ (Fig. 5). It should be noted that during the reexpression experiments normal and NT-PMN released similar amounts of lysozyme into supernatants (not shown) (4). released similar amounts of lysozyme into supernatants (not shown) (4). Thus, while degranulation may explain the partial recovery of binding by NT-PMN (20), it can not account for the differences observed between the two cell types (Fig. 5). It seems unlikely that removal of PMN surface sialic acid inhibits expression of an intracellular, non-lysosomal pool of receptors. The most likely explanation is that NT-PMN exhibit a defect in their ability to recycle a population of FMLP receptors (4). Similar results were observed when receptor reexpression assays were performed in the presence of the glycosylation inhibitor, tunicamycin (Fig. 5). Tunicamycin has been reported to inhibit not only glycosylation, but also protein synthesis (21). Thus, it is likely that neither process plays a role in the receptor reexpression assays.

Inability of NT-PMN to recycle a population of FMLP receptors was associated with inhibition of recovery of their chemotactic responsiveness to FMLP (Fig. 6) (4). In contrast, normal PMN that had been incubated with unlabeled FMLP fully

recovered their ability to respond chemotactically to FMLP (Fig. 6). While it is difficult to establish a direct correlation between kinetics of receptor recovery and chemotaxis (due to inherent differences between the two assays), it appears that inhibition of FMLP-receptor recycling results in inhibition of FMLP-induced PMN chemotaxis. Inhibition of chemotaxis was specific for FMLP, since NT-PMN responded as well as normal PMN when challenged with C5a (3).

It was of interest to determine if removal of PMN surface sialic acid affected the formyl peptide receptor. Removal of sialic acid from the transferrin receptor on K-562 cells alters its intracellular processing (1). Furthermore, it has been shown previously that the FMLP receptor is an asparagine-linked glycoprotein (14). Using HL-60 cells Heiman et al. (22) reported that inhibition of glycosylation results in inhibition of receptor expression on the cell surface and inhibition of FMLP-induced chemotactic responsiveness. Affinity-labeled formyl peptide receptor from normal and NT-PMN exhibited the same molecular weight (Fig. 7). These results provide further evidence for lack of protease contamination in our neuraminidase preparations. Two possible explanations exist for this finding. First, it is likely that removal of sialic acid from the formyl peptide receptor will not alter its electrophoretic mobility on SDS-PAGE. Sialic acid is a small molecule and its contribution to the total molecular weight of the receptor is minimal. Alternatively, sialic acid is not being removed from the receptor molecule but from another, as yet unidentified glycoprotein that plays a role in the recycling of the formyl peptide receptor. The latter seems to be the case with the insulin receptor (2). Removal of surface sialic acid from rat adipocytes inhibits insulin action by blocking recycling of the insulin receptor, without affecting the receptor per se (2).

Although the precise mechanism whereby removal of PMN surface sialic acid inhibits FMLP receptor recycling is unclear, the present work presents a mechanism by which removal of surface sialic acid may inhibit FMLP-induced chemotaxis. Inhibition is stimulus-specific, since removal of surface sialic acid has no effect on either C5a or leukotriene B_4-induced PMN chemotaxis (3). It is feasible that sialic acid residues do not play an important role in the processing of either the C5a or the leukotriene B_4 receptor by PMN.

Summary

Removal of surface sialic acid specifically inhibits human polymorphonuclear leukocyte (PMN) chemotactic responses to N-formyl-methionyl-leucyl-phenylalanine (FMLP). Neuraminidase-treated (NT)-PMN bound and internalized [^{3}H]-FMLP (used as receptor marker) as well as normal PMN. NT-PMN however, retained more [^{3}H]-FMLP-associated radioactivity than normal PMN. Subcellular fractionation studies demonstrated that NT-PMN retained more sedimentable (100,000 x g for 180 min) [^{3}H]-FMLP-associated radioactivity within light golgi-containing fractions than normal PMN. Furthermore, NT-PMN exhibited a defect in their ability to re-express (or recycle) a population of FMLP receptors. Abnormal receptor recycling was associated with inhibition of FMLP-induced PMN chemotaxis. Thus, it appears that recycling of formyl peptide receptors may be necessary for optimal PMN chemotactic responses to FMLP. We postulate that removal of PMN surface sialic acid inhibits FMLP-induced PMN chemotaxis by blocking the reexpression (or recycling) of a population of formyl peptide receptors (perhaps by preventing trafficking of desialated receptors through a light golgi pathway).

TABLE 1.

Subcellular distribution of [^{3}H]FMLP within normal and NT-PMN radioactivity (cpm x 10^{-3}). [a]

	Cytosol	Golgi	Granules	
			Specific	Azurophil
5 Min.				
Normal PMN	46.3	8.2	1.3	0.2 .
NT-PMN	41.8	13.1	1.1	0.15
15 Min	74.5			
Normal PMN	63.8	10.9	5.5	0.31
NT-PMN		19.1	2.0	0.28

[a] Results represent radioactivity present in peak fractions obtained from experiment depicted in Figure 3. Cytosol refers to fraction containing maximum lactate dehydrogenase activity. Golgi refers to light Golgi fraction containing maximum UDP-galactose galactosyl transferase activity, specific granules refer to fractions containing peak values for vitamin B12-binding protein, and azurophilic granules refer to fraction containing maximum *beta*-glucuronidase activity.

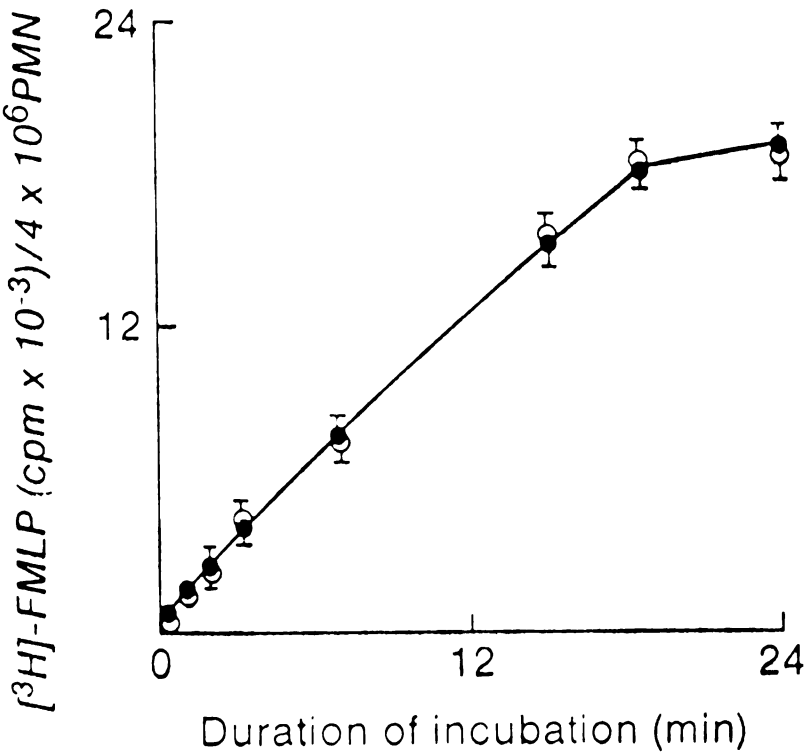

Figure 1

Specific uptake of [^{3}H]-FMLP by normal *(open circles)* and NT-PMN (*closed circles*). Cells (4 x 10^6 PMN) in 0.4 ml HBSS, were incubated with 20 nM [^{3}H]-FMLP at 37^{o}C for varying durations. Following incubation, buffer containing 1000-fold excess unlabeled peptide was added, cells were centrifuged through silicone oil and processed as described in Methods. Results represent mean values (+/- SE) of 3 experiments.

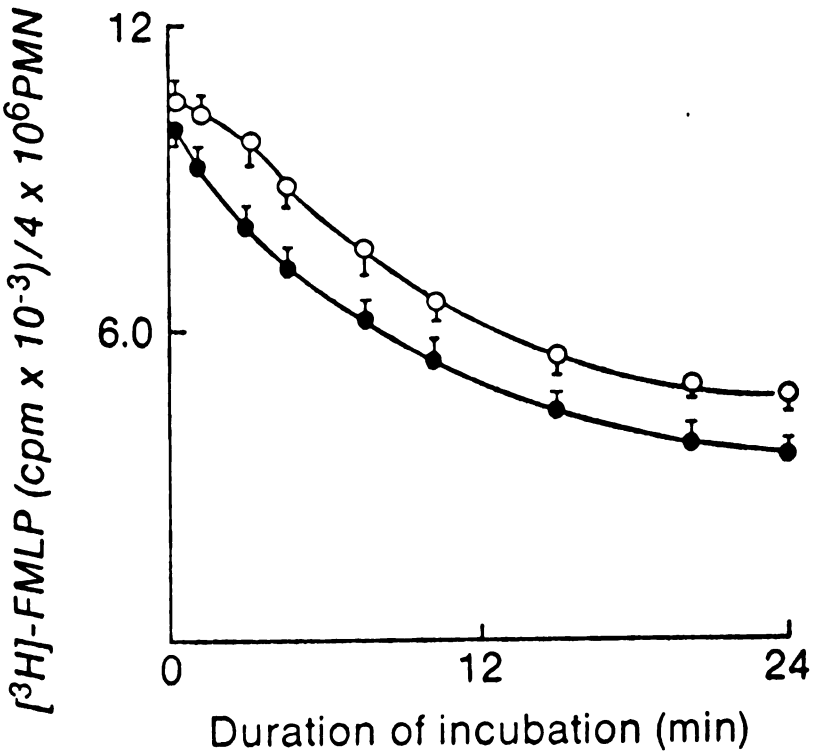

Figure 2

Ability of normal (open circles) and NT-PMN (closed circles) to release [3]-FMLP that had been taken up by the cells. PMN (4 x 10^6 cells) in 0.4 ml HBSS, were incubated with 20 nM [3H]-FMLP at 37°C for 16 min. Reactions were stopped by adding 4°C PBS. PMN were washed twice and resuspended in 0.4 ml of HBSS at 37°C for varying durations. Incubations were terminated by centrifugation through oil and pellets processed as described in Methods. Results represent cell-associated radioactivity, and are mean values (+/-SE) of 3 experiments.

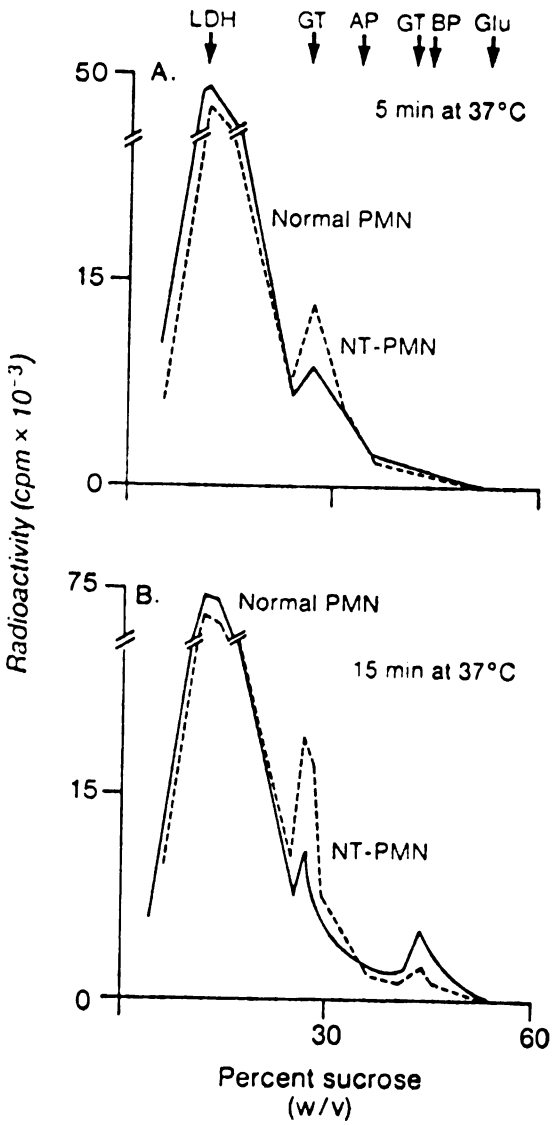

Figure 3

Subcellular distribution of [^{3}H]-FMLP-associated radioactivity within normal and NT-PMN. Cells (6 x 10^8 PMN) were incubated with 50 nM [^{3}H]-FMLP at 37°C as described in *Materials and Methods* and *Results* section, washed, disrupted by N_2 cavitation and fractionated on linear sucrose density gradients, as described in *Materials and Methods*. Distribution of radioactivity after (A) 5 min of incubation with [^{3}H]-FMLP, and (B) 15 min of incubation with [^{3}H]-FMLP. *Solid lines*, normal PMN. *Dashed lines*, NT-PMN. Recovery of radioactivity from gradients (cpm/mg protein): A, Normal PMN: 9.1 x 10^4 NT-PMN: 8.8 x 10^4. B, Normal PMN: 12.0 x 10^6; NT-PMN: 11.7 x 10^4. Subcellular markers: *LDH*, lactate dehydrogenase, *GT*, galactosyl transferase; *AP*, alkaline phosphatase; *BP*, vitamin B_{12}-binding protein; *Glu*, beta glucuronidase.The recovery of specific markers was identical for normal and NT-PMN. One of three experiments with essentially identical results.

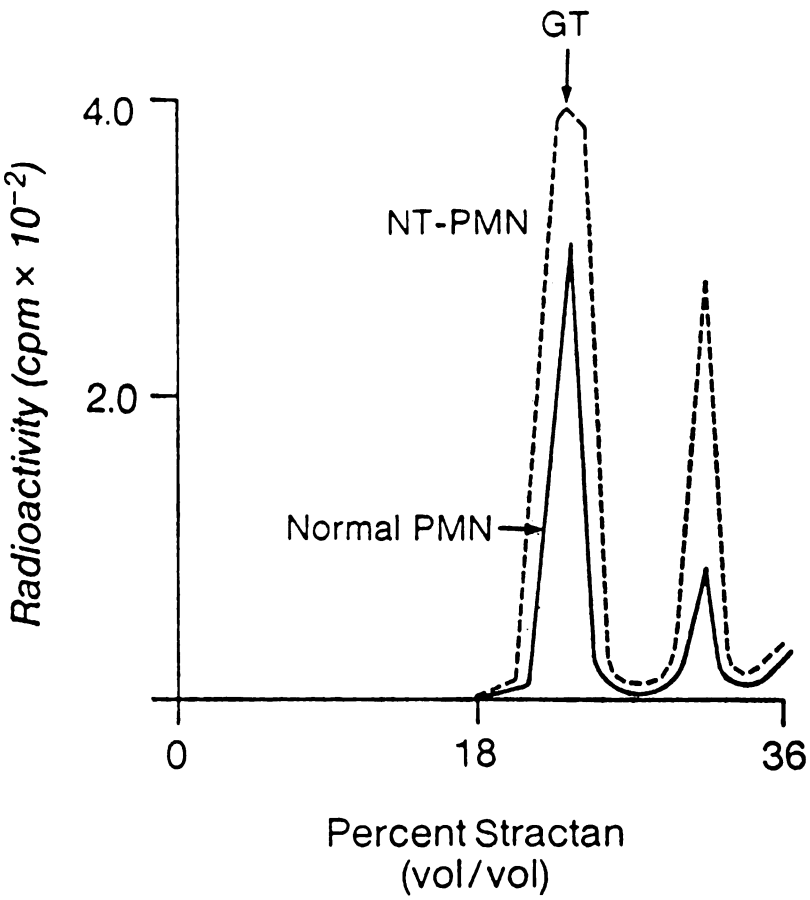

Figure 4

Distribution of [^{3}H]-FMLP-associated radioactivity within peak golgi containing fractions obtained from normal and NT-PMN that had been incubated with [^{3}H]-FMLP for 15 min at 37°C (Fig. 3,B). Aliquots (containing identical amounts of protein) were applied to discontinuous stractan gradients, and fractionated as described in *Methods and Materials*. *Solid line*, normal PMN. *Dashed line*, NT-PMN. GT, galactosyl transferase.One of two experiments with essentially identical results shown

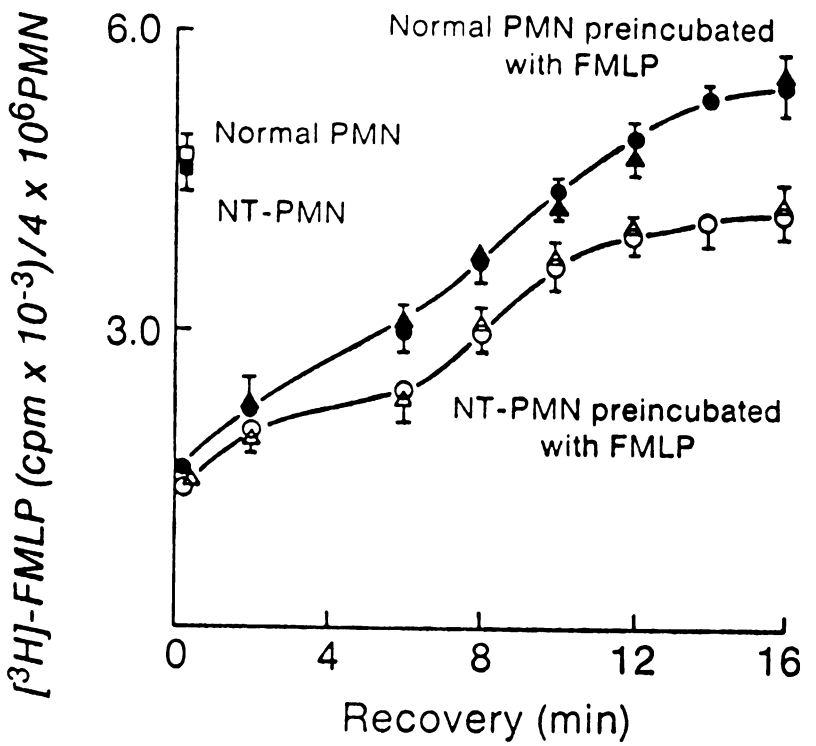

Figure 5

Kinetics of FMLP receptor reexpression by normal and NT-PMN. Cells (5 x 10^6 PMN/ml) suspended in HBSS were preincubated with 50 nM unlabeled FMLP for 5 min at 37°C, in the presence and absence of tunicamycin (10^{-6}M). Reactions were stopped by adding equal volumes of 4°C PBS, and cells were washed once. After washing, normal and NT-PMN were resuspended in HBSS (in the presence and absence of 10^{-6}M tunicamycin), and aliquots (0.25 ml containing 4 x 10^6 cells) incubated at 37°C for varying durations. At each time point, reactions were stopped by adding equal volumes of 4°C Hanks' buffer containing 20 nM [^{3}H]-FMLP, and processed as described in Methods. Receptor reexpression by: normal PMN (*closed circles*); normal PMN plus tunicamycin (*closed triangles*); NT-PMN (*open circles*); NT-PMN plus tunicamycin (*open triangles*). Binding of [^{3}H]-FMLP by normal PMN (*open squares*) and NT-PMN (*closed squares*) that had not been preincubated with unlabeled FMLP. Results represent mean values (+/− SE) of 3 experiments.

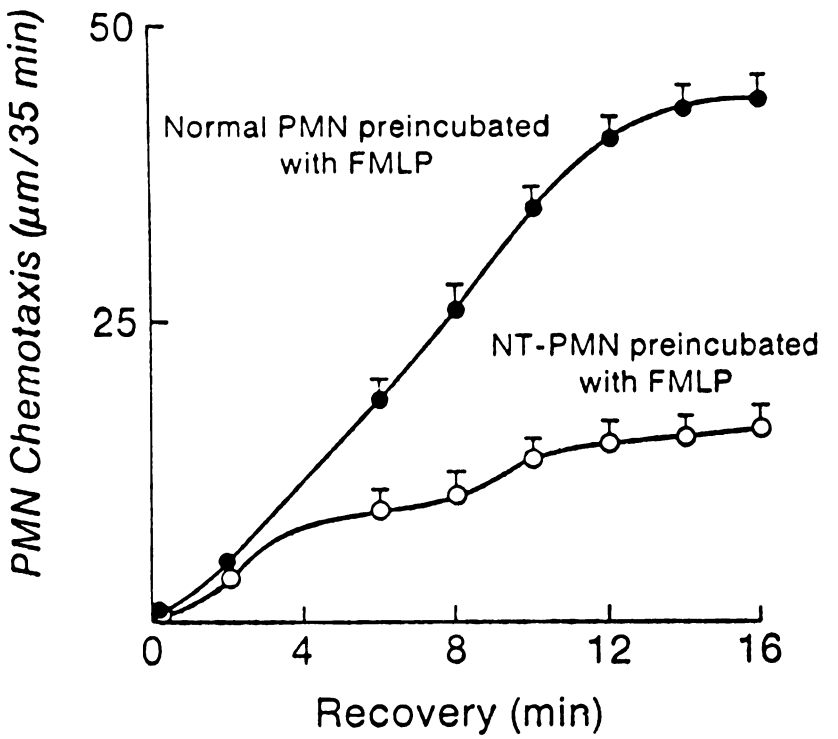

Figure 6

Chemotactic responsiveness of normal and NT-PMN during receptor reexpression. Cells were treated as described in legend to Fig. 5. At varying durations during receptor reexpression, reactions were stopped by adding 1.0 ml 4°C PBS. PMN were washed once and used in the chemotactic assays. FMLP was used as a chemoattractant at a final concentration of 10^{-8}M. FMLP-induced chemotactic responses of normal PMN that had not been preincubated with unlabeled FMLP was 38.2 2.5 um/35 min. C5a (0.5 ng/ml)-induced chemotactic responses of normal and NT-PMN that had been preincubated with unlabeled FMLP and allowed to recover for 16 min at 37°C were 27.8 +/- 3.2 and 28.0 +/- 2.3 um/35 min, respectively. Results are expressed as net PMN migration and represent mean values (+/- SE) of 3 experiments. Stimulated random motility of normal and NT-PMN was identical for the two cell types, before and after preincubation with unlabeled FMLP.

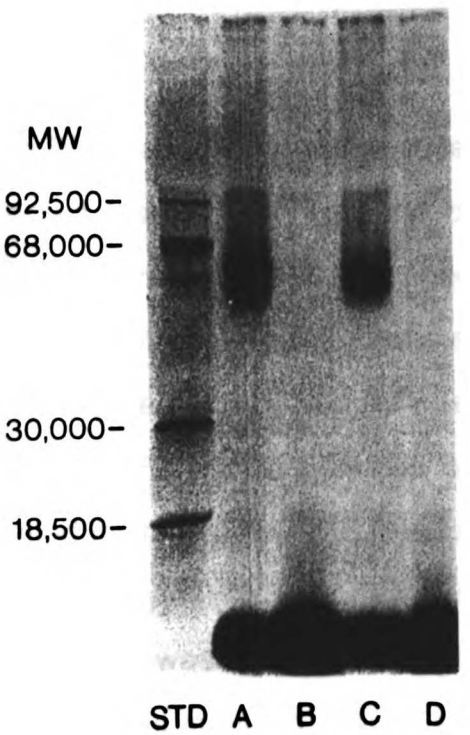

Figure 7

Autoradiogram of normal and NT-PMN plasma membranes to which ^{125}I-FP has been cross-linked. Approximately 10 ug of protein, containing identical amounts of radioactivity, were applied to each lane. STD, molecular weight standards; A, normal PMN plasma membrane plus ^{125}I-FP; B, normal PMN plasma membrane plus ^{125}I-FP plus 1,000-fold excess unlabeled FP; C, NT-PMN plasma membrane plus ^{125}I-FP; D, NT-PMN plasma membrane plus ^{125}I-FP plus 1,000-fold excess unlabeled FP.The apparent increase in radioactivity in *lane A* is due to decrease width of the lane, as compared to *lane C*.

CHAPTER V

RECYCLING OF FORMYL PEPTIDE RECEPTORS BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES.

Introduction

Exposure of human PMN to the synthetic peptide FMLP stimulates these cells to migrate in a directed fashion (i.e., respond chemotactactically). (1). This process is initiated by the binding of FMLP to specific receptors present on the PMN membrane (2). In chapters III and IV, it was suggested that FMLP-induced PMN chemotaxis may require the recycling (or re-expression) of a population of formyl peptide receptors (FPR) . In previous studies form this laboratory, evidence was presented indicating that FMLP-induced PMN chemotaxis did not require marked changes in cytosolic free calcium or specific granule discharge. FMLP-induced chemotaxis was however inhibited by preventing FPR re-expression (3). These results provided indirect evidence suggesting that PMN are able to re-express FPR (independently from mobilization of an intracellular pool), in a process that required minimal changes in cytosolic free calcium. To examine this possibility directly, affinity labelling of PMN FPR.was performed By stimulating the affinity labeled PMN with the calcium-independent protein kinase C activator, phobol myristate acetate (PMA), under low calcium conditions, the first direct evidence indicating that PMN do recycle FPR has been shown.

Materials and Methods.

Preparation of Leukocyte Suspensions.

Platelet-poor leukocyte suspensions containing 97-99% PMN were prepared from venous blood obtained from healthy adult volunteers, as described in chapter II. All solutions used were sterile-filtered and most of the isolation steps were performed at 4°C, using low (0.01 mM) Ca^{2+} buffer (phosphate 10 mM-buffered 140 mM NaCl, pH 7.4) (PBS) to minimize PMN activation (16).

Iodination of Formyl Peptide.

N-formyl-Nleu-Leu-Phe-Tyr (FP) (Sigma Chemical Co., St Louis, MO) was radioiodinated with carrier-free Na[^{125}I] (Amersham, Arlington Heights, IL) by the chloramine T method, as described in chapter VI. Specific activity of [^{125}I]-FP was approximately 600 Ci/mmol.

Binding of [^{125}I]-FP to PMN.

Binding of [^{125}I]-FP to PMN was assessed as described in chapter III. Briefly, PMN (2 x 10^6cells) and [^{125}I]-FP (0.1-60 nM) were incubated at 4°C for 15 min in 0.4 ml of PBS, in the presence and absence of 100-fold excess unlabeled FP. A collection method involving centrifugation through silicone oil was used to separate free from bound peptide .

Affinity-Labeling of PMN FPR.

PMN (10^7 cells/ml), suspended in PBS containing 5.0 mM EDTA, were incubated with 20 pmols of [^{125}I]-FP for 15 min at 4°C to achieve equilibrium binding (15, 19). After incubation, ethylene glycolbis succinimidyl succinate (EGS) (Pierce Chemical Co., Rockford, IL) (0.15 mg/ml) was added, and mixtures incubated for an additional 30 min at 4°C under continuous mixing

conditions. At the end of incubation, PMN were pelleted by centrifugation (150 x g for 8 min, 4°C) and washed three times with cold buffer. Washed cells were resuspended in either PBS, PBS containing 1.0 mM EDTA, or PBS supplemented with 1.0 mM $CaCl_2$, and used for assays. In some instances, affinity-labeled PMN were solubilized using 0.5 ml PBS containing 5.0 mM EDTA, 5.0 mM diisopropylfluorophosphate (DFP, Sigma) and 2.0% (w/v) octylglucoside (Calbiochem, San Diego, CA).

Papain digestion of extracellular receptors.

For the papain digestion studies, affinity labelled PMN (equal number in all reactions) were incubated at 4°C for 15 min. with 5.0 U/ml papain, (Sigma Chemical Co. St. Louis), (final volume 40 ul). Reactions were terminated by the addition of 1.0mM (final concentration) cystatin (Sigma Chemical Co. St. Louis.) Tubes were placed on ice for 10 minutes, after which PMN were solubilized and samples subjected to SDS-PAGE.

SDS-PAGE.

Samples were subjected to SDS-PAGE under reducing conditions using 7-17% gradient slab gels (as described in chapter III). After SDS-PAGE, gels were dried and exposed (48-72 hr at -70°C) to Kodak XR-1 film (Kodak Co., Rochester, NY), using a Cronex intensifying screen (DuPont, Wilmington, DE). In some instances, autoradiograms were read by soft laser densitometry using a Model 620 videodensitometer (BioRad, Richmond, CA) equipped with a 2.01 version of Analyst software (BioRad).

RESULTS AND DISCUSSION.

Binding of [^{125}I]-FP to PMN.

[^{125}I]-FP bound to PMN in a specific and saturable fashion, (Fig. 1,A), exhibiting an ED_{50} of approximately 3.0 nM. Scatchard analysis of binding (Fig. 1,A inset) was consistent with the presence of high (30,600 sites, dissociation constant [K_D] 0.14 nM) and low (146,00 sites, K_D 2.0 nM), affinity sites, (5). Binding of [^{125}I]-FP to PMN was competitive, in the presence of either unlabelled FP (ED_{50} 8.0 nM), or unlabeled FMLP (ED_{50} 600 nM), (Fig.1B). The ED_{50} of inhibition exhibited by unlabeled FP and FMLP were similar to the ED_{50} of these peptides required to induce lysosomal enzyme release from cytochalasin B-treated PMN (not shown) . Thus, FP and FMLP interact with the same receptor on the PMN membrane.

Affinity labeling of PMN FPR.

PMN FPR was affinity labeled using [^{125}I]-FP and EGS, as described in *Methods* . After labeling, the PMN were solubilized using octylglucoside and aliquots were subjected to SDS-PAGE followed by autoradiography. Autoradiograms of affinity-labeled PMN revealed the presence of a single broad band exhibiting a Mr of 50-65,000 daltons (Fig. 2A, lane1). Labeling was specific and bind was competitive in the presence of either 50-fold excess unlabeled FP or 500 fold excess of unlabeled FMLP (Fig.2A, lane3).

Papain digestion of extracellular receptor.

PMN [^{125}I]-FPR can be digested with papain (by limited digestion) to a species exhibiting a Mr of approximately 32-34000 daltons,that retains the

crosslinked labeled peptide (Fig.2,B) (6). Consequently, papain digestion of extracellular [^{125}I]-FPR was used to determine if PMN recycle FPR. Intracellular [^{125}I]-FPR is protected from papain digestion and exhibits its usual Mr of 50-65,000 daltons by autoradiography. Upon recycling (or re-expression), to the cell membrane, [^{125}I]-FPR would become papain-sensitive and be cleaved to a species of Mr of 32-34,000 daltons.

Affinity labeled PMN ($1x10^7$ cells/ml) were incubated in PBS at 37^{o}C for varying periods of time (5-40 min.). At each time point, aliquots (0.5 ml.) were removed, and placed on ice, and 0.5 ml. of cold buffer containing papain was added. Digestion was allowed to proceed for 15 minutes, with termination of digestion achieved by addition of cystatin. After digestion PMN were pelleted by centrifugation, washed, solubilized with octlyglucoside and subjected to SDS-PAGE followed by radioautography. In the absence of PMA (Fig. 3, right) no undigested (i.e. papain resistant) [^{125}I]-FPR was detected over the 40 minute incubation period. Degraded material (some of which probably represents internalized and degraded [^{125}I]-FPR) was present after 10 minutes in the absence or presence of PMN (Fig.3).

In the presence of PMA (Fig.3, left), undigested, (i.e., Mr 50-65,000 daltons, papain resistant) [^{125}I]-FPR was present after 5 minutes and again after 15, 30, 35, and 40 minutes. The amount of undigested [125I]-FPR at 35 minutes was less than that observed at 30 and 40 minutes indicating partial re-expression of internalized [^{125}I]-FPR.

The most likely explanation for our findings is that PMN recycle [^{125}I]-FPR upon stimulation with PMA. PMA induced PMN to internalize [^{125}I]-FPR after 5

minutes(i.e., papain resistant) of incubation. Internalized [125I]-FPR was re-expressed after 10 minutes (papain-sensitive), re-internalized after 15 minutes (papain resistant) and so on. the 4-5 cycles of FPR recycling (over 40 minutes) correspond closely to what we calculated previously as the recycling required for optimal FMLP-induced PMN chemotaxis (chapter III). Furthermore, recycling occurs in PBS, a buffer that contains only 10 uM Ca^{++} (7), providing confirmation of other studies performed in this laboratory (3).

The fact that recycling occured only upon stimulation of PMN with PMA suggests a role for protein kinase C in the process. Because FPR was cross-linked, experiments using FP as stimulus are not feasible. Nevertheless, recycling of [^{125}I]-FPR did not occur when PMN were stimulated with optimal concentrations of the complement derived peptide C5a (not shown).

Summary

The findings reported here provide the first direct evidence indicating that human PMN do recycle FPR. The data provided above are similar to data reported using photoaffinity labeled insulin receptors (8), and indicate that dissociation of ligand-receptor complexes is not an absolute requirement for FPR recycling by human PMN.

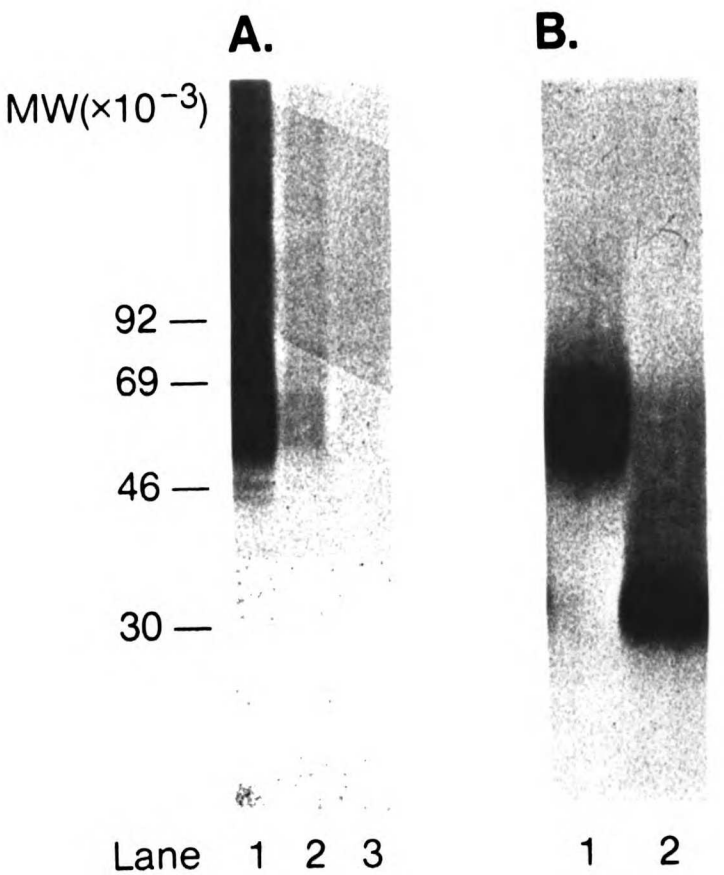

Figure 1.

A. Binding of [^{125}I]-FP to PMN. Cells (2x10^6/ml) were incubated within creasing concentrations of [^{125}I]-FP at 4^{o}C for 15 minutes in the presence and absence of 100-fold excess unlabeled FP. reactions were terminated by centrifugation through silicon oil. Inset: Scatchard plot analysis of [^{125}I]-FP binding to PMN. B. competition of binding of [^{125}I]-FP (5.0 nM) to PMN by increasing concentrations of either unlabeled FP (closed circles) or FMLP (open triangles). Reactions were carried out as described in panel A.

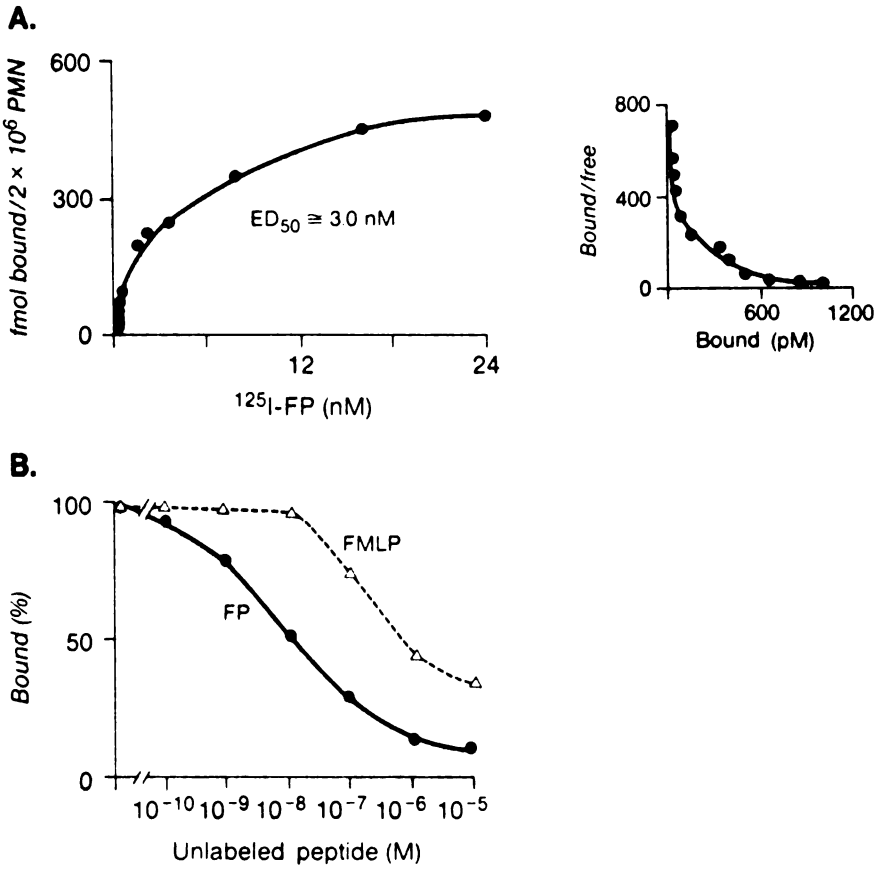

Figure 2.

A. Autoradiogram of affinity labeled PMN FPR. Lane 1: affinity labeled PMN FPR. Lane 2: identical reactions performed in the presence of 50-fold unlabeled FP. Lane 3: identical reactions performed in the presence of 500-fold excess unlabeled FMLP. B. Autoradiogram demonstrating the ability of papain to digest PMN [^{125}I]-FPR. Lane 1: PMN [^{125}I]-FPR, no papain. Lane 2: PMN [^{125}I]-FPR incubated with 5.0 U of papain for 15 min. at 4^{o}C.

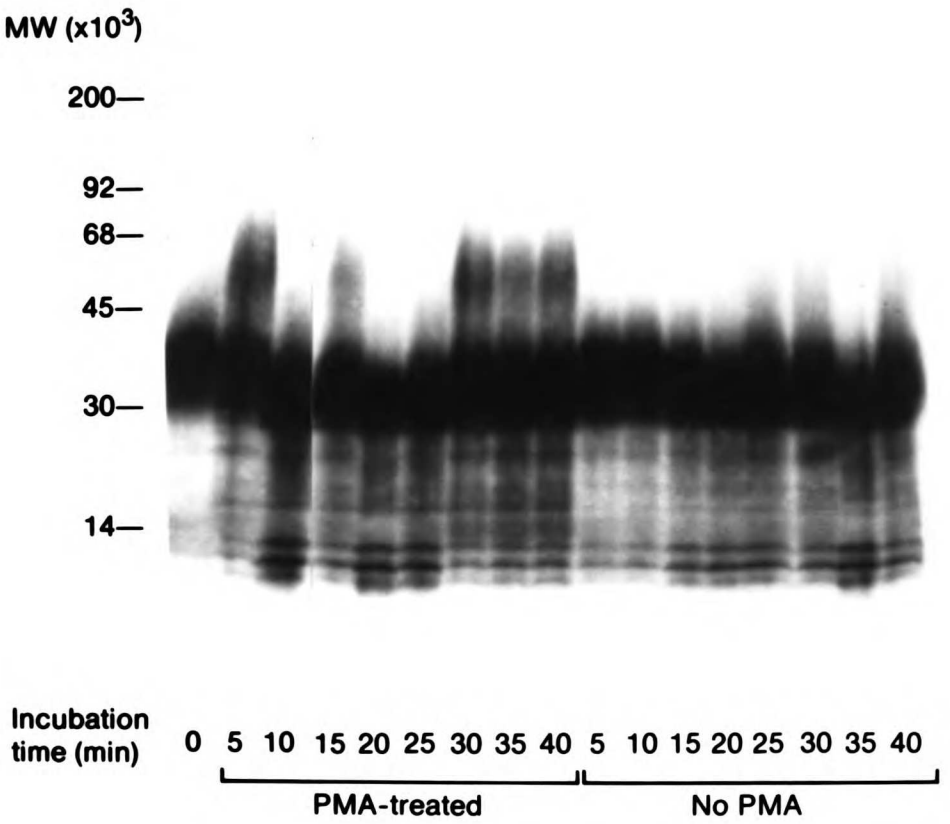

Figure 3.

Recycling of [^{125}I]-FPR by PMN. Affinity labeled PMN (10^7 cells in 1.0 ml.PBS) were incubated at 37°C for varying periods of time in the presence and absence of PMN (10 nanograms per 3 x 10^6 cells). At each time point an aliquot was removed, placed on ice and papain was added. After 15 min. of incubation at 4°C, reactions were stopped by the addition of cystatin. Tubes were kept at 4°C for 10 min., after which PMN were washed, solubilized and samples subjected to SDS-PAGE and autoradiography.

CHAPTER VI

HUMAN POLYMORPHONUCLEAR LEUKOCYTES CONTAIN A POPULATION OF FORMYL PEPTIDE RECEPTORS ANCHORED TO PLASMA MEMBRANE VIA PHOSPHATIDYLINOSITOL.

Introduction.

During the past few years a novel mechanism by which proteins can be anchored to outer plasma membranes has been described (1). This mechanism involves a covalent linkage between protein and an oligosaccharide moiety which is, in turn, glycosidically linked to phosphatidylinositol (1, 2). These class of membrane glycophospholipids, known as glycosyl phosphatidylinositols, have been reported to mediate the anchoring of a variety of proteins to plasma membrane (1-8). Recently, these types of molecules have been implicated also, in the generation of mediators (and signalling function) by the hormone insulin (9, 10). These investigations have been made possible by the isolation and purification of the highly specific calcium-independent bacterial phospholipase, phosphatidylinositol phospholipase C (PIPLC) (11). Treatment of cells with PIPLC results in cleavage of the phosphatidylinositol glycan anchor with release of membrane anchored proteins into supernatants (2, 11).

When human polymorphonuclear leukocytes (PMN) are exposed to formyl peptides these cells are stimulated to migrate in a directed fashion (i.e., respond chemotactically), a process that is initiated by the binding of formyl peptide to specific cell surface receptors (12-14). While investigating the linkage of formyl peptide receptors (FPR) to PMN plasma membrane, it was noted that these cells contain populations of FPR that can be cleaved by PIPLC. Most of the PIPLC-

sensitive PMN FPR is located within an intracellular pool. Chemotactic studies suggest that one function of PIPLC-sensitive FPR may be to prevent early deactivation of PMN chemotactic responsivess to formyl peptide.

Materials and Methods.

Preparation of Leukocyte Suspensions.

Platelet-poor leukocyte suspensions containing 97-99% PMN were prepared from venous blood obtained from healthy adult volunteers, as described in chapter II All solutions used were sterile-filtered and most of the isolation steps were performed at 4°C, using low (0.01 mM) Ca^{2+} buffer (phosphate 10 mM-buffered 140 mM NaCl, pH 7.4) (PBS) to minimize PMN activation (16).

Iodination of Formyl Peptide.

N-formyl-Nleu-Leu-Phe-Tyr (FP) (Sigma Chemical Co., St Louis, MO) was radioiodinated with carrier-free Na[^{125}I] (Amersham, Arlington Heights, IL) by the chloramine T method, as described previously (17, 18). Specific activity of ^{125}I-FP was approximately 600 Ci/mmol.

Binding of ^{125}I-FP to PMN.

Binding of ^{125}I-FP to PMN was assessed as described previously (15, 19). Briefly, PMN (2 x 10^6cells) and [^{125}I]-FP (0.1-60 nM) were incubated at 4°C for 15 min in 0.4 ml of PBS, in the presence and absence of 100-fold excess unlabeled FP. A collection method involving centrifugation through silicone oil was used to separate free from bound peptide (15, 19).

Affinity-Labeling of PMN FPR.

PMN (10^7 cells/ml), suspended in PBS containing 5.0 mM EDTA, were

incubated with 20 pmols of [^{125}I]-FP for 15 min at 4^{o}C to achieve equilibrium binding (15, 19). After incubation, ethylene glycolbis succinimidyl succinate (EGS) (Pierce Chemical Co., Rockford, IL) (0.15 mg/ml) was added, and mixtures incubated for an additional 30 min at 4^{o}C under continuous mixing conditions. At the end of incubation, PMN were pelleted by centrifugation (150 x g for 8 min, 4^{o}C) and washed three times with cold buffer. Washed cells were resuspended in either PBS, PBS containing 1.0 mM EDTA, or PBS supplemented with 1.0 mM $CaCl_2$, and used for assays. In some instances, affinity-labeled PMN were solubilized using 0.5 ml PBS containing 5.0 mM EDTA, 5.0 mM diisopropylfluorophosphate (DFP, Sigma) and 2.0% (w/v) octylglucoside (Calbiochem, San Diego, CA).

PIPLC Treatment of Affinity Labeled PMN.

PIPLC was purified from *Bacillus thuringiensis,* as described (20). One unit (U) PIPLC was defined as the amount of enzyme required to hydrolyze one umol of tritium-labeled phosphatidylinositol per min, at 37^{o}C. Specific activity of the enzyme preparation was 325 U per ml. Affinity-labeled PMN (2 x 10^8 cells/ml) suspended in buffer were incubated with 0 to 20 U/ml PIPLC for 15-60 min at varying temperatures (4-37^{o}C). At the end of incubation, cells were pelleted by centrifugation at 15,000 x g for 30 sec. Supernatants were removed, dialyzed against 200 vol of 0.1 M NaAc, pH 6.5 overnight followed by lyophilization. Cell pellets were solubilized in 0.2 ml of PBS containing 2.0% (w/v) octylglucoside. Lyophilized supernatants were suspended in 0.02 ml sodium dodecyl sulfate (SDS) sample buffer containing 1.0% beta-mercaptoethanol and analyzed by SDS-PAGE. Aliquots (0.02 ml) of solubilized cell pellets were diluted 1:1 with SDS sample buffer and subjected to SDS-PAGE.

SDS-PAGE.

Samples were subjected to SDS-PAGE under reducing conditions using 7-17% gradient slab gels (18). After SDS-PAGE, gels were dried and exposed (48-72 hr at -70°C) to Kodak XR-1 film (Kodak Co., Rochester, NY), using a Cronex intensifying screen (DuPont, Wilmington, DE). In some instances, autoradiograms were read by soft laser densitometry using a Model 620 videodensitometer (BioRad, Richmond, CA) equipped with a 2.01 version of Analyst software (BioRad).

PMN Chemotaxis and Lysosomal Enzyme Release.

Stimulated random motility (chemokinesis) and directed migration (chemotaxis) of PMN were measured using a minor modification of the "leading front" of PMN as described in chapter III.

Extracellular release of the lysosomal marker enzymes lysozyme and beta-glucuronidase, as well as the cytoplasmic marker enzyme lactate dehydrogenase (LDH), was assessed as described previously (15), using cytochalasin B-treated PMN.

Other Reagents.

Phenylmethylsulfonylfluoride (PMSF), and *Cl. Perfringens* phospholipase C was obtained from Sigma. N-glycosidase F (N-glycanase) was from Genzyme.

Results

Initially we determined the ability of [^{125}I]-FP to bind to PMN (Fig. 1,A). Binding of [^{125}I]-FP (0.1-40 nM) to PMN was measured at equilibrium (4°C, 15 min) (15, 19). [^{125}I]-FP bound to PMN specifically, exhibiting an ED_{50} = 2.0 nM. The ED_{50} of [^{125}I]-FP binding to PMN corresponded to the concentration of

unlabeled FP required to induce 50% release of the lysosomal marker enzymes lysozyme and beta-glucuronidase from cytochalasin B-treated (15) PMN (not shown). Scatchard analysis of binding revealed the presence of high (binding sites 30,600, dissociation constant [K_D] 0.14 nM) and low (binding sites 136,200 dissociation constant [K_D] 2.0 nM) affinity receptors per cell. Competition studies (Fig. 1,B) demonstrated that binding of [^{125}I]-FP to PMN could be effectively competed by excess unlabeled FP, with an ED_{50} = 12 nM. Similar results were obtained when displacement experiments were performed (not shown).

Affinity-labeling of PMN FPR, using [^{125}I]-FP and EGS resulted in labeling of a broad band exhibiting a molecular weight of 58-64,000 daltons (Fig. 2, Lane 1) (18, 22). Identical experiments performed in the presence of 5 and 10-fold excess unlabeled FP (Fig. 2, Lanes 2 and 3) demonstrated that labeling of PMN FPR was specific. Incubation of octylglucoside-solubilized affinity-labeled PMN with N-glycanase (10 mU, 37^{o}C for 3 hr) decreased the Mr of [^{125}I]-FPR to 32,000 (23).

To determine if PMN [^{125}I]-FPR was sensitive to PIPLC, affinity-labeled cells (2 x 10^8 PMN/ml) suspended in 0.01 mM [Ca^{2+}] were incubated for 15 min at 37^{o}C in the presence of increasing concentrations (1-7 U/ml) of PIPLC. At the end of incubation PMN were removed by centrifugation (15,000 x g for 30 sec). Cell-free supernatants were processed as described in Methods, and analyzed by SDS-PAGE and autoradiography (Fig. 3). PIPLC induced a concentration-dependent release of PMN [^{125}I]-FPR into supernatants (Fig. 3), a process unaffected by the presence of either 5.0 mM DFP or 1.0 mM PMSF. Small amounts of [^{125}I]-FPR were detected in supernatants of affinity-labeled PMN

incubated in 0.01 mM [Ca^{2+}] in the absence of PIPLC (i.e., spontaneous release) (not shown). It should be noted that incubation of PMN with PIPLC did not result in cell death (as determined by trypan blue exclusion and release of LDH into supernatants), and released [^{125}I]-FPR remained in supernatants after centrifugation at 100,000 x g for 90 min. Furthermore, incubation of affinity-labeled PMN with *Cl. Perfringens* PLC (at concentrations that did not cause cell death) did not result in release of [^{125}I]-FPR.

To investigate spontaneous release of [^{125}I]-FPR into supernatants by affinity-labeled PMN, cells (2 x 10^8 PMN/ml) were suspended either in 0.01 or 1.0 mM [Ca^{2+}] and incubated for 30 min at 37°C in the presence and absence of PIPLC (6.0 U/ml). Supernatants were processed as described above and analyzed by SDS-PAGE and autoradiography (Fig. 4). In the presence of 0.01 mM [Ca^{2+}] affinity-labeled PMN released [^{125}I]-FPR spontaneously (Fig. 4), a process that was accentuated when 1.0 mM [Ca^{2+}] was used (Fig. 4). Spontaneous release of ^{125}I-FPR was not due to cell death, was not inhibited by either DFP or PMSF and released [^{125}I]-FPR remained in supernatants after high speed centrifugation. Extracellular Ca^{2+} had no effect on PIPLC-mediated release of [^{125}I]-FPR from affinity-labeled PMN (Fig. 4). Densitometric analysis of the autoradiogram shown in Fig. 4 demonstrated that, after subtraction of spontaneous release, PIPLC-mediated release of [^{125}I]-FPR was virtually identical for affinity-labeled PMN suspended in either 0.01 or 1.0 mM [Ca^{2+}].

Experiments were performed in which we examined the effect of temperature and micromolar concentrations of extracellular Ca^{2+} on spontaneous and PIPLC-mediated release of [^{125}I]-FPR from affinity-labeled PMN (Fig. 5). Affinity-labeled PMN (2 x 10^8 cells/ml) were suspended in 0.01 mM [Ca^{2+}] in

the presence and absence of 1.0 mM EDTA, and incubated at varying temperatures for 15 min with and without PIPLC (6.0 U/ml). After incubation, supernatants were processed as described above and autoradiograms subjected to soft laser densitometry. PIPLC induced a temperature-dependent, but [Ca^{2+}]-independent, release of [^{125}I]-FPR (Fig. 5). Release of [^{125}I]-FPR by PIPLC could be detected at 10°C and increased almost linearly between 10 and 25°C (Fig. 5). Higher temperatures (37°C) did not result in further release of [^{125}I]-FPR by PIPLC (Fig. 5). Spontaneous release however, occurred only if 0.01 mM [Ca^{2+}] was present and could not be detected at temperatures below 25°C (Fig. 5). This was true even if spontaneous release was assessed over longer incubation times (up to 60 min).

It was of interest to determine if stimulation of affinity-labeled PMN with FP would induce release of [^{125}I]-FPR. Affinity-labeled PMN (2×10^8 cells/ml) were suspended in 0.01 mM [Ca^{2+}] and incubated at 37°C for 10 min in the presence of either buffer, 10^{-10}M FP, or 10^{-9}M FP. After incubation, cells were pelleted by centrifugation (15,000 x g for 1 min), supernatants processed as described in Methods and subjected to SDS-PAGE and autoradiography (Fig. 6). As expected, affinity-labeled PMN incubated in low Ca^{2+} buffer spontaneously released small amounts of [^{125}I]-FPR (Fig. 6, Lane 1). Stimulation of PMN with FP (10^{-10}M, Fig. 6, Lane 2; 10^{-9}M, Fig.6, Lane 3) enhanced [^{125}I]-FPR release from these cells. FP-stimulated release of ^{125}I-FPR from affinity-labeled PMN was not due to cell death (as assessed by LDH release) and released [^{125}I]-FPR was soluble. Furthermore, FP-stimulated release of [^{125}I]-FPR was not inhibited by the presence of 1, 10 phenanthroline (not shown).

PMN possess a pool of intracellular FPR that can be upregulated by exposure of the cells to the phorbol ester, PMA (24, 25). To determine sensitivity of surface and intracellular FPR to PIPLC, PMN (3 x 10^6 cells/ml) suspended in 0.01 mM [Ca^{2+}] were exposed to PMA (10 ng/ml) for 5 min at 37°C. This concentration of PMA was chosen because it does not damage the cells (i.e., PMN do not release LDH and do not form microaggregates, as determined by phase microscopy). After upregulation, cells were suspended in cold buffer, washed twice, and PMN FPR was affinity-labeled. Following labeling, PMN were washed, resuspended in 0.01 mM [Ca^{2+}] (2 x 10^8 PMN/ml) and incubated for 15 min at 37°C in the presence of increasing concentrations of PIPLC (0.6-20 U/ml). Control PMN were subjected to the same manipulations in the absence of PMA. After incubation, supernatants were processed as described and autoradiograms read by soft laser densitometry (Fig. 7).

PIPLC induced release of [^{125}I]-FPR from control, affinity-labeled PMN, in a linear, concentration-dependent fashion (Fig. 7). In contrast, PMA-pretreated PMN exhibited two rates of [^{125}I]-FPR release by PIPLC (Fig. 7). There was an initial steep release observed when 0.7-2.5 U/ml PIPLC was used (Fig. 7). A second rate of [^{125}I]-FPR release from PMA-pretreated PMN was observed when 8.0-20 U/ml PIPLC was present (Fig. 7) that paralleled that observed with control PMN.

Experiments were performed to further characterize the [^{125}I]-FPR that paralleled that observed with control and PMA-treated PMN by PIPLC. Molecular sieve chromatography of supernatants containing [^{125}I]-FPR from PIPLC-treated control PMN, performed on Ultrogel AcA 44 (that had been pre-equilibrated with 0.01 mM [Ca^{2+}] buffer) and eluted in the absence of

detergents, revealed the presence of a symmetric peak of radioactivity exhibiting an approximate molecular weight of 58-62,000 (Fig. 8). Analysis of peak fractions (after pooling and concentration) by SDS-PAGE and autoradiography showed the presence of [^{125}I]-FPR, demonstrating that PIPLC-released receptor was indeed soluble. Similar results were obtained when supernatants containing [^{125}I]-FPR released by PIPLC from PMA-pretreated PMN were used (not shown).

To examine further the effect of PIPLC on PMN FPR, experiments were performed to determine the effect of the enzyme on the equilibrium binding of [^{125}I]-FP to PMN. PMN (2 x 10^7/ml in PBS) were subjected to various manipulations followed by incubation (37^{o}C, 15 min) in the presence and absence of PIPLC (1.0 U/ml). At the end of incubation, reactions were stopped by the addition of equal volumes of 4^{o}C PBS and tubes placed on ice for 10 min. PMN were washed twice with cold buffer and their ability to bind [^{125}I]-FP under equilibrium conditions (4^{o}C, 15 min) determined.

Incubation of PMN, in the presence and absence of PIPLC, for 15 min at 4^{o}C had no effect on the ability of these cells to bind [^{125}I]-FP (Fig. 9,A). Scatchard analysis of binding were consistent with the presence of high and low affinity receptors (Table I). The lack of effect of PIPLC (at 4^{o}C) on [^{125}I]-FP binding to PMN support the results presented in Fig. 5 using affinity-labeled cells.

Incubation of control PMN at 37^{o}C for 15 min resulted in receptor upregulation (Fig. 9,B). Upregulation was due to expression of high and low affinity sites without significant changes in their [K_D] (Table I). The majority of binding was attributable to low affinity receptors. Incubation of PMN at 37^{o}C for 15 min in the

presence of PIPLC resulted in a diminution of [^{125}I]-FP binding to the cells (Fig. 9,B), which was due mostly to a decrease in the number of low affinity sites (Table I) without changes in [K_D].

Experiments were performed to assess the effect of PMA-mediated upregulation on receptor expression and sensitivity to PIPLC. PMN were incubated with PMA (as described above) for 5 min at 37^{o}C. After incubation, reactions were stopped by addition of equal volumes of cold buffer and cells washed 3 times with 4^{o}C PBS. Washed cells were resuspended in PBS and incubated for 15 min at 37^{o}C in the presence and absence of PIPLC, after which equilibrium binding was assessed as described above.

While there was an increase in the number of low affinity receptors (Fig. 9,C) (Table I), PMA-treated control PMN exhibited a number of high affinity receptors that was similar to that observed at 4^{o}C (Table I) but lower than the number expressed by cells incubated at 37^{o}C alone (Table I). Treatment of these cells with PIPLC resulted in a diminution of [^{125}I]-FP binding (Fig. 9,C) which was due to a decrease in low affinity sites (Table I). It has been reported previously that PMA is capable of down-regulating a population of PMN FPR (26). Consequently, experiments were performed in which we attempted to prevent PMA-induced receptor down-regulation by pretreating PMN with the fungal metabolite cytochalasin B. PMN were preincubated with cytochalasin B (5.0 ug/ml), prior to the addition of PMA. After incubation with PMA (5 min, 37^{o}C), cells were processed as described above and equilibrium binding determined.

Incubation of cytochalasin B-treated control PMN with PMA resulted in enhanced specific binding of [^{125}I]-FP (Fig. 9,D) as compared with PMA alone (Fig. 9,C). Upregulation was due to expression of high and low affinity receptors

without changes in their [K_D] (Table I). The number of high affinity receptors was similar to that expressed by cells incubated at 37°C alone (Table I). Incubation of cytochalasin B-PMA-treated PMN with PIPLC resulted in decreased binding of [^{125}I]-FP by the cells (Fig. 9,D), which was due to a diminution in high and low affinity receptors without changes in their [K_D] (Table I).

Finally, experiments were performed to determine the effect of PIPLC treatment on the chemotactic responsiveness of control and PMA-pretreated PMN to increasing (10^{-11}-10^{-8}M) concentrations of FP. Control and PMA-pretreated PMN (2 x 10^8 cells/ml) suspended in 0.01 mM [Ca^{2+}] were incubated for 15 min at 37°C in the presence and absence of PIPLC (6.0 U/ml). After incubation, PMN were washed with cold buffer, resuspended in 1.0 mM [Ca^{2+}] buffer containing 2.0% (w/v) bovine serum albumin, and used for the chemotactic assays (Fig. 10).

Control PMN that had not been preincubated with PIPLC migrated chemotactically to suboptimal (10^{-11}M) and optimal (10^{-10}) concentrations of FP (Fig. 10,A). Higher FP concentrations (10^{-9}-10^{-8}M) resulted in diminished PMN migration, a phenomenon known as deactivation (15, 19). Treatment of control PMN with PIPLC resulted in increased chemotactic responsiveness to 10^{-11}M FP and a more pronounced deactivation response (Fig. 10,A).

In the absence of PIPLC, PMA-pretreated PMN (Fig. 10,B) were less responsive to FP than untreated control PMN (Fig. 10,A). PMA-pretreated PMN exhibited suboptimal chemotactic responsiveness when 10^{-11}-10^{-10} M FP was present in the lower chambers, and optimal chemotactic responses were observed when 10^{-9}M FP was used (Fig. 10,B). Deactivation was detected when 10^{-8}\M FP was present. Interestingly, incubation of PMA-pretreated PMN

with PIPLC significantly (10-fold) increased their chemotactic responsiveness to FP (Fig. 10,B). These cells behaved similarly to PIPLC-treated control PMN (Fig. 10,A).

Discussion

Results presented here indicate that PMN possess a population of FPR that is PI-linked. It is unlikely that the [^{125}I]-labeled material released by PIPLC is not PMN FPR. [^{125}I]-FP bound to PMN in a specific, saturable (Fig. 1) and displaceable fashion (i.e., characteristics of receptor mediated ligand binding) (13). Crosslinking of PMN with [^{125}I]-FP and EGS resulted in labeling of a broad band (Mr 58-62,000) (18, 22), a process competed by the presence of 5-fold excess unlabeled FP (Fig. 2). Furthermore, digestion of octylglucoside-extracted [^{125}I]-FPR with N-glycanase reduced its Mr to 32,000, indicating that PMN FPR contains an asparagine-linked oligosaccharide chain(s), as previously described (23).

Release of [^{125}I]-FPR from PMN by PIPLC was not due to either cell death or microvesiculation, since PMN did not release LDH into supernatants during the incubation and released receptor remained in supernatants after centrifugation at 100,000 x 90 min. Further evidence that released [^{125}I]-FPR was soluble was obtained by molecular sieve chromatography, in the absence of detergents (Fig. 8). These results indicate that PIPLC releases [^{125}I]-FPR from affinity-labeled PMN. Interestingly, labeled PMN spontaneously released [^{125}I]-FPR into supernatants in a [Ca^{2+}]-dependent fashion (Figs. 4 and 5) a process that was enhanced by stimulation of affinity-labeled cells with FP (Fig. 6). These results are similar to those reported recently for the PI-linked immunoglobulin G receptor (i.e., FcR III) on PMN (27), suggesting that shedding of FPR may play a

role in regulating PMN function. Spontaneous and stimulated release of [^{125}I]-FPR could be inhibited by chelation of [Ca^{2+}], (but not by 1, 10 phenanthroline) suggesting that PMN may contain a glycosyl-PI-PLC (2).

Intracellular FPR appeared to be more sensitive to PIPLC than surface FPR (Fig. 7). Low concentrations of PIPLC liberated more [^{125}I]-FPR from PMA-upregulated than control PMN (Fig. 7). While it is difficult to quantity exactly the amount of FPR released from PMN by PIPLC, densitometric analysis comparing supernatants and pellets of affinity-labeled control and PMA-pretreated PMN revealed that PIPLC released approximately 10% of control PMN [^{125}I]-FPR and 35-45% of PMA-pretreated PMN [^{125}I]-FPR. It is possible that some feature either on the PMN membrane or glycolipid moiety may render surface FPR more resistant to PIPLC. An alternative explanation would be that a significant proportion of the intracellular pool of FPR is expressed in the form of a PI-linked protein. Incubation of octylglucoside solubilized [^{125}I]-FPR, obtained from control and PMA-upregulated PMN, with N-glycanase resulted in a species Mr 32,000 (not shown) suggesting that the polypeptide backbone of intracellular and surface FPR is similar.

Further evidence that PIPLC releases a population of PMN FPR was obtained by equilibrium binding studies (Fig. 9, Table I). Under most conditions, treatment of PMN with PIPLC affected binding in the low affinity range (Table I). The fact that changes in binding were observed (Fig. 9) without major changes in [K_D] (Table I) argues against a nonspecific effect of the enzyme. These results, however, can not be strictly compared with those using affinity-labeled PMN. First, incubation of PMN at 37^{o}C (in the absence of prior treatment with EGS) results in upregulation of receptors (Fig. 9,B). Under these conditions, treatment

of PMN with PIPLC resulted in a diminution of [^{125}I]-FP binding (15% in the high affinity end and 25% in the low affinity end). Second, it appears that treatment of PMN with PMA (in the absence of EGS) results in down-regulation of a population of receptors (Fig. 9,C) (Table I). This phenomenon is quite apparent at the high affinity end but it may occur at the low affinity end and be obscured. This phenomenon probably is not a significant factor after treatment of PMN with EGS. However, if one attempts to immobilize expressed receptors by using cytochalasin B (Fig. 9,D) (Table I) it appears that PIPLC can release a population of high and low affinity receptors from PMN. Interestingly, the number of high affinity receptors left after PIPLC treatment (using cytochalasin B and PMA) was similar to the observed at 4^{o}C (Table I). These results support the notion that upregulated receptors are more sensitive to PIPLC than native surface receptors.

Functional studies revealed that treatment of control PMN with PIPLC increased their chemotactic responsiveness to FP (Fig. 10,A). PMA-pretreatment resulted in diminished PMN chemotactic responses to FP (Fig. 10,B). However, incubation of PMA-pretreated PMN with PIPLC resulted in restoration of their chemotactic responsiveness to levels similar to those observed using PIPLC treated control PMN (Fig. 10,A and B). Thus, it appears that one function of PIPLC-sensitive FPR may be to prevent early deactivation of PMN chemotactic responses to formyl peptides. Since it appears that PI-linked proteins have increased lateral mobility than transmembrane proteins (28), clustering of PI-linked FPR to the front of the migrating cell may effectively decrease the concentration of stimulus available for binding of formyl peptide to transmembrane receptors. This possibility may account for the effect of PIPLC on FP-induced migration of PMA-treated PMN in spite of higher receptor

number. Such process may allow the PMN to migrate along higher gradients of stimulus (i.e., concentrations that would otherwise result in deactivation). Another explanation for the effect of PIPLC on PMN chemotactic responsiveness is that the enzyme may liberate other surface molecules that result in increased adhesiveness of the cell to surfaces. Regulation of surface density of PI-linked FPR (and perhaps other PI-linked proteins) by a PMN phospholipase may be an effective mean of modulating their chemotactic responsiveness to formyl peptide and of localizing the cell to sites of acute inflammation

Summary

While investigating the linkage of formyl peptide receptors (FPR) to PMN plasma membrane, we found that PMN contain a population of FPR that can be released upon treatment with phosphatidylinositol-specific phospholipase C (PIPLC). PMN FPR was affinity-labeled, specifically, by incubating (4^oC, 30 min) cells (10^7/ml) with 20 pmol of [^{125}I]-N-formyl-Nleu-Leu-Phe-Tyr ([^{125}I]-FP) and EGS (0.15 mg/ml). Incubation of affinity-labeled PMN with PIPLC resulted in the release of soluble [^{125}I]-FPR into supernatants, in a concentration-dependent fashion. Release of [^{125}I]-FPR by PIPLC was unaffected by either the presence of protease inhibitors or chelation of extracellular Ca^{2+}. Treatment of PMN with the phorbol ester PMA resulted in increased [^{125}I]-FP binding. At low concentrations, PIPLC released more [^{125}I]-FPR from PMA-upregulated PMN than from control PMN. Equilibrium binding studies demonstrated that treatment of PMN with PIPLC resulted in a diminution of [^{125}I]-FP binding to cells.

Incubation of affinity-labeled PMN, in the absence of PIPLC, revealed that these cells spontaneously released [^{125}I]-FPR in a reaction requiring

extracellular Ca^{2+}(perhaps due the presence of endogenous glycan-PI-PLC). Spontaneous release of ^{125}I-FPR could be enhanced by stimulation with formyl peptide.

Chemotactic studies suggested that one function of PIPLC-sensitive [^{125}I]-FPR may be to prevent early deactivation of PMN in response to formyl peptides, a function that could be regulated by a PMN PI-specific phospholipase.

TABLE 1.

Binding of [125I]-FP to PMN. [a]

Experiment Condition.	High Affinity		Low Affinity.	
	Sites	K_D [nM]	Sites	K_D [nM]
4°C	30,800	0.2	115,151	4.2
4°C, PIPLC	31,400	0.18	116,753	4.6
37°C	84,700	0.6	344,000	5.2
37°C, PIPLC	73,829	0.48	262,007	3.0
PMA	27,911	0.38	404,857	9.5
PMA, PIPLC	41,711	0.39	314,448	4.7
CYTO B + PMA	83,042	0.7	608,440	7.5
CYTO B + PMA, PIPLC	47,635	0.4	463,005	6.0

[a] One of three experiments using PMN from different donors.Results are calculated from experiments presented in Figure 9.

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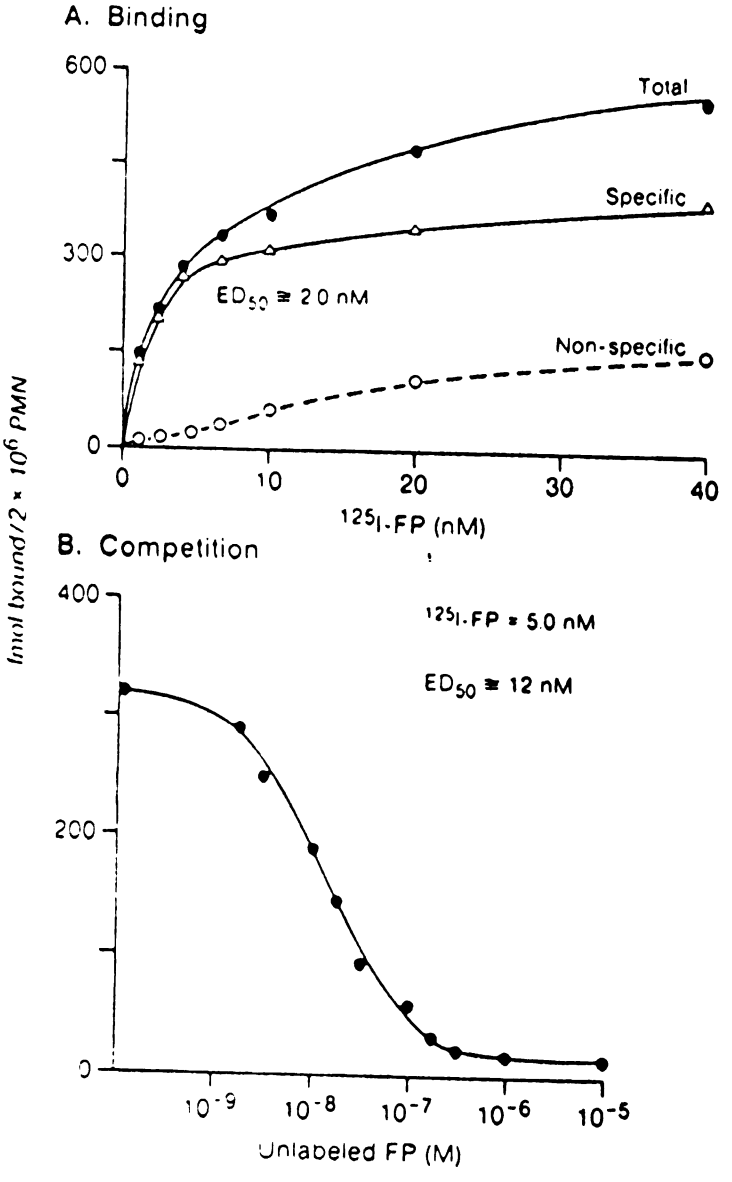

Figure 1

A. Binding of ^{125}I-FP to PMN. Cells (2 x 10^6/ml) were incubated with increasing concentrations of ^{125}I-FP at 4°C for 15 min (to achieve equilibrium binding), in the presence and absence of 1000-fold excess unlabeled FP. Reactions were terminated by centrifugation through silicon oil. B. Competition of binding of ^{125}I-FP (5.0 nM) by increasing concentrations of unlabeled FP. Reactions were carried out as described for panel A.

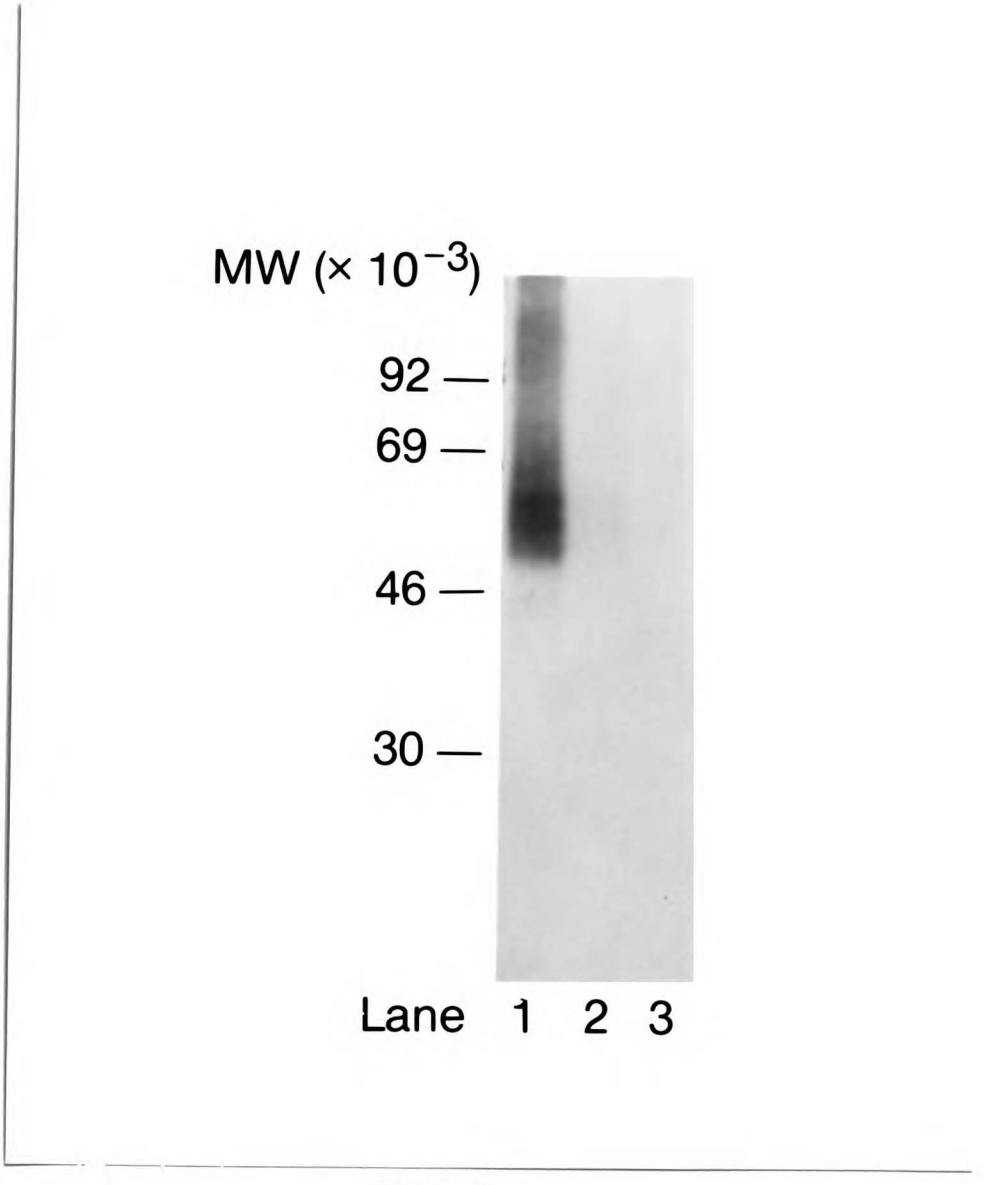

Figure 2

Autoradiogram of affinity labeled PMN FPR. PMN FPR was labeled as described in *Methods and Materials* and solubilized with 2.0% (w/v) octylglucoside in PBS.Solubilized FPR preparations were subjected to SDS-PAGE (7-17% slab gel) and analyzed by autoradiography. Lane 1: affinity labeled PMN FPR; Lanes 2 and 3: identical reactions performed in the presence of 5 and 10-fold excess unlabeled FP, respectively, to demonstrate specificity of crosslinking.

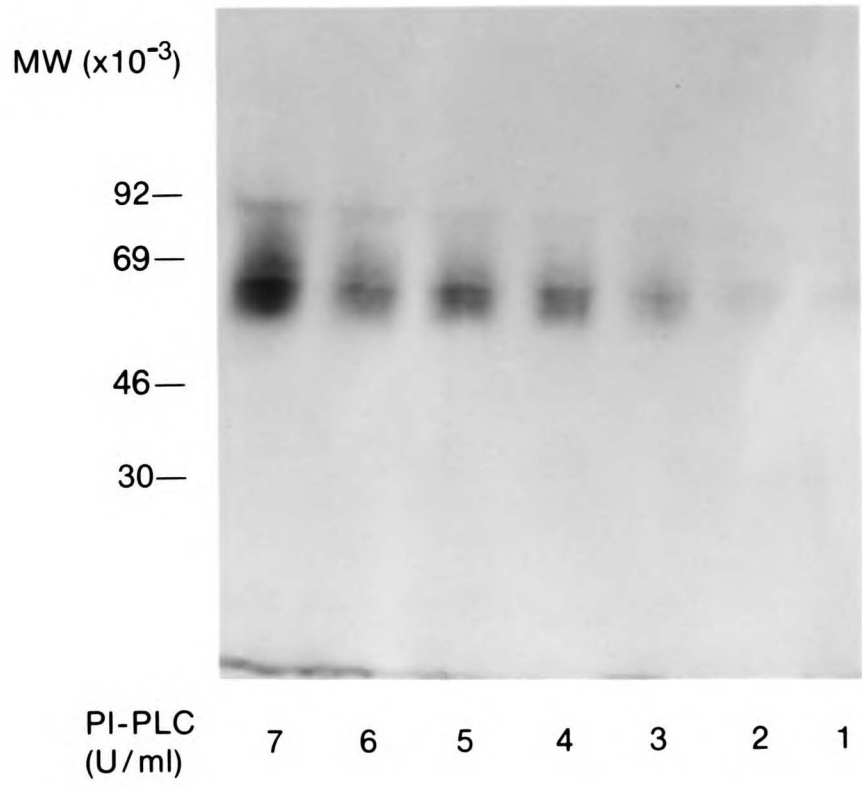

Figure 3

Autoradiogram depicting cleavage of ^{125}I-FPR from affinity labeled PMN by PIPLC. Affinity labeled PMN (2 x 10^8/ml) were incubated for 15 min at 37°C in the presence of increasing concentrations of PIPLC. At the end of incubation, cells were pelleted by centrifugation, supernatants processed as described in *Methods and Materials,* and subjected to SDS-PAGE and autoradiography.

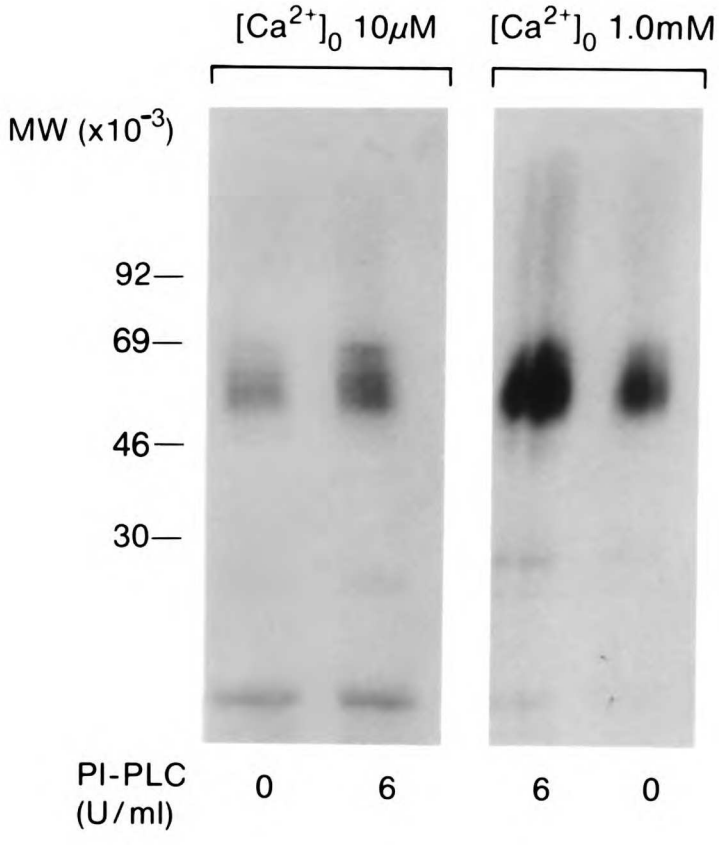

Figure 4

Autoradiogram demonstrating the effect of extracellular Ca^{2+} on spontaneous and PIPLC-mediated release of ^{125}I-FPR from affinity labeled PMN. Affinity labeled PMN (2 x 10^8/ml) were suspended in buffer containing either 10 uM Ca^{2+} or 1.0 mM Ca^{2+}, and incubated for 30 min at 37°C in the presence and absence of PIPLC (6.0 U/ml). At the end of incubation, cells were pelleted and supernatants analyzed by SDS-PAGE and autoradiography.

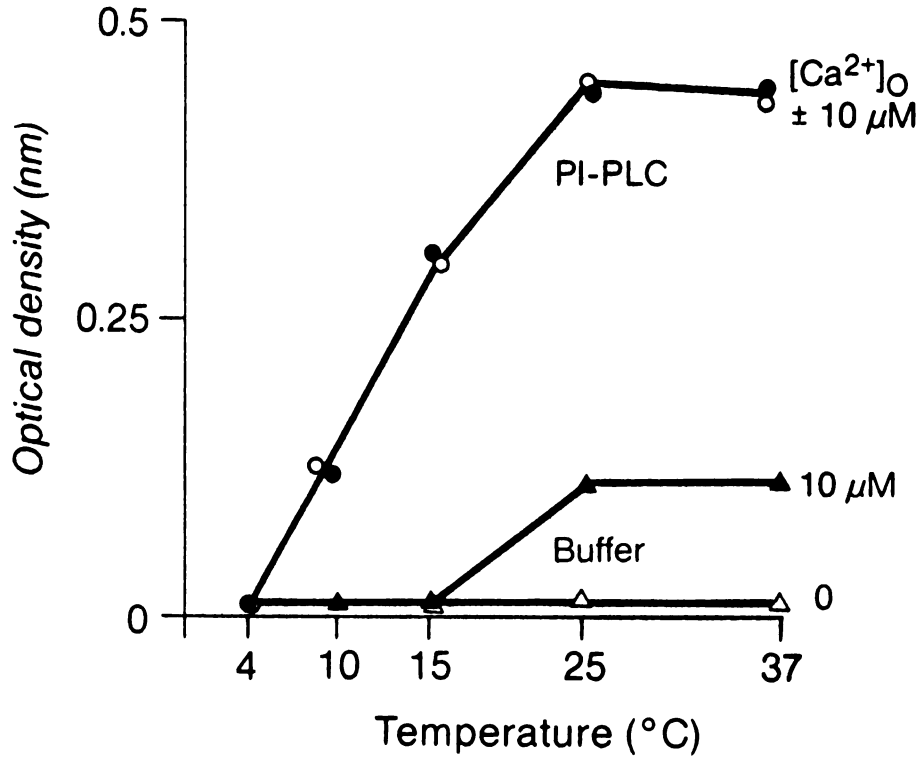

Figure 5

Densitometric analysis of the effect of temperature and micromolar concentration of extracellular Ca^{2+} on spontaneous and PIPLC-mediated release of ^{125}I-FPR from affinity labeled PMN. Affinity labeled PMN (2 x 10^8/ml) were suspended in buffer containing either 10 uM Ca^{2+} or 10 uM Ca^{2+} plus 1.0 mM EDTA, and incubated at varying temperatures (4-37°C) for 15 min in the presence and absence of PIPLC (6.0 U/ml). At the end of incubation supernatants were processed as described in *Methods and Materials*, subjected to SDS-PAGE and autoradiography, and autoradiograms analyzed by soft laser densitometry. PIPLC release of ^{125}I-FPR in the presence of either 10 uM [Ca^{2+}] (minus spontaneous release in 10 uM [Ca^{2+}](open circles) or 10 uM[Ca^{2+}] plus 1.0 mM EDTA (closed circles). Spontaneous release of ^{125}I-FPR by PMN incubated in the presence of either 10 uM [Ca^{2+}](closed triangles) or 10 uM [Ca^{2+}] plus 1.0 mM EDTA (open triangles).

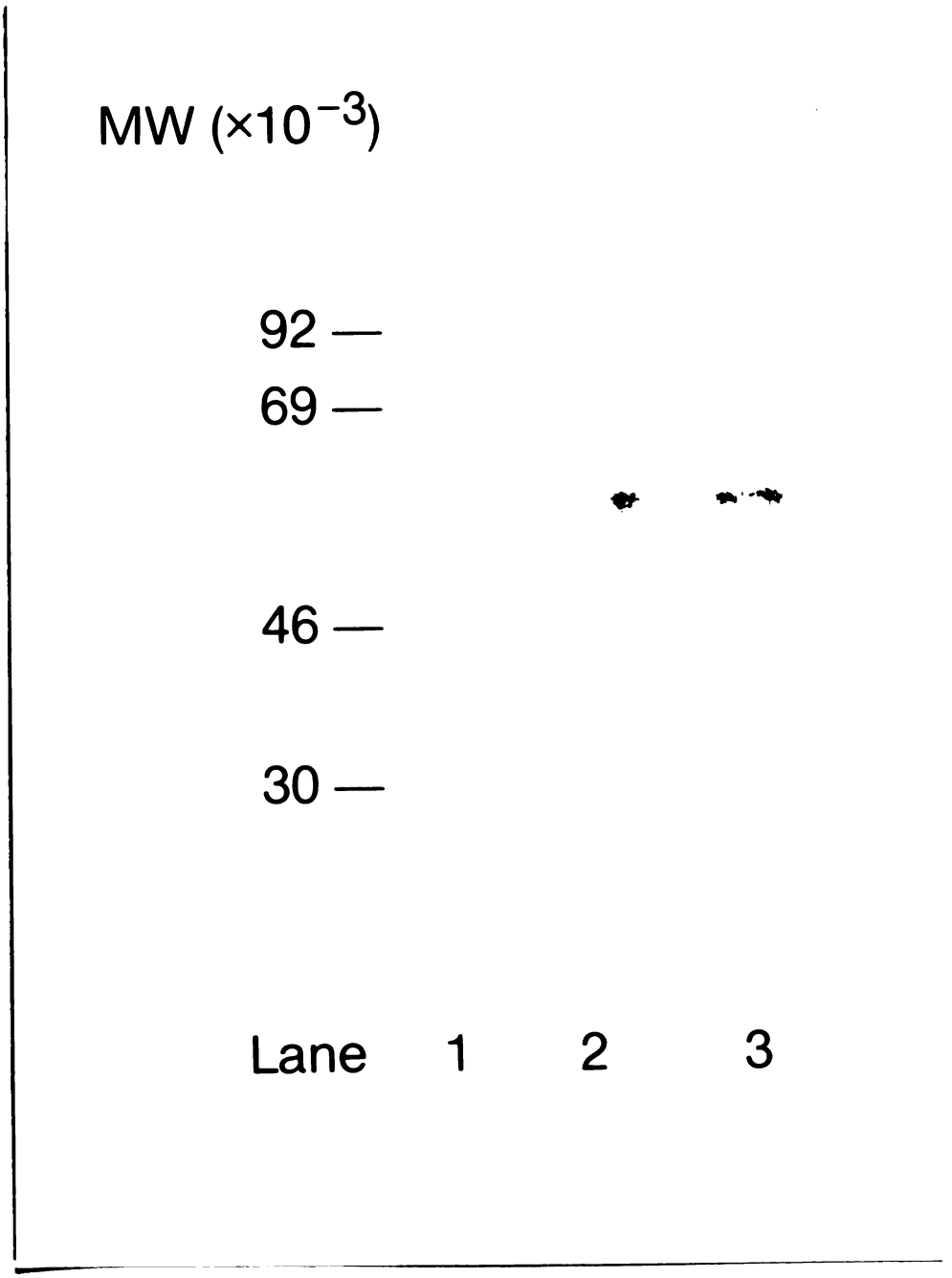

Figure 6

Effect of FP on the release of ^{125}I-FP by affinity labeled PMN, in the absence of exogenous PIPLC. Affinity labeled PMN were incubated for 10 min at 37°C in 0.01 mM [Ca^{2+}] buffer in the absence or presence of FP. After incubation, cells were pelleted by centrifugation and supernatants processed as described in *Methods and Materials*. Lane 1: buffer; Lane 2: buffer + 10^{-10}M FP; Lane 3: buffer + 10^{-9}M FP

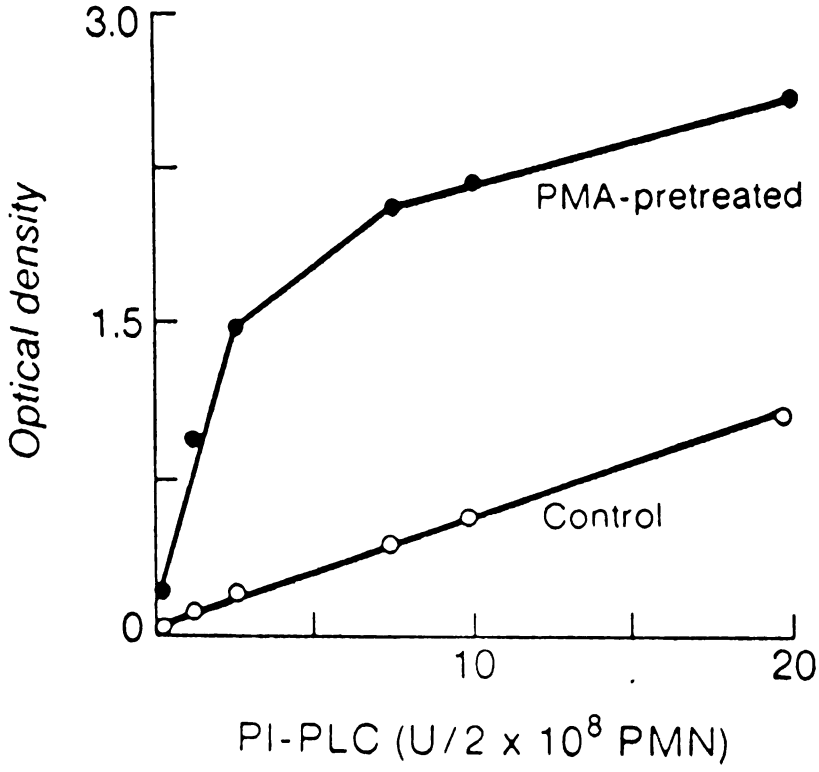

Figure 7

Effect of PMA-pretreatment on the release of ^{125}I-FPR from PMN by PIPLC.

PMN were incubated for 5 min at 37°C in the presence of either buffer or buffer plus PMA (10 ng/ml), as described in Methods. At the end of incubation, PMN were washed and FPR was affinity labeled as described. Following labeling, PMN were washed, resuspended in 0.01 mM [Ca^{2+}] buffer and incubated for 15 min at 37°C in the presence of increasing concentrations of PIPLC. After incubation, cells were pelleted by centrifugation, and supernatants processed as described in legend to Figure 5.

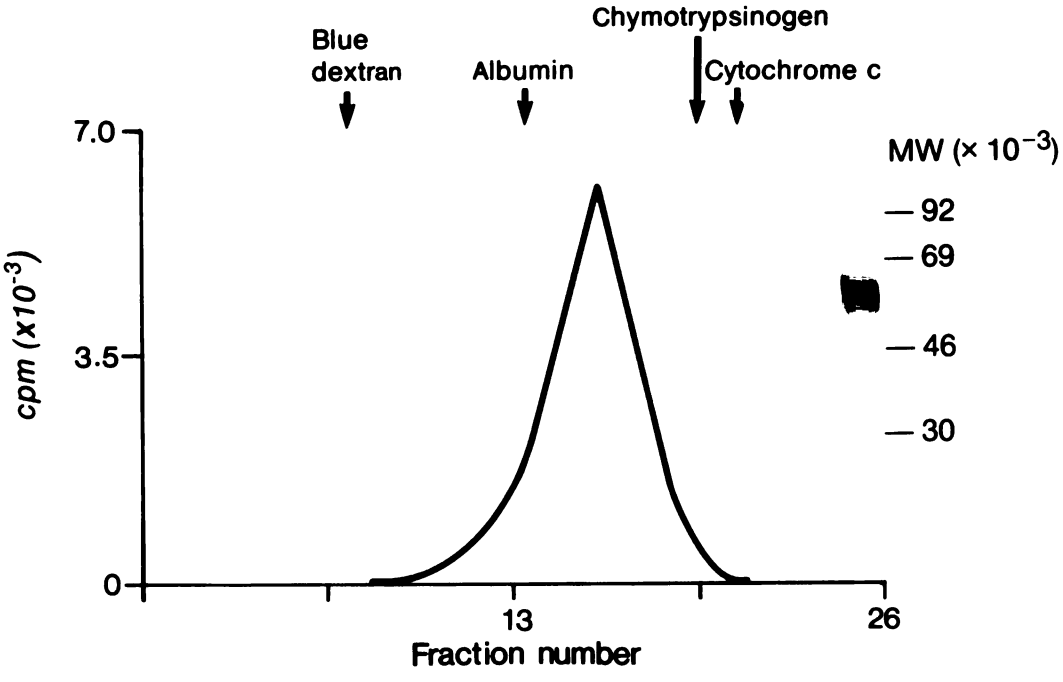

Figure 8

Molecular sieve chromatography of ^{125}I-FPR released from affinity labeled PMN by PIPLC. Affinity labeled PMN (2 x 10^8/ml) were incubated at 37°C for 15 min with PIPLC (6.0 U/ml) in 0.01 mM [Ca^{2+}] buffer. At the end of incubation cells were pelleted by centrifugation and supernatants lyophilized as described in *Methods and Materials*. Lyophilized supernatants were resuspended in 0.1 ml of PBS and applied to a 1 x 20 cm column of AcA 44 that had been preequilibrated with PBS. Elution was performed with PBS. Fractions (0.5 ml) were collected and radioactivity determined. Fractions containing peak radioactivity were dialyzed against 0.1 M NaAc pH 6.5, lyophilized, resuspended in SDS sample buffer and subjected to SDS-PAGE and autoradiography. Insert: autoradiogram of material obtained from peak fractions.

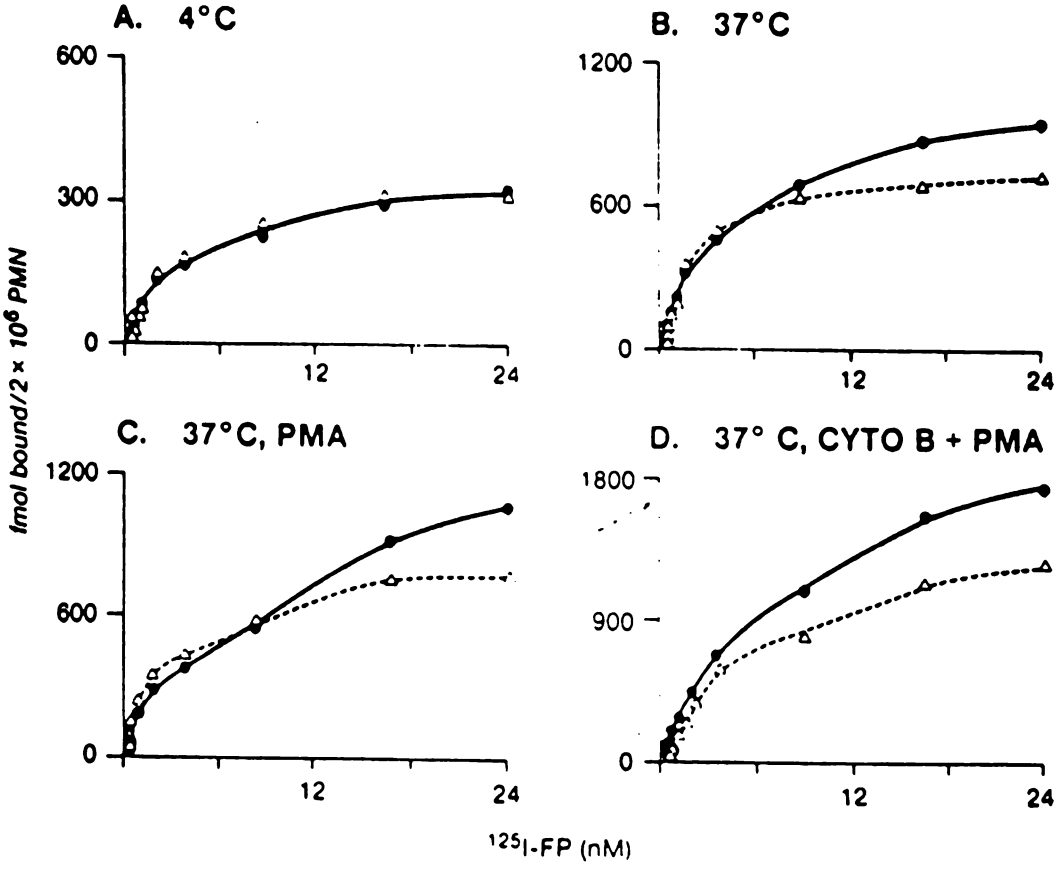

Figure 9

Effect of PIPLC on binding of ^{125}I-FP to PMN. Cells (2.0 x 10^6/ml) were incubated with increasing concentrations of ^{125}I-FP at 4°C for 15 min in the presence and absence of 100-fold excess unlabeled FP. A. Specific binding of ^{125}I-FP to PMN after incubation for 15 min at 4°C in the presence (▲) and absence (●) of PIPLC. B. Specific binding of ^{125}I-FP to PMN after incubation for 15 min at 37°C in the presence (▲) and absence (●) of PIPLC. C. Specific binding of ^{125}I-FP to PMA-treated PMN after incubation for 15 min at 37°C in the presence (▲) and absence (●) of PIPLC. D. Specific binding of ^{125}I-FP to cytochalasin B-PMA-treated PMN after incubation for 15 min at 37°C in the presence (▲) and absence (●) of PIPLC. One of three experiments with similar results.

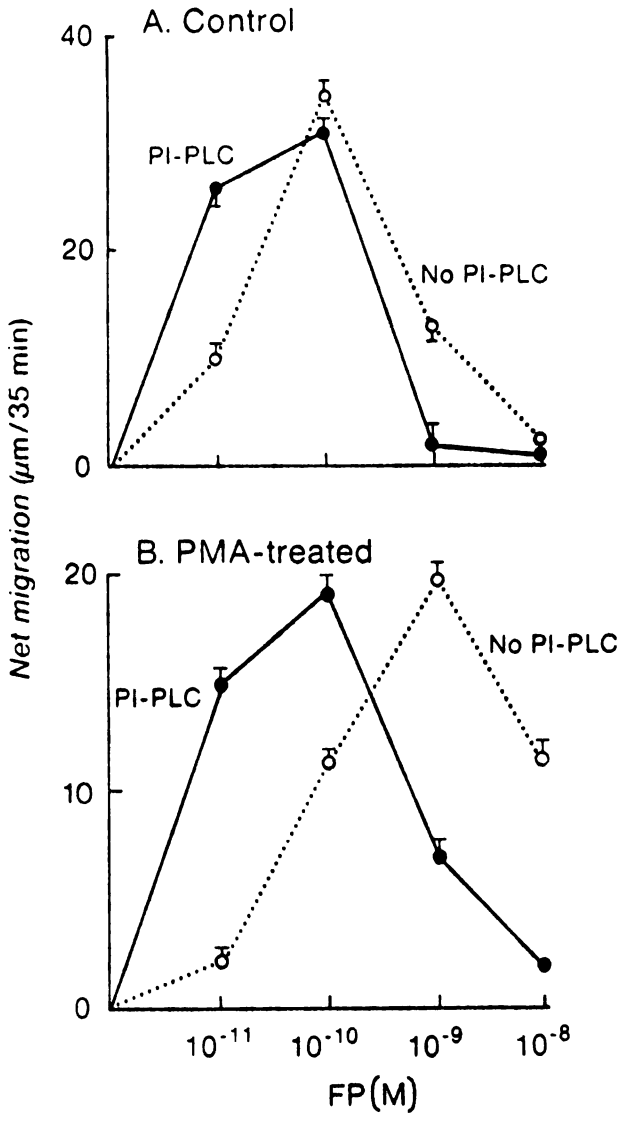

Figure 10

Effect of PIPLC on the chemotactic responsiveness of control and PMA-pretreated PMN. A. Control PMN (closed circles) PIPLC (6.0 U/ml) treated, (open circles) no PIPLC. B. PMA-pretreated PMN (closed circles) PIPLC treated, (open circles) no PIPLC.

CHAPTER VII

EPILOG

This thesis examined two testable hypotheses. First, that PMN contain a population of formyl-peptide receptors that are recycled on stimulation, (binding) by the peptide ligand. Second, that there exist a heterogeneous population of receptors, some of which are anchored in the membrane by phosphatidylinositol: such receptors may modulate FMLP-induced functions upon stimulation by formyl peptides, namely chemotactic and possibly lysosomal enzyme release and superoxide anion generation.

There have been many studies on the modulation of PMN chemotaxis, and it is well recognized that chemotaxis is initiated by the binding of the chemotractant to its specific membrane receptor. A flurry of events ensue, including ionic fluxes between the cell and the extracellular fluid, reorientation of actin fibers in the cell, release of lysosomal enzymes and the generation of reactive oxygen radicals. Ultimately, chemotaxis occurs and PMN migrate along an increasing concentration gradient of chemotractant peptide. This directed migration is dependent on the cells ability to 'sense' this concentration gradient, a function that is contingent on the presence of receptors on the cell surface to sense the chemotractant (i.e. FMLP). Thus, it is appears necessary that for chemotaxis to occur a continued expression of receptors must take place.

The continued expression of surface receptors that were internalized following binding to ligands may occur by several different processes. First, cells may be metabolically very active, so as to continually synthesize receptor, either during periods of stimulation as well as periods of non-stimulation. Second, in cells which are short lived, and with limited metabolic activity continued expression of surface receptors may occur because of a large pool of intracellular stored receptor. Finally, in cells with high metabolic activity as well as cells with low metabolic activity, continued receptor availability may occur as a result of receptor recycling. Mature PMN are cells with a short half-life and limited metabolic activity. Protein synthesis is limited in the mature PMN and indeed inhibition of protein synthesis does not inhibit chemotaxis in the mature PMN. Continued expression of surface FMLP receptors in PMN during the process of chemotaxis may thus occur either by continued availability of a 'stored receptors' or by recycling of receptors. This paper presents evidence that FMLP receptors are recycled during FMLP-induced PMN chemotaxis. Evidence is presented based on studies of specific inhibition to FMLP-induced chemotaxis and studies on surface digestion of covalent affinity linked ligand-receptor complexes.

Specific inhibition of FMLP-induced PMN chemotaxis occurs when surface glycoproteins are altered either by removal of surface sialic acid residues with neuraminidase or by the binding of N-acetyl glucosamine residues by the non-agglutinating derivative of the lectin wheat germ agglutinin (WGA-D). Experiments in chapters III and IV demonstrate that changes in receptor modulation occurs at two levels; first, there is a decrease in the number of receptor expressed at the cell surface,and second, that an accumulation of FMLP, (presumably bound to its receptor) occurs in a compartment of similar

density and enzyme activity (galactosyl-transferase) as the light Golgi compartment. It should be emphasized that manipulation of sugars in no way blocks binding of the ligand to its receptor, rather that decrease receptor is expressed at the cell surface, hence leading to the speculation that intact receptor glycosalation may be necessary for the ligand-receptor to stimulate PMN chemotaxis.

The exact role of the Golgi in the processing of FMLP receptors is uncertain at this time, although other receptor systems, namely the insulin, and transferrin receptors , also involve the Golgi as part of their recycling pathway (1,2). It has been postulated from studies with these receptors systems that recycling through the Golgi system may allow for post-transitional repair of receptors, or refurbishing of receptors in response to physiological stimuli (3,4). Indeed the alteration of the receptor glycosalation, may hinder their transport through the Golgi,leading to the accumulation of receptor within the Golgi. It is interesting to speculate that unlike other proteins, the migration of the FMLP-receptor within the PMN, more specifically may be receptor mediated, i.e. Golgi may possess binding sites (or receptors) for the FMLP-receptor.

It is important to note that while advances with other receptor systems using chemicals such as monensin, and chloroquine (5,6), (which alter the uncoupling of receptor-ligand complexes), have yeilded important information on the recycling modulation, absolute proof of receptor recycling is contingent on.the isolation and characterization of the receptor and the development of a monoclonal antibodies to the receptor. Through advanced techniques such as iterative fractionation, and mathematical models utilizing fluorescent-labeled

antibodies to receptors great strides have been made in our understanding of cell sorting of other receptors systems (7).

A more direct demonstration of FMLP recycling was performed by papain cleavage of affinity linked N-formyl peptide-receptor complexes (chapter V). From these experiments, a estimate of the recycling half life was determined, which was similar to that reported for the transferrin receptor. The above set of experiments also yielded information with regards to the limited role of intracellular calcium in formyl-peptide chemotaxis and about the possible role of protein kinase c in the transduction of events in chemotaxis. Of importance in this set of experiments was the implication of protein kinase c in FMLP-induced PMN chemotaxis, implying that upregulation of receptors (by treatment of cells with PMA) is imperative for the recycling of affinity labelled receptors. Also of interest was the observation that receptor-ligand dissociation was not necessary for recycling of receptors. It would be of interest to perform these experiments with a monoclonal antibody against the receptor once available.

It should be evident from chapter V, and form previous chapters that it is highly likely that a heterogeneous population of FMLP receptors exist. Studies presented in chapter VI of this thesis indicate that a subset of peptide receptor are anchored in the cell plasma membrane by phosphatidylinositol (PI). These receptors when treated with phosphatidylinositol-specific phospholipase C, were cleaved off.and a decrease in chemotaxis was observed as well as a decrease in enzyme release was noted.(not shown in this dissertation) It is uncertain what role the PI anchored receptors play *in vivo*.These experiments provide an impetus for further research on the anchoring of proteins in the

plasma membrane and the variation of signal transduction as a result of protein membrane configuration.

Recent speculation that a subpopulation of FMLP receptors may contain multiple trans-membrane domains, is further evidence that the FMLP receptor is heterogeneous. What has yet to be determined beyond doubt is whether the PI-linked receptor is indeed a receptor vs a binding site. In experiments performed in chapters V and VI evidence is presented that the covalent-linked FMLP receptor (FPR), does indeed demonstrate characteristics of a receptor rather than a binding site. It is thus of paramount importance that the FMLP receptor be isolated in order to answer some of these pressing questions. Speculation that a receptor(s) with a heterogeneous structure and function may not be unreasonable.

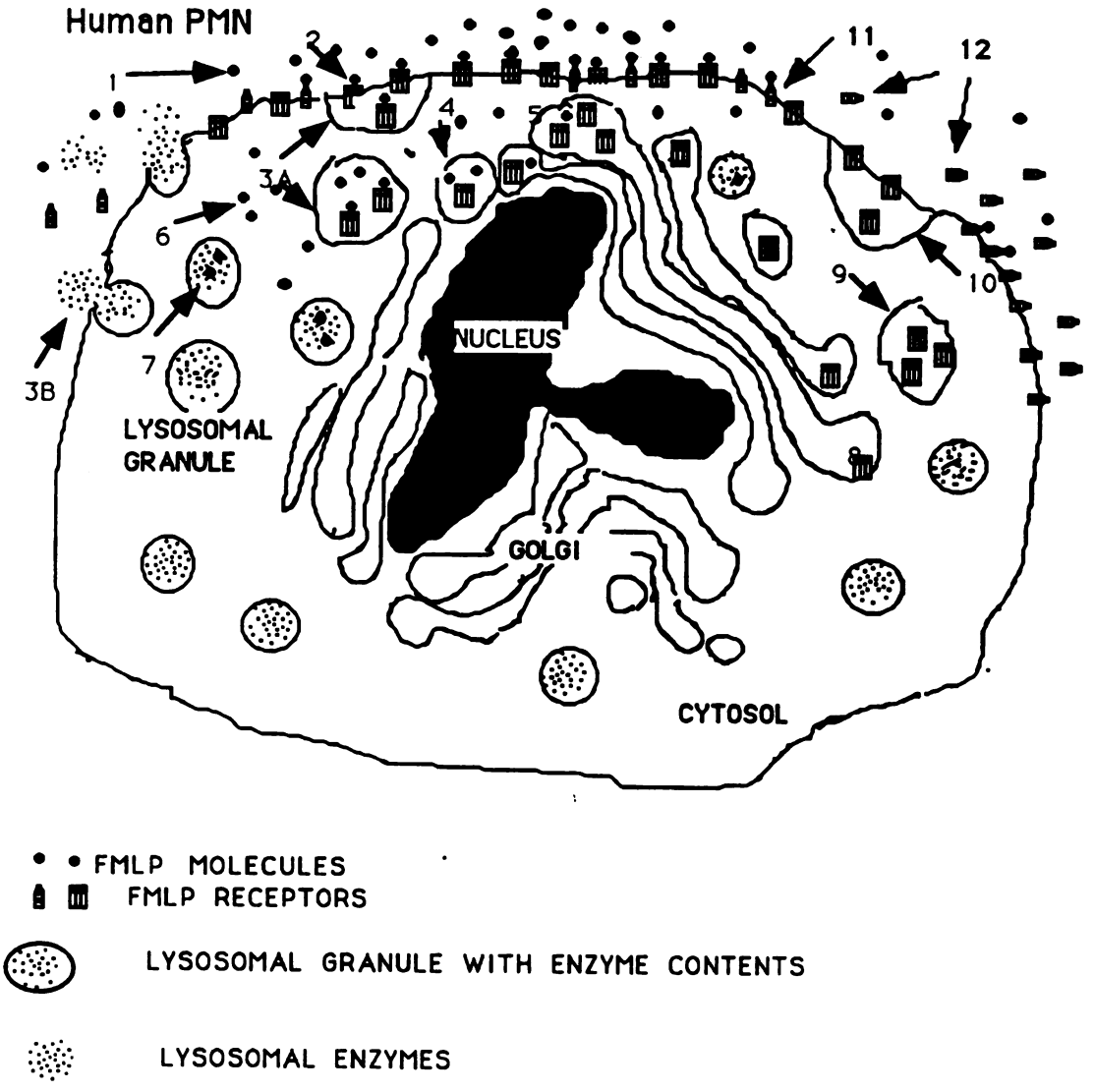

Figure 1.

Formyl-peptide receptor recycling and processing by human PMN. Free FMLP (1), binds to specific receptors on the PMN plasma membrane (2). Following binding of the ligand to its receptor, enzyme release occurs and receptor-ligand complexes are internalized(3A,3B). Receptor-ligand complexes are transported to the Golgi apparatus 940, where they are segregated to the light Golgi compartment (5). Dissociation of receptor-ligand complexes probably occurs in a pre-Golgi or light Golgi compartment, with free FMLP found in the cytoplasm and to a lesser extent in the lysosomal granules.(6,7). FMLP receptors are probably refurbished in the Golgi and recycled back to the plasma membrane (8,9,10). Manipulation of terminal sugar residues with a derivative of wheat germ agglutinin,of with Neuraminidase, causes an accumulation of FMLP receptors within the Golgi, or pre-Golgi compartment. It appears that FMLP receptors that recycle are primarily involved in and modulate chemotaxis. Some receptors (possibly formyl-peptide binding sites), appear to be anchored in the membrane by phosphatidlyinositol,the latter may be cleaved by treatment with phosphatidylinositol-specific phospholipase C (11,12). *(Numbers in brackets refer to notations in diagram and not to bibliography).*

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