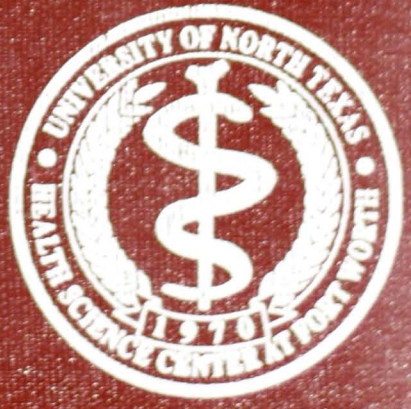
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The cDNA for human interleukin-8 (IL-8) was subcloned from a bacterial source into the eukaryotic baculoviral vector expression system. Recombinant human IL-8 (rhIL-8) was synthesized and secreted from *SF*9 cells following infection of a recombinant virus harboring the full-length IL-8 structural gene. Recombinant human interleukin-8 was purified (>600 fold) to homogeneity using preparative HPLC. The rhIL-8 preparation retained all of the physical, immunological, and biochemical properties of the natural product (monocyte-derived IL-8). Baculovirus vector expression coupled to preparative HPLC proved to be a very efficient method for large-scale recombinant interleukin production.

Biochemical mechanisms that mediate IL-8 receptor-stimulated activities are poorly understood. In this study, I have explored the intracellular mechanism(s) induced by IL-8 in differentiated HL-60 cells. IL-8 induced a rapid and transient activation of phospholipase A_2 in differentiated HL-60 cells. A consequence of phospholipase A_2 activation was the release of arachidonic acid and the generation of lysophospholipids from membrane phospholipids. The IL-8 stimulated-arachidonic acid release was pertussis toxin and phospholipase A_2 inhibitor sensitive, and protein kinase C independent. In contrast to another neutrophil chemotactic factor, fMLP, IL-8 did not stimulate the activation of phospholipase C and phospholipase D. When comparing the phosphorylation events induced by IL-8 and fMLP, I found that these two chemotactic factors triggered different protein phosphorylation profiles. Tyrosine phosphorylation of proteins was not detected following IL-8 stimulation in HL-60 cells. However, IL-8 stimulated the rapid

autophosphorylation of calcium/calmodulin-dependent protein kinase II (CaM kinase II). These results strongly suggest that the IL-8 receptor is closely coupled to the activation of PLA_2 and that CaM kinase II is an integral component of IL-8 receptor signal pathway.

INTERLEUKIN-8: BACULOVIRUS EXPRESSION AND THE RECEPTOR SIGNAL TRANSDUCTION PATHWAY

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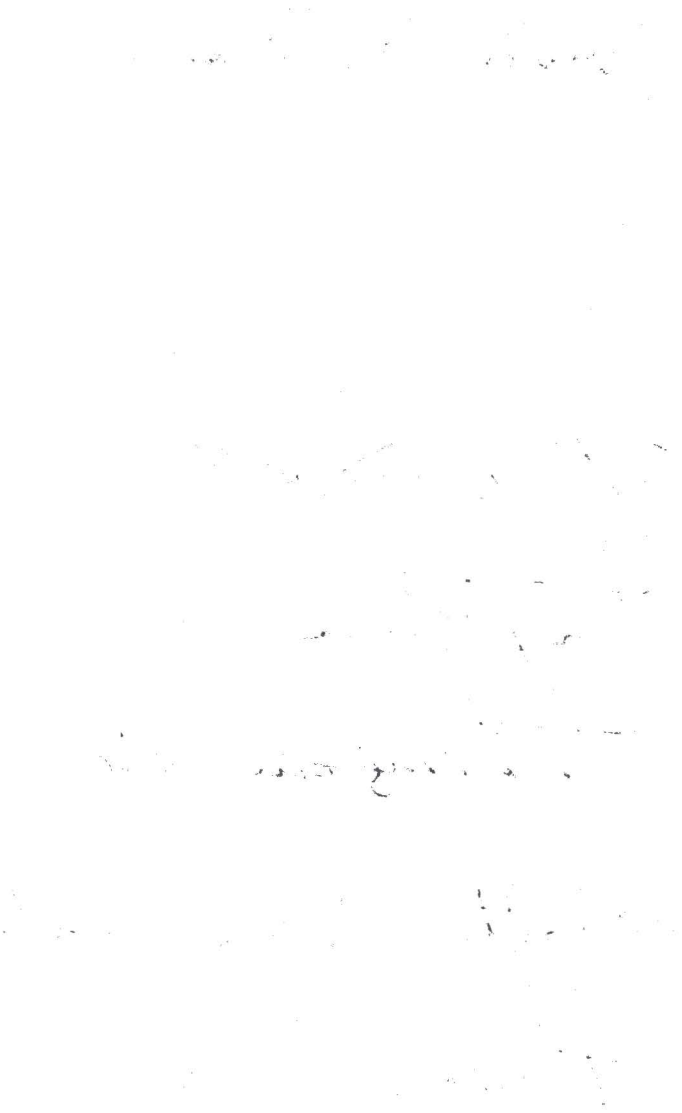

INTERLEUKIN-8: BACULOVIRUS EXPRESSION AND THE RECEPTOR SIGNAL TRANSDUCTION PATHWAY

DISSERTATION

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ABBREVIATIONS

AA	arachidonic acid
AcNPV	*Autographa californica* nuclear polyhedrosis virus
Ap	Ampicillin
ATP	Adenosine 5'-triphosphate
BSA	bovine serum albumin
CaM kinase II	Ca^{2+}/calmodulin-dependent protein kinase II
CHO	chinese hamster ovary
CIAP	calf intestine alkaline phosphatase
DAG	diacylglycerol
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
ECV	extracellular virus particles
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
fMLP	N-formyl-Met-Leu-Phe
HBSS	Hank's Balanced Salts Solution
PEPES	N-(2-hydroxyetheyl)piperazine-N'-2-ethane-sulfonic acid
HETE	hydroxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
IL-8	interleukin 8
IL-8R1	interleukin 8 receptor 1

IL-8R2	interleukin 8 receptor 2
IP_1	inositol-1-monophosphate
IP_2	inositol-1,4-bisphosphate
IP_3	inositol-1,4,5-triphosphate
IP	inositol phosphate
LTB_4	leukotriene B_4
MAP-2	microtubule associated protein-2
MLCK	myosin light chain kinase
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PEt	phosphatidylethanol
PI	phosphatidylinositol
PIP	phosphatidylinositol-1,4-bisphosphate
PIP_2	phosphatidylinositol-1,4,5-triphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol myristate acetate
rhIL-8	recombinant human interleukin 8
SDS	sodium dodecyl sulfate
SF9	*Spodoptera frugiperda* insect cell
TFA	trifluoroacetic acid
7-TMS	seven-transmembrane segment
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TPCK	L-1-tosylamino-2-phenylethylchloromethyl ketone

CHAPTER I

INTRODUCTION

Chemotactically Activated Human Neutrophils and HL-60 cells-In higher eukaryotes, such as mammals, communication between cells of different origins is essential. This intercellular communication mediates the complex systemically controlled biological responses. Intercellular communication between cells having different origins and functions is a frequently occurring event in the human immune system. Hematopoiesis, cell and humoral immunity, immune surveillance functions, inflammatory response, delayed hypersensitivity , chemotaxis and other immune processes such as phagocytosis, helper, suppressor, or killer cell functions, often involve mechanisms which require extensive intercellular communication. The mechanisms by which human immune cells communicate are as diverse as the processes which they propagate. However, one common recurrent theme characterizes each of these communicative processes. This shared mechanism involves the synthesis, secretion, and cellular recognition of functionally relevant immune cytokines or interleukins as they are now called. Human interleukins serve an essential functional role in the initiation of complex intercellular communication processes during cell mediated immune response and immune surveillance. This type of communication is frequently observed in cells of either myeloid or lymphoid lineage. The cytokines operative in the human immune system have been divided into several groups. The groupings are usually based upon cytokine function. Examples of these functional groups include hematopoietic proliferation factors (growth factors), interferons, cell differentiation factors, cell progression factors, chemotactic factors, necrosis factors and

cytokines with pluripotential function for more than a single cell target, i.e., the interleukins. Each of these cytokines is a small soluble protein (usually less than 20 kDaltons) which mediates its biological response through the formation of a ligand-receptor complex. Although most of the immune interleukins are pleiotropic by nature, recent findings suggest that members of this group of immune cell mediators often function with some redundancy. Different interleukins share some functional specificities. These mediators often act synergistically to amplify a general biological response within target cells. This duplication of functional specificity affords the target cell the ability to control the range of its biological response, by activating and routing multiple cell surface receptor signaling into a single convergent transduction pathway. This redundancy appears to be nature's way of fine tuning a biological response within a cell for a wide range of differing cell environments. This appears to be true for immune cell chemotaxis.

Inflammation is a complicated process which involves ordered communication between several immune and non-immune cell types. This process appears to be regulated by a variety of cytokines. From a systemic point of view, the inflammatory response is characterized by the orderly recruitment of immune cells to sites of infection or tissue damage through chemotaxis. Chemotaxis is defined as the vectorial migration of cells in response to a chemical signal. The agents present at the site of inflammation which mediate this process are termed chemoattractants or chemotactic factors. Neutrophils are among the first immune cells to arrive at the site of inflammation and play a vital role in host defense by ingesting and destroying microbial and other potentially toxic agents. The participation of neutrophils at the site of inflammation is associated with induction of their cytocidal and bactericidal activities induced by different stimulus agonists, a process that is often called phagocytic cell activation. Neutrophils can be activated by the binding of individual chemotactic factors to their respective cell surface receptors (1). One obvious response of

the chemotactically stimulated neutrophil is the shape change characterized by the rapid formation of large, thin cytoplasmic lamellae. Cellular shape changes are frequently paralleled by changes in F-actin content of the cell. This suggests that the formation of lamellae during the process of chemotaxis depends upon actin polymerization (2). Chemotactic factors often induce both exocytosis and respiratory burst metabolism (3, 4). Chemotactically-induced granule exocytosis leads to the release of several stored secretory enzymes, and to remodeling of the neutrophil plasma membrane (Fig. 1). Surface densities of the complement receptors (CR1 and CR3), as well as, cytochrome b_{558}, a component of the NADPH oxidase, all are markedly enhanced through the fusion of the intracellular membrane of subcellular organelles with the plasma membrane (3). Induction of respiratory burst metabolism is a common event in chemotactically-stimulated immune phagocytes. This process results from the assembly and activation of the NADPH oxidase complex, a multicomponent flavocytochrome enzyme that catalyzes the one-electron reduction of molecular oxygen to form superoxide anion (O_2^-) (Fig. 1). This oxygen radical is the precursor for a series of microbicidal oxidants which include hydrogen peroxide and hypochlorous acid (4).

The HL-60 cell line is derived from promyelocytic leukemia cells of myeloid origin (108). These cells can be chemically-differentiated into macrophage or neutrophil phenotypes depending on the choice of the chemical differentiating agents. A variety of compounds induce monocytic/macrophage-like differentiation of HL-60 cells. Typical inducers include 1, 25 dihydroxyvitamine (vitamine D_3) and phorbol esters (TPA). HL-60 cells that are induced to differentiate by these compounds exhibit distinct "monocyte/macrophage" characteristics (108). On the other hand, several other inducers including dimethyl sulphoxide (DMSO), retinoic acid, and dibutyryl cyclic AMP induce differentiation of HL-60 cells into neutrophil-like cells. On treatment with these compounds, HL-60 cells display morphological, enzymatic, functional, and surface

membrane antigen characteristics of mature granulocytes. Receptors for chemotaxis are among the first cell surface markers to appear during the differentiation process (108). Differentiated HL-60 cells become competent to chemotaxis, superoxide production, and degranulation in response to chemotactic factor stimulation.

Several neutrophil-specific chemotactic factors have been identified and characterized in recent years. The best known include formyl-Met-Leu-Phe peptide (fMLP) (21), complement fragment C_{5a} (22), platelet-activating factor (PAF) (23), and leukotriene B_4 (LTB_4) (24). C_{5a} is formed either in the plasma or inflammatory exudates upon complement activation. fMLP is a product derived from either bacteria or mitochondria of damaged tissues. PAF and LTB_4 are products of the lipoxygenase system, released by activated neutrophils or other phagocytic cells. All chemotactic factors mentioned above initiate their biological responses in neutrophils through specific cell surface receptors. These responses include the activation of the motility system, chemotaxis, exocytosis, and respiratory burst metabolism. The majority of the receptors for human neutrophil chemotaxis have been well-characterized by molecular cloning. They include: two isoforms of fMLP receptors (25, 26), which differ only by two amino acids, the human PAF receptor, and the human C_{5a} receptor (27, 28). All chemotactic receptors that have been cloned code for proteins containing seven hydrophobic membrane spanning sequences that alternate with eight hydrophilic stretches. The hydrophobic domains each consisting of 21 to 25 amino acids, have predicted α-helical conformations, and are sufficiently large to span the plasma membrane. These features suggest that the chemotactic receptors may belong to the G protein-coupled seven-transmembrane-segment (7-TMS) receptor superfamily (1).

Structure and Function of Interleukin-8-Interleukin-8 (IL-8), a newly identified chemotactic factor, was identified and characterized independently by three laboratories in

1987 (5, 6, 7). This cytokine was first named Monocyte Derived Neutrophil Chemotactic Factor (MDNCF) and later renamed interleukin-8 because it was shown to be synthesized in several different cell types and to induce chemotaxis in more than one cell type (8). IL-8 is the only human interleukin which demonstrates chemotactic properties. Cell types that synthesize and secrete IL-8 include monocytes, macrophages, lymphocytes, endothelial cells, fibroblasts, and neutrophils (8). IL-8 has been shown to be synthesized in response to a variety of different stimuli (8). Two most common stimuli include interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). Monocytes, macrophages, and endothelial cells express IL-8 mRNA in response to lipopolysacharrides (LPS) and several other inflammatory agents (9).

The IL-8 gene has nucleotide sequence domains that are similar to those of several other cytokines. The IL-8 gene was shown to contain four exons and three introns in a structure quite similar to the genes that encode for interleukin 2, 4, 5 (IL-2, 4, 5,), interferon γ, Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), and tumor necrosis factor (TNF) (10) (Fig. 2). No sequence homology was found in the 5' flanking regions of the IL-8 gene when compared to hematopoietic cytokines IL-2, IL-3, GM-CSF, and G-CSF. In searching for possible 5' regulatory sequences, potential binding sites (consensus sequences) for several nuclear transcriptional enhancer binding proteins have been identified. These putative elements include the glucocorticoid response and the heat shock elements (11). Currently, a model of IL-8 gene regulation that proposes the necessity of cooperative interaction among *cis*-regulatory enhancer binding factors, including nuclear factor-κB to induce IL-8 gene expression in response to TNF-α or IL-1, is being explored (12).

cDNA for human IL-8 encodes a 99 amino acid precursor which is cleaved to form the mature protein. The length of the mature protein has been disputed. Polypeptide lengths from 69 to 79 amino acid have been reported (5, 13, 14). A consensus has recently

been reached on the existence of two forms: 72 and 77 amino acids in length (5). Endothelial cells predominantly release the 77 amino acid form (13). Human monocytes and lymphocytes primarily release the 72 amino acid peptide (5). Human IL-8 has been shown to contain four cysteine residues with no site for potential N-glycosylation (10). NMR spectroscopy has been useful in elucidating the secondary and tertiary structure of IL-8. The IL-8 molecule consists of a triple stranded anti-parallel β-sheet with a carboxyl terminal α-helix. The α-helix appears to lie on the β-sheet (15).

IL-8 was first identified by its ability to attract neutrophils to the site of infection. Other biological responses include the induction of lysosomal enzymes release and activation of respiratory burst metabolism (6, 16). Both the 77 and 72 amino acid forms of IL-8 strongly promote degranulation in cytochalasin-B treated neutrophils (14). One laboratory (18a) demonstrated an increase in superoxide anion production in the presence of IL-8, which is similar in onset and duration to that elicited by fMLP (17). Our laboratory and others have disputed such findings (18). IL-8 induced exocytosis leads to changes in surface proteins in human neutrophils which is mediated through the fusion of intracellular membranes with the plasma membranes. Increased expression of MAC-1 (CD11/CD18) on neutrophils was seen in response to IL-8 and may contribute to increased adhesion of neutrophils to vascular endothelial cells and may account for the margination of neutrophils to IL-8 injection sites (19). Interestingly, neutrophil adhesion to IL-1 activated human endothelial cell monolayers was inhibited both by the 72 and 77 amino acid isoforms of IL-8. The 72 amino acid form inhibited neutrophil adhesion to endothelial monolayers more efficiently (14).

Receptors for IL-8 were first identified by binding studies performed with human neutrophils. Several groups reported the existence of a single class of high-affinity receptors with K_d values between 0.2 and 4 nM, and densities of 20,000 to 90,000 sites

per cell (178, 179). At 37 ^{o}C, bound ^{125}I-labeled IL-8 is rapidly internalized (180). More than 90% of the ligand-bound receptors are endocytosed within 10 min, and the receptors are recycled as indicated by their re-expression on the cell surface even in the presence of cycloheximide (180). Differentiated HL-60 cells also display cell surface IL-8 receptors with a density of 8,000 sites per cell (181). This observation has been confirmed in our laboratory.

Two distinct human IL-8 receptors (IL-8R1 and IL-8R2) were cloned in 1991 (29, 30). Holmes *et al.* (29) isolated a cDNA coding for a 350-amino acid protein (IL-8R1) from human neutrophil cDNA library by expression screening for ^{125}I-labeled IL-8 binding in COS-7 cells. Murphy and Tiffany (30) isolated a cDNA coding for a 360-amino acid protein (IL-8R2) by screening a cDNA library from HL-60 cells with a synthetic oligonucleotide probe for the rabbit IL-8 receptor, rbF3R, and expressed the functional receptor by cDNA microinjection in *Xenopus Laevis* oocytes. The cDNAs for IL-8R1 and IL-8R2 code for polypeptides with *Mr* of approximately 40 kDa and a sequence identity of 77% (29). The IL-8 receptor structure is similar to that of fMLP. Primary cDNA sequence of the IL-8 receptor is 79 % identical to that of fMLP receptor. Like the receptor for fMLP, the IL-8 receptor contains seven putative transmembrane domains of 20-25 amino acids each with presumed α-helical structure, which are typical for G-protein-coupled receptors.

Baculoviral Expression of Recombinant Human IL-8-The biological mechanism(s) behind IL-8 receptor mediated cellular activities is poorly understood. To pursue this subject and to fully explore the underlying mechanism(s) require relatively large quantities of recombinant human IL-8 (rhIL-8). The demand for large quantities of recombinant IL-8 led our laboratory to develop a readily available source for biologically active recombinant human IL-8. This work represents my first research objective and the early focus of my dissertation.

Expression of human cDNA in a bacterial expression systems often leads to difficulties in producing recombinant proteins with the correct conformation and biological activity. Signal sequences are not removed and the required post-translational processing does not take place in the bacteria, resulting in poor or non-existing biological activity. Yeasts, such as *Saccharomyces cerevesiae*, have often been shown to be a more effective expression systems than their bacterial counterparts for recombinant mammalian protein production. Transformed yeast cells produce recombinant proteins more closely resembling to their mammalian source. However, the post-translational modifications in yeast are not identical to the natural processes occurring in mammalian cells. Yeast tends to over-glycosylate (polymannosylate) recombinant proteins. The search for more native-like recombinant proteins led to the development of mammalian expression systems. Typically these systems are based on chinese hamster ovary cell culture system (CHO). The activity of recombinant proteins produced by mammalian cell cultures is relatively high but low yields and associated difficulties in controlling and regulating culture conditions have hampered the full acceptance of this expression system.

Within the last few years, a new recombinant DNA (rDNA) technology which overcomes many of the limitations inherent in bacterial, yeast, and mammalian systems, was developed. This new system was based on the specific properties of a family of insect viruses known as "baculoviruses" first developed by Summers *et al.* (113). The baculovirus *Autographa californica nuclear polyhedrosis virus* (AcNPV), the prototype virus of the family *Baculoviridae*, has a wide host range and infects more than 30 species of *lepidopteran* insects (114). The genome of AcNPV consists of double-stranded, circular supercoiled DNA approximately 128 kilobases (kb) in length (115, 116). During AcNPV infection, two forms of viral progeny are produced : extracellular virus particles (ECV), and occluded virus particles (OV) (117). The latter are embedded in proteinaceous

viral occlusions, called polyhedra. A polyhedrin protein with a molecular weight of 29,000 is the major viral encoded structural protein of the viral occlusion which accounts for up to 50% of the total "Coomassie stainable" protein of the insect cell detected on SDS-Polyacrylamide Gels during a lytic phase of infection (118). The life cycle of the AcNPV in *SF9* cells is summarized in Fig 3. Viral particles enter the cell by endocytosis or fusion, and the virus DNA is uncoated at the nuclear pore or in the nucleus. DNA replication occur at about 6 hours post-infection and by 10 hours post-infection extracellular virus is released from the cells by budding. Polyhedrin protein can be detected by 12 hours post-infection but viral occlusions are not detected until 18-24 hours post-infection. Extracellular virus levels reach a maximum between 36-48 hours post-infection but the polyhedrin proteins continue to accumulate for 4-5 days until the infected cells lyse.

This new Baculovirus Expression System (BES) has several advantages over previously used expression systems. First, the environment for recombinant protein production, the baculovirus provides a eukaryotic environment that is generally conductive to the proper folding, disulfide bond formation, oligomerization, and many other post-translational modifications required for biological activity of some eukaryotic proteins. Second, the exceptionally strong polyhydrin promoter yields very high heterologous protein expression. This is the most distinguishing feature of baculovirus expression system. Because of the strong polyhydrin promoter, the highest expression levels reported using baculovirus expression vectors have reached a value of up to 25%-50% of the total cellular protein. Although this level of expression has rarely been observed for the more than 300 recombinant proteins expressed in baculovirus to date. Third, the simplicity of technology, baculovirus system is helper-virus independent and therefore relatively simple to use. Chemically-defined serum-free medium has been developed and used to maintain the *SF9* insect cells, which makes the purification of recombinant proteins easier.

Based on the above mentioned advantages, we subcloned the entire structural portion of the human IL-8 gene from a bacterial gene construct into a shuttle vector, specific for the baculoviral vector expression system. The overall goals of this portion of my research include: (i) production of large amounts (>1 mg/liter culture) of recombinant human IL-8, (ii) isolation of chemotactically active recombinant human IL-8 (rhIL-8) whose biological properties match those of the monocyte-derived natural product, and (iii) development of an efficient purification protocol for rhIL-8 involving preparative HPLC technology.

Receptor Signal Transduction Pathway of IL-8 and Other Chemotactic Factors-Several elements of the receptor signal transduction pathway leading to activation of neutrophils by various chemotactic factors have been identified. A model of signaling pathway triggered by the fMLP receptor is schematically represented in figure 4. The human neutrophil response is initiated by the binding of fMLP to its receptor (4). Agonist-receptor interaction is the first event in receptor mediated signal transduction. The receptor-agonist complex must persist to sustain the cellular response.

The involvement of G proteins in chemotactically induced signal transduction was originally suggested by the inhibitory effect of *Bordatella Pertussis* toxin (4). Further evidence has come from the observations that fluoride and, in electropermeabilized cells, the non-hydrolyzable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTPγS), which directly activates G proteins, can mimic receptor-dependent stimulation (34). Adenosine 5'-diphosphate-ribosylation by pertussis toxin blocks the function of G_i-type protein and prevents the transduction of agonist signals (34). The IL-8 receptor-induced transmembrane signaling mechanism(s) also involves G protein(s). The IL-8 induced-chemotaxis in human neutrophils is sensitive to pertussis toxin (17). IL-8 markedly stimulates GTP hydrolysis and GTPγ[^{35}S] binding to human neutrophil plasma membranes. Both activities are strictly specific for guanine nucleotides. Binding of

GTPγS to G protein in the presence of Mg^{2+} has previously been shown to coincide with G protein activation (35).

Hydrolysis of membrane phospholipids has been shown to be the first step in the formation of a wide variety of bioactive lipids. Recent studies suggest that three types of phospholipases are involved at some level during neutrophil activation. These include phospholipase A_2 (PLA_2), phospholipase C (PLC), and phospholipase D (PLD) (Fig. 4) (181). The schematic model for the action of these phospholipases on phospholipids is shown in figure 5.

The phosphatidylinositol (PI)-specific PLC isozyme requires Ca^{2+} for optimal activity. This isozyme preferentially hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) but also acts on PI, phosphatidylinositol 4-monophosphate (36, 37). The cleavage of PIP_2 yields two second messengers, inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) (Fig. 5). IP_3 is released into the cytosol and binds to specific receptors on intracellular Ca^{2+} storage organelles (38). The binding of IP_3 induces the release of Ca^{2+} and leads to the characteristic rise in cytosolic [Ca^{2+}] (39, 40, 41). The other second messenger, diacylglycerol, remains associated with membrane and activates protein kinase C (PKC) (36, 37).

Activation of PLC by fMLP occurs by a G protein mediated mechanism. This was demonstrated using neutrophil membranes, in which PLC, in the presence of Ca^{2+}, can be stimulated with GTPγS or fluoride (37, 42, 43, 44). Pre-treatment of neutrophils or isolated membrane with *B. pertussis* toxin (to ADP-ribosylate G_α) prevents PLC activation (109, 110, 111, 112). A similar role for PLC in IL-8 receptor mediated signal transduction in neutrophil remains unclear at the present time. One group reported that IL-8 stimulated polyphosphoinositide hydrolysis and generated IP_3 in human peripheral blood lymphocytes (45). But an expected increase in intracellular Ca^{2+} was not observed in

response to IL-8 stimulation in that study.

The pathway(s) involved in the signal transduction of the chemotactic receptor-mediated activation of the respiratory burst metabolism in human neutrophils is not completely understood. Initially, several studies focused on the importance of the activation of PLC during the activation of the neutrophil and the subsequent production of the second messengers, inositol 1,4,5-triphosphate and 1,2-diacylglycerol. Intracellular 1,2-diacylglycerol resulting from this hydrolysis may be responsible for the activation of the respiratory burst by protein kinase C (182). However, several studies have indicated that receptor-mediated activation of respiratory burst can occur in the absence of PIP_2 hydrolysis (183). Moreover, there was not a clear correlation between the accumulation of DAG and O_2 consumption (184, 185). Hence, protein kinase C activation appears to be an important activation mechanism of the respiratory burst metabolism, but may not constitute the only activation mechanism.

A role for PLD activity in neutrophil signal transduction has been suggested by several recent studies (51, 69, 70, 71, 72). Phospholipase D acts primarily on phosphatidylcholine (PC) to generate phosphatidic acid (PA) (73). This phospholipid metabolite represents a potential second messenger that may be **directly** involved in the activation of NADPH oxidase (74, 75). Cleavage of phosphatidic acid yields diacylglycerol, the physiological activator of protein kinase C, as discussed earlier (76, 77). Diacylglycerol production by this pathway is slow and protracted when compared with that resulting from hydrolysis of PI (51, 70). Neutrophil PLD has been shown to be Ca^{2+}-dependent (80). Stimulation of the neutrophil with fMLP in the absence of extracellular Ca^{2+} results in only a partial activation of PLD. Stimulation in Ca^{2+} depleted neutrophils results in no activation of PLD activity (80, 81).

The activation of PLA_2 also plays an important role in inflammatory processes. In HL-60 cells and neutrophils, PLA_2 hydrolyzes phospholipids and 1-alkylphospholipids,

generating two main precursor molecules, arachidonic acid and 1-0-alkyl-*sn*-glycerol-3-phosphorylcholine (lyso-PAF) (Fig. 5) (51, 52, 53). The free arachidonic acid is subsequently oxidized by cyclooxygenase and lipooxygenase to generate prostaglandins and leukotrienes, respectively (54, 55, 56, 57). Lyso-PAF is acylated by an acetyl-CoA transferase to 1-O-alkyl-2-sn-glycerol-3-phosphorylcholine, PAF (58, 59). Leukotriene B_4 and PAF are not only products of neutrophil activation, but also activate the oxidative processes in the human neutrophil via G protein-dependent chemotactic receptors.

Stimulus-dependent activation of PLA_2 is routinely measured by the release of radiolabeled arachidonic acid or its oxidized metabolites (56, 59, 60, 61). The isozyme(s) and the phospholipid substrate pools of stimulated neutrophils, have not yet been determined. In addition, the signal transduction mechanisms leading to PLA_2 activation are unknown. A G protein-dependent activation has been suggested in this process, because both non-hydrolyzable GTPγS and fluoride stimulate the release of arachidonic acid in intact and permeabilized neutrophils (62, 63). However, it is not clear whether PLA_2 activity is dependent on the direct interaction with G protein subunit(s). Some evidence suggests that IL-8 may induce PLA_2 activity in neutrophils as IL-8 does stimulate the arachidonic acid-lipoxygenase pathway and releases LB4 and 5-HETE in activated neutrophils (68). Most recent evidence suggests that PLA_2-derived lipid mediators may regulate a number of neutrophil responses including degranulation and adhesion (68b). Inhibitors of PLA_2 blocked degranulation and MAC-1 (CD11/CD18) up-regulation in human neutrophils when challenged with IL-8.

The transient rise in cytosolic free Ca^{2+} is due to release from intracellular stores and/or influx through the plasma membrane (82). A rise in cytosolic Ca^{2+} appears to be essential for neutrophil activation. However, internal Ca^{2+} pools maybe sufficient to satisfy some functions, since neutrophils conduct exocytosis and oxygen burst normally

when the extracellular Ca^{2+} has been chelated (83). The IL-8-induced Ca^{2+} influx is apparently due to the cooperative mobilization of both intra- and extracellular Ca^{2+} (84). This process is assumed to be mediated by G proteins (17, 85). Pre-treatment with pertussis toxin largely inhibited the IL-8 induced changes in $[Ca^{2+}]$ as the increase was substantially inhibited in the absence of extracellular Ca^{2+}, and the effect of IL-8 on $[Ca^{2+}]$ was reduced after quenching with Mn^{2+}. The Ca^{2+} channel opened by IL-8 seems to be essential for the induction of chemotaxis as the L-channel blockers totally blocked IL-8 induced chemotaxis in neutrophils (86).

A direct consequence of a rise in Ca^{2+} is the activation of selected kinases. One of the best characterized calcium-dependent protein kinases is protein kinase C. A role for PKC and protein phosphorylation in the fMLP induced signal transduction pathway was suggested by results that demonstrated the stimulatory effects of phorbol esters, diacylglycerol, coupled to the inhibition demonstrated by staurosporine, sphingosine bases and other protein kinase C inhibitors (86, 87, 88, 89). The respiratory burst response induced by phorbol ester and diacylglycerol, however, was much slower in onset than that elicited by receptor agonists. This indicates that PKC activation is only a part of the total signal transduction process (90, 91). Evidence for the direct involvement of PKC in IL-8 signal pathway has been suggested recently (92). One group reported that IL-8 elicited respiratory burst could be blocked by PKC inhibitors, 17-hydroxywortmannin (H7) and staurosporine (93). Another group reported that IL-8 induced human lymphocyte chemotaxis was inhibited by PKC inhibitors, H7 and sphingosine. A conflicting result was reported by Petersen, *et al.* (94), who have shown that a novel protein kinase C inhibitor , 3-Hexadecyl-mercapto-2-methoxymethylpropyl-1-phosphocholine (BM 41440), did not inhibit IL-8-stimulated lysosomal release. Moreover in this study, they have shown that IL-8 did not activate PKC when assayed by the direct measurement of PKC activity in human neutrophils.

Another major Ca^{2+} responsive kinase is calcium/calmodulin-dependent protein kinase II (CaM kinase II), a multifunctional Ca^{2+}/calmodulin-dependent protein kinase. This enzyme has been known to be involved in regulating a variety of cellular functions including neutrotransmitter synthesis, carbohydrate metabolism, cytoskeletal function, and gene expression (95). The activities of this CaM kinase II have been identified in variety of tissues and cells including brain, skeletal muscle, spleen, kidney, lung, liver, heart, and erythrocytes (96). Recently, calcium induced Ca^{2+}/calmodulin-dependent protein kinase activity was demonstrated in human neutrophils (97). CaM kinase II may play a role in the mechanism(s) underlying rhTNFα-induced activation of Ca^{2+}-activated Cl^- current in human neutrophils. With intracellular application of the Ca^{2+} chelator 1,2-bis(2-amino-phenoxy)ethane-*N,N,N',N'*-tetraacetate (5mM), the calmodulin antagonist , CaM kinase II-(290-309), or the inhibitory peptide , CaM kinase II-(273-302), Cl^- current was no longer activated by rhTNFα. Intracellular application of the control peptide, CaM kinase II-(284-302) or the protein kinase C inhibitory peptide PKC-(19-36) did not block the rhTNFα-induced activation of Ca^{2+} activated Cl^- current in human neutrophils. Huang *et al.* have recently identified the activity of this enzyme in human neutrophils directly based on the kinase activity toward site II of synapsin and syntide 2 (a synthetic substrate for CaM kinase II activity) and by immunoblotting of neutrophil protein with antibody against CaM kinase II (98). Chemotactic factor (fMLP) induces a Ca^{2+}influx and activates several Ca^{2+}/Calmodulin-dependent substrates including calcineurin and myosin light chain kinase (MLCK) in neutrophils (99). Direct evidence for a role of CaM kinase II in chemoattractant-induced signaling in neutrophils has not been reported. However a common CaM kinase II substrate, microtubule-associated protein-2 (MAP-2), has been activated by chemotactic factor in human neutrophils (100).

Activation of human neutrophils by fMLP triggers a complex series of protein

phosphorylation reaction including serine/threonine and tyrosine phosphorylation. The best-known enzyme acting on serine and threonine residues is protein kinase C. Protein phosphorylation patterns in neutrophils stimulated with fMLP are similar to those obtained after stimulation with phorbol esters (88, 90, 101, 102). The cytosolic component of the NADPH oxidase, p47-*phox*, contains several recognition sites for protein kinase C and becomes rapidly phosphorylated after challenge with either receptor agonist or phorbol ester (103, 104). A rapid increase in the phosphotyrosine content of several proteins is observed in neutrophils after stimulation with chemotactic agonist (fMLP) (105, 106, 107), but the mechanism of tyrosine kinase activation under these condition is not known. Unlike receptors for growth factors and some other transmembrane proteins (186), the 7-TMS receptor lacks intrinsic tyrosine kinase domains, and there is no indication that it associates with any identified tyrosine kinases. IL-8 stimulation of neutrophils does induce phosphorylation events. Matsusshima and coworkers (187) observed that IL-8 treatment caused rapid phosphorylation of cytosolic 48-kd and 65-kd proteins that have been reported to be phosphorylated by other chemotactic factors. These results suggested that these phosphoproteins may be involved in signal transduction for cytoskeleton protein polymerization.

In summary, although it has been demonstrated that the signal transduction mechanisms triggered by the IL-8 receptor may involve a G protein(s), a transient rise in cytosolic free Ca^{2+}, activation of PKC, and several phosphorylation events, the intracellular mechanisms that mediate IL-8 induced-cellular responses are still poorly understood. Upon the successful expression and purification of IL-8 from Baculovirus Expression System, the central focus of my research was to explore the IL-8 receptor signaling pathway. Early post-receptor events of fMLP signal transduction are reasonably well understood. Transduction of the signal generated by the fMLP-receptor complex is known to proceed through a heterotrimeric GTP binding protein and to be associated with

activation of PLC, PLA_2, and PLD. Since the biological properties and receptor structures of IL-8 and fMLP are similar, in this study, I asked whether IL-8 receptor-mediated signal pathway was similar to that of fMLP receptor. To answer this question, the activation of PLA_2, PLC, and PLD induced by IL-8 and fMLP in differentiated HL-60 cells were tested. In addition, the mechanisms of the activation of phospholipase were determined. The involvement of protein kinase activity in IL-8 signaling pathway was investigated.

CHAPTER II

EXPERIMENTAL PROCEDURES

Cells and Viral Stocks-Spodoptera frugiperda(*Sf*9) armyworm insect cells, *Autographa californica* nuclear polyhedrosis virus (AcNPV), and pVL1392-3 (Fig. 6) were gifts from the laboratory of Dr. Max Summers (Texas A&M University, College Station, TX) (119). The bacterial vector construct, (gMDNCF2-1)pUC19-1.7-5, harboring the full length structural portion of the human IL-8 gene (120) was a gift from Dr. Kouji Matsushima at the National Cancer Institute. *E. coli* DH5α (*supE*44, *hsdR*17, *recA*1, *endA*1, *gyr A*96, *thi*-1, *relA*1) cells were obtained from Gibco/BRL (Gaithersburg, MD). Human Leukemic cell line (HL-60 cells) was gift from Dr. Harry Malech at the National Institute of Health.

Chromatographic materials-Chromatography was performed on a Waters 600E preparative HPLC equipped with a variable wavelength detector and controlled by computer interfaced Baseline Powerline interfacing software. Preparative HPLC columns used were TSK-SP-5PW (SP cation exchange resin), 21.5 x 150 mm, Protein-Pak 125, 10 x 30 mm (3 columns in series), and Protein-Pak C-4, 5 mm (reverse phase matrix), 7.8 x 150 mm (Waters, Inc.). SDS-PAGE was carried out using ultra pure acrylamide and bis acrylamide from Gibco/BRL (Gaithersburg, MD).

Other materials-Restriction endonucleases and tissue culture materials were purchased from Gibco/BRL (Gaithersburg, MD) and used as recommended by the supplier. fMLP, aristolochic acid, (Phorbol 12-Myristate 13-Acetate) PMA, staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H7) and unlabeled lipids were from

Sigma (S. Louis, MO). (γ-^{32}P)ATP (6000 Ci/mmol), [^{3}H]arachidonic acid (210 Ci/mmol), [^{3}H]inositol (23.45 Ci/mmol), and [^{14}C]lysopalmitol phosphatidylcholine (57.00 Ci/mmol) were from Du-Pont (Boston, MA) All reagents for amino acid composition analysis were obtained from Pierce Inc. Fluorescent substrates for oxygen burst metabolism assay were from Molecular Probes. Chromatographic solvents were from Burdick and Jackson Laboratories. All other reagents were of A.C.S. grade or better and supplied by Sigma (St. Louis, MO).

Sf9 Cell Culture-Insect *Sf9* cells were cultured in chemically defined serum free Gibco media, *Sf*900 containing 50 mg of gentamycin sulfate and 2.5 mg amphotericin B (Fungizone) per liter. Cells were grown in T flasks (175 cc) containing 30 ml of *Sf*900 media and maintained at 28°C in a sterile air atmosphere. Viral infections were made using 30 flasks (175 cm^2) of *Sf*9 cells at 70% confluence. Each flask (2.0 x 10^7 cells) was infected with 1 ml of recombinant virus stock (>10^5 pfu/ml) diluted into 10 ml of culture media.

Subcloning human IL-8 into the Baculovirus Shuttle Vector, pVL1392-The structural portion of the human IL-8 gene was restriction endonuclease excised from a bacterial vector construct following plasmid transfer (transfection) and gene amplification in *E. coli* DH5a cells. The subcloning strategy, schematically illustrated in Fig. 7, involved *Bam*H1 digestion of (gMDNCF2-1)-pUC19-1.7-5 to yield a 900 bp restriction fragment. cDNA fragments were separated on a 0.7% ultra-pure low temperature melting agarose gel by electrophoresis in Tris/Borate/EDTA buffer as described by Sambrook *et al.* (121). The agarose slice containing the cDNA insert was melted and contaminating proteins were removed by conventional organic extraction followed by ethanol precipitation as described by Sambrook *et al.* (121). Following *Bam*H1 and CIAP digestion, the cleaved shuttle plasmid, pVL1392 (Fig. 6), was electrophoretically isolated on a low melting agarose gel

and contaminating proteins were removed as described above. Ligation was performed using 0.5 μg of insert and 5.0 μg of shuttle plasmid cDNA by the protocol of King *et al.* (123). An aliquot of the ligation mix was electrophoretically analyzed to ensure complete ligation. *E. coli* DH5a cells were transformed with the ligation mixture and the harbored interleukin gene was amplified in the transformants in the presence of the selectable marker, ampicillin (75 mg/ liter of LB broth). cDNA IL-8 insert was integrated into the polylinker region of the Baculovirus shuttle vector pVL1392 at a unique *Bam*H1 site located a few bases downstream from the start site for RNA synthesis and positioned immediately behind the strong Baculoviral polyhedron promoter. Subcloned transformants were evaluated for correct gene orientation by asymmetric cutting with appropriate restriction endonucleases and frozen as 20% glycerol stocks. A single transformant was grown in a 2 liter culture and the shuttle plasmid construct containing IL-8 was purified using the mild alkaline lysis method for cDNA purification as described by Sambrook *et al.* (121). The purified construct was again evaluated for correct insert orientation, plasmid purity, and cDNA mass and frozen at -20° C as 20% glycerol stocks. These -20° C stocks were routinely used to produce recombinant virus.

Recombinant Baculovirus Production-The production and purification of recombinant Baculovirus stock harboring the human IL-8 cDNA was performed according to the protocol developed by Invitrogen Corp. (San Diego, CA). Following amplification in *E. coli*, the purified shuttle vector-human IL-8 construct was incubated for 2 h at 28° C with wild-type Baculovirus in sub-confluent cultures of the armyworm insect cell line, *Sf*9. After removal of excess virus and DNA, fresh media was added to a final volume of 5 ml per 60 mm well in six well plates. The cultures were incubated at 28° C for 7-10 days and the cell supernatant solution was harvested by centrifugation at 1500 x *g* for 20 min to remove lysed cell debris. The mixed viral stock solution was stored aseptically at 4° C awaiting further viral subcloning. Serial dilutions (10^{-1} to 10^{-8}) of the mixed viral stock

were incubated with sub-confluent cultures of *Sf*9 cells for 2 h. The excess virus was removed and individual cultures of virus-infected *Sf*9 cells were overlaid with 1.25% low-melting agarose. Five to seven days later the cultures were stained with neutral red dye and recombinant plaques were selected on an inverted microscope. Selected recombinant plaques were amplified and subcloned as described above. This process was repeated two additional times. The end results of this procedure yielded several monoclonal recombinant viral stocks free of any contaminating wild type plaques. Several positive clones were evaluated using a conventional ELISA assay described below 5-7 days post infection.

Chromatographic methods-All buffers were pre-filtered through 0.22 mm Millex filters (Millipore, N.Y.) and degassed prior to use. All chromatography was performed under an Argon purging atmosphere and protein was monitored at 280 nm for all columns except for the reverse phase delta pak C-4 column which was monitored at 218 nm. The protocol for the purification of recombinant human IL-8 synthesized in the Baculovirus vector expression system is outlined in Fig. 11. Chromatographic elution conditions for each of the preparative HPLC columns were as described in the figure legends (Fig. 12-16). Even numbered column fractions were subjected to gradient gel electrophoresis. SDS-PAGE was performed in the presence of β-mercaptoethanol by a modification of the method of Laemmli (124). The modification consisted of lowering the bis acrylamide:acrylamide ratio from the standard 1.0%:28% (w/w) to 0.5%:40% (w/w). Samples subjected to SDS-PAGE were visualized by silver staining using the method of Merill *et al.* (125)

Anti-IL-8 ELISA using antibody generated against monocyte derived IL-8-Aliquots (100 µl) of even numbered column elution fractions were delivered to flat bottomed 96 well Falcon tissue culture plates and assayed for $_{rh}$IL-8 using a conventional ELISA (123) capable of detecting 0.1-0.4 ng of the recombinant interleukin per well.

Following antigen binding and blocking of the non-specific sites with diluted milk, plates were washed and incubated with rabbit anti-human IL-8 antibody at a 1:500 dilution for 2 h. The excess antibody was washed and the primary immunoconjugate was incubated for 2 h with a peroxidase-conjugated second antibody, goat anti-rabbit IgG at a 1:500 dilution. Following removal of the second conjugated antibody and final wash, the ELISA color was developed by adding 100 ml of a freshly prepared 1 mg/ml solution of ABTS in 10 mM sodium acetate, pH 5.0 containing 40 ml of 3.0% hydrogen peroxide. Samples were analyzed using a Bio-Rad ELISA plate reader at 415 nm. Positive (purified commercial IL-8 from monocytes) and negative (non-specific antigen, no antigen, no first and/or second antibody added) controls and all fraction eluates were assayed in triplicate.

Human neutrophil isolation-Human neutrophils were isolated from donor blood (50 ml) of healthy adult male as described by the method of Dooley *et al.* (126). Briefly, fresh human blood was obtained by venepuncture from the healthy adult and collected into EDTA-anticoagulated tube. 15 ml of 63% Percoll were added to 50-ml conical centrifuge tube; 15 ml of 72% Percoll were then underlaid through a catheter attached to syringe, with care to avoid the introduction of air bubbles which might disturb the discontinue gradient. Undiluted, EDTA-anticoagulated blood (15ml) was then layered on top of each Percoll gradient which was then centrifuged for 30 min at 500 *g* at the room temperature. The neutrophil-rich band was aspirated, and the cells were washed 2 times in cold Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}, counted, and resuspended in appropriate media. Cell viability as assessed by Trypan Blue exclusion was >99%.

Human neutrophil chemotaxis assay-Column fractions showing activity by ELISA were evaluated for their ability to induce human neutrophil chemotaxis by the agarose method (127). Briefly, agarose was dissolved in sterile, distilled water by heating in a boiling water bath for 10 to 15 min. After cooling to $48^{0}C$ in a water bath, the agarose was mixed with an equal volume of prewarmed 2 x MEM supplemented with 10% heat-

inactivated serum. 5 ml of the agarose medium were delivered to each 60x15 mm tissue culture dish and allowed to harden. The dish then transfered to the refrigerator for 30 to 60 min to facilitate cutting of the wells. Six series of the three 2.4 mm in diameter and spaced 2.4 mm apart were cut in each plate using a plexiglass template and stainless steel punch. The agarose plugs were plucked out with the aid of needle. A model for chemotaxis assay is shown in figure 8. recombinant human IL-8 was serially diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% bovine serum albumin (BSA). Neutrophils were suspended in DMEM supplemented with 10% FBS. The center wells of each three-well series received a 10 µl of the cell suspension containing $2.5x10^5$ purified neutrophils. The outer wells received 10 µl of diluted IL-8 fraction. The inner wells received a 10 µl of non-chemotactic control medium, DMEM. The completed dishes were incubated at 37^0C in a humidified atmosphere containing 5% CO_2 in air. After incubation for 2 hours, the wells were fixed with the agarose in place by addition of 3 ml absolute methanol for 30 min and 3 ml of 47% buffered formalin for 30 min. After fixation the gel were removed intact and the plate were stained with Wright's stain and air dried. Quantitation of chemotaxis was done by measurement (in centimeters) of the linear distance the cells had moved from the the margin of the well toward the IL-8 (chemotactic distance) and the linear distance the cells had moved from the margin of the well toward the control medium (random mobility distance). The chemotactic index is defined as follows:

$$\text{Chemotactic index} = \frac{\text{chemotactic distance - random motility distance}}{\text{random mobility distance}}$$

Superoxide anion production assay-The method of Allen *et al.* (128) was

employed to assess superoxide anion production by human neutrophils stimulated either by FMLP, a synthetic chemotactic peptide (positive control), or purified rhIL-8, or column fractions containing rhIL-8. The stimuli were added to $2x10^6$ neutrophils in 1 ml phenol red free HBSS containing 85 µM cytochrome C. After incubation for 1 hour at 37^0C, the reaction was stopped by the addition of 300 U superoxide dismutase (SOD). The cells then centrifuged at 200 x g for 10 min at 4 ^{0}C and absorbance of the supernatant was read at 550 nm in a spectrophotometer (Bechamen, DU-68). The results were expressesed as nanomoles of reduction of cytochrome c per $2x10^6$ neutrophils using the extinction coefficient: E_{550} nm=2.1 x 10^4 $M^{-1}cm^{-1}$.

Oxygen burst metabolism assay-Oxygen burst metabolism was determined using dihydrodichlorofluorescein as a sensitive fluorogenic chromophore to follow the oxidation of the non-fluorescent substrate, dihydrodiacetylflouroescein, to its fluorescent product, dihydrofluorescein as developed by Wymann *et al.* (129). This assay consumes hydrogen peroxide secreted by human neutrophils exposed to the chemotactic agent and requires saturating amounts of exogenous horseradish peroxidase to be present during the oxidation of the substrate. The assay detects oxygen burst metabolism in the picomole range of sensitivity.

Amino acid analysis-Protein samples were subjected to denaturing SDS-PAGE and electrophoretically transferred to Immobilon P membrane (Millipore Corp., New Jersey) by the method of LeGendre and Matsudaira (130) using a Bio-Rad electrophoretic transfer apparatus. The transferred proteins were stained for two minutes with Coomassie Brilliant Blue in 50% methanol, destined in 50% methanol, and the stained band carefully cut away from the Immobilon P support. Samples were hydrolyzed *in vacuo* at 110° C for 48 and 72 h in 6 N HCl. Hydrolyzed samples were assayed for amino acid composition by the method of Dong and Gant (131). Values were normalized for total molar abundance and compared to values for the mature form of human IL-8 based on

cDNA sequence (120).

HL-60 Cell Culture and Labeling-HL-60 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum at 37 ^{0}C in a 5% (v/v) CO_2 atmosphere at 95% humidity. Cells were differentiated to neutrophil-like cells by inclusion of 500 μM dibutyryl cAMP in the growth media for 48 hours as described (108).

To label cells with [^{3}H] inositol, RPMI 1640 were supplemented with 5 μCi/ml [^{3}H]inositol for 48 hours when cells were treated with 500 μM dibutyryl cAMP. Cell labelling reach to equilibrium after 48 hours incubation (data not shown). To label cells with [^{14}C]alkylyso-PC, HL-60 cells at a concentration of 1x10^{7}cells/ml were labeled with 2 μCi/ml [^{14}C]alkyllyso-PC for 4 hours at 37 ^{o}C in RPMI-1640 medium after cell differentiation. [^{3}H]arachidonic acid labelling of the HL-60 cells was achieved by incubation of cells (1x10^{7}cells/ml) with 1 μCi/ml [^{3}H]arachidonic acid for 4 hours at 37 ^{o}C in RPMI-1640 complete medium. Preliminary experiments demonstrated that arachidonic acid was preferentially incorporated into phosphatidylcholine (PC) and to a lesser extent, into phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). Equilibrium labeling of the arachidonate-containing lipids was achieved following a 4 h inclusion (results not shown). In some experiment, the culture medium was further supplemented with indicated concentration of pertussis toxin, cholera toxin, aristolochic acid, staurosporine, H7 and PMA at the indicated times.

At indicated time following incubation, HL-60 cells were washed twice with the Hepes Saline Buffer and then suspended at a desired concentration for the assays of PLC, PLA_2, and PLD as described below. The composition of the Hepes Saline Buffer is 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM $NaHCO_3$, 5mM Glucose, 0.1% BSA, and 20 mM Hepes (pH 7.2).

Measurement of PLC Activation-After 48 hours labeling, the cells were washed

twice and suspended at a concentration of $5x10^6$ cells/ml with Hepes Saline Buffer. The cell suspension 0.2 ml was treated at 37 °C for 10 min with 10 mM LiCl to inhibit inositol phosphatase. The cells then incubated with same volume of 100 ng/ml IL-8 or 1 μM fMLP for indicated times at 37 °C. The reaction was stopped by the addition of 1.88 ml of chloroform/methanol/HCl (100/100/2), and 0.62 ml each of chloroform and water were added to separate the phases. Following centrifugation at 1,000xg for 5 min, a 1.8 ml aliquot of the upper aqueous phase was mixed with 3.2 ml of water and placed on a column of Dowex AG-X8-formate. The column was washed with 8 ml of water and then 20 ml of 60 mM ammonium formate /5 mM disodium tetraborate to elute [^{3}H]inositol and [^{3}H]glycerolphosphoryinositol. [^{3}H]inositol-1-monophosphate (IP) was eluted with 8 ml of 200 mM ammonium formate/100 mM fomic acid. The [^{3}H]inositol-1,4-bisphosphate (IP_2) was then eluted with 8 ml of 400 mM ammonium formate/100 mM formic acid and the [^{3}H]inositol triphosphate (IP_3) was eluted with 8 ml of 1000 M ammonium fomate/100 formic acid. Fractions of 2 ml were taken and counted on scintillation counter.

Measurement of PLD Activation-Alkyl [^{14}C] lyso-PC-labeled HL-60 cells ($1x10^7$) were suspended in 0.45 ml of Hepes Saline buffer. The cells then incubated in the presence of 0.5% ethanol (v/v) and 5 mg/ml cytochalasine B for 5 min. The reaction was initiated by addition of 50 ml IL-8 (100ng/ml) or FMLP (1 mM). After the indicated time of incubation, 0.5 ml of chloroform/methanol/acetic acid (100/200/2) was added to stop the reaction. The standard samples (10 mg each) of PA and PEt were added. Viability of the cells, as assessed by trypan blue exclusion, was not affected by ethanol.

The phases were separated by the procedure of Bligh and Dyer (108a). The lower chloroform phase was dried and spotted on Silica Gel plates. The plates were developed by using a solvent system consisting of the organic phase of chloroform/methanol/acetic acid (65/15/2, by volume). In the solvent system, PA (R_f=0.37) and PEt (R_f=0.62)

were separated from each other and from other phospholipids (R_f=0-0.2). The lipids were located by staining with iodine vapor or by autoradiography. The amount of radioactivity co-migrating with a phosphaditylethanol standard was determined by liquid scintillation counting after scraping the gel into a scintillation vial.

Measurement of PLA2 Activation-The cell suspension ($2x10^7$ cells in 0.2 ml), which had been cultured for 4 h with [^{3}H]arachidonic acid and then washed twice by the Hepes saline Buffer not containing [^{3}H]arachidonic acid as described above, was incubated for 10 min at 37 ^oC with same volume of the regular Hepes saline buffer containing IL-8 (100 ng/ml) or fMLP (1μM). The reaction, stopped by the addition of 0.9% NaCl/2 mM EDTA, was immediately centrifugated at 1000 x g for 1 minute. Aliquots of the supernatant (0.5 ml) were removed and radioactivity quantitated by liquid scintillation spectrometer. The identity and purity of arachidonic acid was assessed by thin layer chromatography.

Lipid Analysis (isolation and separation of lipids)-[^{3}H]Arachidonate-labeled cells stimulated with IL-8 as described above, were quenched with chloroform/methanol (1:2, v/v). To a 0.5 ml aliquot of the total cell suspension or its supernatant were added 3 ml of chloroform/methanol/HCl (100:200:2,v/v/v), 1 ml chloroform, 1 ml of 2 M KCl, and 0.34 ml of water successively for the purpose of extracting lipids. The mixture was then shaken vigorously and separated into two phases by centrifugation at 1000 x g for 5 minutes. The lower phase (1.5 ml) was evaporated and used for separation of lipids by thin-layer chromatography on Silica Gel plate. Neutral lipids were separated in a solvent consisting of hexane/diethyl ether/acetic acid (80/20/1, v/v/v) and phospholipids were separated in a solvent consisting of chloroform /methanol /acetic acid /water (75/45/3/1,v/v) as described (56). *Rf* values of the authentic sample were 0.11 for phosphatidylcholine, 0.21 for phosphatidylserine, 0.33 for phosphatidylinositol, and 0.55 for phosphatidylethanolamine respectively.

Assay of CaM kinase II activity-The CaM kinase II activity assay was performed by the methods described previously (134, 135, 136, 137). HL-60 cells ($5x10^5$) were preincubated in KRB medium for 10 min at 37°C, and then incubated in KRB containing 100 ng/ml IL-8 or control (0.1% BSA) for various time. Media was then removed and HL-60 cell quickly washed (200 µl) and sonicated in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 1.0 mM EDTA, 2.0 mM DTT, 10 mM sodium pyrophosphate, 0.4 mM ammonium molybdate, 0.1 mg/ml leupeptin). Ten (10) µl of homogenate (approximately 25 µg protein) was immediately assayed at 30°C for 30 sec in tubes containing 40 µl of a reaction mixture which gave a final concentration of 50 mM PIPES, 10 mM $MgCl_2$, 0.1 mg/ml BSA (Fraction V), 10 µM autocamtide-2, 20 µM [γ-^{32}P]ATP(6000 Ci/mmol), and either 0.5 mM $CaCl_2$/5 µg/ml calmodulin for Ca^{2+}-stimulated activity or 0.9mM EGTA for Ca^{2+}-independent activity. Termination of the reaction was achieved by the rapid addition of 25 µl ice cold 15% TCA and immediate vortexing. Tubes were allowed to stand 20 min at 4°C to precipitate large proteins followed by 1 min centrifugation at 7000 x *g*. Thirty (35) µl of supernatant was spotted onto 5 cm x 2 cm strips of phosphocellulose paper (Whatman P81). The paper strips were washed 5 times for 20 min with constant shaking in distilled water. The paper strips were subsequently dried at 120°C for 20 min, and $^{32}P_i$ incorporation into autocamtide-2 by liquid scintillation counter.

Cell permeabilization procedure-HL-60 cells were permeabilized by streptolysin O (SLO). The toxin was first activated with 10 mM dithiothreitol for 10 min. HL-60 cells ($1x10^7$) were permeabilized at 37°C in 1 ml of HBSS containing 1 unit of SLO for 5 min. The percentage of permeabilized cells was assayed by the loss of their ability to exclude Trypan Blue. Under condition used, >70% cells took up Trypan Blue. Cells were used immediately after permeabilization.

Detection of protein phosphorylation in response to stimulation of permeabilized cells- Protein phosphorylation was detected by a method described by Evens *et al.* (138). HL-60 cells were permeabilized by the indicated procedure and suspended in HBSS after 2 min at 37^{0}C, 40 μM sodium Orthovanadate and 100 μM phenylmethanesulphonyl fluoride was added for further 5 min Phosphorylation reaction was initiated by the addition of IL-8 or fMLP with 5 μCi [γ-^{32}] ATP to 10 μl cells ($5x10^{6}$). The volume of the final incubation mixture was 20 μl. Labeling continued for 3 min and the reaction was stopped by the addition of 20 μl of an mixture containing 50 μM unlabeled ATP. Tubes were centrifuged at 1,500 x g for 5 min at 4^{0}C. Detergent-soluble fraction was analyzed by electrophoresis in 10% polyacrylamide gels in the presence of SDS under reducing condition. The gel was fixed , stained with Commassi Blue to reveal molecular weight markers and dried under heat and vacuum. Dried gel were autoradiographed for 24 hours using Kodak X-Omat AR film (rochest, NY) at -70^{0}C using intensifying screens to detect phosphorylated proteins.

Immunoblot detection of tyrosine phosphorylation of proteins in HL-60 cells- Immunoblotting was carried out as described (139). HL-60 cells ($1x10^{7}$/ml) in 200 μl RPMI 1640 medium were stimulated with IL-8 (100 ng/ml) or fMLP (1 μM) or treated with RPMI-1640 medium for three min. The reaction was stopped by quick centrifugation (5000 x g for 15 sec). The pellets were resuspended in 100 μl HBSS and lysed in a 50 μl solution containing 0.25 M Tris (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 10% glycerol, and 0.5% bromophenol blue. One hundred microliters of each cell lysate is electrophoresed on a 10% polyacrylamide gel, transfered to nitrocellulose, and immunoblotted with the anti-phosphotyrosine antisera. The antibodies bound to the nitrocellulose membrane were detected by ^{125}I-labelled goat-anti-rabbit antibody. Blots were developed on Kodak X-Omat film overnight at -70^{0}C.

Protein determinations-Protein concentration was determined by a method

described by Bradford *et al.* (140).

Data Presentation-All experiments were performed at least three times in duplicate or triplicate. The results of multiple observations are presented as the means±S.D. or as the representative value of results from three or more experiments.

CHAPTER III

RESULTS

Subcloning of human IL-8 and recombinant virus production-We wanted to synthesize and purify human IL-8 in a eukaryotic vector expression system. We chose to express the human IL-8 gene in the Baculovirus Expression System. Human IL-8 was subcloned into a shuttle vector of the Baculovirus Expression System using standard molecular biology techniques. Briefly, the structural portion of the human IL-8 gene was removed from a bacterial construct using restriction endonucleases, isolated by agarose gel electrophoresis. The contaminating proteins was removed by organic extraction, and the gene insert concentrated by ethanol precipitation. This fragment was then ligated into the baculovirus shuttle plasmid, pVL1392 as outlined in Fig 6. This new shuttle vector construct was first transfected into the competent *E. coli* strain, DH5α. Transformants were selected, evaluated for gene orientation, and their plasmid DNA amplified using a large scale DNA preparation protocol. These shuttle plasmid constructs are under the control of the strong polyhedrin promoter. New shuttle vector constructs were then co-transfected with wild type virus DNA into insect cell line, *SF9,* for recombinant virus production. During the co-transfection process, successful generation of new recombinant virus harboring the IL-8 gene and expressing recombinant IL-8 requires that wild-type viral DNA and shuttle plasmid DNA undergo the process of homologous recombination. The methodology for the overall process utilizes standard molecular biology protocols and has been extensively detailed in recent reviews and monographs (119). Following successful homologous recombination and outgrowth of recombinant viral plaques, each recombinant

viral plaque was purified to monoclonality through three separate plaque isolation procedures as described under Methods (119). Initially, plaque selection was performed visually, by the method called occlusion-negative plaque selection. Briefly, those clones lacking the polyhedrin protein and not generating cell occlusions were selected. Wild-type baculovirus-infected cells contain viral occlusion (Fig 9). The morphology of recombinant baculovirus infected *SF9* cells is shown in Fig. 10. Few to no occlusion bodies were observed in the recombinant virus-infected cells. Infected-*SF9* cells increase 20-40% in diameter relative to uninfected cells (Fig. 9). Recombinant IL-8 viral plaques in *SF9* culture were assessed for IL-8 expression by ELISA using anti-IL-8 antibody. The viral titer of recombinant viruses harboring human IL-8 cDNA was amplified for each isolate in *Sf9* by three separate viral infection which were harvested late near the lytic stage of infection. Harvested recombinant viral stocks were stored at $4^{o}C$ and later used to infect large scale cultures of *Sf9* cells for production of recombinant human IL-8.

Purification of recombinant human IL-8-Purification of rhIL-8 employing preparative HPLC was performed as outlined in Fig. 11. Conditioned media from a one liter infected culture containing 450 mg of total protein was harvested 5 days post infection by centrifugation at 1500 x *g* for 10 min and loaded onto a ToyoPearl TSK-CM 650 column. The elution profile of the cell supernatant from the fast-flow carboxymethyl ion exchange column is shown in Fig. 12. The majority of the protein in the conditioned media did not bind to the column and eluted early in the void volume. A UV absorbing protein peak containing rhIL-8 eluted late in the gradient elution profile at a concentration of 400 mM NaCl representing 1000 mho conductivity. This fraction contained >1.0 mg of rhIL-8 as evaluated by ELISA and silver staining as described in methods. Biological activity of rhIL-8 was monitored using freshly isolated human neutrophils by the agarose chamber chemotaxis assay (see Fig. 8) as described under Methods. The purification step involving

the fast flow column removed about 80% of contaminating proteins. The ELISA positive fractions (160-180) were pooled, concentrated, dialyzed against Buffer A of the SP column and loaded onto a TSK-SP-5PW column. As shown in Fig. 13, rhIL-8 eluted late in the linear gradient portion of the column profile (fractions 113 to 119). The fractions containing rhIL-8 were once again identified by ELISA and silver staining following SDS-PAGE. Results from ELISA have also been included in Fig. 13. Fractions 113 to 119 were pooled, dialyzed against Buffer A and loaded to a reverse phase Delta-Pak RP-C4 (300A) column. The elution profile of rhIL-8 from the reverse phase column is shown in Fig. 14. Importantly, this column resolved 10 or more protein contaminants which had previously co-eluted with IL-8 on the SP column. Fractions 34-39 were shown to contain > 0.5 mg of antibody reactive rhIL-8. The pooled fractions were dialyzed against a buffer used for HPLC 3X Protein-Pak-125-PW gel filtration column, concentrated to a volume of 1 ml, and then loaded onto the column. As shown in Fig. 15, a major UV absorbing peak containing antibody reactive rhIL-8 was eluted between fractions 39-45. The major protein in these fractions proved to be rhIL-8 as judged by ELISA and silver staining following SDS-PAGE with an estimated molecular weight of 8-12 kDa. Fractions 39-45 were pooled, concentrated, and dialyzed against the loading buffer of a second reverse phase column. The sample was loaded onto the Delta Pak RP-C4 (300A) column and a shallow linear gradient was generated. Three UV absorbing peaks eluted in the linear portion of the gradient as shown in Fig. 16. rhIL-8 eluted as a single symmetrical peak. As shown in Fig.17, fractions 57-58 contained pure rhIL-8 as visualized by silver staining following SDS-PAGE. From these data, it is clear that the pooled fractions 57 and 58 contain a single protein with an estimated molecular weight of 8.7 kDa. The rhIL-8 and other resolved proteins from the RP-C4 column were individually pooled and evaluated for their ability to: a) stimulate chemotaxis of human neutrophils, b) induce oxygen burst metabolism in human neutrophils, and c) react with polyclonal antibody generated against

IL-8 purified from human monocytes.

Biochemical and Immunological Characterization of rhIL-8-The results of the purification of rhIL-8 are summarized in Table I. Recombinant interleukin-8 from a liter of infected culture was purified over 600 fold yielding in excess of 500 μg of rhIL-8 . Purified rhIL-8 was subjected to amino acid analysis and compared with the predicted composition of the mature form of monocyte derived human IL-8 are presented in Table II. Amino acid analysis demonstrated that 14 out of fifteen amino acids analyzed showed an amino acid residue abundance within 2 or less residue equivalence for that predicted from the cDNA sequence. Eight amino acids analyzed proved to have a residue abundance within a single molar equivalence to that expected. Finally, five amino acid residues out of fifteen demonstrated an amino acid residue composition identical to that predicted from cDNA. Results of the immunological characterization of purified rhIL-8 and a comparison to other chemotactic agents are shown in Table III. The recombinant interleukin was consistently immunoreactive against rabbit antibody generated against monocyte derived human IL-8 at all stages of purification. Purified rhIL-8 demonstrated a strong chemotactic response in the agarose chemotaxis assay and was shown to exhibit a chemotactic index similar to fMLP.

Effect of IL-8 and fMLP on IP and IP3 generation-Phosphoinositide turnover plays an important role in transmembrane signaling of a wide variety of extracellular signals (36, 37, 38). Phosphoinositides are mainly composed of three major species of phospholipids: PI, PIP, and PIP_2. In response to a variety of extracellular signals, these phosphoinositides can be hydrolyzed by the action of phospholipase C to produce DAG and the respective phosphorylated inositol, IP_1, IP_2, and IP_3 (36). Among these products, DAG and IP_3 often serve as messengers for protein kinase C activation and intracellular Ca^{2+} mobilization, respectively (38). As discussed earlier, fMLP has been

shown to stimulate PLC activity in human neutrophils and HL-60 cells (42, 43, 44). Protein kinase C and Ca^{2+} has been shown to be involved in the regulation of fMLP-induced cellular activities in neutrophils (83, 84, 86, 89). IL-8 has recently been shown to stimulate the phosphatidylinositol (PI)-specific phospholipase C activity and release of IP_3 in human peripheral blood lymphocytes (45). Both chemotactic factors (IL-8 and fMLP) have been shown to generate similar biological effects in human neutrophils (188). To examine whether IL-8 stimulates PLC activation in HL-60 cells, We compared the effects of IL-8 and fMLP stimulation on IP and IP_3 generation in differentiated HL-60 cells. When pre-labelled HL-60 cells were stimulated with IL-8 or fMLP, saturable generations of inositol phosphates (IP) and IP_3 were observed only in fMLP-stimulated cells (Fig 18, 19). fMLP-induced generation of IP and IP_3 was a rapid cellular event. Release of these phospholipid metabolites was significant ($p<0.05$) by 10 seconds and reached its maximum by 30 seconds. To our surprise, rhIL-8 repeatedly failed to induce IP and IP_3 release in HL-60 cells. No difference was observed in IP and IP_3 generation between IL-8-stimulated and control cells (treated with Hepes-Buffered Saline) throughout the time course. Similar results were observed using freshly isolated human neutrophils. When pre-labelled human neutrophils were stimulated with IL-8 (100 ng/ml) over the times indicated (Fig 20), no detectable IP_3 increase was observed compared to the control cells.

Incubation of [^{3}H]inositol pre-labelled HL-60 cells with various concentrations of fMLP resulted in the release of IP_3 in a concentration dependent manner (Table 4). The concentration of fMLP necessary for the maximum release of IP and IP_3 was identical. The concentration of fMLP giving the maximal IP_3 generation was determined to be 1 μM in HL-60 cells. This value is in good agreement with earlier reports (132). The concentration of IL-8 required for maximal response in human neutrophils is about 10 nM (142, 143). Incubation of pre-labelled HL-60 cells at concentrations of IL-8, several fold below or above 10 nM did not result in any measurable IP_3 generation. These observations

indicate that IL-8 or fMLP stimulation of HL-60 cells results in different effects on PLC - mediated phospholipid metabolism and imply potentially different cellular targets for each of their respective signal transduction mechanisms.

Effect of IL-8 and fMLP on PEt generation-As stated earlier, hydrolysis of phosphatidylcholine by PLD has been recognized as an important source of second messenger molecules in many cell types (51, 69, 70). Phosphatidic acid (PA) generated from PLD activation has been suggested to be involved in the activation of the NADPH oxidase in human neutrophils (71, 72). In addition to the hydrolytic activity that generates phosphatidic acid, PLD also catalyzes a transphosphatidylation reaction in the presence of primary alcohols. This unique reaction is characterized by the transfer of the phosphatidyl moiety of the phosphatidylcholine to the primary alchohol producing phosphatidyl alcohols. Generation of phosphatidylethanol (PEt) in the presence of ethanol is unique to PLD activation among other phospholipid hydrolyzing enzymes. Strong evidence has been accumulated that fMLP stimulates PLD-catalyzed hydrolysis and transphosphatidylation of phospholipids in human neutrophils and HL-60 cells (70, 71, 72). To investigate whether IL-8 stimulates PLD activity in HL-60 cells, the effects of the IL-8 and fMLP exposure to HL-60 cells for the potential generation of PEt was compared. When HL-60 cells pre-labelled with [^{14}C]lyso-PC were stimulated with fMLP (1μM) in the presence of 0.5% (v/v) ethanol, the synthesis of PEt was clearly evident after separation of the lipids by TLC (Fig.21). PEt, which had a R_f value of 0.6 under the conditions used, was confirmed by comparison of its mobility to PEt standard. fMLP induced PEt synthesis only in the presence of ethanol. In contrast, IL-8 (100ng/ml) did not stimulate PEt synthesis in the presence or absence of ethanol (Fig. 21). These results suggest that the signaling pathway for phospholipid metabolism differ for PLD activation between IL-8 and fMLP receptors in HL-60 cells. Further evidence for support of this idea came from the time course of PEt

generation induced by IL-8, fMLP or BSA. As shown in figure 22, addition of fMLP (1μM) to HL-60 cells in the presence of cytochalasin B (5μg/ml) and ethanol (0.5%, v/v) caused a rapid generation of [^{14}C]PEt, the rate of PEt synthesis began to decline after 40 seconds of stimulation. Maximal level of PEt synthesis was reached at about 60 seconds. Since PEt can not be metabolized by HL-60 cells (70), the amount of [^{14}C]PEt remained virtually unchanged after 1 min. The rapid accumulation of [^{14}C]PEt indicates that fMLP induced PLD activation is rapid and transient in nature. In contrast, IL-8 failed to induce PEt generation in the presence of cytochalasin B and ethanol throughout the entire time course in HL-60 cells. No differences were observed in PEt generation between cells exposed to IL-8 and cells which were not exposed. Cytochalasin B has been shown to enhance many chemotactic factor-induced neutrophil responses (189). Although cytochalasin B is known to perturb the cytoskeletal organization in neutrophils, the actual mechanism of this priming process is unclear. Taken together, the above observations strongly suggest that IL-8 stimulation does not generate the second messenger, PA, through the induction of PLD activity and does not activate NADPH oxidase through this pathway in neutrophils or/and differentiated HL-60 cells.

Effect of IL-8 and fMLP on arachidonic acid release-As described earlier, in addition to activation of PLC and PLD, fMLP has been shown to induce the activation of PLA_2 in neutrophils and HL-60 cells (51, 52, 53). fMLP-stimulated PLA_2 activation was evaluated by measuring the release of the radiolabeled arachidonic acid in neutrophils in response to fMLP. To investigate the effect of IL-8 stimulation on PLA_2 activity in HL-60 cells, cells were prelabeled with [^{3}H] arachidonic acid and then challenged with IL-8 or fMLP. Addition of IL-8 or fMLP to HL-60 cells caused a rapid release of arachidonic acid from pre-labelled phospholipids. To determine the kinetics of [^{3}H]arachidonic acid release, measurement of extracellular [^{3}H]arachidonic acid released was carried out over the time course (Fig 23). [^{3}H]Arachidonic acid release was significantly elevated after 2 min

stimulation, maximal about 10 min in response to IL-8 stimulation. Maximal increases in arachidonic acid release in the presence of IL-8 were 3.6 fold over the control. Similar results were obtained with fMLP stimulation as shown in figure 23. The results shown in figure 24 demonstrate that HL-60 cells respond in a dose-dependent manner to IL-8 with a 50% Efficiency Concentration (EC_{50}) value of 1.5 ng/ml. These data indicate that both fMLP and IL-8 induce arachidonic acid release in HL-60 cells and further suggest that arachidonic acid may play a role in both chemotactic receptor-mediated signaling pathways.

Effect of PLA2 inhibitor aristolochic acid on IL-8 stimulated [3H]arachidonic acid release-Agonist-stimulated arachidonic acid release from phospholipids is generally considered to proceed by two main pathways. One involves direct activation of PLA_2. The second pathway involves the action of PLC on inositol phospholipids, leading to the generation of arachidonic acid-rich DAG, which is then deacylated by DAG lipase to generate free arachidonic acid. One way to distinguish between these two pathways is to use specific inhibitors of PLA_2. Aristolochic acid, a nitrophenanthrene derivative isolated from *Aristolochia* species, binds directly to PLA_2 to alter the α-helical content of the protein and inhibit enzymatic activity. Aristolochic acid inhibits a spectrum of calcium-dependent, neutral-active PLA_2s, including those from human synovial fluid and platelets, as well as snake venom (144, 145). Recently it has been demonstrated that aristolochic acid inhibits agonist-stimulated PLA_2 activity in human neutrophils (146). To determine whether IL-8-activated PLA_2 activity can be inhibited by aristolochic acid, HL-60 cells were treated with increasing concentrations of aristolochic acid prior to stimulation with IL-8. Results of such an experiment are seen in Fig 25. Aristolochic acid markedly inhibited release of [^{3}H]arachidonic acid induced by IL-8 in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) value of 100 μM. Results from these experiments suggest that stimulated activation of phospholipase A_2 is directly involved in IL-8-stimulated

arachidonic acid release in HL-60 cells. Therefore, the possibility of an arachidonic acid release by PLC activation and subsequent deacylation of diacylglycerol in response to IL-8 has been ruled out.

Effect of staurosporine, H7 and PMA on [^{3}H]arachidonic acid release induced by IL-8-The activity of phospholipase A_2 has been reported to be regulated by several different mechanisms. These include G proteins, Ca^{2+} mobilization, and protein kinase C activation (147, 190). To explore the possibility that IL-8-stimulated arachidonate release might be dependent upon prior activation of phospholipase A_2 by a C-kinase mechanism, PKC was either down-regulated by chronic PMA treatment, or inhibited by pretreatment with protein kinase C inhibitors, staurosporine and H7. Staurosporine prevents the translocation of cytosolic PKC to the membrane (148). H7 represents a novel class of protein kinase C inhibitors and is much less active against calmodulin-dependent kinase (149). Protein kinase C activity in HL-60 cells was down-regulated by prior (16-h) exposure to 300 μM PMA (150). Cells in the presence or absence of PMA showed no difference in IL-8-stimulated arachidonic acid release (Fig. 26). When cells were pretreated with protein kinase inhibitors, staurosporine and H7 (Fig. 27), no difference was observed in the IL-8-stimulated arachidonic acid release between untreated cells and cells treated with the respective inhibitors. These observations rule out the possibility that PKC plays a direct role in IL-8-induced arachidonic acid release.

Effect of pertussis and cholera toxins on [^{3}H]arachidonate release induced by IL-8-As discussed earlier (page 13), fMLP has been shown to stimulate the release of arachidonic acid via a G protein coupled mechanism in human neutrophils and HL-60 cells (63, 65, 66, 67). Pertussis toxin ADP-ribosylates the inhibitory guanine nucleotide-binding regulatory protein (G_i) and inhibits fMLP-induced activation of phospholipase A_2 in HL-60 cells (63, 65). To investigate the potential role of a G protein coupled mechanism

in IL-8 stimulated PLA_2 activation, HL-60 cells were treated with G-protein probes, i.e., pertussis and cholera toxin. Pretreatment of HL-60 cells with pertussis toxin completely prevented IL-8 induced [^{3}H] arachidonic acid release (Fig 28). Pre-treatment of HL-60 cells with cholera toxin did not have any effect on IL-8 induced arachidonic acid release (Fig 28). Our results are in agreement with previous reports that IL-8 induced chemotaxis in human neutrophils were pertussis toxin sensitive and cholera toxin insensitive (3). This suggests that the G_i/G_0 protein is involved in regulating PLA_2 activity in HL-60 cells stimulated by IL-8.

Effect of Ca^{2+} on IL-8-induced [^{3}H]arachidonic acid release-Ca^{2+} is an important activator of PLA_2, PLC and PLD activities. Ca^{2+} ionophores induce maximal arachidonic acid release in human neutrophils and HL-60 cells (151). To explore the role of Ca^{2+} in IL-8 induced arachidonic acid release, prelabelled HL-60 cells were stimulated with IL-8 under different [Ca^{2+}] conditions. When both intra- and extracellular Ca^{2+} were chelated with EGTA, no IL-8-induced arachidonic acid release was observed (Fig. 29). When extracellular Ca^{2+} was chelated, IL-8 induced arachidonic acid release from prelabelled HL-60 cells, but the cells released arachidonic acid at a slower rate and to a lower maximal threshold (2.5 fold increase v.s. 3.4 fold increase) (Fig. 29). The rate of IL-8-induced arachidonic acid release was greatly reduced in the absence of extracellular Ca^{2+}. These results suggest that Ca^{2+}mobilization plays an important role in IL-8-stimulated PLA_2 activation. External Ca^{2+} mobilization may be the trigger for the IL-8 mediated-G coupled signaling pathway.

Effect of IL-8 on phospholipid breakdown-To examine the phospholipid source of the arachidonic acid released, IL-8-stimulated changes in the [^{3}H]arachidonic acid labeling of phospholipids in the HL-60 cells were determined. Figure 30 shows that arachidonic acid was incorporated into phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylserine (PS). No additional attempt

was made to resolve the inositol phospholipids. The results shown in these figures suggest that IL-8-stimulated [^{3}H]arachidonic acid release were from PC and PE. The release was rapid, and significant by 10 seconds ($p=0.023$).

Autonomous CaM kinase II activity in HL-60 cells-CaM kinase II has been known to be involved in regulating wide a variety of cellular functions including neutrotransmitter synthesis, carbohydrate metabolism, cytoskeletal function, secretion, and gene expression (95). CaM kinase II has been identified in human neutrophils by immunoblotting (98). To investigate the possibility of existence of CaM kinase II activity in HL-60 cells, autonomous CaM kinase II activity was assessed using a CaM kinase II autophosphorylation peptide, autocamtide-2. Autocamtide-2 phosphorylation has been reported as being specific for CaM kinase II (152). Activation of this enzyme by Ca^{2+}/Calmodulin results in a rapid autophosphorylation of Thr 286 or The 287 (on the α- and β- subunits, respectively) (153). The consequence of this autophosphorylation is the generation of an "autonomous" Ca^{2+} and calmodulin independent activity. A model for this mechanism is illustrated in figure 31. Protein concentration dependent autonomous CaM kinase II activity was demonstrated in HL-60 cell homogenate (Figure 32). Since experiments were carried out in the presence of 1 mM Ca^{2+}, the Ca^{2+}-dependent enzyme activity was maximally induced. This suggests that CaM kinase II does exist in HL-60 cells.

Direct evidence for a role of CaM kinase II in IL-8 signaling in neutrophils and/or HL-60 cells has not been reported. However, CaM kinase II can be activated by Ca^{2+} channel regulated by TNF in human neutrophils (97) and IL-8 has been shown to stimulate Ca^{2+} influx through an L-type channel mechanism in the plasma membrane of human neutrophils (191). To determine whether IL-8 activate CaM kinase II autonomous activity in HL-60 cells, the ability of IL-8 to promote the generation of autonomous CaM kinase II

activity was assessed by using autocamtide-2 peptide. HL-60 cells were first stimulated with IL-8 (100 ng/ml) and then homogenized. The 'autonomous' Ca^{2+} and calmodulin independent autophosphorylation was measured (CaM kinase II activity) as described in methods. In these experiments the autonomous activity is expressed as a proportion of the Ca^{2+}-dependent activity to normalize activity to total amount of enzyme present in the homogenate. As illustrated in Fig 33, IL-8 (100 ng/ml) induced a rapid and profound increase in autonomous CaM kinase II activity indicating a rapid activation of CaM kinase II activity by IL-8 stimulation. Autonomous CaM kinase II activity in IL-8 stimulated HL-60 cells increased rapidly relative to control, peaking at 1 min. Maximal stimulation at 1 min was 3.5+0.8 fold over control (BSA). In contrast to its rapid appearance, increased autonomous CaM kinase II activity was prolonged and declined slowly. This result clearly indicates that IL-8 activates CaM kinase II activity which is likely due to increased Ca^{2+}-influx and mobilization of intracellular Ca^{2+}pools.

Protein phosphorylation induced by IL-8 and fMLP- In order to determine whether IL-8 and fMLP induce the same pattern of protein phosphorylation and to identify IL-8 induced-phosphorylated proteins, protein phosphorylation was detected in response to IL-8 and fMLP in permeabilized HL-60 cells. As illustrated in Fig 34, there was markedly different protein phosphorylation pattern between IL-8 and fMLP stimulation in HL-60 cells. As reported earlier (187), IL-8 phosphorylates 48- and 64 -kd proteins in neutrophils. These proteins were also identified in IL-8 and fMLP-induced phosphorylation in HL-60 cells. In addition to 48- and 64-kd proteins, several other proteins were also phosphorylated in IL-8 and fMLP stimulated cells. IL-8 triggered the phosphorylation of p75,p34, and p21 which were not found during fMLP stimulation. On the other hand, fMLP phosphorylated p32 and p38 exclusively. The differences in phosphorylation pattern induced by IL-8 and fMLP strongly suggest that IL-8 and fMLP induce different elements in their respective signaling pathways in HL-60 cells.

Time course of tyrosine phosphorylation in response to IL-8-Protein tyrosine phosphorylation is a common mechanism of intracellular signaling in many types of cells (105, 106). Several tyrosine phosphorylation events have been assigned to fMLP stimulation (107). fMLP induced rapid tyrosine phosphorylation of 80 and 40 kDa proteins in neutrophils (107). To determine whether IL-8 also induce tyrosine phosphorylation in HL-60 cells, immunoblotting was performed in HL-60 cells stimulated with IL-8 over the time course. HL-60 cells were stimulated with IL-8 at various times. Cells then lysed in a solution containing sodium dodecyl sulphate (SDS). Lysates were resolved on polyacrylamide gels, transfered to nitrocellulose membranes, and probed with an antibody against phosphotyrosine. Figure 35 shows the tyrosine phosphorylated proteins in HL-60 cells, but no detectable changes were observed over the control or between different time points. This suggests that IL-8 induces a different signaling pattern from fMLP which does not involve tyrosine phosphorylation.

CHAPTER IV

DISCUSSION

Human IL-8 is the only interleukin shown to elicit chemotactic properties for human neutrophils and stimulates a series of functional activities involved in host defense. These activities include chemotaxis, exocytosis, remodeling of surface proteins, synthesis of leukotrienes, and potentially the induction of oxygen burst metabolism (192). The central focus of this dissertation was to explore the biochemical mechanisms which elicit these IL-8 receptor-mediated cellular responses. IL-8-driven neutrophil chemotaxis and activation are normal physiological immune responses during host defense. Over-response of neutrophils to IL-8 stimulation may be the basis of the pathogenesis of several infectious and non-infectious diseases which include rheumatoid arthritis, psoriasis, asthma, and immune vasculitis (192). Therefore, understanding of IL-8 receptor signal pathway may provide the biochemical targets for design of drugs or inhibitors capable of treating these diseases.

Baculoviral Expression of Recombinant Human IL-8-To pursue the central goal of my research and explore the IL-8 receptor-mediated signaling mechanisms required relatively large quantities of IL-8. This demand led us to express and purify recombinant human IL-8 in a baculovirus expression system. The baculovirus expression system has several major advantages over other expression systems. The polyhedrin promoter-driven expression of high level of recombinant proteins makes this system most attractive. Another advantage of this system is the eukaryotic environment in which the baculovirus expression system produce the expressed recombinant proteins that exhibit most natural

properties. Finally, baculovirus is help-virus-independent. *SF9* cells can be maintained in serum-free medium which makes interleukin purification much easier. The development of an HPLC purification protocol was based on the simplicity of technology. The goals of the first objective of my dissertation, to produce large amounts (>1.0 mg of interleukin/liter of conditioned media) of recombinant human IL-8, to obtain chemotactically viable recombinant human IL-8, and to develop a purification protocol for $_{rh}$IL-8 involving preparative HPLC have been successfully achieved. The question concerning whether or not the Baculovirus vector expression system is capable of producing a biologically viable (chemotaxis active) human IL-8 in large amounts has been addressed in Tables I and III. It is evident that $_{rh}$IL-8, synthesized and secreted from recombinant viral infected *Sf9* cells, was produced in amounts exceeding 500 μg/liter of post infection conditioned media. Recombinant interleukin-8 retained all of the biological properties and chemotactic activity observed for monocyte derived mature product. When used in picomolar concentrations, purified $_{rh}$IL-8 displayed a chemotactic index similar to those value obtained for strong chemotactive peptides such as fLMP. Since the chemotactic assay was performed in the presence of serum, this assay resembled in vivo conditions more closely than that performed in saline buffer. In vivo neutrophils are regulated by a variety of stimuli at same time. Different stimuli may act on neutrophils synergistically and lower the concentration of each stimulus required for individual stimulation. Under such conditions, the concentration of IL-8 required for neutrophil chemotaxis should be relatively close to physiological concentrations. We found that rhIL-8 induced biological activities in neutrophils in the picomolar concentration range and not nanamolar range as reported earlier (17). It is reasonable to assume that picomolar concentration of IL-8 may be in the range of physiological levels. The results of characterization of rhIL-8 summarized in Table II and Table III leave little doubt that the silver stained protein observed in Fig. 17

isolated by the purification protocol has an amino acid composition and immunological properties expected for the post-translationally processed recombinant human IL-8.

Purified recombinant interleukin-8 has the ability to induce oxygen burst metabolism when used in the picomolar amounts and assayed using the sensitive fluorescence assay. In agreement with a recent investigation from the laboratory of Matsushima (157) and in conflict with earlier reports (158), we have confirmed that the rhIL-8 stimulation of human neutrophils for induction of oxygen burst metabolism and superoxide anion formation could not be measured using the relatively insensitive cytochrome C reduction assay. Under conditions where IL-8 consistently failed to stimulate neutrophils for oxygen burst metabolism, positive controls, such as the addition of fLMP peptide, did stimulate superoxide anion formation when assayed using the cytochrome c reduction assay (see Table III). rhIL-8 indued oxygen burst metabolism only in cells that were preincubated with concanavalin A (Con A). Con A, known as an activator of neutrophils, is a plant lectin derived from jack bean and binds to cell-surface glycoproteins with a high specificity for glucopyranoside and mannopyranoside. However, since there are many glycoproteins on neutrophil plasma membranes, including many receptors of neutrophil activators, the specific binding sites for Con A remain unclear. It has been demonstrated that Con A greatly potentiated and primed fMLP-induced oxygen burst in neutrophils. Recently Ohta, *et al* reported that Con A caused an increase in tyrosine phosphorylation of several proteins in neutrophils. However, the mechanism by which Con A primed and potentiated IL-8 induced oxygen burst metabolism in neutrophils is unclear. Moreover, rhIL-8 could always be easily identified in each of the chromatographic eluates by ELISA using antibody generated against human monocyte IL-8.

Taken together, the above results demonstrate that the Baculovirus Vector Expression system is an excellent choice for recombinant expression of rhIL-8. We believe

the baculovirus expression system could be a good choice for the production of many other human interleukins. This vector expression system may be utilized for the production of varying quantities of recombinant interleukin including both small and large scale research amounts of interleukins. Employing preparative HPLC technology has permitted us to quickly purify rhIL-8. Since the scale of interleukin production is not limited, we now have an easy to use vector expression system using a recombinant virus population that will support the purification of vector expressed rhIL-8 in the 10-100 mg range. Such a system has allowed us to pursue studies for IL-8 receptor-induced biochemical mechanisms requiring relative amounts of actively recombinant IL-8.

IL-8 Receptor Signal Transduction Pathway-Although it has been demonstrated that the receptor signaling triggered by IL-8 may involve roles for G protein(s), transient rise in cytosolic free Ca^{2+}, activation of PKC, and phosphorylation events, the intracellular mechanisms that mediate IL-8 induced responses are still poorly understood. In contrast, the early post-receptor events of fMLP signal transduction are reasonably well understood. Transduction of the signal generated by the fMLP-receptor complex is known to proceed through a heterotrimeric GTP binding protein and to be associated with activation of PLC, PLD and PLA_2. Since the biological properties and receptor structure of IL-8 are similar to those of fMLP, in this study, I have asked whether IL-8 and fMLP share similar receptor signal transduction mechanisms. To answer this question, (1) the activation of PLA_2, PLC, and PLD induced by IL-8 and fMLP in differentiated HL-60 cells was tested and the mechanism(s) of the activation of phospholipase was determined, (2) The involvement of protein kinase activity in IL-8 receptor signaling pathway was also investigated.

PLC activation is one of the most common mechanism used by cytokines and hormones to elicit their biological effects (193). Activation of receptors linked to the hydrolysis of PI elicit the generation of DAG and IP_3. IP_3 increase intracellular free Ca^{2+}

from intracellular stores. Thus, the bifurcation of this pathway leads to the generation of at least two second messengers, DAG and Ca^{2+}. Although there is 79% homology between IL-8 and fMLP receptor, their receptor signaling pathways seem to differ in PLC activation. In our studies, IL-8 did not induced IP and IP_3 generation in HL-60 cells and neutrophils. This suggests that PLC activation is unlikely to be involved in the IL-8 receptor signal pathway. However, previous studies have demonstrated that IL-8 stimulated polyphosphoinositide hydrolysis in human peripheral blood lymphocytes (45). But an obvious increase in intracellular Ca^{2+} was not observed in response to IL-8 stimulation in that study. A recent study showed that the IL-8 receptor is coupled to G_o proteins in human lymphocytes (46). This G_o protein is apparently absent in differentiated HL-60 cells and human neutrophils (47). These results suggest that the IL-8- receptor may function through different type of pertussis toxin sensitive G protein(s) in HL-60 cells and lymphocytes. Therefore, IL-8 induced signal pathway could be different in these two cell types.

The precise functional roles of PLC-generated two second messengers, DAG and Ca^{2+}, in activated neutrophils, have not been defined. However, they certainly regulate neutrophils by different mechanisms. A rise in intracellular Ca^{2+} is not required for shape change, which is perfectly normal in Ca^{2+} depleted cells, but is essential for granule exocytosis and induction of the respiratory burst (195). The shape change is also unaffected by PKC inhibitors, staurosporine and H-7, which suggests that DAG-activated PKC are not necessary for neutrophil motility (196). A difference in the control of exocytosis and the respiratory burst was demonstrated with staurosporine, which inhibits the burst but not the release of granule enzymes (194). In spite of the link in the generation of DAG and Ca^{2+} (from PLC activation), neither second messenger is unique to the PLC signaling system; each can be generated from other signalings by appropriate receptor stimulation. For example, DAG can be produced without a concurrent change in IP_3 and

intracellular Ca^{2+} by activation that coupled to PLD. Similarly, intracellular Ca^{2+} can be increased via receptor-ligated Ca^{2+} channels and voltage-sensitive Ca^{2+} channels without an accompanying change in DAG. Thus, although IL-8 does not activate PLC, Ca^{2+} and DAG could be generated from other IL-8-induced signaling pathways and similar responses can be elicited in neutrophils.

The identity of PLC isoforms involved in neutrophil activation is unclear. Immunological studies and cDNA analysis have revealed that several PLC isoforms are present in human neutrophils. They include PLC β1, 2 and γ 1,2 (48, 49). PLC-γ family has been shown to be regulated by tyrosine phosphorylation, not by G protein mechanism. fMLP-stimulated PLC activity can be depleted by permeabilization with streptolysin O, implying that the PLC enzyme is cytosolic and can leak through plasma membrane pores. The PLC β1 isoform was shown to be regulated by G_q family, the pertussis insensitive family (159). Recently, it has been demonstrated that PLC is activated by βγ-subunits of G proteins rather than by $G_{i\alpha}$.

Another phospholipase that exists in neutrophils and plays an important role in neutrophil activation is phospholipase D. PLD has two distinct enzymatic activities. The first one is to hydrolyze phospholipids to generate the free polar head group and phosphatidic acid. The second activity is to catalyze a transphophatidylation reaction in the presence of primary alcohol and produce phosphatidylalcohol (160, 161). The generation of phosphatidylethanol (PEt) in the presence of ethanol is the unique indication of PLD activation. As we observed earlier, HL-60 cells differ in PEt generation in response to IL-8 and fMLP. fMLP-stimulated rapid generation of PEt was obtained on TLC plates. Since the PEt can not be metabolized by cells, accumulation of PEt was observed in HL-60 cells over the time course. The microfilament disruptor, cytochalasin B, enhances PLD activation and oxidative burst of neutrophil in response to chemotactic factor like fMLP.

The basis of the action of cytochalasin B is poorly understood, but is most likely to be involved in changes in the cytoskeletal network. The granules can get complete access to the plasma membrane when cytochalasin B is present (189). In the presence of cytochalasin B, no detectable PEt was generated in response to IL-8 in HL-60 cells. PA generated from PLD activation plays an important role in oxidative burst (74, 75). The results showing that IL-8 failed to induce PLD activation could explain why IL-8 failed to induce oxygen burst directly in human neutrophils.

The mechanisms by which fMLP activate PLD activity in neutrophils and HL-60 cells remain unclear. It was believed earlier that fMLP receptor coupled G proteins may activate PLD directly (70, 71). The most recent report challenged the earlier findings (189a). Cockcroft et al. reported that PLD is a downstream effector of ADP-ribosylation factor in HL-60 cells. Since PLD can be activated by both GTPγS and fluoride, it support the view that ADP-ribosylation factor activation occurs after activation of a G protein.

Neutrophils release arachidonic acid and its metabolites in response to fMLP stimulation. The released arachidonic acid and its metabolites through the action of PLA_2 are believed to play an important role in various physiological and pathophysiological processes. The results presented in this study demonstrate that a phospholipase A_2 acting upon PC and PE is stimulated in HL-60 cells in response to IL-8. This conclusion is supported by several lines of evidence. First, IL-8-induced release of [^{3}H]arachidonate is both time-and dose-dependent in HL-60 cells (Fig. 23, 24). Second, the phospholipase A_2 inhibitor, aristolochic acid, completely inhibited release of [^{3}H]arachidonate induced by IL-8 (Fig. 25). Third, close examination of the cellular phospholipids demonstrated that the fatty acid was released from both PC and PE upon IL-8 stimulation (Fig. 30).

A 110-kDa PC-specific phospholipase A_2 has been purified and cloned from another human leukemic cell line, U937 cell (162, 163). This cytosolic PLA_2 has been shown to be activated by submicromolar concentrations of calcium. It contains a 45-

amino-acid Ca^{2+} binding domain and a putative Ca^{2+}-dependent lipid binding sequence. In permeabilized HL-60 cells, the ability of IL-8 to stimulate PLA_2 diminishes if the cytosolic is allowed to leak out first (data not shown). This would be indicative of presence of a cytosolic PLA_2 activity in HL-60 cells. More recently, it was shown that human neutrophils contain a cytosolic PLA_2 with biochemical properties analogous to that of U937 $cPLA_2$ (164) This neutrophil $cPLA_2$ is immunologically indistinguishable from U937 $cPLA_2$. Whether both IL-8 and fMLP stimulate the same PLA_2 activity and the identity of this PLA_2 are questions which remain to be answered.

The transient nature of the PLA_2 response is compatible with its potential participation in signalling by IL-8 in HL-60 cells. The lasting activation of PLA_2 activity, still observed after 10 min in IL-8 stimulated cells (Fig. 2), could be accounted for by consequent platelet activating factor or leukotriene B_4 activation of PLA_2 by release of intracellular Ca^{2+} stores (165).

As described earlier, agonist-stimulated arachidonic acid release is considered to proceed through two main pathways. One involves PLA_2 action on phospholipids releasing arachidonic acid from the *sn*-2 position. The second pathway involves DAG generation. DAG generated from the activation of PLC and/or PLD can release arachidonic acid through the action of DAG lipase. Free arachidonic acid is released from the stimulated cells without activation of PLA_2. The activation of PLA_2 is probably required for IL-8 stimulated arachidonic acid release in HL-60 cells, since the PLA_2 inhibitor, aristolochic acid, completely inhibited IL-8-stimulated arachidonic acid release. Furthermore, we have found that IL-8 did not stimulate the activation of PLD and PLC activity in HL-60 cells.

PLA_2 activation has been reported to be regulated by several mechanisms including Ca^{2+}, PKC, and G proteins (147, 190). Cytosolic Ca^{2+} levels have long been proposed

to play a role in the regulation of intracellular PLA_2 (166). In fact, many studies have demonstrated that Ca^{2+} ionophores can induce maximal release of arachidonic acid from cellular phospholipids suggesting that receptor activation is not a pre-requite for activation of PLA_2. Although, IL-8 has been reported to stimulate Ca^{2+} channel in neutrophils, we observed that IL-8 still induced AA release with the depletion of extracellular Ca^{2+} in HL-60 cells. The mechanism(s) by which IL-8 induces increase in intracellular Ca^{2+} and its relation with PLA_2 activation needs to be further investigated.

Protein kinase C may be another important component in the regulation of intracellular PLA_2, although in many cells, activation of PKC alone is not sufficient to induce liberation of arachidonic acid release from cellular phospholipids (197). Activators of PKC such as phorbol diester and DAG enhance the release of arachidonic acid induced by Ca^{2+}-ionophores and/or agonists in many cellular systems (198). Inhibitors of PKC, and down-regulation of PKC by pretreatment of cells with PMA, on the other hand, reduce receptor-stimulated release of arachidonic acid. However, involvement of protein kinase C on phospholipase A_2 activation seems unlikely, since specific protein kinase C inhibitors, staurosporine and H7, did not inhibit IL-8 induced arachidonic acid release. HL-60 cells, in which PKC was down-regulated by pretreatment with PMA for 16 hrs, showed no significant difference in the IL-8-stimulated release of arachidonic acid compared with control cells. However, these results did not exclude the possibility that a subclass of PKC activity insensitive to these inhibitors exists in HL-60 granulocytes.

IL-8 receptor signaling is mediated by pertussis toxin sensitive G protein(s). If the activation of phospholipase A_2 activity is coupled directly to IL-8 receptor occupation, it is likely that a G protein may mediate this interaction. The IL-8 receptor has been shown to couple to at least one G protein which is insensitive to cholera toxin and sensitive to pertussis toxin (17). G protein regulation of phospholipase A_2 has been suggested in a

variety of cell types (167, 168). The activation of PLA_2 may be stimulated by G proteins independent of Ca^{2+} and/or PKC signaling pathway. Pertussis toxin inhibits fMLP induced arachidonic acid release in HL-60 cells and neutrophils (66, 67), a result compatible with phospholipase A_2 activation by G protein. In our experiments, we demonstrated that IL-8-induced release of arachidonic acid was sensitive to pertussis toxin and insensitive to cholera toxin, which is compatible with report that IL-8 receptor was coupled to pertussis toxin sensitive G protein (17). Although PLA_2 activity may be regulated directly by G protein, the type of G protein which regulates this activation and whether it is identical with the G protein that regulates PLC remains to be clarified. The mechanism of G protein mediated-activation of PLA_2 activity in HL-60 cells is unknown.

Studies demonstrating G protein control of arachidonic acid release in HL-60 cells are consistent with the notion that receptor-mediated activation of PLA_2 resulting in arachidonic acid and its metabolites production may represent an independent signal transduction pathway. Fatty acids may have messenger-like role(s) within cells. It has been reported that arachidonic acid can induce the release of intracellular Ca^{2+} in HL-60 cells (169), platelets (170), and pancreatic islets (171). These studies have shown that arachidonic acid induced the release of Ca^{2+}, sequestered in the endoplasmic reticulum at micromolar concentration, comparable to those required for IP_3 to exert such an effect. It may be possible, therefore, that arachidonic acid plays an important role in IL-8-stimulated calcium mobilization. Furthermore, arachidonic acid and its lipoxygenase metabolites were shown to modulate the activity of ion channels in nerve (172) and muscle cells (173, 174). More interestingly, arachidonic acid was shown to regulate Ca^{2+} channels and induce Ca^{2+} influx in neutrophils (199). Although the mechanism of IL-8 induced opening of Ca^{2+} channel is unclear, PLA_2 activation may play an important role. Arachidonic acid has also been proposed to activate certain isozymes of protein kinase C, in particular γ, in DAG independent manner (175, 176). In addition, archidonic acid and prostaglandins

were shown to have differential effects on the activity of RAS-GAP (GTPase activating protein) suggesting that eicosanoids may participate in the control of the state of cellular Ras (177). As such, stimulated PLA_2 activity may be important , not just in affecting short term signals within cells, but also in regulating longer term cellular responses.

Protein phosphorylation is a common mechanism of intracellular signaling in virtually all types of cells. Upon selective introduction of phosphate group on serine, threonine, or tyrosine residues, the charge and conformation of the substrate protein are markedly altered, leading to changes in enzymatic activity, binding properties, intracellular distribution, etc., and consequently to modification in signaling. Protein phosphorylation events are catalyzed by protein kinases. They are subdivided into two classes on the basis of their target amino acid: serine or threonine on the one hand, and tyrosine on the other. The catalytic difference is reflected by class-specific structural domains in the putative active site of the enzymes.

Binding of chemotactic factors to their receptors on neutrophils triggers a complex series of protein phosphorylation reactions including serine/threonine and tyrosine phosphorylation. Several phosphoproteins having molecular masses of 82, 66, 64, 58, 50, 48, and 27 kDa have been detected following stimulation with fMLP (200). Phosphoproteins of 64, 48, 42, 40, 38, and 32 kDa were also identified in our experiment stimulated with fMLP (Fig 28). This discrepancy may be due to the difference in phosphorylation conditions. HL-60 cells stimulated with IL-8 display a greatly different phosphorylation profile. In addition to 48- and 64 kDa proteins, IL-8 triggered the phosphorylation of p75, 35, and 21 which were not detected following fMLP stimulation. The 64 kDa protein is the major phosphorylated protein induced by IL-8 and fMLP in neutrophils (202). It has been characterized by Shibata, *et al* (201). The results of phosphoamino acid analysis shows that p64 is phosphorylated by a serine kinase in human

neutrophils (201). fMLP and IL-8-stimulated phosphorylatin of p64 was inhibited by preincubation N-(6-aminohexy)-5-chloro-1-naphthalenesulfonamide (W-7, a Ca^{2+}/Calmodulin-dependent kinase inhibitor) or staurosporine, and p64 was phosphorylated with PMA stimulation. These results suggest that protein kinase C and/or Ca^{2+}/Calmodulin dependent kinase may mediate the phosphorylation of p64.

Protein-tyrosine kinase has been suggested to be involved in cellular proliferation or transformation of cells because of their close association with products of several viral transforming genes and a number of growth factor receptors (186). However, several investigators have reported that the increase of protein-tyrosine phosphorylation occurred in neutrophils (105, 106, 107). fMLP causes tyrosine-phosphorylation of 80-and 40 kDa proteins in neutrophils (105). Tyrosine-phosphorylation of these proteins was inhibited by pertussis toxin treatment. The identity of the these two tyrosine phosphorylated proteins is unclear. To examine whether IL-8 also stimulates tyrosine phosphorylation in HL-60 cells, immunoblotting was performed in HL-60 cells stimulated with IL-8 over the time course. It was found that IL-8 did not induce tyrosine phosphorylation changes in HL-60 cells. Protein tyrosine phosphorylation has been suggested to play an important role in activation of the NADPH oxidase and in superoxide anion generation. The phosphotyrosine phosphatase inhibitor, orthovanadate, has been reported stimulate tyrosine phosphorylation and oxygen consumption in neutrophils (203). Furthermore, fMLP-induced tyrosine phosphorylation and superoxide production was almost completely inhibited by tyrosine kinase inhibitors. The mechanism by which chemotactic factor (fMLP) stimulates tyrosine phosphorylation in neutrophils is unclear. Recently Uings, *et al* reported that tyrosine phosphorylation is involved in receptor coupling to PLD but not PLC in human neutrophils (204). Now it becomes more apparent that both PLD activation and tyrosine phosphorylation may be involved in the activation of the NADPH oxidase. In contrast, IL-8 did not stimulate these processes. These results may further explain the inability of IL-8

to induce oxygen burst metabolism in human neutrophils.

CaM kinase II is a serine/threonine kinase. CaM kinase II activity in neutrophils was assessed by using a CaM kinase II autophosphorylation segment, autocamtide-2. The production of a Ca^{2+}-independent activity is the unique indication of CaM kinase II autophosphorylation. The assay using this peptide substrate representing the region in which autophosphorylation occurs allowed an accurate quantitative evaluation of the CaM kinase II activity. Calmodulin is present in high concentrations in neutrophils (205), but its exact role calmodulin in neutrophil activation remains unclear. CaM kinase II has been detected by immunoblotting with antibody against CaM kinase II in human neutrophils (98). Further proof of the presence of the kinase in neutrophils was provided during this investigation by direct measurement of autonomous CaM kinase II activity (Fig 26). The kinase activity present in neutrophils was protein-dependent. Since 1 mM Ca^{2+} and calmodulin were present in the incubation buffer, this kinase activity represents the maximal stimulation.

The time course for IL-8-induced autonomous CaM kinase II activity (Fig 29) provided the first evidence that CaM kinase II activity may be activated by IL-8 in HL-60 cells. CaM kinase II activity was increased rapidly and remained high over a sustained period. Once the enzyme is activated by Ca^{2+}/Calmodulin, it becomes Ca^{2+}/Calmodulin independent. This suggest that CaM kinase II may remain active after intracellular Ca^{2+} has returned to basal levels, resulting in prolonged phosphorylation of it's substrates. The physiological role of CaM kinase II in neutrophils is not clear. One possible function might be the regulation of cytoskeletal movement which is essential for chemotaxis and exocytosis. Since the common substrates of CaM kinase II, MAP-2 and MLCK (myosin light chain kinase) are activated in fMLP induced chemotaxis in neutrophils. A similar mechanism may be utilized by IL-8 stimulated cells. Another potential substrate is

calcineurin (protein phosphatase 2B). Inhibition of this enzyme totally blocked fMLP induced chemotaxis in human neutrophils (206).

Although it was shown that existence of this CaM kinase II activity in HL-60 cells and that IL-8 activated CaM kinase II in a temporal manner. More experiments are needed to confirm these preliminary results: (1) CaM kinase II inhibitors inhibiting IL-8 induced responses (chemotaxis or exocytosis) in neutrophils; (2) anti-CaM kinase II antibody precipitating CaM kinase II in HL-60 cells; (3) pertussis toxin examining its effect on IL-8 induced CaM kinase II activity.

Cross-talk between intracellular signaling systems is recognized as one of the major means for cells to regulate and orchestrate a variety of responses to different stimuli. Cross-talk is usually defined as a modulatory interaction between two distinct channels of information transfer. In the context of signal transduction, cross-talk may occur by three modes: (1) if a constituent of one pathway is modulated by a constitutent of another pathway; (2) if two pathways converge upon a common target or (3) if a messenger of one pathway is converted to a messenger of another pathway. Evidence for cross-talk between CaM kinase II and PLA_2 has been demonstrated in neutral tissue (synaptic terminals) (207). It was found that purified CaM kinase II inhibited PLA_2 activity in lysed nerve endings, and this effect was prevented by treating the lysates with a selective peptide inhibitor of CaM kinase II (208). On the other hand, CaM kinase II was inhibited by products of PLA_2 activity, arachidonic acid and its lipoxygenase metabolites. This negative cross-talk between CaM kinase II and PLA_2 suggests that these two receptor-mediated cascades may exert mutually antagonistic action upon each other. Alternatively it may represent a mechanism of negative feedback control in these cells. As demonstrated earlier, IL-8 activates both PLA_2 and CaM kinase II in HL-60 cells. The existence of cross-talk between PLA_2 and CaM kinase II in HL-60 cells and its physiological consequences of this cross-talk need to be further investigated.

In summary, contrary to my expectation, this study shows that IL-8 and fMLP do not share a common receptor signal transduction pathway. A model of fMLP receptor signaling pathway is shown in figure 4. A putative model of IL-8 receptor signal transduction pathway that represents findings provided in this dissertation is displayed in Fig. 36. We have found that both IL-8 and fMLP induced a rapid and transient activation of PLA_2 in differentiated HL-60 cells. The consequence of phospholipase A_2 activation was the release of arachidonic acid and the generation of lysophospholipids from membrane PC and PE. The IL-8 stimulated-arachidonic acid release was pertussis toxin and phospholipase A_2 inhibitor sensitive, and PKC independent. These results strongly suggest that occupation of the IL-8 receptor is coupled to activation of PLA_2 via a G protein mechanism as shown in our model (Fig 36). In contract to fMLP, IL-8 did not stimulate the activation of PLC and PLD. In good agreement with earlier reports (Fig 4), fMLP did induce the activation of PLC and PLD activity in HL-60 cells. When comparing the phosphorylation events induced by IL-8 and fMLP, we found that these two chemotactic factors triggered different protein phosphorylation profiles. Tyrosine phosphorylation of proteins has not been detected following IL-8 stimulation in HL-60 cells. Furthermore IL-8 stimulated the rapid autophosphorylation of calcium/calmodulin-dependent protein kinase II (CaM kinase II). It indicates that this enzyme is an integral component of IL-8 receptor signal pathway.

Cells respond to changes in their environment and exert their biological functions through the interaction between ligands and cell surface receptors. The cellular responses are intricately controlled by cascades of biological events (steps) induced by ligand receptor activation in cells. Differences in cellular signaling induced by IL-8 and fMLP receptor imply differences in neutrophilic functions induced by them. As discussed earlier, neutrophils do respond to IL-8 and fMLP differently. For example, IL-8 is a more potent

chemotactic factor than fMLP (17). Picomolar IL-8 can induce chemotaxis response for human neutrophils. Unlike fMLP, IL-8 does not induce superoxide production directly in neutrophils. Under certain conditions, IL-8 even does not induce exocytosis in neutrophils (209). Although the detailed biochemical mechanisms of these neutrophil activities remain unclear, preliminary elucidation of IL-8 receptor signaling pathway may provide some explanations for these differences. Failure in the activation of PLD and induction of tyrosine phosphorylation by IL-8 may lead to its inability to induce oxygen burst metabolism. PLA_2 activation and/or the activation of CaM kinase II may play important roles in IL-8 induced chemotaxis. Taken together, I propose that IL-8 plays an important role in neutrophil homing events rather than bacterial or viral killing events. By homing, IL-8 may drive mature neutrophils from bone marrow into blood circulation and finally to the site of inflammation. At the same time, neutrophils are primed by IL-8. Woznick *et al* (208) recently reported that IL-8 primes NADPH oxidase and PLA_2 activation induced by fMLP in human neutrophils. In addition IL-8 enhanced the fMLP-stimulated increase in $[Ca^{2+}]$. Most interestingly, leukemic cells from acute myeloblast leukemia (AML) were shown to express functional IL-8 receptors. IL-8 induced cytosolic free calcium changes in these myeloid leukemic cells expressing the IL-8 receptor (209). This indicates that IL-8 may be activating the same signaling pathway in myeloid leukemic cells as in neutrophils. Therefore, it is conceivable that one of the two IL-8 receptors, presumably the IL-8 receptor-2, may be involved in functions that are unrelated to the recruitment of inflammatory cells. Most recently, it was shown that IL-8 suppressed colony formation of immature subsets of normal myeloid progenitor cells stimulated by GM-CSF and stem cell factor (210). Therefore, it is my expectation that neutrophil homing may not only be the biological effect of IL-8, and that a search for a broader spectrum of IL-8 activities in hematopoiesis should be further explored.

Figure 1. **Schematic representation of phagocytic exocytosis and respiratory burst.** The NADPH oxidase is selectivly activated in the wall of the vacuole, generating O_2^- and hydrogen peroxide in the vacuolar lumen. Enzymes are also released into the vacuole by degranulation of cytoplasmic granules.

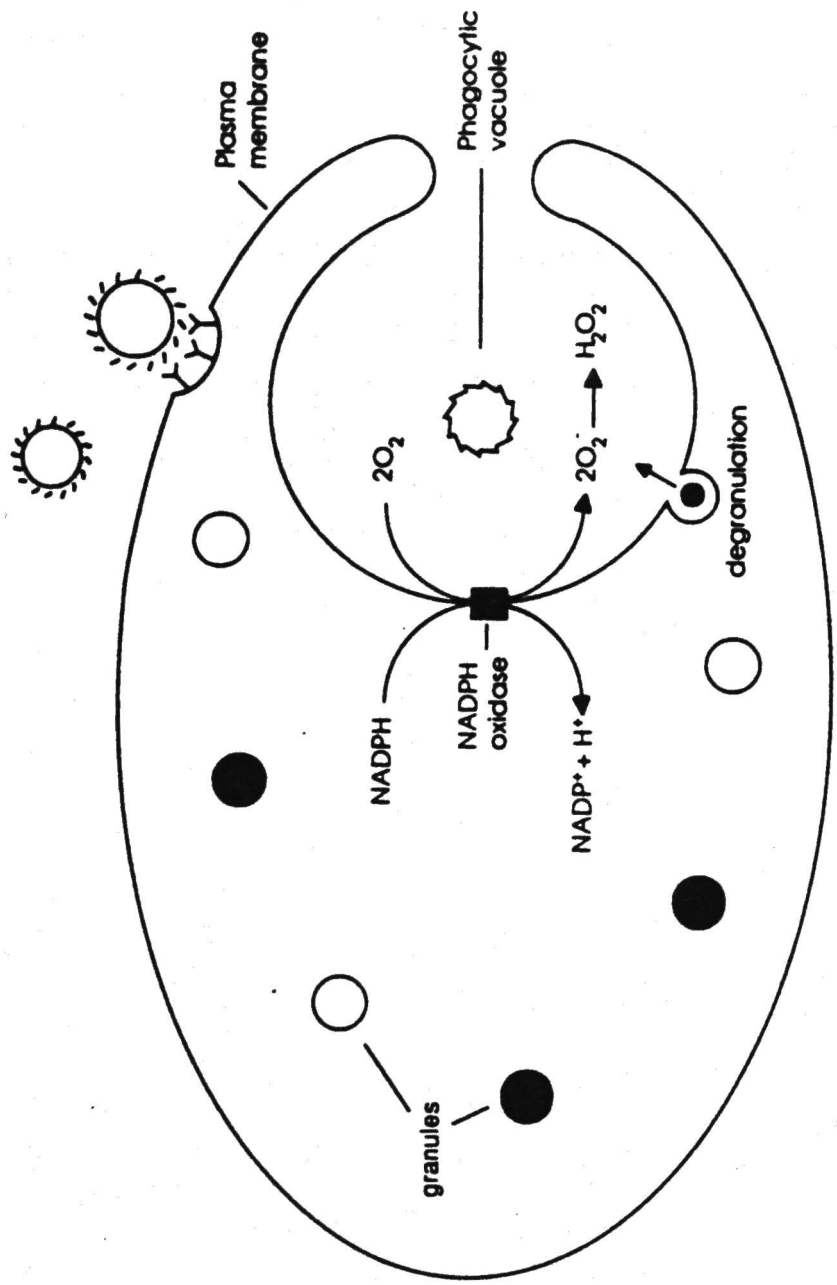

Plasma membrane
Phagocytic vacuole
$2O_2$
$2O_2^{\cdot}$
H_2O_2
degranulation
NADPH
NADPH oxidase
$NADP^+ + H^+$
granules

Figure 2. **The nucleotide sequence and deduced amino acid sequence of human genomic IL-8 DNA.** Nucleotides are numbered starting at the beginning of a previously reported cDNA (120). The "TATA" and "CAT" boxes are underscored. Presumed polyadenylation signal sequences are also underscored.

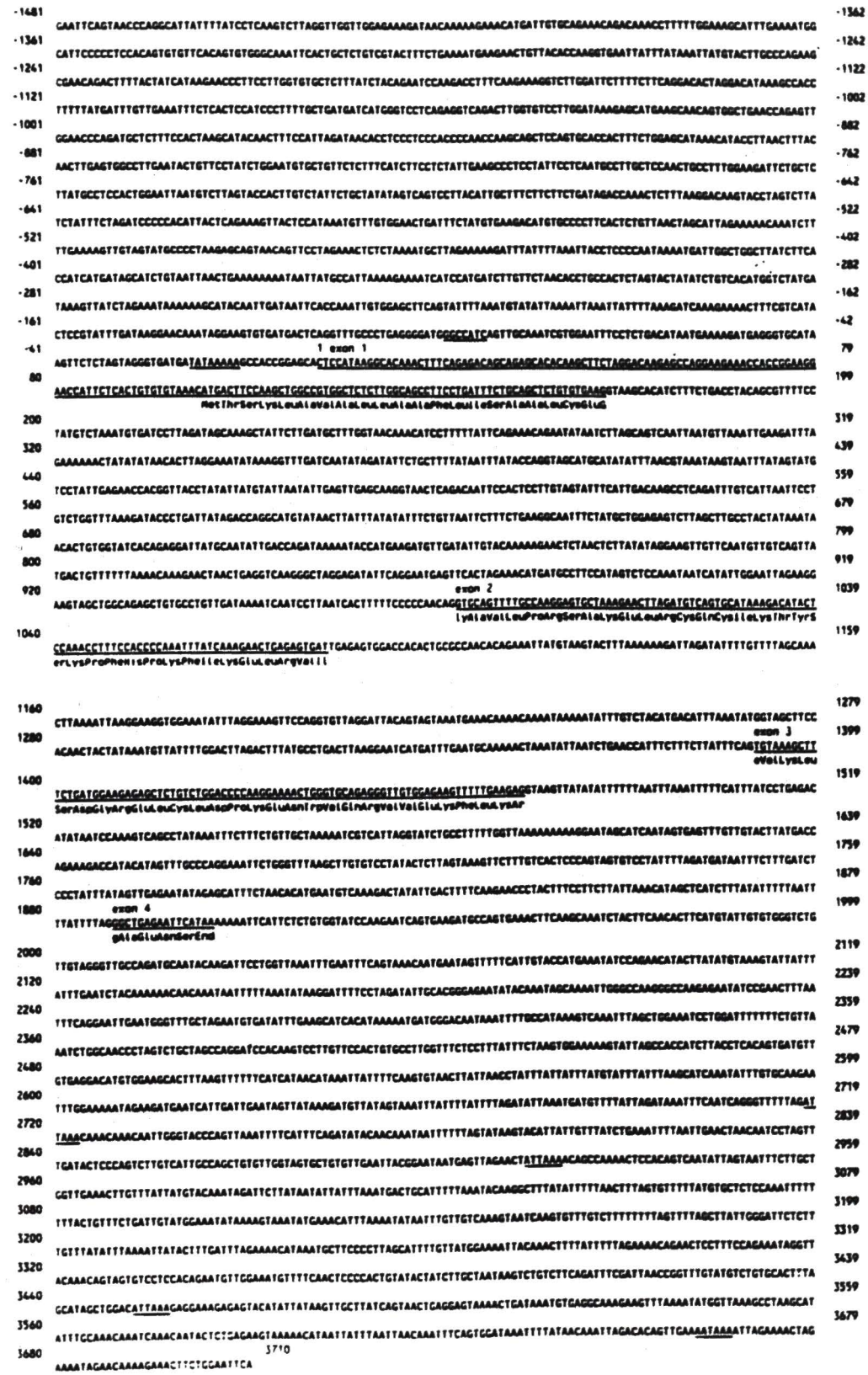


Figure 3. **Baculovirus life cycle.** The schematic depicts of the unique biphasic life cycle of a typical baculovirus. In the environment a susceptible insect ingests the viral occlusions from a food source. The crystal dissociates in the gut of the susceptible insect to release the infectious virus particles which invade the gut cells, penetrate to the nucleus and uncoat. Viral DNA replication is detected by 6 hours. By 10-12 hours post infection extracellular virus bud from the surface to infect other cells and tissues. Late in infection (18-24 hours post infection) the polyhedrin protein assembles in the nucleus of the infected cells and virus particles become embedded in the proteinacous occlusions. The viral occlusions accumulate to large numbers and the cell lyses. The viral occlusions are responsible for horizontal transmission among susceptible insects, the extracellular virus is responsible for secondary and cell to cell infection in cultured cells or the insect host (119).

Baculovirus Life Cycle

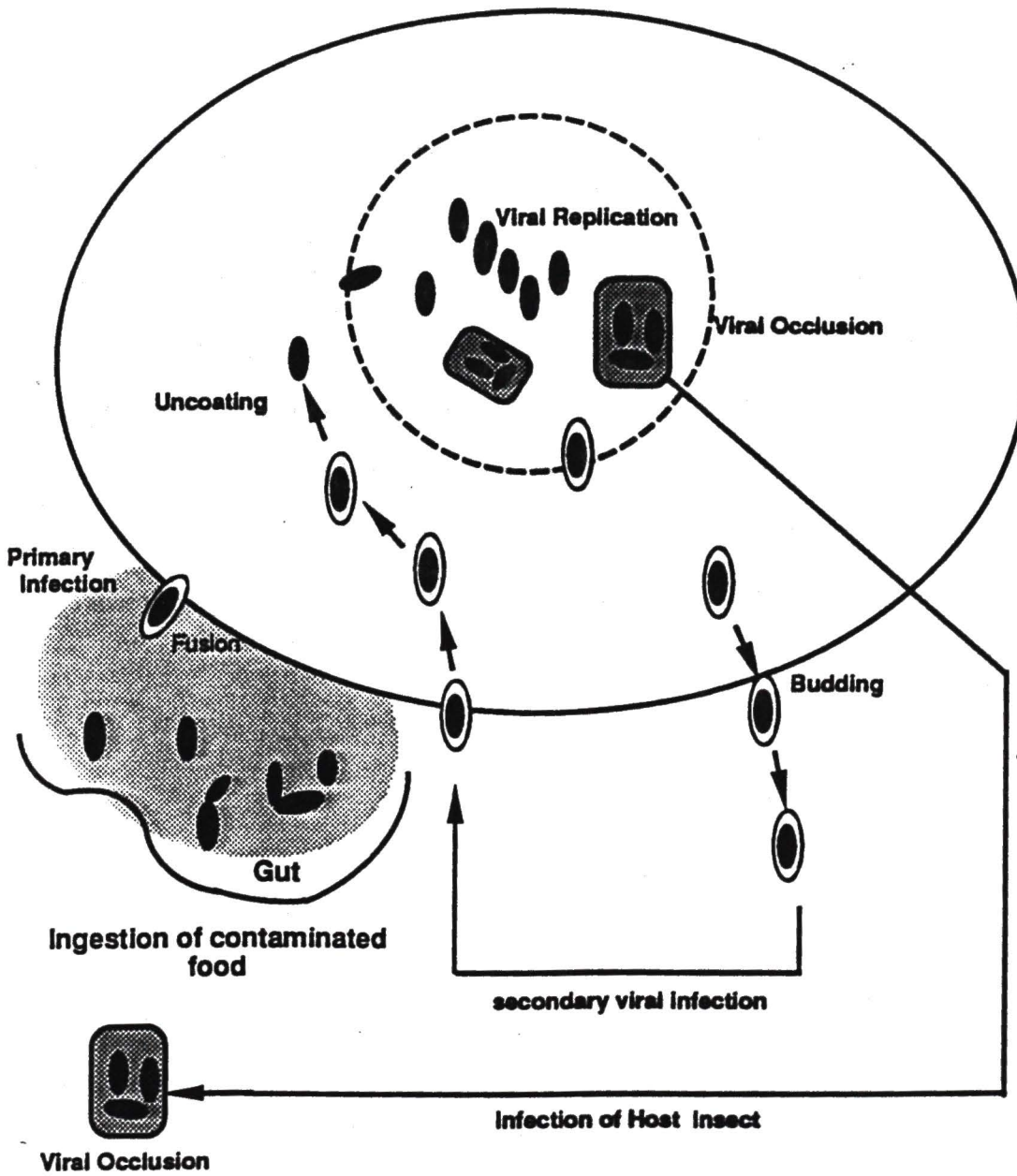


Figure 4. **Multiple pathways triggered by the fMLP receptor in neutrophils.** Rapid activation of three phospholipases are known to be regulated by the fMLP receptor. The primary event is the PLC activation and the subsequent increase in cytosol Ca^{2+} and protein kinase C activity which in turn, triggers PLD and PLA_2 downstream. Alternatively, the G protein(s) can also directly activate PLD and PLA_2. Synergistic activation of the downstream phospholipases occur due to the simultaneous activation of a G protein and an increase in Ca^{2+} and protein kinase C activity. G_p, G_a, G_e, are the G-protein(s) that regulate PLC, PLA_2, and PLD respectively. A, agonist; R, receptor.

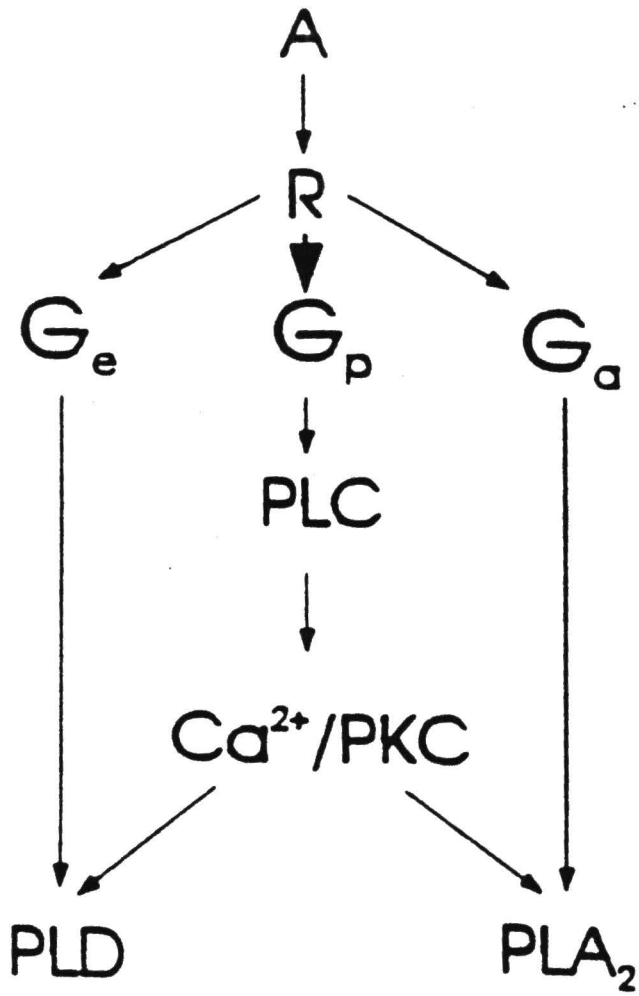
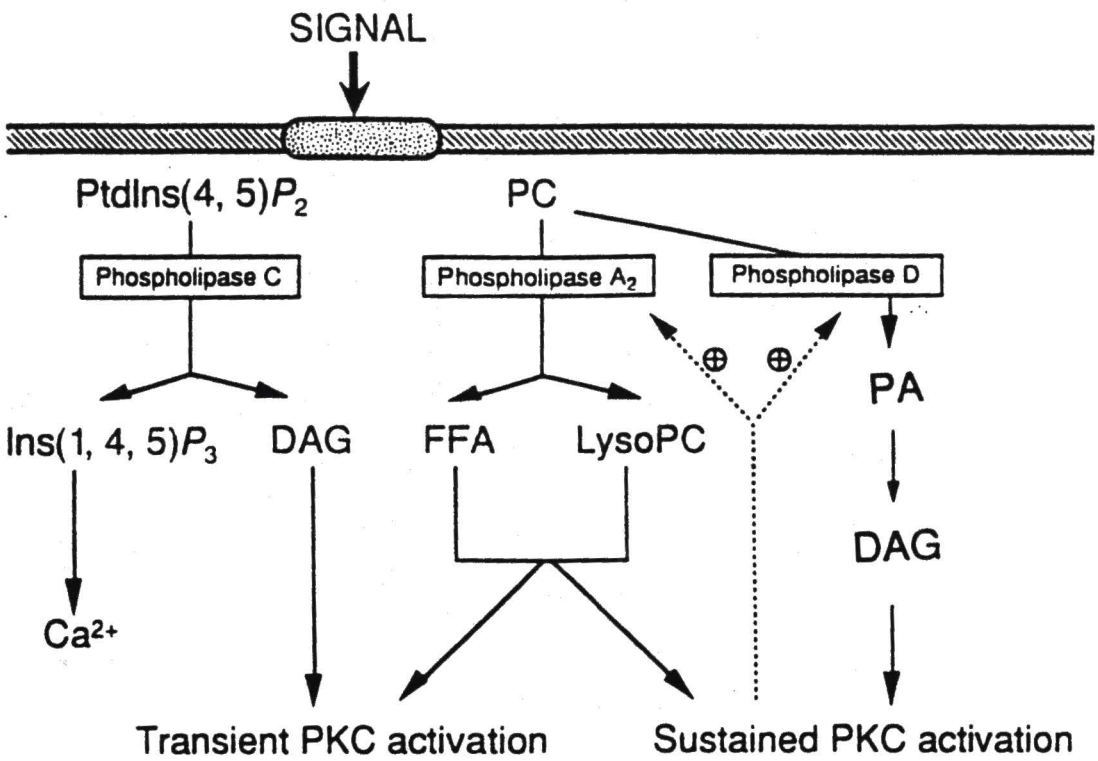

A
R
G_e
G_p
G_a
PLC
Ca^{2+}/PKC
PLD
PLA_2

Figure 5. **Cellular phospholipid breakdown by signal-activated phospholipases.** (A) PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; Ins(1,4,5)P_3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; FFA, *cis*-unsaturated fatty acid; LysoPC, lysophosphatidylcholine; PKC, protein kinase C. Dashed lines and the crosses inside circles indicate the positive feedback effect of PKC on the activities of phospholipases. (B) Glycerophospholipids consist of a hydrophobic DAG moiety, linked via a phosphodiester bond to a polar headgroup which differs among phospholipid classes.


SIGNAL
PtdIns(4, 5)P_2
PC
Phospholipase C
Phospholipase A_2
Phospholipase D
PA
Ins(1, 4, 5)P_3
DAG
FFA
LysoPC
DAG
Ca^{2+}
Transient PKC activation
Sustained PKC activation

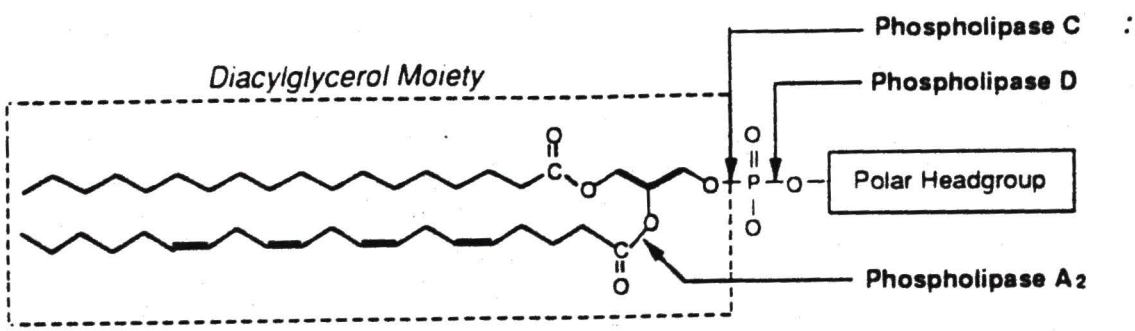

Phospholipase C
Diacylglycerol Moiety
Phospholipase D
Polar Headgroup
Phospholipase A_2

Figure 6. **AcNPV shuttle vector pVL 1392.** The circular restriction map is the AcNPV shuttle vector (transfer vector) pVL 1392. Number at restriction sites indicate distance in base pairs (bp) from the Hind III end of the pUC8 backbone; the arrow marks the polyhedrin sequence; Ap^r, ampicillin resistance gene.

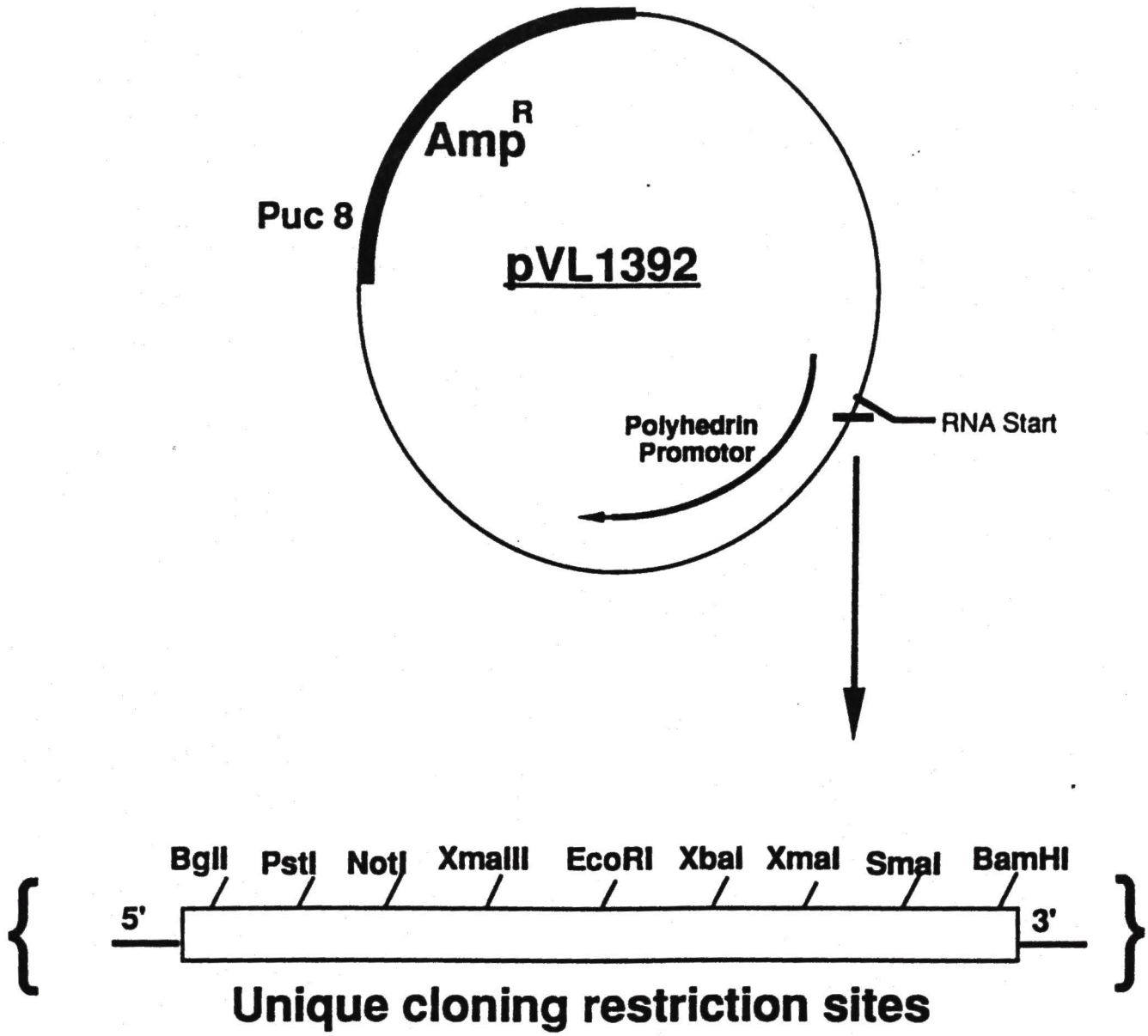

Amp R
Puc 8
pVL1392
Polyhedrin Promotor
RNA Start
BglI
PstI
NotI
XmaIII
EcoRI
XbaI
XmaI
SmaI
BamHI
5'
3'
Unique cloning restriction sites

Figure 7. **Subcloning strategy for human IL-8 gene.** Subcloning of the structural portion of human IL-8 cDNA from a bacterial plasmid source to the Baculovirus shuttle plasmid pLV1392.

SUBCLONING STRATEGY FOR HUMAN IL-8

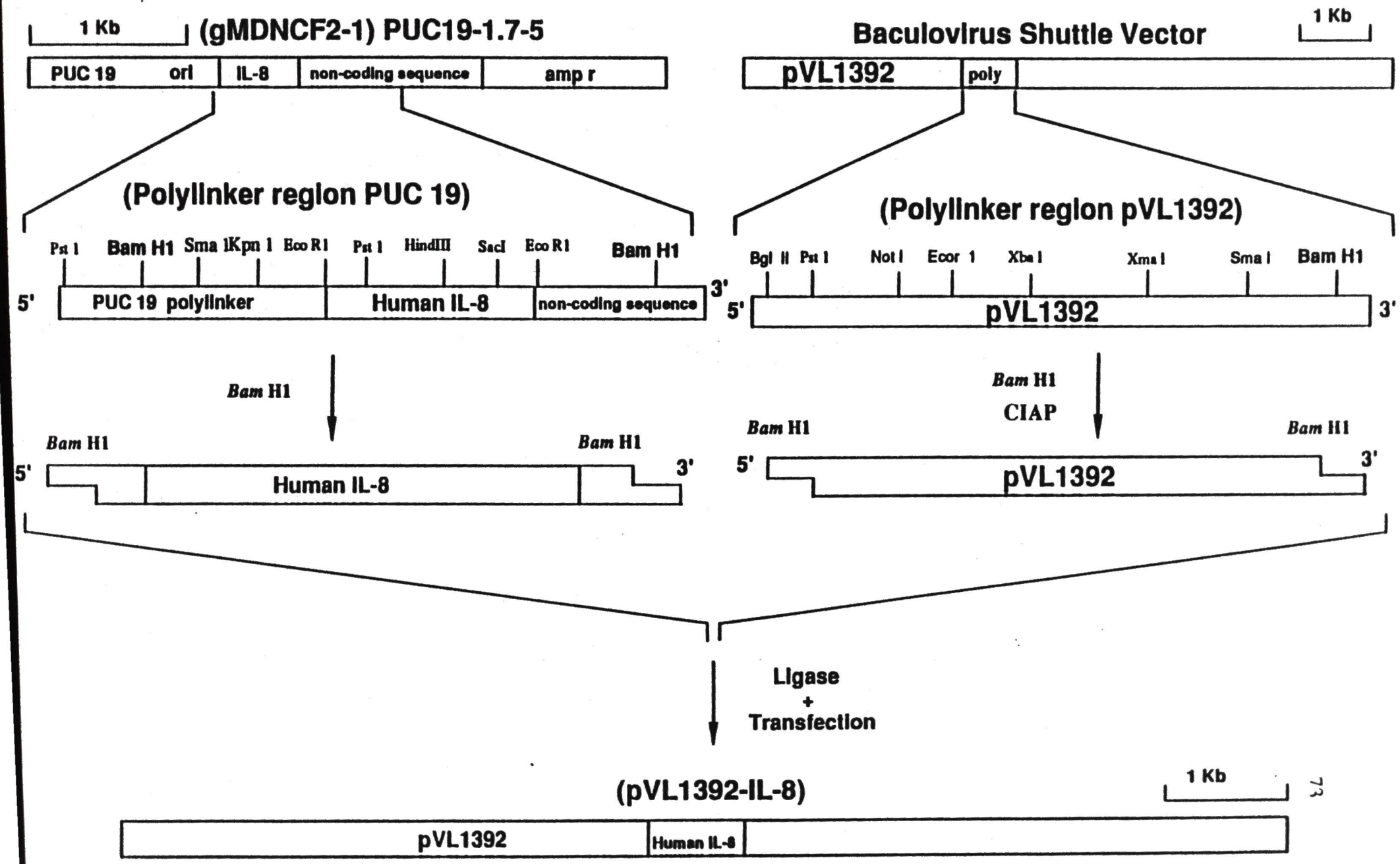


Figure 8. **Model for chemotaxis assay.** This figure shows dimensions of a tissue culture dish and punch employed to form and align wells in the agarose gel. A maximum of six replicates of the triplicate wells are made to eliminate overlap of adjacent gradients.

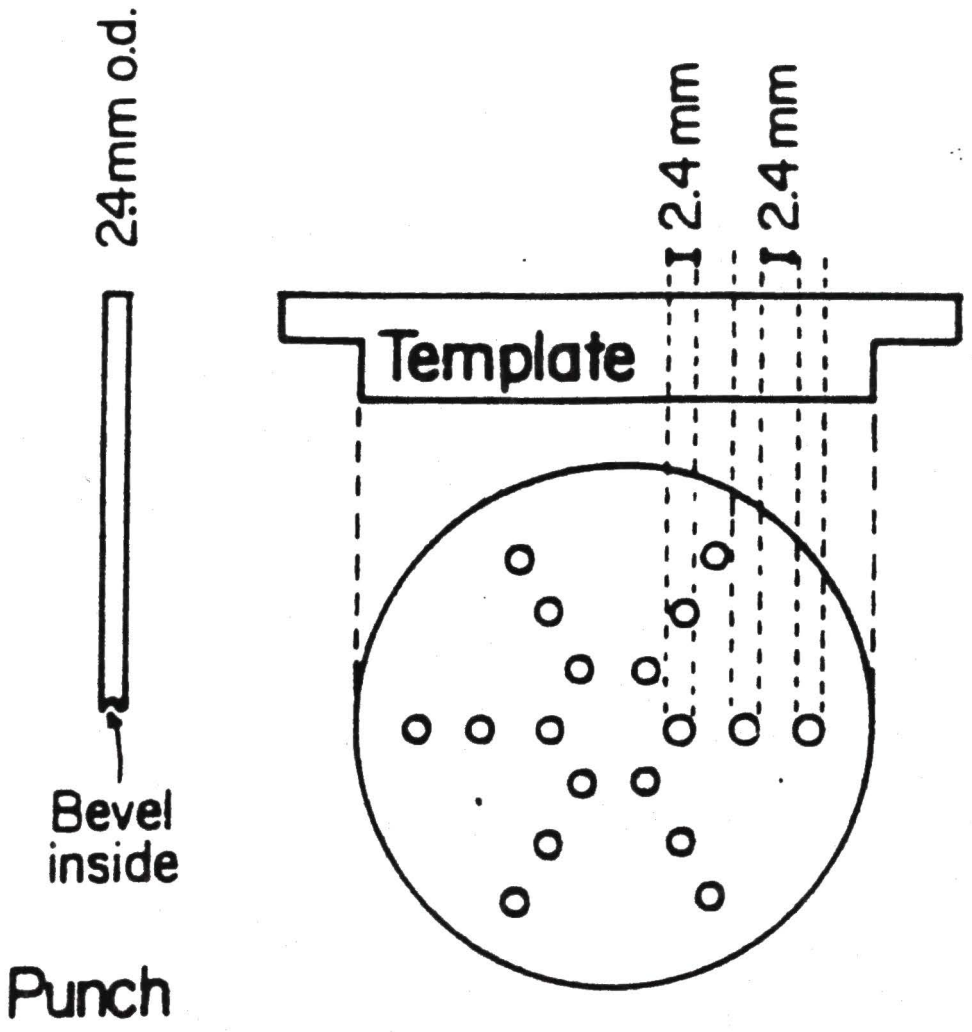

2.4mm o.d.
Bevel inside
Punch
2.4 mm
2.4 mm
Template
60 x 15 mm
Tissue Culture Dish

Figure 9. **Appearance of uninfected, AcNPV, and recombinant AcNPV-infected SF9 cells.** SF9 cells were co-transfected by wild-type viral DNA and shuttle vector. (A) Uninfected; (B) Wild-type infected or (C) recombinant virus infected cells are shown 72 hours post infection. The morphological differences of these cells are described in Results section (page 32).

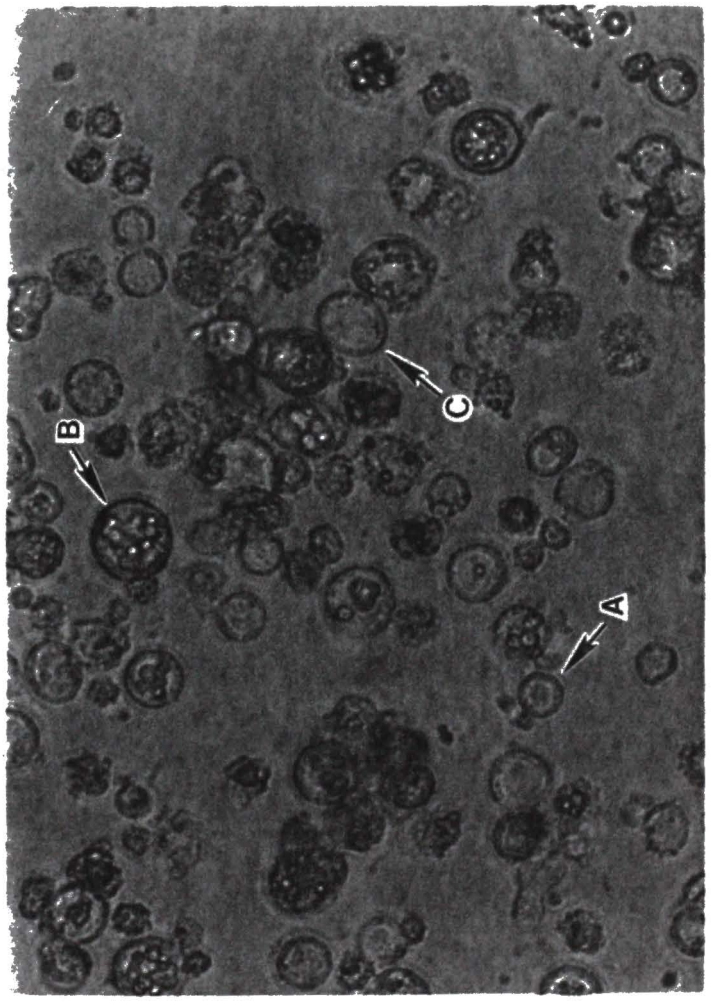

A
B
C

Figure 10. **Appearance of infected cells with recombinant viruses.** Recombinant virus infected SF9 cells are shown at 72 hours post infection.

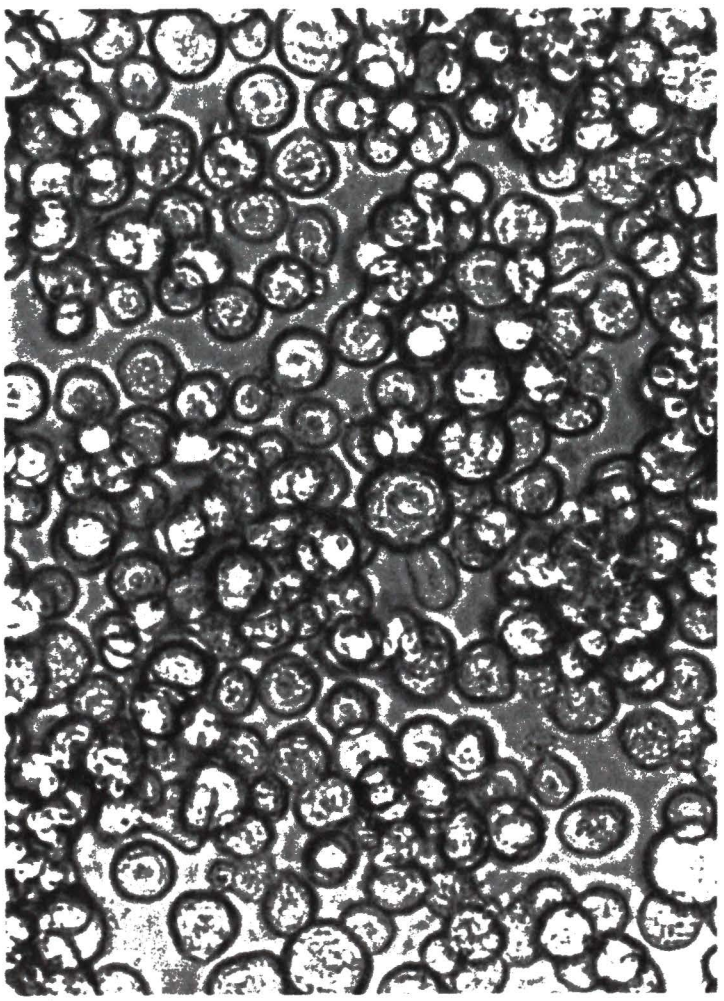


Figure 11. **Scheme showing an outline of the purification protocol for recombinant human IL-8.**

PURIFICATION OF HUMAN RECOMBINANT IL-8

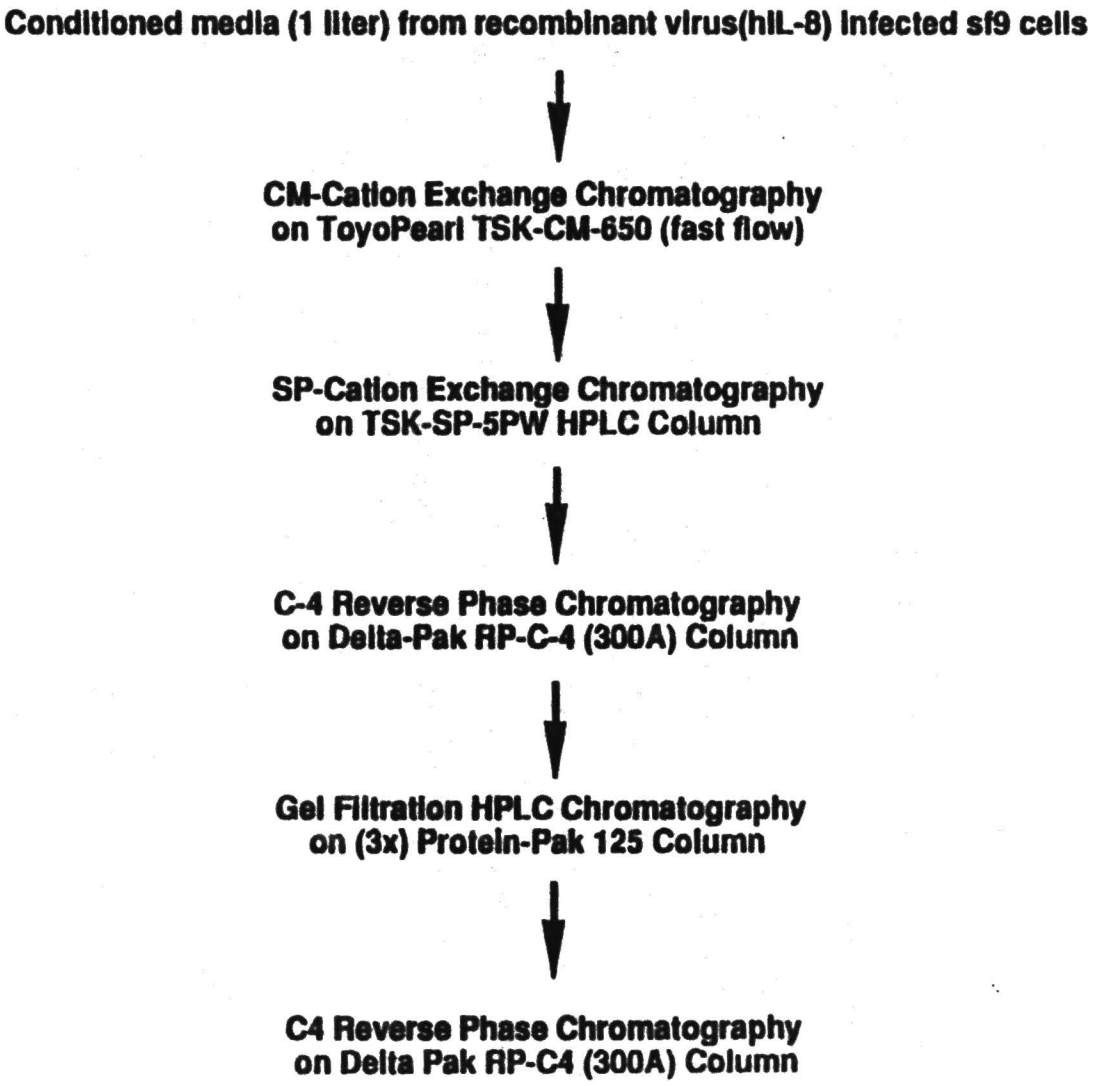


Figure 12. **Cation exchange (CM) chromatography of five day post infection conditioned media.** The sample (200 ml) harvested from conditioned media (ten days post infection) was dialyzed, concentrated and loaded onto the ToyoPearl TSK-CM 650 column (75 mm x 250 mm) (800 ml total bed volume) using Buffer A, 20 mM MES, pH 6.0, and Buffer B, 20 mM MES, pH 6.0 containing 500 mM NaCl under the following elution conditions: isocratic elution in 100% Buffer A for 500 ml, linear gradient to 1000 ml in 100% Buffer B followed by isocratic elution in 100% Buffer B for 300 ml. The eluate was monitored at 280 nm and 8 ml fractions were collected.

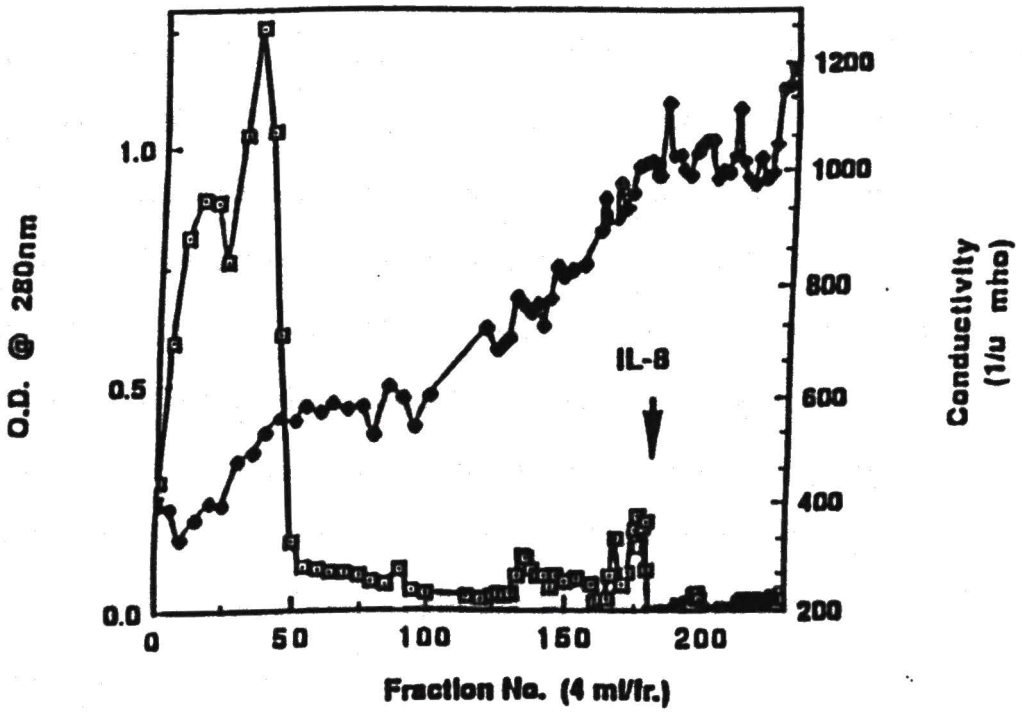

O.D. @ 280nm
1.0
0.5
0.0
0
50
100
150
200
Fraction No. (4 ml/fr.)
IL-8
1200
1000
800
600
400
200
Conductivity
(1/u mho)

Figure 13. **Cation exchange (SP) chromatography of five day post infection conditioned media.** Chromatography separation following recombinant virus infection of the human IL-8 gene. Chromatography on the TSK-SP-5PW column was as follows: Buffer A, 20 mM MES, pH 6.0; Buffer B, 20 mM MES, pH 6.0 containing 500 mM NaCl. The active fractions from the CM column were concentrated (40 ml) and dialyzed against Buffer A. The sample was loaded onto the TSK-SP-5PW column pre-equilibrated with Buffer A with two successive 20 ml injections. Maintaining a flow rate of 2.0 ml/min the sample was eluted under the following conditions: isocratic elution in 100% Buffer A for 20 min, linear gradient from t=20-180 min in 100% B, isocratic elution in 100% Buffer B till t=210 min. The eluate was monitored at 280 nm and 4 ml fractions were collected. The results of an ELISA on all even numbered fractions have been included in the elution profile of the SP column.

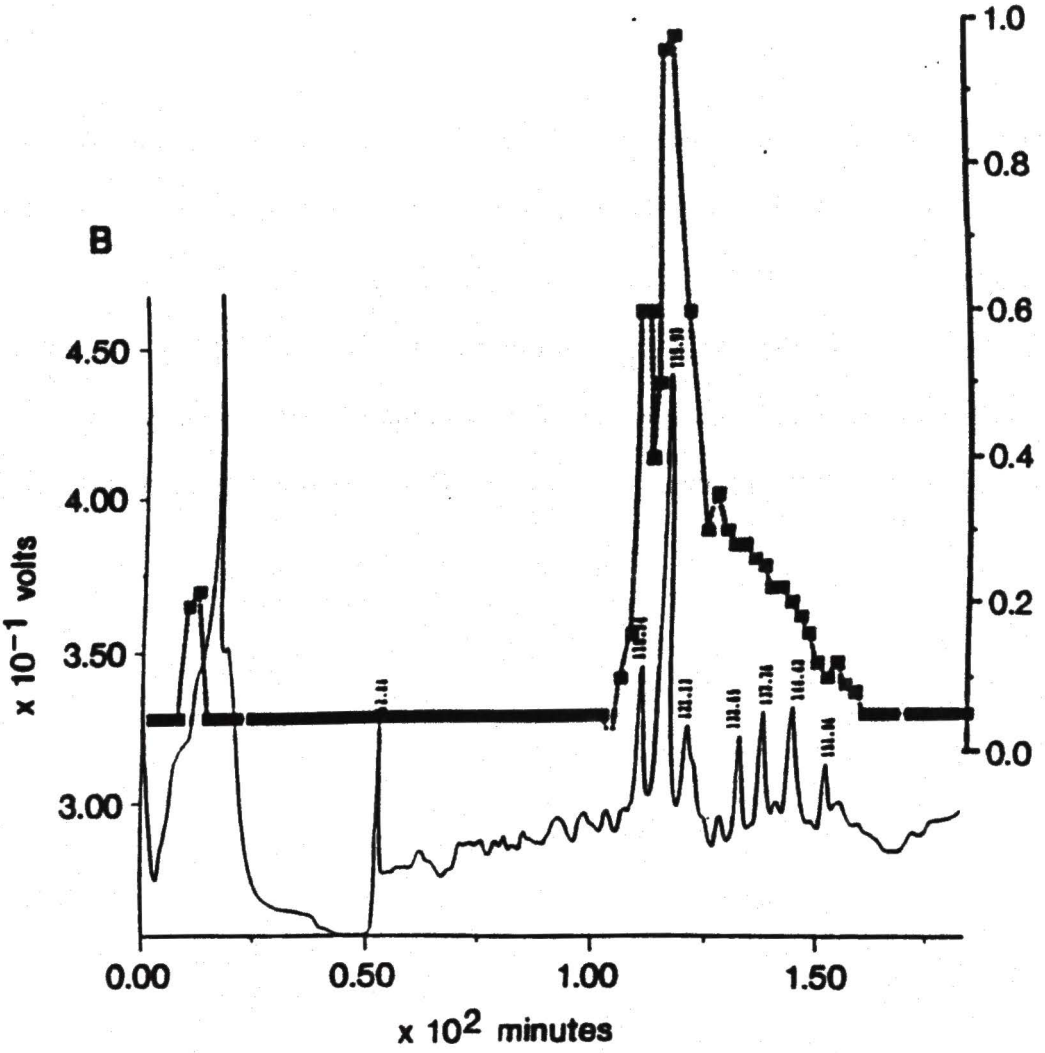
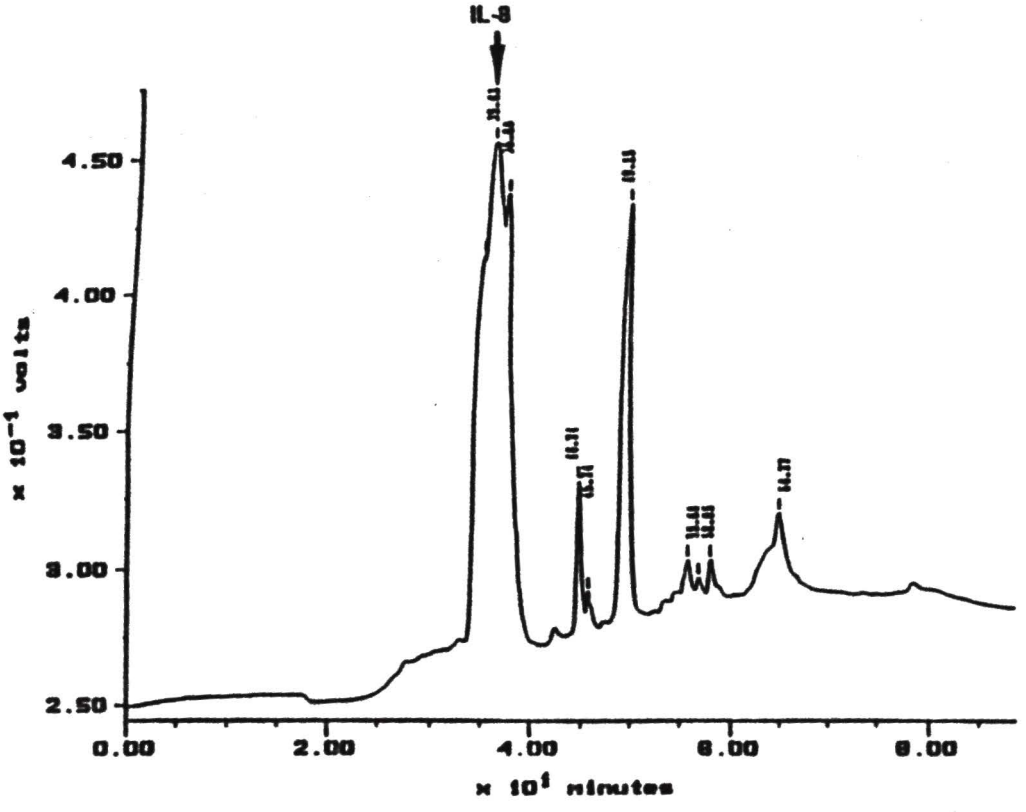

B
4.50
4.00
3.50
3.00
x 10⁻¹ volts
1.0
0.8
0.6
0.4
0.2
0.0
0.00
0.50
1.00
1.50
x 10² minutes

Figure 14. **Reverse phase chromatography of recombinant human IL-8 containing fractions.** Chromatography on the reverse phase Delta Pak RP-C4 (300A) column (4.9 x 150mm) was as follows, Buffer A:.0.1% TFA in H_2O, Buffer B: 60% Acetyl Nitrile in H_2O containing 0.1% TFA. Elution conditions were 0.5ml/min flow rate collecting 0.5 ml fractions using the following gradient conditions: isocratic elution in 100% Buffer A for t=0-20 min, linear gradient to 100 B, t=20-80 min, isocratic 100% Buffer B, t=80-90.


IL-8
4.50
4.00
3.50
3.00
2.50
x 10⁻¹ volts
0.00
2.00
4.00
6.00
8.00
x 10¹ minutes

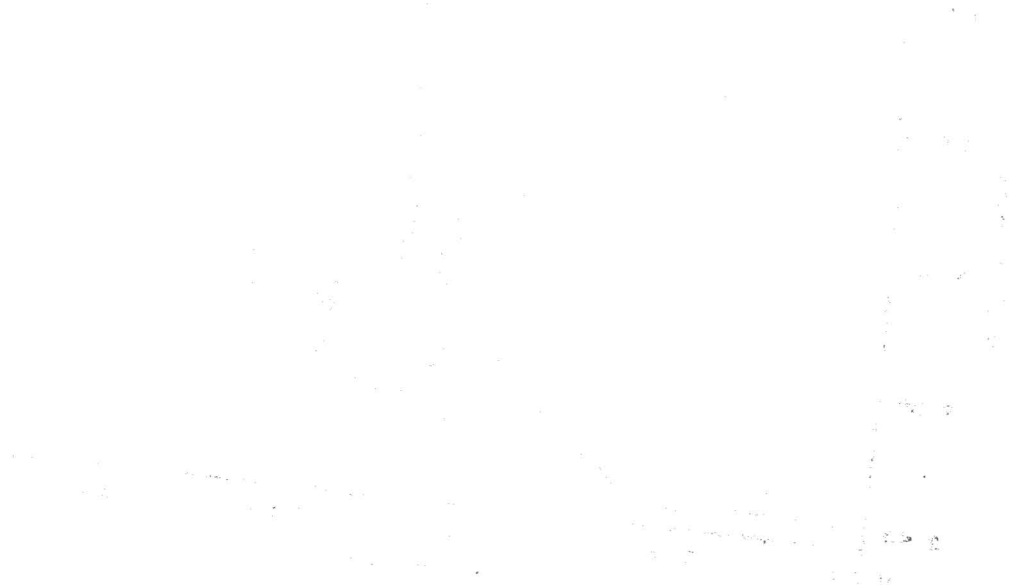


Figure 15. **Gel filtration HPLC chromatography of recombinant human IL-8 containing fractions**. Chromatography on Protein-Pak-125-PW column was as follows, Elution Buffer: 20mM MES, pH6.0, 100mM NaCl, pH 6.0 containing 100μg ovalbumin. Elution conditions were 0.5 ml/min flow rate in elution buffer for t=0-90 min.

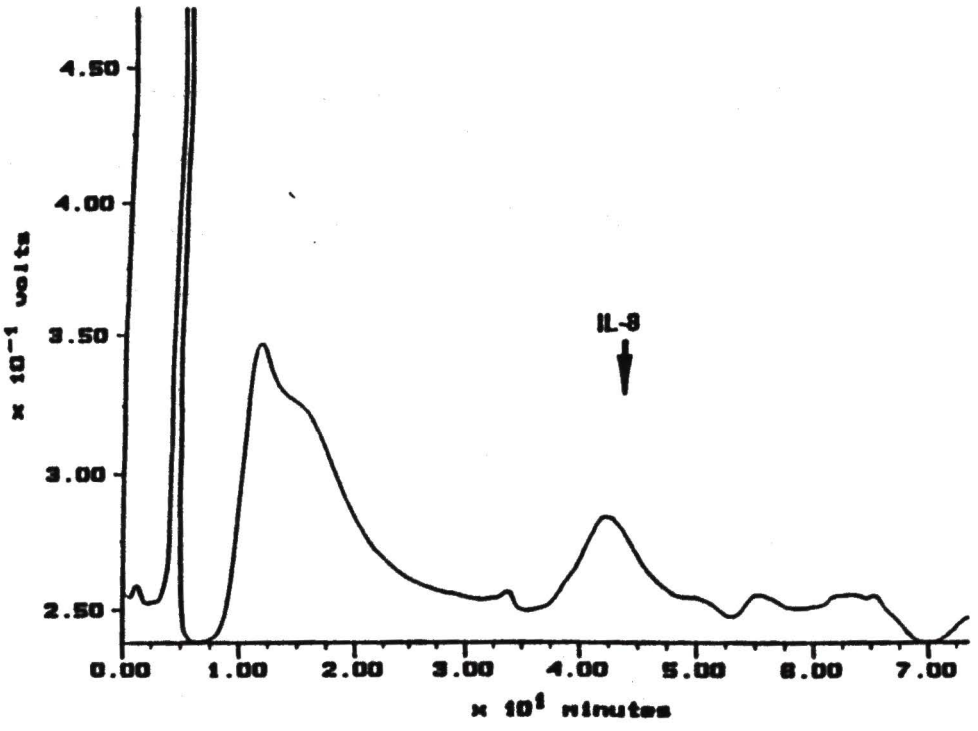

4.50
4.00
3.50
3.00
2.50
x 10⁻¹ volts
IL-8
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
x 10¹ minutes

Figure 16. **Chromatography on a reverse phase Delta Pak RP-C4 (300A) column (4.9 x 150mm).** Chromatography was run using a shallow gradient as follows, Buffer A: 10% acetyl nitrile containing 0.1% TFA in H_2O, Buffer B:60% acetylnitrile in H_2O containing 0.1% TFA. Elution conditions were 0.5ml/min flow rate using the following gradient conditions: isocratic elution in 100% Buffer A for t=0-20 min, linear gradient to 70% B, t=20-80 min, isocratic 70% Buffer B, t=80-90. The results of an ELISA on even numbered fractions has been included in the elution profile

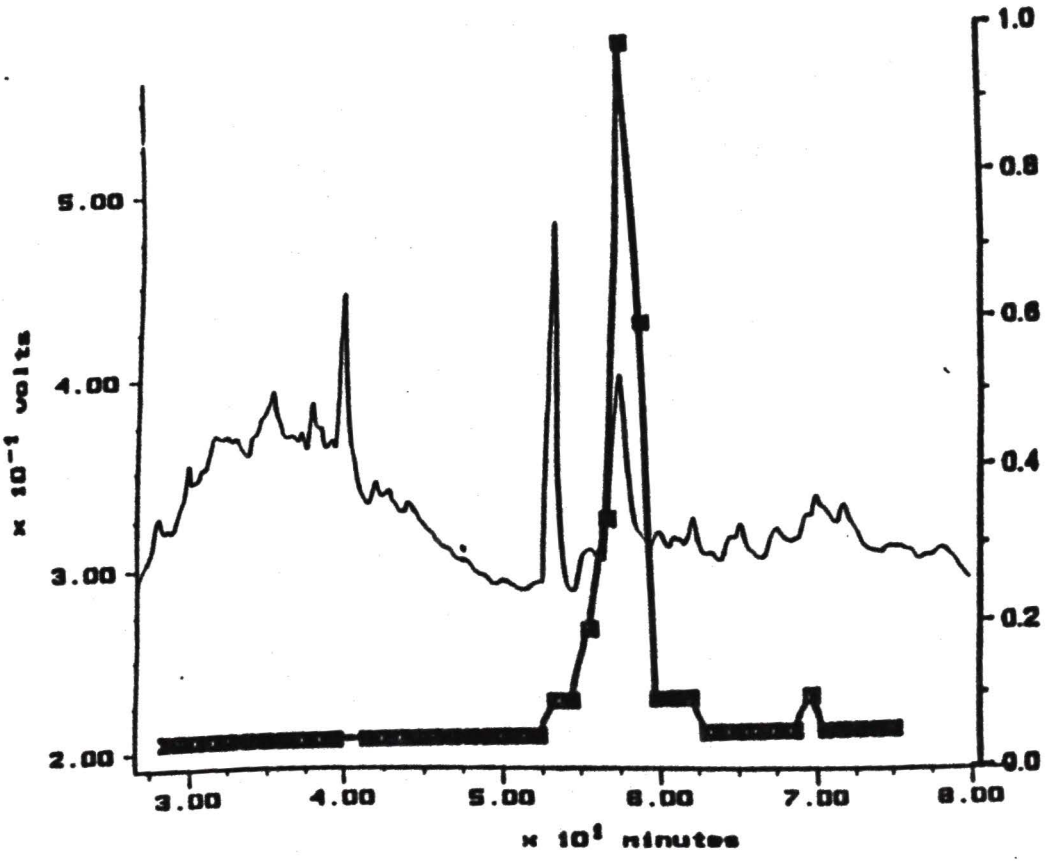

5.00
4.00
3.00
2.00
x 10⁻¹ volts
1.0
0.8
0.6
0.4
0.2
0.0
3.00
4.00
5.00
6.00
7.00
8.00
x 10¹ minutes

Figure 17. **SDS-PAGE and silver staining of rhIL-8.** a gradient gel 10-20 % acrylamide performed as described in methods, lane 1, aliquot (50ul) of pooled fractions 57 and 58 from the shallow gradient reverse phase column profile shown in fig 16, lane 2, blank, lane 3 molecular weight standards.

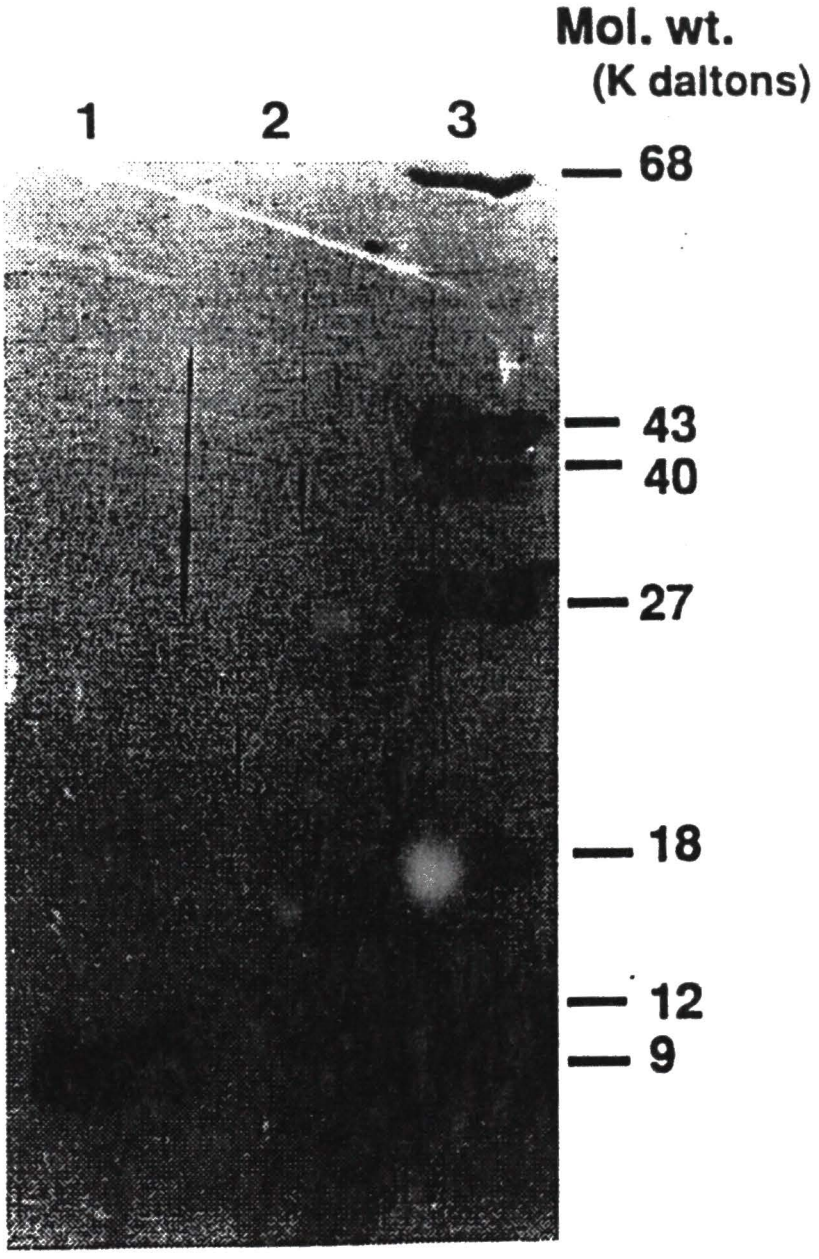

Mol. wt.
(K daltons)
1
2
3
68
43
40
27
18
12
9

Figure 18. **Kinetics of IP generation in HL-60 cells in response to fMLP and IL-8**. The differentiated HL-60 cells, treated as described under Experimental Procedures, were radiolabeled and stimulated with 100 ng/ml of IL-8 or 1 μM FMLP or Hepes-buffered saline for the stated time. IP was eluted from a Dowex AG-X8 formate column with 1000 mM ammonium formate/100 formic acid. The results are expressed as means±S.D. of the three separate experiments.

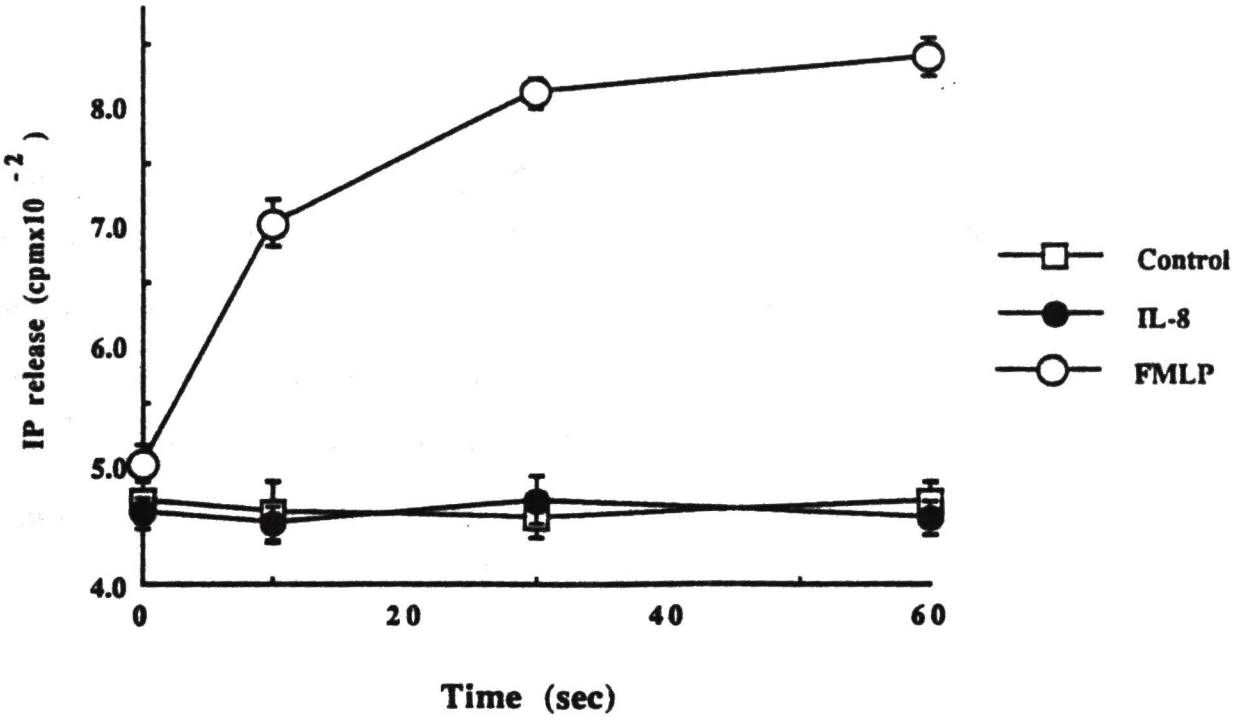

IP release (cpmx10 -2)
8.0
7.0
6.0
5.0
4.0
0
20
40
60
Time (sec)
Control
IL-8
FMLP

Figure 19. **Kinetics of IP_3 generation in HL-60 cells in response to fMLP and IL-8.** The [^{3}H]inositol labelled HL-60 cells were stimulated with 100 ng/ml of IL-8 or 1 μM FMLP or control (Hepes-buffered saline) for the stated time. IP_3 was eluted from a Dowex AG-X8 formate column with 1000 mM ammonium formate/100 formic acid after IP_1 and IP_2 had been eluted. The results are expressed as means±S.D. of three separate experiments.

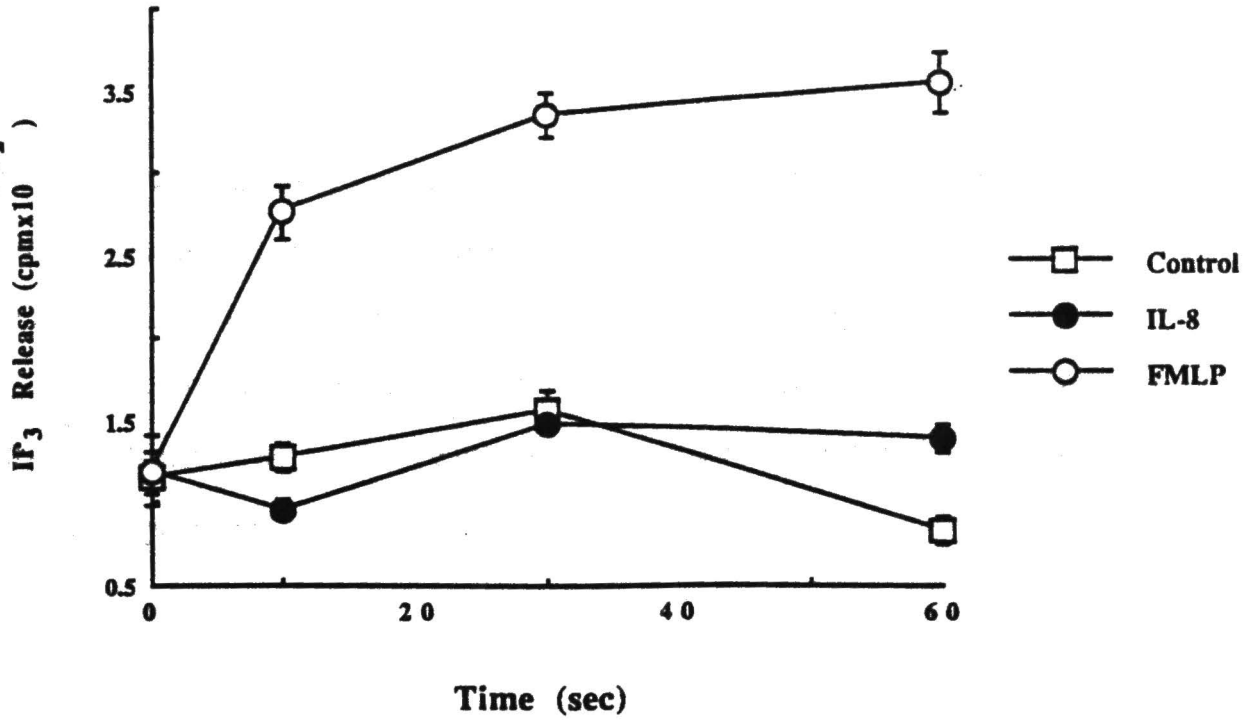

IF_3 Release (cpmx10^{-2})
3.5
2.5
1.5
0.5
0
20
40
60
Time (sec)
Control
IL-8
FMLP

Figure 20. **Kinetics of IP_3 generation in IL-8 stimulated human neutrophils.** The [^{3}H]inositol labelled human neutrophils were stimulated with 100 ng/ml of IL-8 or 1 μM FMLP or control (Hepes-buffered saline) for the stated time. IP_3 was eluted from a Dowex AG-X8 formate column with 1000 mM ammonium formate/100 formic acid after IP_1 and IP_2 had been eluted. The results are expressed as means±S.D. of the three separate experiments.

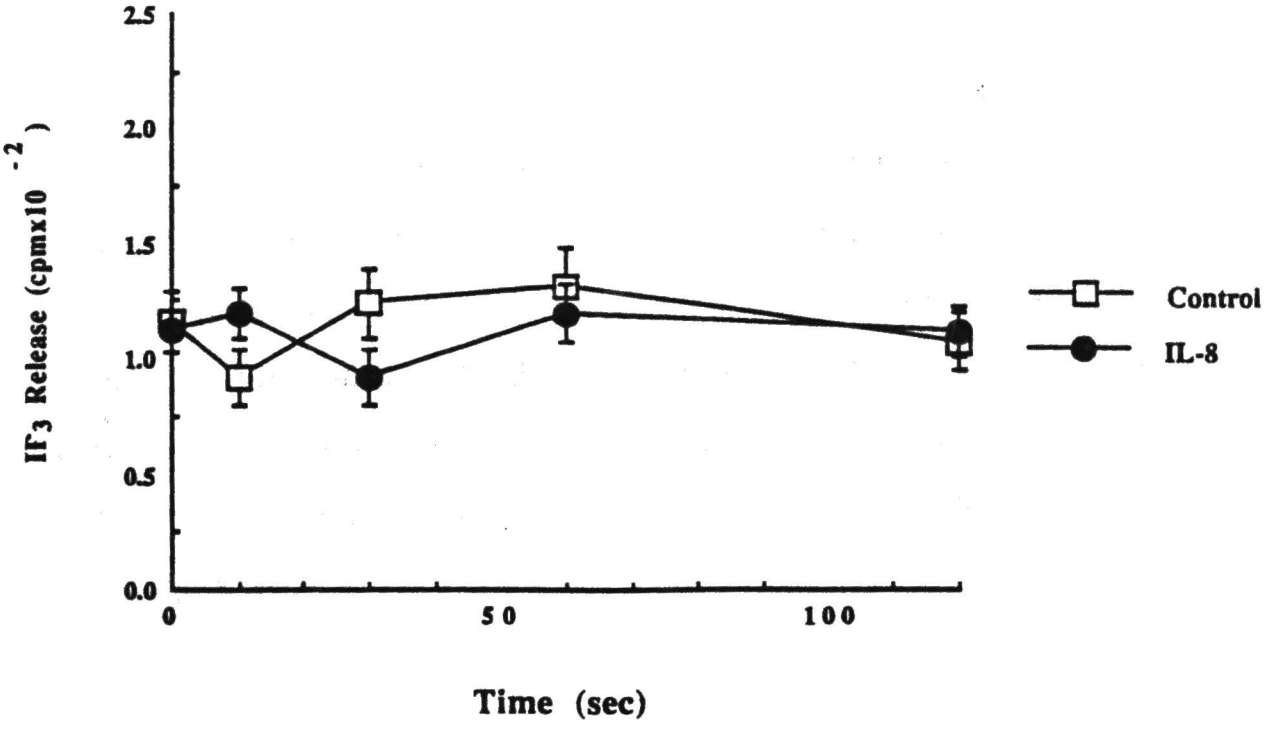

2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
IP3 Release (cpmx10 -2)
Time (sec)
Control
IL-8

Figure 21. **Activation of PLD activity in HL-60 cells.** HL-60 cells were labelled with [^{14}C]lyso-PC as described in Experimental Procedures. The cells ($1x10^7$) were preincubated in the presence of cytochalasin B (5 μM/ml) with or without ethanol (0.5%, v/v), and subsequently stimulated with IL-8 (100ng/ml), fMLP (1 μg) or BSA. After 5 min the reaction was stopped, lipids were extracted and the labelled products were analyzed for 1-O-[^{14}C]alkyl-phosphatidylethanol by TLC. Results are means±S.D.of three different experiments.

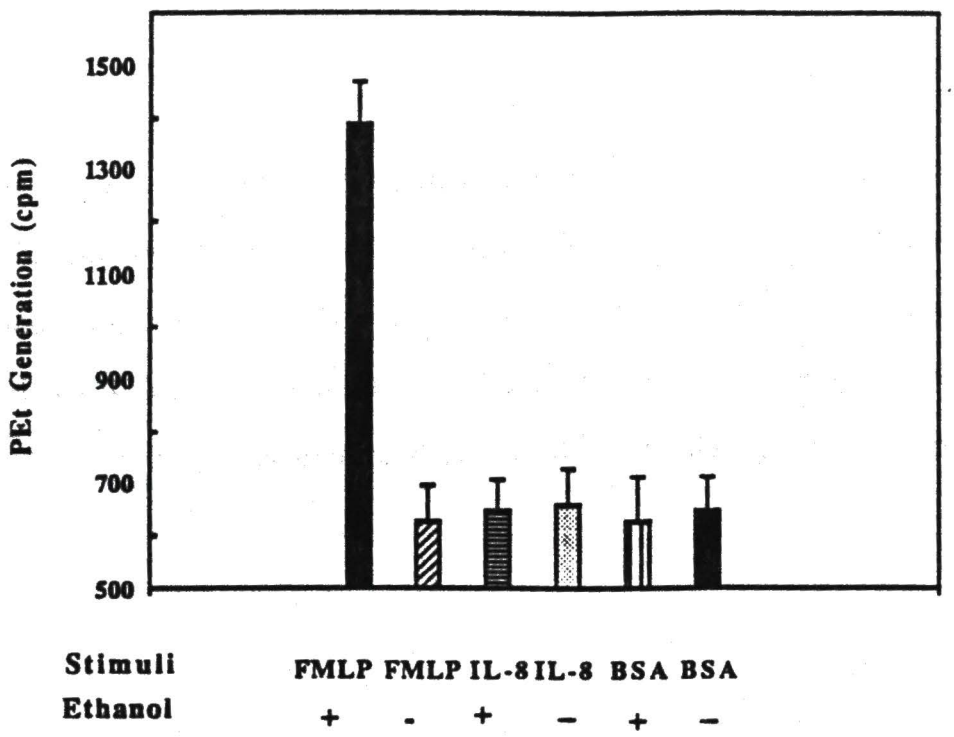

PEt Generation (cpm)
1500
1300
1100
900
700
500
Stimuli
Ethanol
FMLP FMLP IL-8 IL-8 BSA BSA
+ - + - + -

Figure 22. **Kinetics of PEt generation in fMLP and IL-8 stimulated HL-60 cells.** The differentiated HL-60 cells, treated as described under Experimental Procedures, were radiolabeled with [^{14}C]lyso-PC and stimulated with 100 ng/ml of IL-8 or 1 μM FMLP or Hepes-Buffered Saline for the stated time. PEt was separated from other phospholipids by TLC. The results are expressed as means±S.D. for a single typical experiment

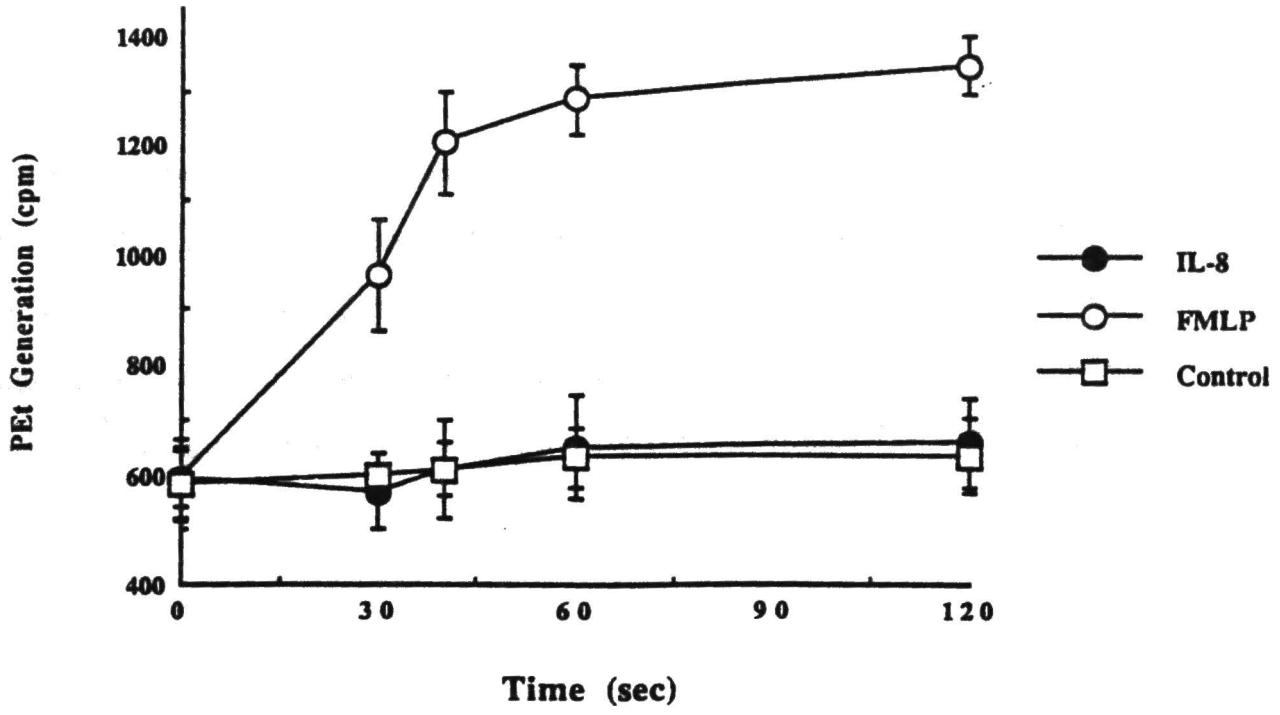

PEt Generation (cpm)
1400
1200
1000
800
600
400
0
30
60
90
120
Time (sec)
IL-8
FMLP
Control

Figure 23. **Kinetics of arachidonic acid generation in fMLP and IL-8-stimulated HL-60 cells.** The differentiated HL-60 cells, treated as described under "Materials and Methods" were radiolabeled and stimulated with 100 ng/ml IL-8 or treated with Hepes-buffered medium for the stated times. Arachidonic acid was confirmed by Silica Gel thin layer chromatography plates. The results are expressed as means±S.D. of three separate experiments.

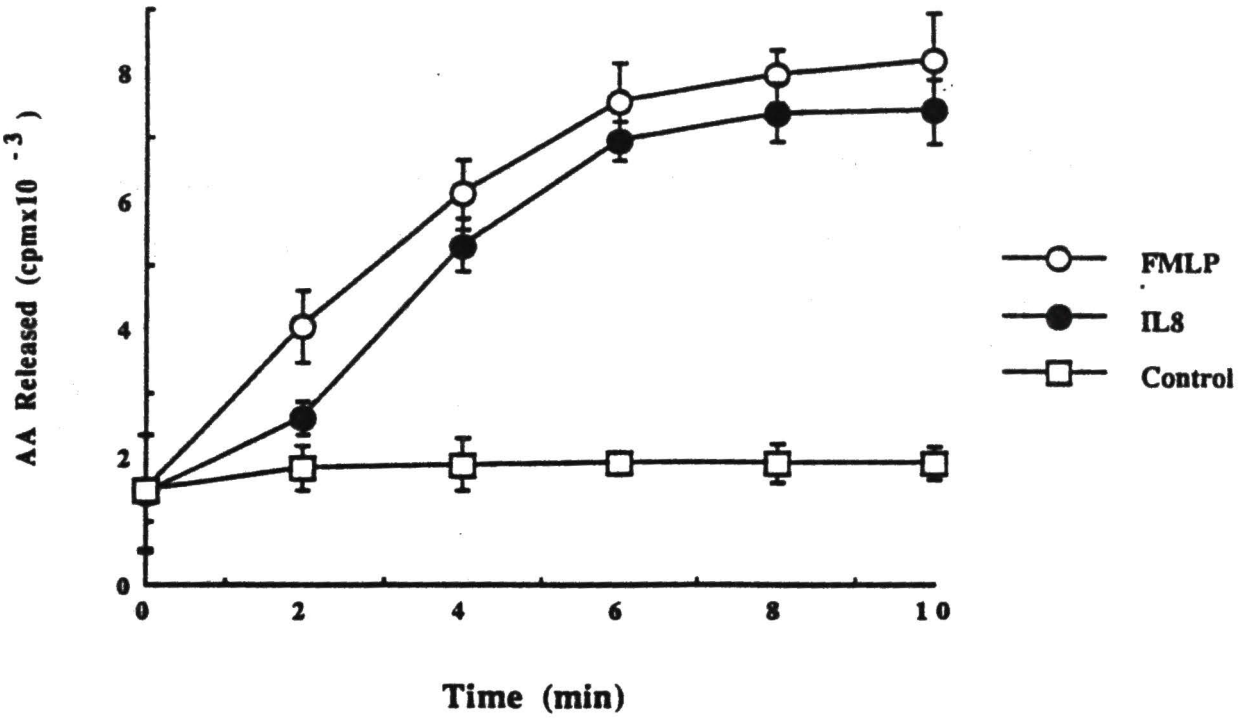

AA Released (cpmx10 -3)
0
2
4
6
8
0
2
4
6
8
10
Time (min)
FMLP
IL8
Control

Figure 24. **Dose-dependence of arachidonic acid generation in IL-8-stimulated HL-60 cells.** [^{3}H]arachidonic acid-labeled HL-60 cells were stimulated with increasing concentration of IL-8 for 8 min. The results are expressed as means±S.D. of three separate experiments.

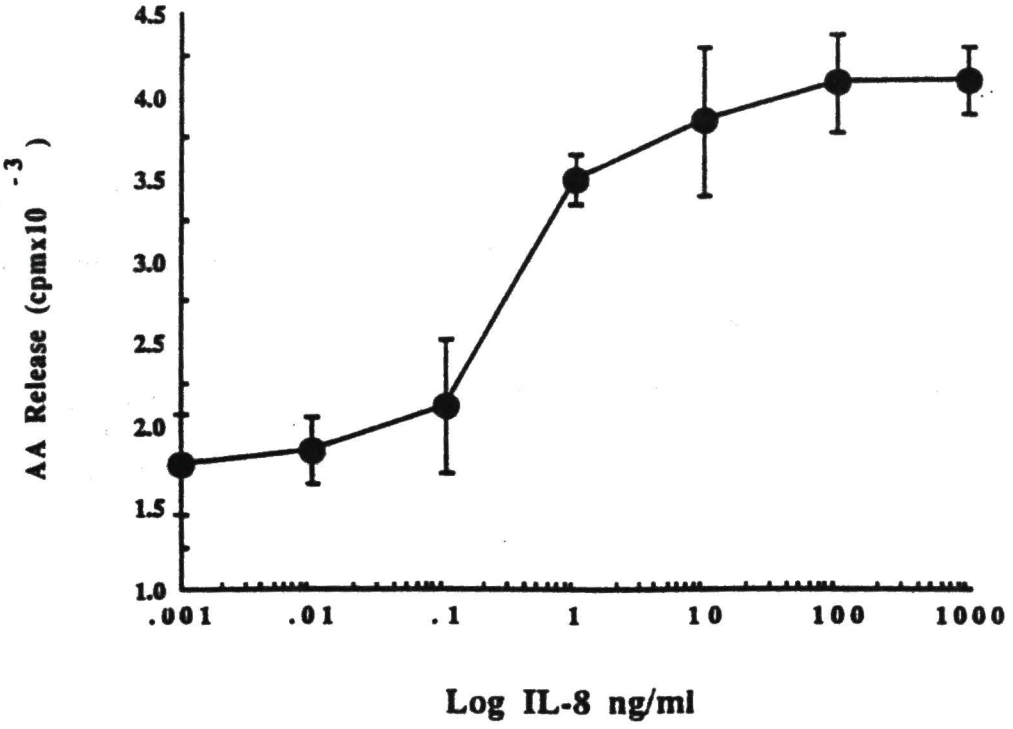
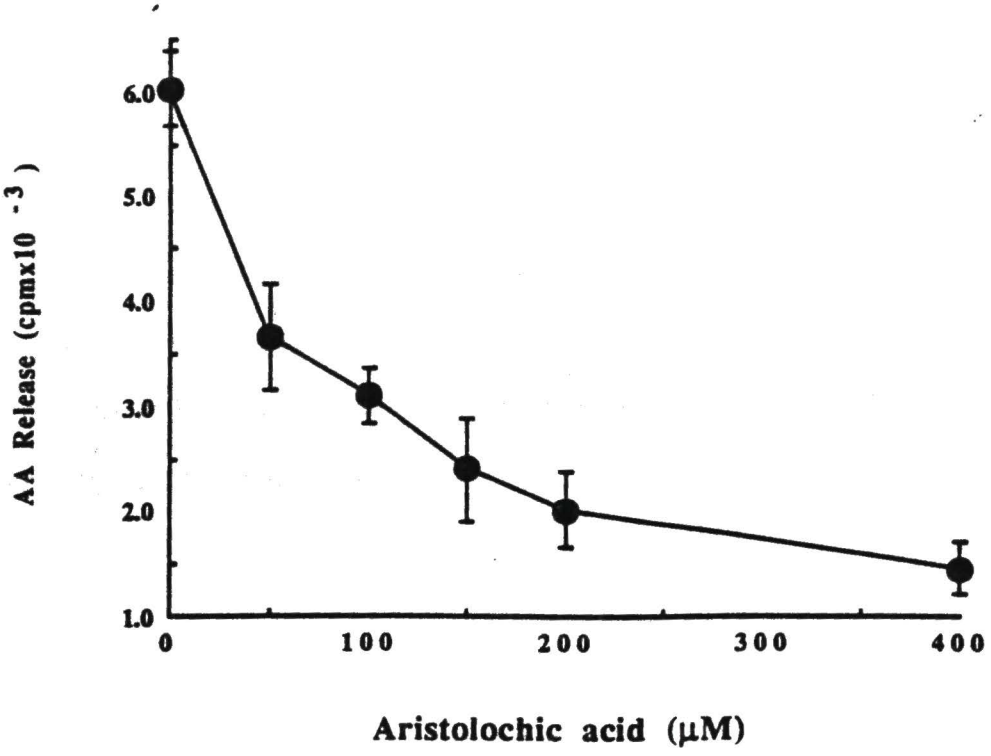

AA Release (cpmx10 -3)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.001
.01
.1
1
10
100
1000
Log IL-8 ng/ml

Figure 25. **Effect of aristolochic acid treatment on IL-8 stimulated-arachidonic acid release.** HL-60 cells, labeled [^{3}H]arachidonic acid, were incubated with Hepes-buffered medium containing the indicated concentrations of aristolochic acid for 15 min prior to stimulation with 100 ng/ml IL-8 for 8 min. Results are expressed as Means±S.D. for a single typical experiment where n=3.


AA Release (cpmx10 -3)
6.0
5.0
4.0
3.0
2.0
1.0
0
100
200
300
400
Aristolochic acid (μM)

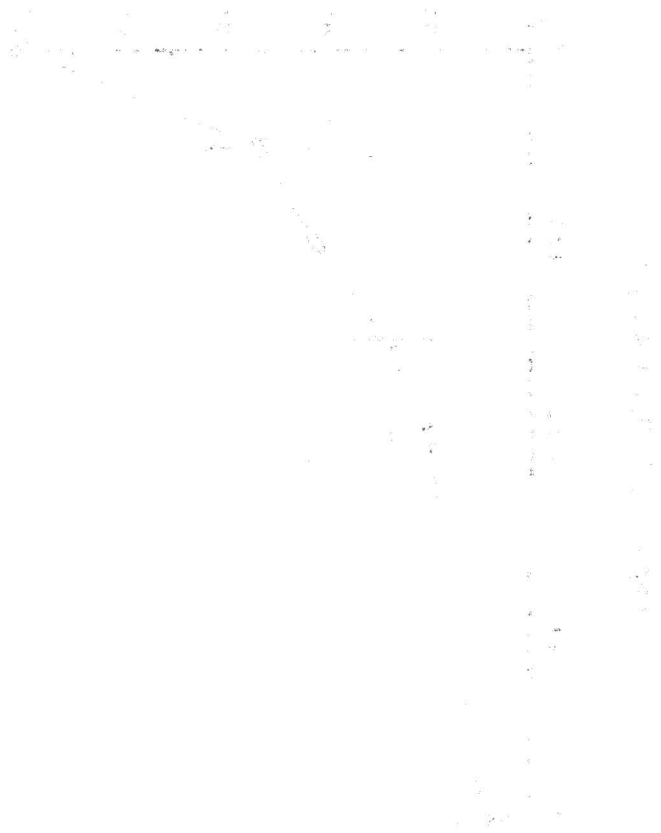


Figure 26. **Effect of PMA treatment on IL-8-stimulated arachidonic acid release.** HL-60 cells, labeled with [^{3}H]arachidonic acid , were incubated with PMA 300 mM for 16 hours prior to stimulation with 100 ng/ml IL-8. Arachidonic acid release was measured in cells pretreated with PMA and untreated cells. Results are expressed as means+S.D. for a single typical experiment where n=3.

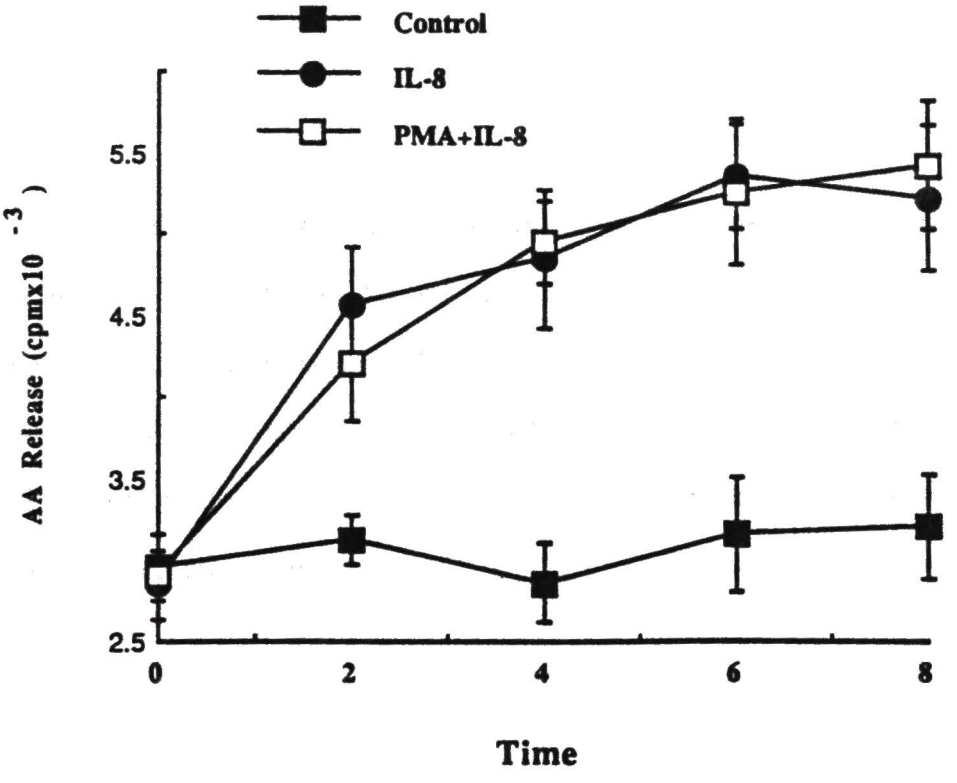

Control
IL-8
PMA+IL-8
AA Release (cpmx10 -3)
5.5
4.5
3.5
2.5
0
2
4
6
8
Time

Figure 27. **Effect of staurosporine and H7 treatment on IL-8-stimulated arachidonic acid release.** HL-60 cells, labeled with [^{3}H]arachidonic acid , were incubated with staurosporine (10^{-6} M) for 15 min, and H7 (50 ng/ml) for 4 hours prior to stimulation with 100 ng/ml IL-8. Arachidonic acid release was measured in cells pretreated with staurosporine and untreated cells. Results are expressed as means+S.D. for single typical experiment where n=3.

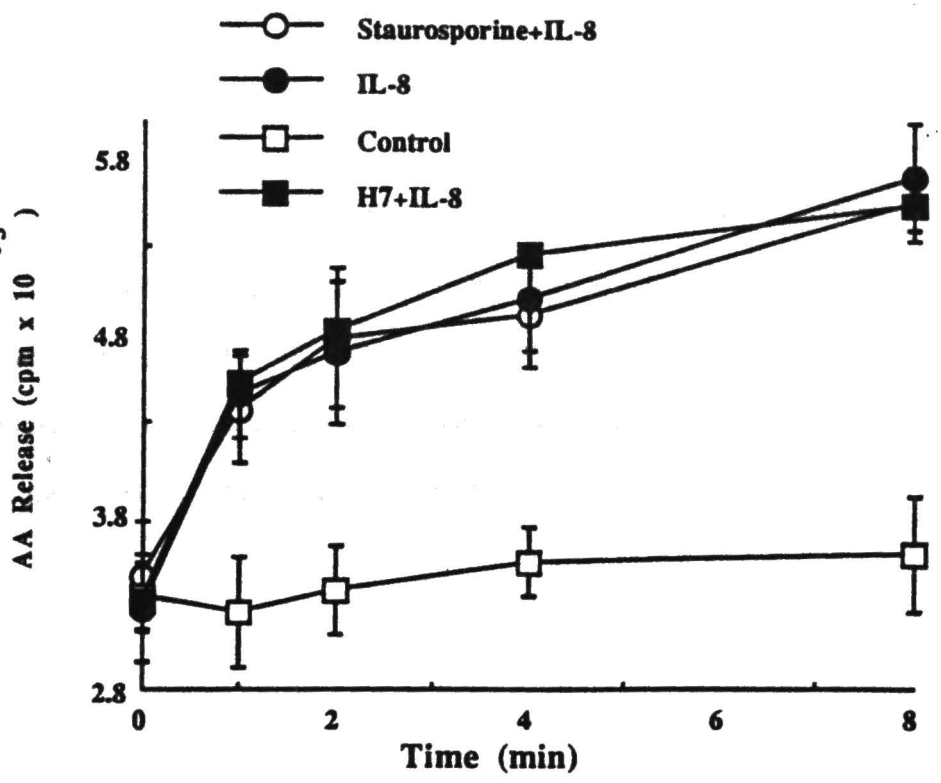

Staurosporine+IL-8
IL-8
Control
H7+IL-8
AA Release (cpm x 10 -3)
5.8
4.8
3.8
2.8
0
2
4
6
8
Time (min)

Figure 28. **Effect of toxin treatment on IL-8-stimulated arachidonic acid release.** HL-60 cells were incubated with 500 ng/ml Pertussis toxin or 1000 ng/ml Cholera toxin for 4 h prior to the experiments. Stimulation with IL-8 was performed as described previously. Arachidonic acid release was measured in cells pretreated with PTX, CTX, and untreated cells. Results are expressed as means+S.D. from single typical experiment.

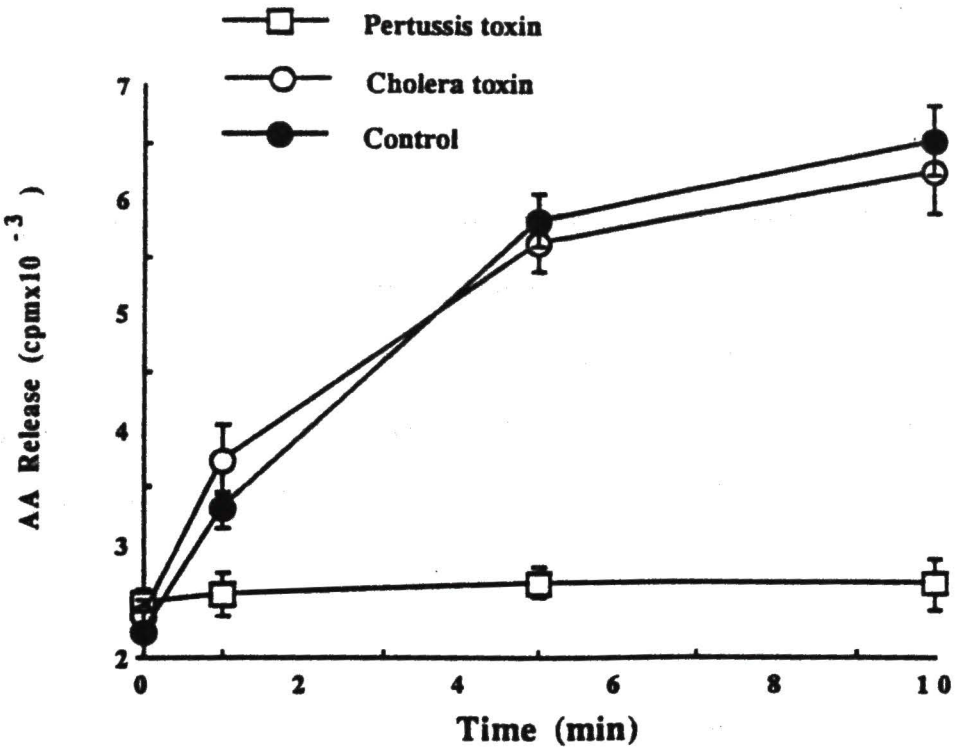

Pertussis toxin
Cholera toxin
Control
AA Release (cpmx10 -3)
7
6
5
4
3
2
0
2
4
6
8
10
Time (min)

Figure 29. **Effect of Ca^{2+} on IL-8-induced arachidonic acid release.** [^{3}H]Arachidonic acid-labeled HL-60 cells were washed and stimulated with IL-8 in medium containing 1mM Ca^{2+}, or medium in which the extracellular and/or intracellular medium have been buffed with EGTA. Results are expressed as means±S.D. from a typical experiment.

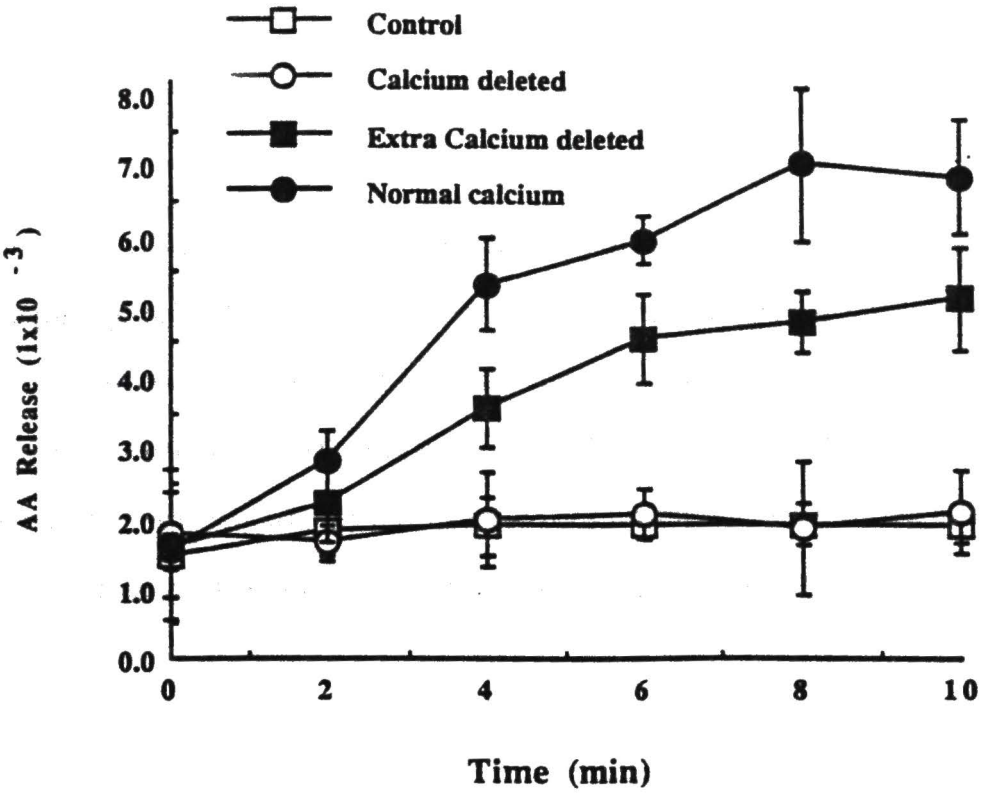
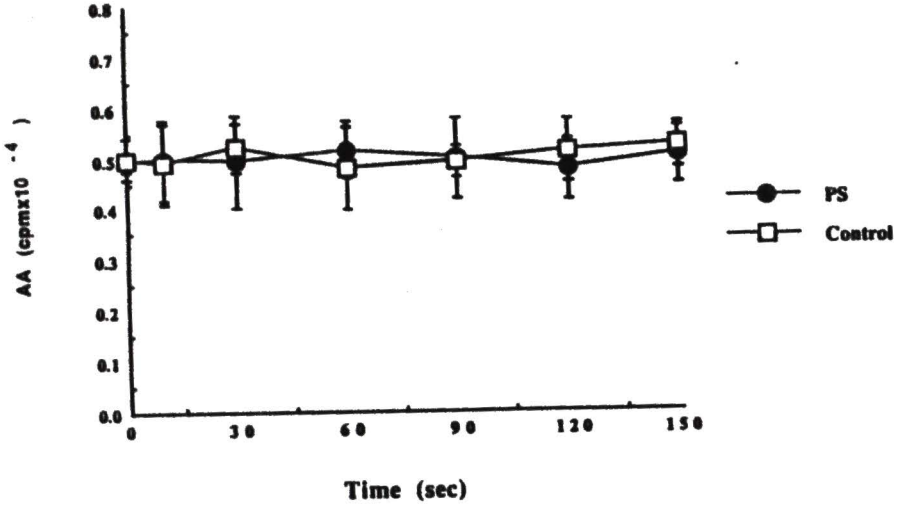
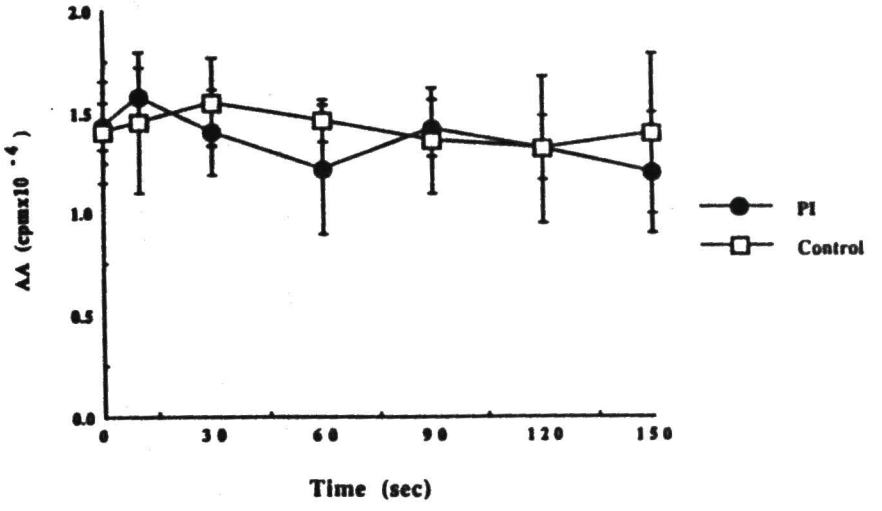
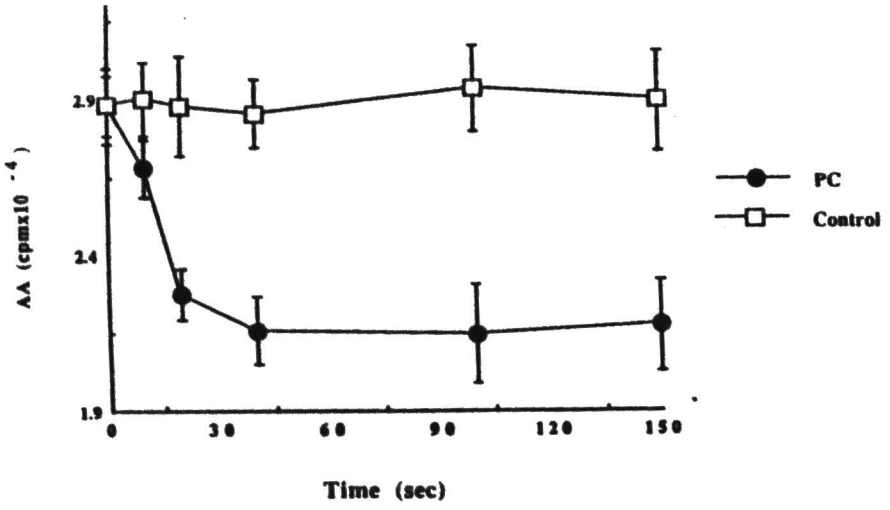

Control
Calcium deleted
Extra Calcium deleted
Normal calcium
AA Release (1x10 -3)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
2
4
6
8
10
Time (min)

Figure 30. **IL-8-stimulated changes in the [^{3}H]arachidonic acid labeling of phospholipids in HL-60 cells.** HL-60 cells labeled with [^{3}H]arachidonic acid were incubated with either Hepes-buffered medium or 100 ng/ml IL-8 for the Stated times. Cellular phospholipids were extracted as described. Changes in the arachidonic acid content of phosphaditylserine in response to this treatment were detected using thin layer chromatography. The results are expressed as Means+S.D. of five experiments.


AA (cpmx10 -4)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
30
60
90
120
150
Time (sec)
PS
Control


AA (cpmx10 -4)
2.0
1.5
1.0
0.5
0.0
0
30
60
90
120
150
Time (sec)
PI
Control


AA (cpmx10 -4)
2.9
2.4
1.9
0
30
60
90
120
150
Time (sec)
PC
Control

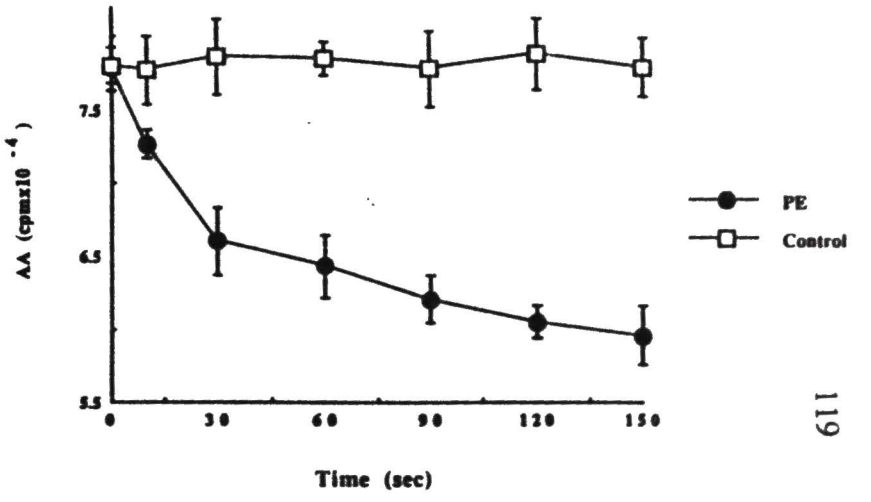

AA (cpmx10 -4)
7.5
6.5
5.5
0
30
60
90
120
150
Time (sec)
PE
Control

Figure 31. **Model for the regulation of CaM kinase II by Ca^{2+} and calmodulin.** Addition of Ca^{2+}/calmodulin stimulates intersubunit autophosphorylation, resulting in the trapping of calmodulin and conversion of the enzyme to a Ca^{2+} independent state.

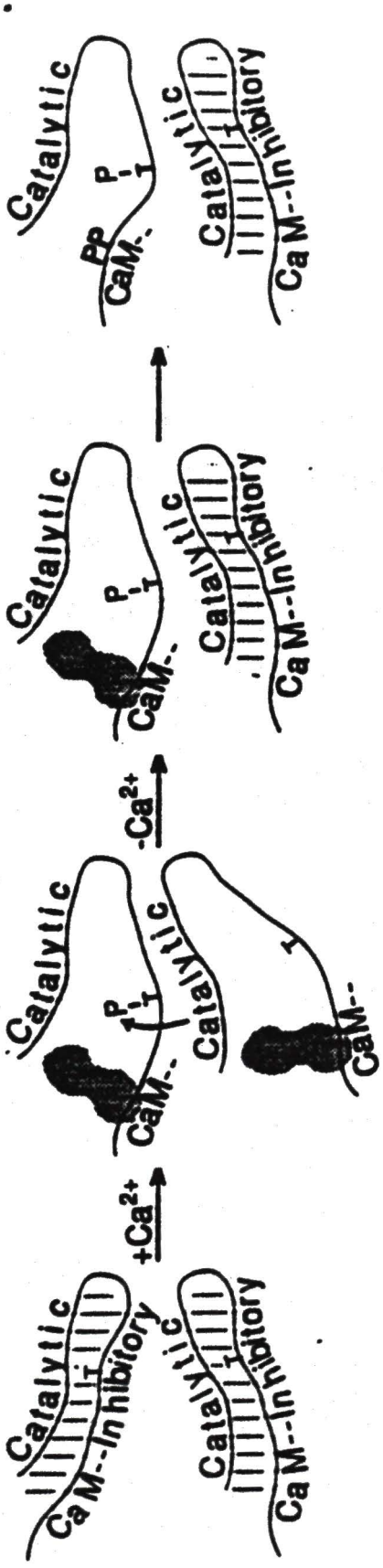
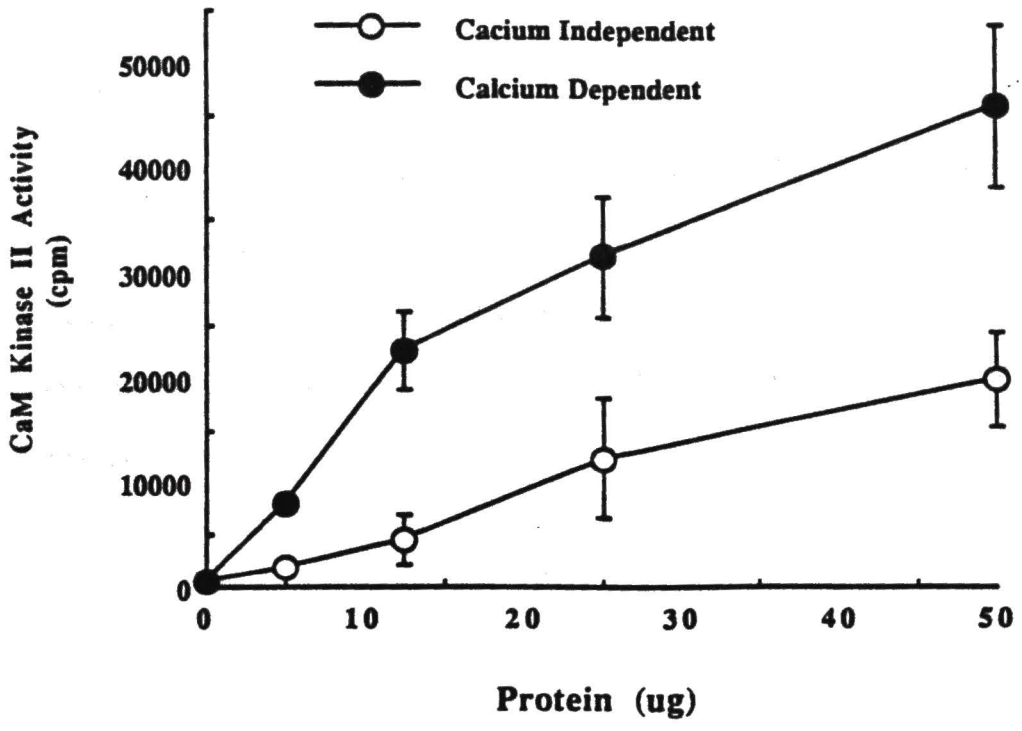

Catalytic
CaM--Inhibitory
+Ca2+
-Ca2+
CaM--

Figure 32. **protein concentration range for CaM kinase II autonomous activity assay.** HL-60 cells were homogenized by sonication and protein concentration determined by Bradford assay as described in Experimental Procedures. Assay (25µl) for both Ca^{2+}/Calmodulin-dependent and independent activity were immediately conducted in tubes containing between 0 and 50 µg homogenate protein.


Cacium Independent
Calcium Dependent
CaM Kinase II Activity
(cpm)
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
Protein (ug)

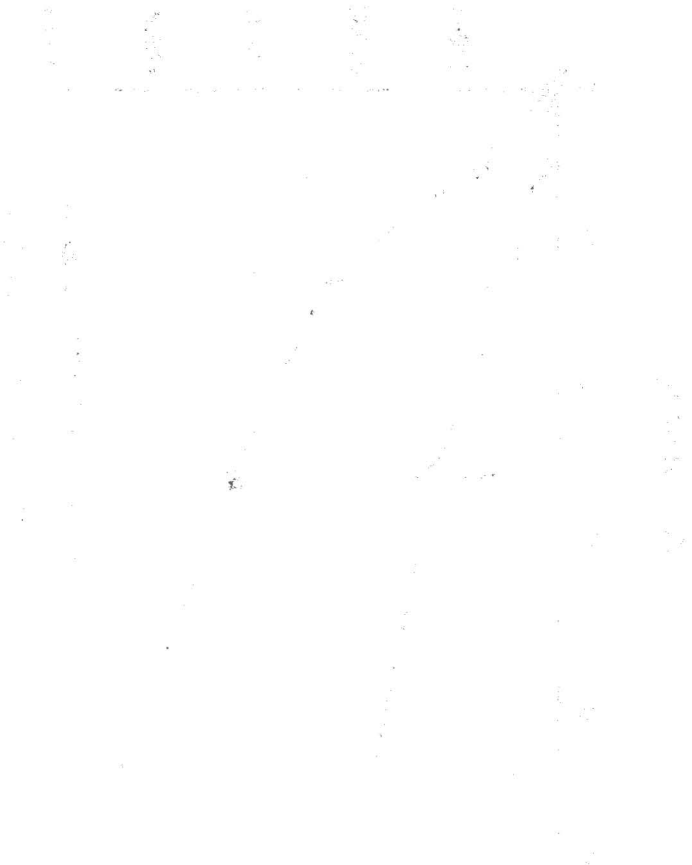


Figure 33. **Kinetics of IL-8-induced autonomous CaM kinase II activity.** HL-60 cells ($5x10^5$ cells per tube) were incubated with IL-8 (100 ng/ml) or control (BSA) for the indicated times. Autonomous CaM kinase II activity was determined as described in Experimental Procedures. Results are expressed as means+ S.D. for a single typical experiment.

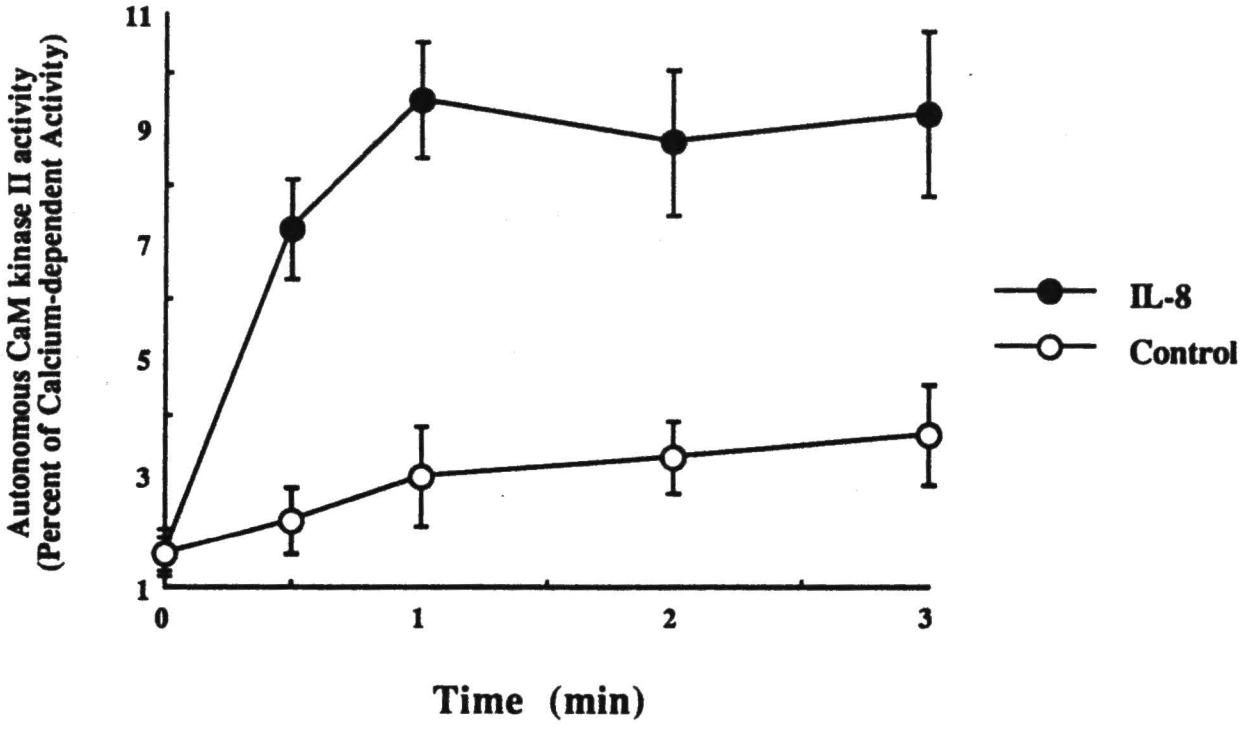

Autonomous CaM kinase II activity
(Percent of Calcium-dependent Activity)
11
9
7
5
3
1
0
1
2
3
Time (min)
IL-8
Control

Figure 34. **Protein phosphorylation in HL-60 cells induced by IL-8 and fMLP** . Permeabilized HL-60 cells were incubated with IL-8 or fMLP or HBSS buffer for 3 min. Cells were then solubilized, electrophoresed, and autoradiographed as described in Methods.

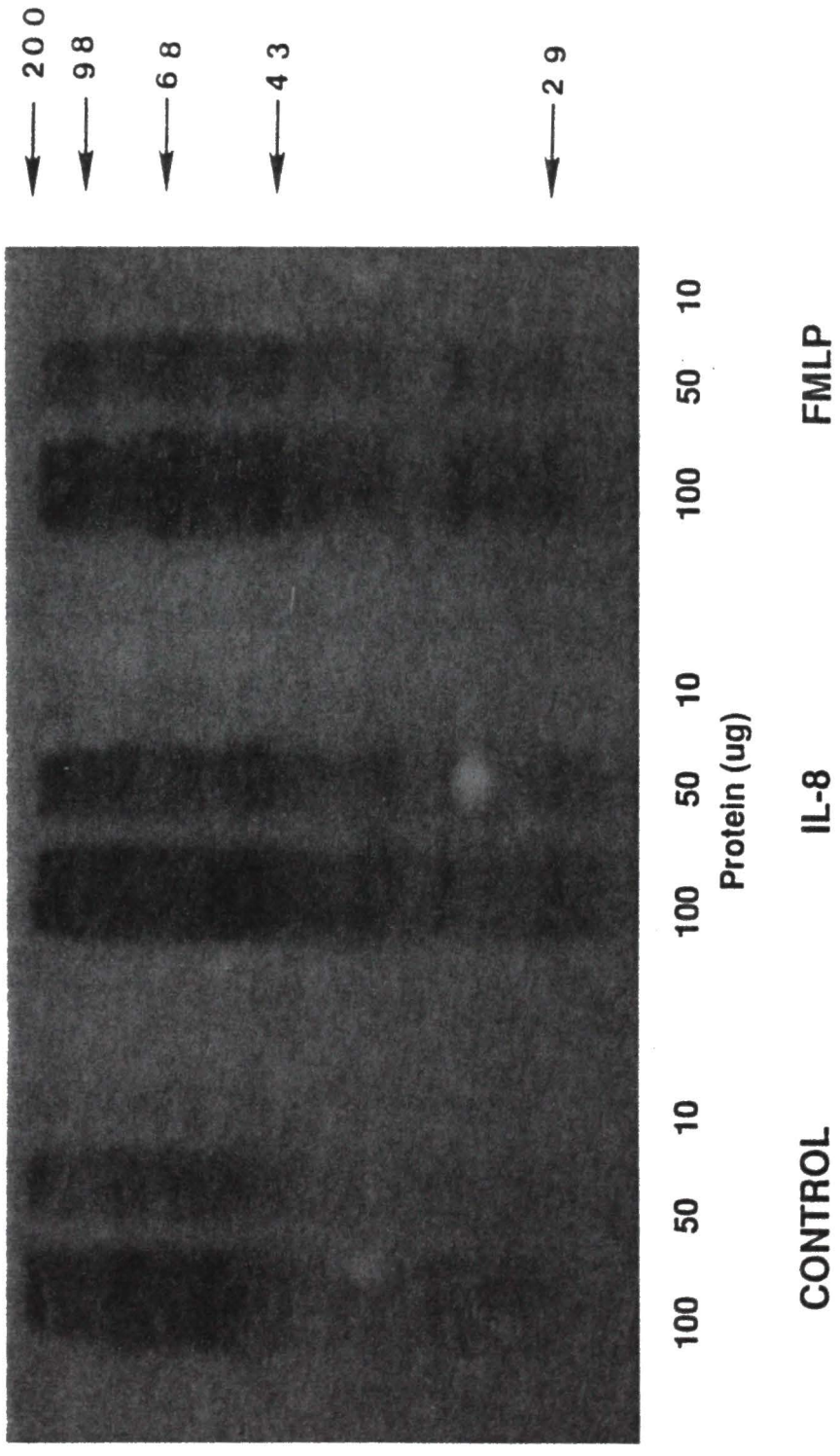

200
98
68
43
29
100 50 10 100 50 10 100 50 10
Protein (ug)
CONTROL
IL-8
FMLP

Figure 35. **Tyrosine phosphorylation in HL-60 cells in response to IL-8.** HL-60 cells were incubated with either HBSS buffer for 1 min or with 100 ng/ml IL-8 for increasing periods of time. Western blotting using a monoclonal antibody directed against phosphotyrosine proteins was then performed as described in Methods.

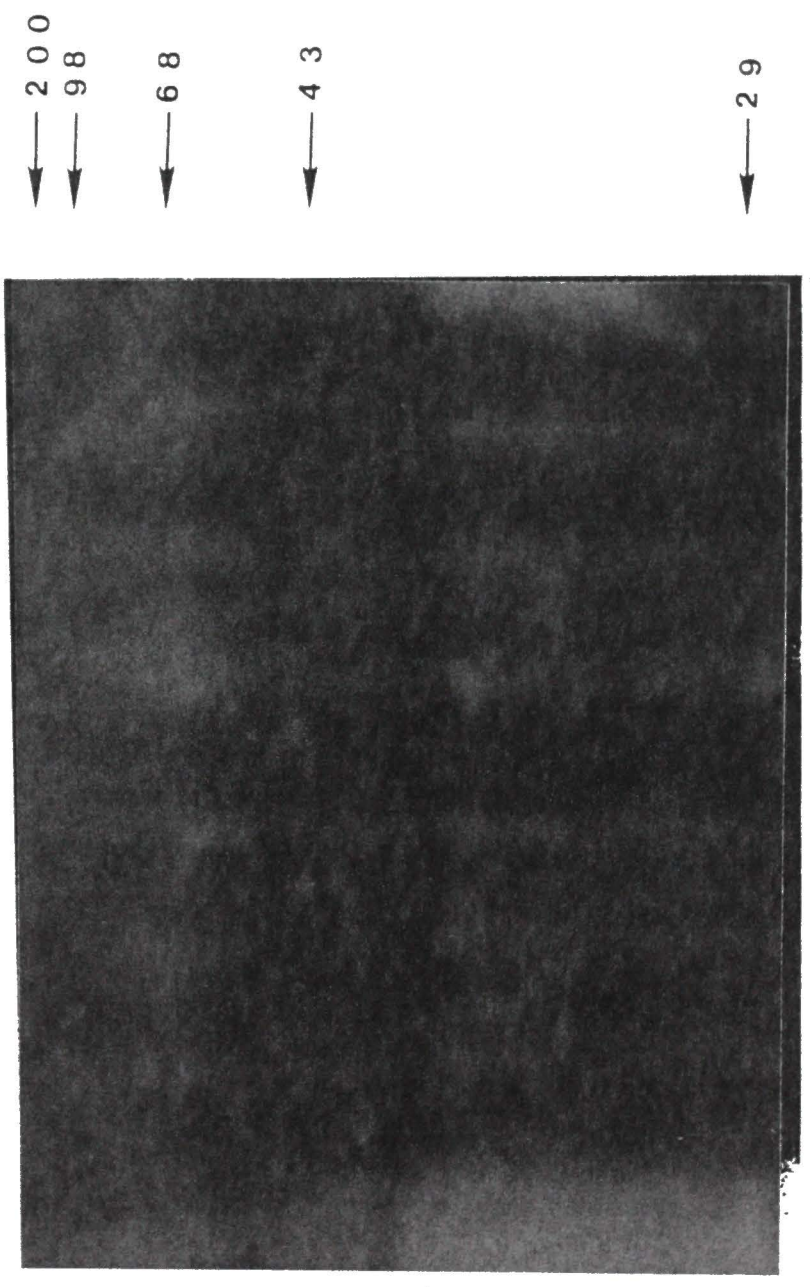

200
98
68
43
29
C
0
0.5
1
2
3
5
Time (min)

Figure 36. **Model for the IL-8 receptor signal transduction pathway.**

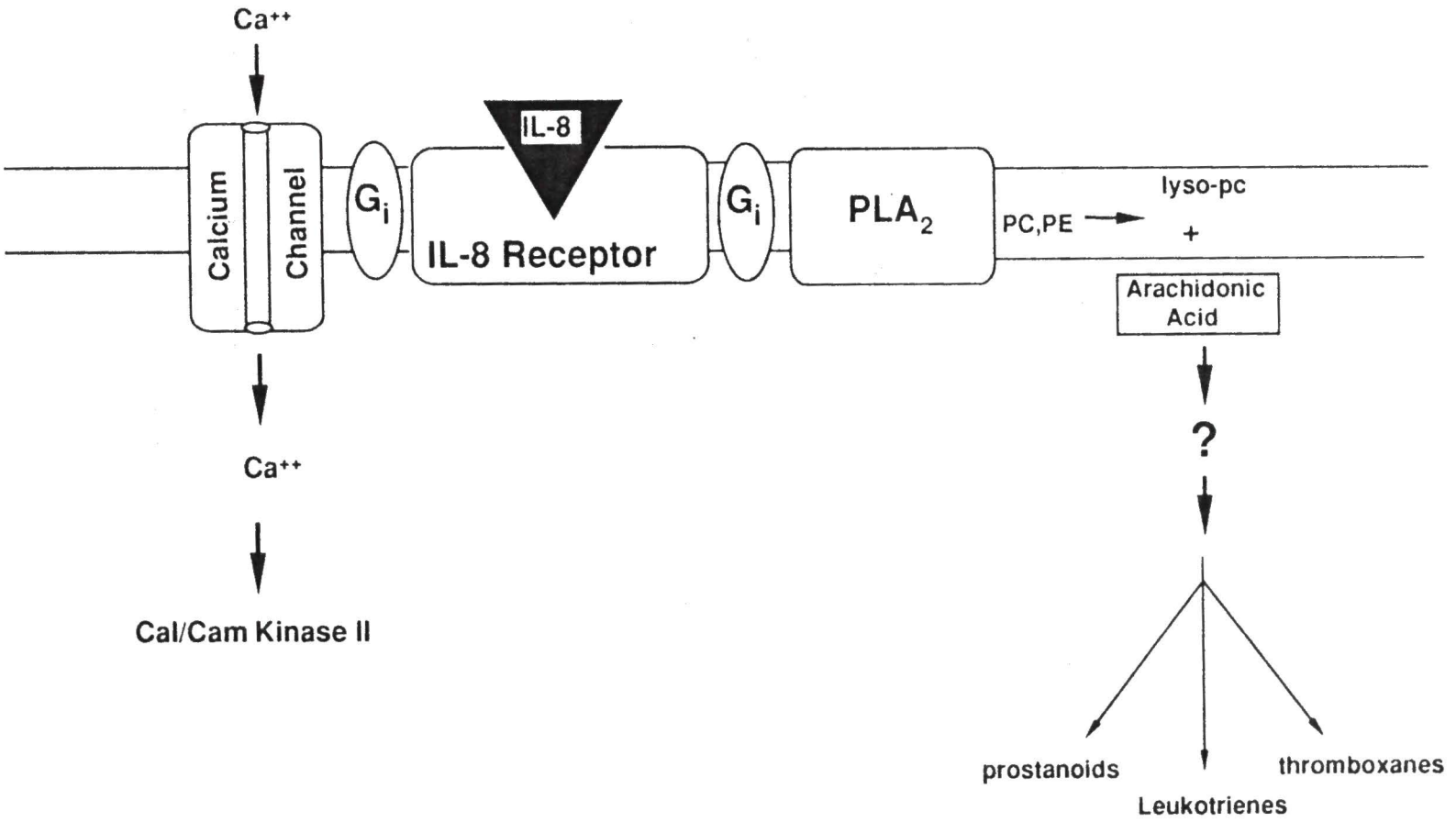

Ca++
Calcium
Channel
Gi
IL-8
IL-8 Receptor
Gi
PLA2
PC,PE
lyso-pc
+
Arachidonic Acid
?
Ca++
Cal/Cam Kinase II
prostanoids
Leukotrienes
thromboxanes

TABLE I

Purification of Human Recombinant rhIL-8 by HPLC

Procedure	Volume (ml)	Total Protein (mg)	rhIL-8 (mg)	Yield (%)
Cell supernatant	1000	450.3	5.5	100.0
ToyoPearl-CM	120	39.6	3.5	64.0
SP-TSK-5PW	31	3.2	1.6	29.0
Deita-Pak C4-RP	3	0.6	0.6	10.9

TABLE I I

Amino Acid Composition of Recombinant IL-8

Amino Acid	IL-8 nmoles*	IL-8 Residues	Expected	Il-8 %	Expected %
ASX	0.250	6.0	5	9.8	8.2
THR	0.162	3.9	2	6.3	3.3
SER	0.325	7.8	5	12.7	8.2
GLX	0.456	10.9	10	17.8	16.4
GLY	0.000	0.0	0	0.0	0.0
ALA	0.150	3.6	3	5.9	4.9
VAL	0.139	3.3	5	5.5	8.2
MET	0.052	1.3	0	2.1	0.0
ILE	0.128	3.1	5	5.0	8.2
LEU	0.193	4.6	6	7.6	9.8
TYR	0.127	3.0	1	5.0	1.6
PHE	0.082	2.0	3	3.2	4.9
HIS	0.091	2.2	2	3.6	3.3
LYS	0.213	5.1	9	8.3	14.8
ARG	0.187	4.5	5	7.3	8.2

TABLE III

Biological Properties of Purified Human Recombinant IL-8

Chemotactant	Mol. Wt. (daltons)	Antibody Reactivity * Anti-human IL-8, (monoctye derived)	Chemotactic Index	Oxygen Burst Metabolism ** cytochrome c assay	Oxygen Burst Metabolism *** fluorescence assay
$_{hr}$IL-8	9200	+++++	1.67	<2.0	177.0
IL-8/NAP-1 (moncyte)	9000	+++++	1.87	(0.015)[a]	(195)[b]
FMLP peptide	354	-/+	2.17	48.4	256.0

* Antibody reactivity was determined using a conventional ELISA assay on plastic in a 96 well falcon Primiara tissue culture plate. The assay used purified rabbit anti-human IL-8 antibody and peroxidase conjugated goat anti-rabbit IgG as discribed in methods. A plus (+) is defined as a change in O.D. of 0.2 absorbance units at 415nm.

** Oxygen burst metabolism was measured using a cytochrome c reduction assay measuring superoxide anion production in the pMole range as discribed in methods. A unit of activity was defined as the ability to reduce 1.0 nmole O_2/sec/10^7 neutrophils/nmole of chemotactant added.

*** Oxygen burst metabolism measured by fluorescence consumes hydrogen peroxide and added exogenous horseraddish peroxidase to oxidize the non-fluorescent substrate dihydrodiactetal fluorecein into its fluorescent product fluorecein. The senstivitly of this assay for peroxide utilization is in the nMole range as discribed in methods. A unit of activity was defined as the ability to reduce 1.0 pmole O_2 /sec/10^7 neutrophils/pmole of chemotactant added.

a Oxygen burst metabolism has been evaluated by Djeu, J.Y. et. al. (34) using the cytochrome c reduction assay measuring superoxide anion production.

b Oxygen burst metabolism has been eviauated by Waltz, A. et. al. (35) using a sensitive chemiluminescence assay.

Table IV. IL-8 and fMLP Concentration Dependence of IP_3 Generation [a]

Stimuli Concentration (μM)	IP_3 Released (cpm)		
	BSA	IL-8	fMLP
0	160±15	155±22	156±25
10^{-4}	159±14	152±16	179±12
10^{-3}	164±23	127±26	208±21[b]
10^{-2}	157±22	132±19	241±18[b]
10^{-1}	172±31	151±23	260±26[b]
1	150±21	124±18	290±23[b]
10	160±12	153±21	288±32[b]

[a] Cells are incubated as described in Methods. Data represent mean±SD of at least three separate experiments.

[b] $p<0.05$, fMLP vs BSA

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