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# EFFECT OF THE LEUMEDIN N-(FLUORENYL-9-METHOXYCARBONYL)-LEUCINE ON THE FUNCTION OF EOSINOPHILS

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A Thesis

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 1995

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# ABSTRACT

Stan Smith, Advisor

N-(fluorenyl-9-methoxycarbonyl)-leucine (FMOC-leucine) is a member of a class of compounds collectively called leumedins. These compounds have been described as having anti-inflammatory properties in several animal models. Specifically, leumedins have been shown to inhibit the upregulation of Mac-1 integrin molecules on neutrophils. The effects of leumedins on the function of human eosinophils have not been described. The effects of FMOC-leucine on the function of human eosinophils were examined in this study. In addition, the effects of FMOC-leucine on the function of neutrophils were examined for comparison to eosinophils. The two functions that were examined are chemotaxis and activation as measured by the inhibition of migration through polycarbonate filters and cluster of differentiation (CD)11b upregulation. Eosinophil enriched suspensions were activated by incubating with 1.0 μM platelet activating factor (PAF) in the presence of FMOC-leucine at concentrations of 0.3 μM, 3.3 μM, or 33.3 μM. The activation of eosinophils was assessed by flow cytometry measurement of CD11b. These data were used to calculate the % inhibition of activation. FMOC-leucine at concentrations of 0.3 μM, 3.3 μM, and 33.3 μM inhibited the activation of eosinophils by 7%, 21% and 67%, respectively. The effect of FMOC-leucine on PAF induced chemotaxis was measured with the use of Transwell plates with 3.0 μm pores. PAF was added to the lower chambers and FMOC-leucine was added to the upper chambers followed by the addition of 100,000 cells and an incubation at 37$^{O}$ C for 3 hours. Net stimulated

movement (NSM) values were calculated for each treatment condition. FMOC-leucine at a concentrations of 0.3 μM, 3.3 μM, and 33.3 μM inhibited the NSM of eosinophils by 3%, 34%, and 100%, respectively. FMOC-leucine was clearly demonstrated to have a significant inhibitory effect on the PAF-induced upregulation of CD11b and PAF-induced chemotaxis of eosinophils. Statistically significant chemotaxis inhibition was achieved with a 3.3μM concentration of the drug ($p<0.01$). Statistically significant inhibition of CD11b upregulation was achieved with a 3.3μM concentration of the drug ($p=0.03$).

"Work expands so as to fill the time available for its completion."
C. Northcote Parkinson

"Everything should be made as simple as possible, but not simpler."
Albert Einstein

"A mind that is stretched to a new idea never returns to its original dimensions."
Oliver Wendell Holmes

This work is dedicated to God, who has given me the strength, knowledge and perseverance to complete this project. To my parents who instilled in me an appreciation of education and nurtured my curiosity from an early age and to my wife who has shown me unwavering support and and endless encouragement during my work on this project.

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# INTRODUCTION

## Granulocyte Function

Polymorphonuclear leukocytes , also referred to as granulocytes, are composed of three distinctive cell types that are all derived from a common intermediately differentiated bone marrow precursor, the promyelocyte. Under the influence of colony stimulating factors and other cytokines, these intermediately developed promyelocytes undergo terminal differentiation to produce neutrophils, eosinophils, and basophils. Terminally differentiated granulocytes are short-lived phagocytic cells that contain lysosomes filled with proteolytic enzymes. They also produce peroxide and superoxide radicals which are toxic to many microorganisms. Some granules also contain bactericidal proteins such as lactoferrin.[1]

An important function of granulocytes is their participation in the inflammatory response, a major component of the body's innate immune defense mechanism. Inflammation comprises many events and is initiated by tissue damage caused by a variety of factors. The various types of tissue damage capable of initiating an inflammatory response include mechanical injury (e.g., laceration), thermal injury (e.g., burn), chemical injury (e.g., exposure to a corrosive chemical), biological injury (e.g., infection by a microorganism), and immunological injury (e.g., hypersensitivity reactions).[1] Inflammation constitutes an important part of both innate and acquired immunity. This process has evolved as a protective response against injury and infection. However, in certain cases such as hypersensitivity, inflammation becomes

the problem rather than the solution to the problem.[1] An inflammatory reaction, well out of proportion to the threat of the insulting material, can cause damage to the host mounting the response without any benefit to the host.

## Chemotaxis and Activation

To participate in an inflammatory reaction in the tissues, the granulocyte must move from the fast flowing center of the blood vessel and roll along the walls of capillaries. Eventually, the cell adheres to vascular endothelial cells. Granulocytes are briefly retained by the vascular basement membrane but then enter the tissue by passing through small openings in this membrane. This process is energy dependent and is greatly enhanced by chemotactic factors.[1] A number of substances generated by the inflammatory process have been demonstrated to be chemotactic factors that serve to amplify the entire process by recruiting phagocytic cells to the site of the immunological challenge.[2]

The first step in the process of granulocyte chemotaxis is the binding of chemotactic factor to specific receptors located on the plasma membrane of the granulocyte.[3] Depending on experimental conditions, chemotactic factor-receptor interaction can initiate a number of cellular functions in the granulocyte. These functions include aggregation, exocytosis of cytoplasmic granules, upregulation of integrins, and a number of other biochemical changes including an increase in oxidative metabolism (respiratory burst).[4] Within seconds of chemotactic factor binding, the granulocyte membrane becomes more fluid, the concentration of cyclic adenosine

monophosphate (cAMP) increases, the electrical charge of the cell changes, and calcium is mobilized into the cytosol. Other findings that have been described within the first few minutes of chemotactic factor-granulocyte interaction include secretion of granular contents, shifts in ion concentrations ($Ca^{++}$, $K^{+}$, and $Na^{+}$), activation of enzymes, cellular swelling, increased microtubule assembly, and increased integrin expression.[5]

One of the first steps in the migration of activated granulocytes to a site of inflammation is adherence to the endothelial cells lining blood vessel walls. This attachment is effected principally via certain heterodimeric adhesion molecules known as integrins.[2] On neutrophils, at least two such integrins play a major role in adherence to endothelium, CD11a/CD18 (leukocyte function antigen or LFA-1) and CD11b/CD18 (Mac-1). The ligand for LFA-1 is intercellular adhesion molecule (ICAM-1) and the ligand for Mac-1 is the complement fragment iC3b. There are a number of adhesion molecules present on the luminal surface of endothelial cells that are postulated to be important in eosinophil adhesion and migration into tissue that are upregulated by the same chemotactic factors that activate leukocytes.[6] These molecules include intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (ELAM-1), and vascular cell adhesion molecule-1 (VCAM-1).[7] The level of expression of these adhesion molecules is dependent on protein synthesis and is upregulated by endotoxin, interleukin-1 (IL-1), tumor necrosis factor (TNF), platelet activating factor (PAF), and other cytokines.[3] *In vitro*, antibodies against

ICAM-1 and ELAM-1 have been shown to inhibit both eosinophil and neutrophil adhesion to interleukin-1-stimulated endothelial cells.[5] However, antibodies to VCAM-1 inhibit only eosinophil adhesion. The ligand for VCAM-1 appears to be very late activation antigen-4 (VLA-4) which is present on eosinophils and lymphocytes but not neutrophils.[8,9]

Leukocyte adhesion to vascular endothelial cells lining the postcapillary venules represents a crucial early event in cellular recruitment to inflammatory sites. The importance of this mechanism is emphasized by patients with a rare autosomal recessive disease termed leukocyte adhesion deficiency (LAD). LAD patients suffer recurrent life-threatening infections due to an inability to recruit neutrophils to sites of infection.[5] The disease is caused by mutations in the common β subunit (CD18) of the leukocyte integrins that result in loss of expression of all three members of the CD18 family of adhesion molecules.[2] The leukocyte integrins are members of a superfamily of adhesion receptors; each member of which is a heterodimer composed of an α and β subunit. The integrins are divided into subfamilies according to their β subunit association, i.e., the β–1 (CD29), β–2 (CD18), and β–3 (CD63) integrins. However, there are at least three other known β subunits that may give rise to other, as yet undefined, subfamilies.[2] Despite a major defect in neutrophil recruitment in LAD patients, lymphocyte extravastation and accumulation appear essentially normal. Of particular interest is the observation that eosinophils are present in the extravascular tissues of some LAD patients, indicating that these cells possess an alternate adhesion

pathway. Recent studies have described a lymphocyte/endothelial cell adhesion pathway, independent of the CD11/18 β–2 integrins and involving the membrane glycoprotein α–4, β–1 (CD49d/CD29) and its ligand, VCAM-1, which is expressed by cytokine-stimulated cultured vascular endothelial cells.[5] A portion of the α–4, β–1 receptor binds fibronectin which is distinct from its role in cell-cell interactions.[1] Eosinophils, but not neutrophils, express α–4, β–1 (VLA-4) and are able to utilize the VLA-4/VCAM-1 pathway for adhesion to vascular endothelium.[21]

The acute activation of neutrophils results in increased affinity of the cells for endothelium in many circumstances. This activation is mediated by neutrophil L-selectin which is constitutively expressed on the cell surface, and within minutes of activation leads to rolling.[26] L-selectin is rapidly shed from the cell surface after activation, while at the same time Mac-1 expression increases. The shedding of L-selectin may serve as a detachment mechanism that allows the neutrophil to shift to β-2 integrin-dependent adhesion and locomotion.[4] LFA-1 is also constitutively expressed on the neutrophil surface and mediates tight adherence and may have particular importance later in inflammatory reactions, after expression of its endothelial ligand, ICAM-1, has been induced. The endothelium also appears to play a role in acute adherence of neutrophils. Endothelial cells contain granule-bound P-selectin, which is rapidly expressed on the cell surface upon activation, and like L-selectin, mediates rolling. Endothelial cells synthesize platelet-activating factor within minutes of stimulation. Platelet-activating factor is not released from endothelial cells as it is

from other cells. Instead, it remains at the cell surface, where it binds receptors on rolling neutrophils and activates them to upregulate Mac-1.[18]

## Eosinophils

Eosinophils are important effector cells in the pathogenesis of allergic diseases, especially asthma. These cells are also important effector cells in non-allergy induced intrinsic asthma. Evidence for a significant role for eosinophils in the pathogenesis of asthma comes from pathological, bronchoalveolar lavage, and physiochemical studies.[10] The lungs of patients who have died during an asthmatic attack exhibit widespread inflammation with prominent eosinophil infiltration.[7] Bronchoalveolar lavage fluids and transbronchial biopsies of asthmatics have increased numbers of eosinophils and eosinophil products.[7] Moreover, eosinophilic inflammation of the airways has been shown to be correlated to several parameters suggestive of disease activity.[5,10,11] In addition, eosinophil products have the potential to mediate many of the pathophysiological changes observed in asthmatics. To reach the site of inflammation in the airways, eosinophils must be able to penetrate endothelial cell barriers in response to chemotactic stimuli. Several mediators and cytokines have been shown to have chemotactic properties for eosinophils.[5] One such mediator, platelet-activating factor (PAF), has been shown to induce eosinophil chemotaxis and adherence to endothelial cells and to have other biologic actions that are potentially important in asthma pathogenesis.[12] Indeed, PAF is postulated to be an important mediator in asthma largely because of its effects on eosinophils. Eosinophils are

important inflammatory cells because they have the capacity to generate and release an array of mediators. These include the toxic granule-associated basic proteins and the membrane derived lipid mediators, leukotrienes and platelet-activating factor.[13] In bronchial asthma these potent substances play a major role in epithelial destruction, mucus hypersecretion, microvascular leakage, and bronchial smooth muscle contraction.[5] However, the mechanism(s) responsible for the preferential accumulation of eosinophils in the lungs of asthmatics remains unclear. Earlier studies addressed the possible existence of selective eosinophil chemoattractants. However, the only potent eosinophil chemotactic mediators described so far are equally active on neutrophils.[5]

T-helper lymphocytes ($T_H$ cells), via their interaction with antigen-presenting cells bearing antigenic peptides associated with major histocompatibility complex (MHC) class II molecules, clearly play a major role in determining which epitopes become targets of the immune response. However, when confronted with an invading organism/substance, the immune system must make a second, perhaps even more important "decision." It must select effector mechanisms and cells appropriate for the invasion. The way in which this selection of effector mechanism is first made remains obscure but it is ultimately associated with $T_H$ cell subsets which release different cytokines. These subsets of $T_H$ cells probably represent different patterns of differentiation from the same precursors, but it is conceivable that they arise from different cell lineages. In the mouse it is suggested that virgin lymphocytes ($T_HP$ cells),

which have not previously been stimulated, release IL-2. Short term stimulation leads to the development of $T_H0$ cells which can release a wide range of cytokines. After chronic stimulation, the specialized $T_H1$ and $T_H2$ types arise.[2] Some cytokines are released by both types (IL-3, GM-CSF, and TNFα) while others are not. $T_H1$ cells release IL-2 and IFNγ while $T_H2$ cells release IL-3, IL-4, IL-5, IL-6, and IL-10.[1] These different patterns of cytokine secretion may direct the response towards different effector mechanisms. For instance, $T_H1$ cells tend to activate macrophages.[2] On the other hand, $T_H2$ cells tend to increase production of eosinophils and mast cells, and to enhance production of antibody, including IgE.[1] These $T_H2$ lymphocytes are partly responsible for the accumulation of eosinophils in the lungs of asthma patients as the products of the lymphocytes are chemotactic for eosinophils.[5]

Currently, at least four cationic proteins are recognized in the granules of the eosinophil.[5] These are stained orange by eosin, an acidic dye. These membrane-bound granules consist of an electron-dense crystalloid core with an electron-lucent matrix surrounding it. The cationic proteins include eosinophil major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP).[14,15] MBP is localized in the crystalline core of the eosinophil granule and is associated with all of the core proteins. EPO, EDN, and ECP are located in the matrix of the granule. *In vitro*, MBP, ECP, and the EPO + $H_2O_2$ + halide system can kill helminthic parasites and are toxic to tumor cells.[5] Although capable of killing parasites and tumor cells, a major negative side effect is that this

system is capable of damaging many normal mammalian cells, including the guinea pig and human respiratory epithelium. Plugs of dead epithelial cells are a diagnostic hallmark of asthma.[5]

Mast cell- and basophil-derived products play a role in the recruitment of eosinophils. These products include eosinophil chemotactic factor of anaphylaxis (ECF-A), histamine, hydroxyeicosatetraenoic acids (HETEs), $LTB_4$, and PAF.[5] The eosinophil chemotactic activities of these mediators have been compared at equal concentrations using human eosinophils purified by percoll density separation.[7] PAF and $LTB_4$ were much more effective than the two ECF-A tetrapeptides and HETEs in causing eosinophil chemotaxis.[5] Studies conducted by Fukuda et al.[16] suggest that PAF is released from human basophils and possibly from lung mast cells by a specific antigen-bound antibody interaction and may be responsible in part for eosinophil migration toward the site of an acute allergic reaction. In addition to chemoattractants from mast cells, lymphokines may contribute to antigen-induced chronic recruitment of eosinophils in the airway.[16] In guinea pigs sensitized by intraperitoneal injection of antigen, Hirotsu et al.[17] observed two distinctive peaks of eosinophil infiltration at the site of intracutaneous injection of the antigen and extracted eosinophil chemotactic factor, which was derived from T-lymphocytes. In the bronchial mucosa, the same two-peaked pattern of eosinophil infiltration was observed.[17]

## Eosinophil Activation

A number of cells have been shown to produce alkyl-acetyl-glyceryl-

etherphosphoryl-choline (PAF), including neutrophils, eosinophils, basophils, macrophages, endothelial cells, and epithelial cells.[1] PAF is well characterized as a potent chemotactic agent for neutrophils and eosinophils.[18] PAF has been shown to increase mucus production in animals, increase microvascular permeability, and to cause pulmonary and bronchial edema. Inhalation of PAF causes bronchoconstriction in both normal and asthmatic individuals. PAF-induced brochoconstriction occurs within several minutes of PAF inhalation and may last up to two hours.[5] Small amounts of PAF produced by mast cells in response to IgE-mast cell interaction appear to start a process which leads to the attraction of granulocytes which themselves are capable of producing PAF. Additionally, PAF has been demonstrated to contribute to degranulation of eosinophils which could easily lead to tissue damage in both the hypersensitive and immunologically normal individual.[1]

Eosinophil granule proteins can cause damage to the bronchial epithelium. The concentration of MBP found in the sputum of some asthma patients is high enough to cause damage to the bronchial epithelium.[19] In order to compare the toxic effects of the granule proteins to the airway, Motojima et al.[20] cultured tracheal rings from guinea pigs with MBP, ECP, or EPO for 48 hours. Native MBP with a reactive sulfhydryl group caused exfoliation of epithelial cells at a concentration of 10 μg/ml, partial ciliostasis at 50 μg/ml, and complete ciliostasis at 100 μg/ml. ECP at a concentration of 100 μg/ml caused exfoliation of epithelial cells. EDN did not show any appreciable toxicity. EPO at 12 μg/ml only caused exfoliation of epithelial cells,

partial ciliostasis at 120 μg/ml and complete ciliostasis at 1,200 μg/ml. When EPO was combined with hydrogen peroxide and iodide, the toxicity was increased by a factor of four compared to the toxicity without hydrogen peroxide and iodide. This EPO-induced damage was completely inhibited by catalase and reduced by the deletion of iodide from the test system.[20]

Granulocytes have membrane receptors for PAF that belong to the superfamily of G protein-linked receptors. In the eosinophil, this G protein-linked receptor stimulates the activation of three separate phospholipases: phospolipase D (PLD), phosphoinositide-specific phospholipase (PLC), and phospholipase $A_2$ ($PLA_2$). It is generally accepted that PLC is coupled to the receptor via a G protein. The mechanism of how cell-surface stimulation leads to the activation of PLD and $PLA_2$ had yet to be firmly established. Substantial evidence has now emerged suggesting that these reactions are also regulated by G proteins.[21]

PLC, PLD, and $PLA_2$ have the potential to deliver intracellular signals that can be integrated to yield the terminal physiological response of the cell. In the case of eosinophils, the physiological responses include adherence, aggregation, chemotaxis, exocytosis of secretory granules, as well as the activation of nicotinamide adenine dinucleotide phosphate oxidase (respiratory burst). Multiple second messengers are generated within seconds of receptor stimulation and these messengers can either converge or diverge to trigger the individual functional responses in the eosinophil. The functions of many of the individual second messengers have been extensively

studied by controlling their endogenous production or by externally adding them into permeabilized cell preparations.[21] From such functional studies, the roles of the individual second messengers are beginning to be understood.

Activation of the PLC pathway causes the generation of two products, inositol triphosphate ($IP_3$) which mobilizes intracellular $Ca^{++}$, and diacylglycerol (DAG) which activates protein kinase C (PKC). Activation of PLD increases the membrane concentration of phosphatide, which may act as a second messenger in its own right, or it may be further metabolized to generate DAG. Activation of $PLA_2$ leads to the release of arachidonate and this also may have a dual function. It can act as a second messenger or it can be a substrate for the synthesis of eicosanoids.[21]

## Granulocyte Chemotaxis

Neutrophils have been shown by Casale et al.[22] to migrate across various barriers including polycarbonate filters, epithelial monolayers, and endothelial monolayers cultured on polycarbonate filters in response to numerous chemoattractants. Formylmethionylleucylphenylalanine (FMLP), leukotriene ($LTB_4$), and platelet-activating factor (PAF) have all been demonstrated to be capable of inducing neutrophil and eosinophil chemotaxis through noncellular, endothelial cell, and epithelial cell barriers in a dose-dependent fashion.[18] However, these chemoattractants have different potencies. Additional differences appear to be related to the barrier that the granulocytes must migrate through. Chemotaxis is greater when polycarbonate filters have endothelial cell monolayers grown over the filters versus

naked filters. Overall, the degree of neutrophil migration toward a particular chemoattractant is clearly influenced by the barrier through which the neutrophils must migrate. Dose-response experiments using noncellular barriers (polycarbonate filters) have shown that PAF is approximately 100-fold less potent than either $LTB_4$ or FMLP in inducing neutrophil chemotaxis.[18]

PAF has been demonstrated to be a potent eosinophil chemoattractant. Dose-response experiments have indicated that 1 μM PAF induces significant eosinophil migration through naked polycarbonate filters as well as endothelial cell monolayers. Between 25 and 40% of the maximal eosinophil migration observed at 180 minutes occurs in the first 60 minutes with either endothelial cell monolayers or naked polycarbonate filters. By 120 minutes, near maximal eosinophil migration is observed. Significant increases in eosinophil migration after 180 minutes is generally not observed.[7] To establish that PAF-induced eosinophil transendothelial migration is a chemotactic and not a chemokinetic process, Casale et al.[7,12] measured eosinophil net stimulated migration through endothelial cell monolayers and naked polycarbonate filters under the following three conditions: 1 μM PAF in upper wells, 1 μM PAF in the lower wells, and 1 μM PAF in both upper and lower wells. PAF was found to induce predominantly chemotactic movement through both naked polycarbonate filters and endothelial cell monolayer-polycarbonate filters. Neither PAF in the upper wells or equal concentrations of PAF in both upper and lower wells induced significant eosinophil migration into the lower wells after a three hour incubation. However,

eosinophil net stimulated movement values were > 60% when PAF was present in the lower wells only.[7,12]

## Leumedins

A series of N-(fluorenyl-9-methoxycarbonyl) amino acids, collectively called leumedins have been described as having antiinflammatory properties. These compounds have been described by Burch et al.[23] and are as follows: L-homophenylalanine (NPC 14686), L-alanine (NPC 14688), Glycine (NPC 14692), L-leucine (NPC 15521), N-(fluorenyl-9-methoxycarbonyl)-L-leucine (NPC 15199), DL-1,2,3,4-tetrahydroxiso-quinoline (NPC 15533), L-tetra-leucine (NPC15573), L-norleucine (NPC15667), N-9H-(2,7-dimethylfluorenyl-9-methoxycarbonyl)-L-leucine NPC (15669), N-[9H-{3 fluorenyl-9-proprionyl}]-L-homophenylalanine (NPC 15895), S-benzyl-beta, beta-dimethyl-N-[9H-{fluorenyl-9-methoxy}carbonyl]-D-cysteine (NPC 15953), and N-[9H-{1-methylfluorenyl-9-methoxy}carbonyl]-L-homophenylalanine (NPC15961). These compounds have been shown to be efficacious in reducing inflammation in several animal models.[19,24,25,27-30] All of the leumedins tested were capable of inhibiting arachidonic acid and oxazolone-induced dermatitis in a mouse model. When administered intraperitoneally, leumedins inhibited this measurable inflammatory response to topically applied arachidonic acid.[25] Because arachidonic acid-induced edema is partly mediated by prostaglandins and leukotrienes, the ability of several of the compounds to inhibit the enzymes in the synthetic pathways of these lipids was assessed by Burch et al.[23] NPC 14692 was

found not to inhibit fatty acid cyclooxygenase or 5-lipoxygenase at concentrations up to 300 μM. NPC 15199 (FMOC-leucine) and NPC 15669 were without effect on cyclooxygenase at concentrations up to 300 μM but were very weak inhibitors of 5-lipoxygenase, with $IC_{50}$ values of 173 μM and 69 μM, respectively.[23] The structure of FMOC-leucine is shown in Figure 1. Leumedins have also been shown to inhibit the reversed passive Arthus reaction, neutrophil accumulation at inflammatory sites, and acute-phase responses.[25] The reverse passive Arthus reaction is initiated by the formation of antigen-antibody complexes in the skin followed by complement activation and recruitment of neutrophils, leading to capillary damage and leakage.[1] In this inflammatory reaction, arachidonic acid metabolites are not important. After intraperitoneal administration, several of the leumedins inhibited the reverse passive Arthus reaction. Phagocytic activity was not inhibited by the leumedins.[2]

Leumedins offer possibilities for the treatment of asthma because they possess anti-inflammatory and immunosuppressive properties.[23] Leumedins have been demonstrated to inhibit the accumulation of neutrophils at inflammatory sites by inhibiting upregulation of Mac-1 integrin molecules on the surface of neutrophils.[27] Neutrophils which are unable to upregulate their Mac-1 are unable to migrate to an inflammatory site. The obvious danger of blocking granulocyte recruitment in the lungs is infection but this risk already exists with the more globally acting corticosteroid and other immunosuppressive therapies which are currently in use.[21]

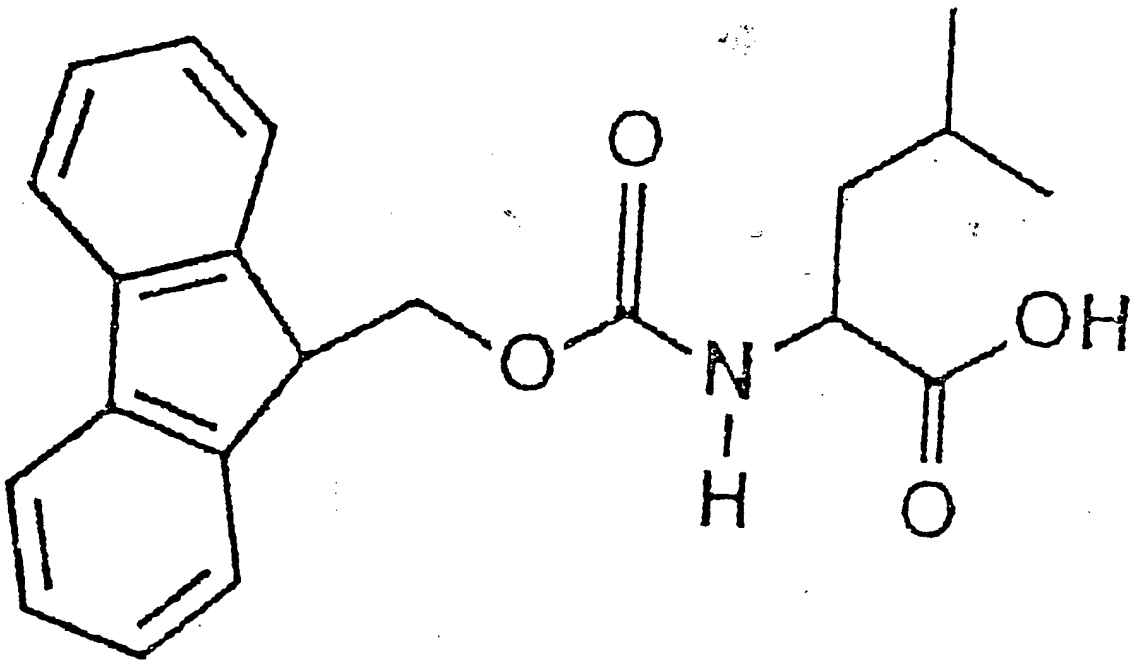


Figure 1. FMOC-leucine. The structure of N-(fluorenyl-9-methoxycarbonyl)-leucine (FMOC-leucine) is shown in this figure. FMOC-leucine is a member of a group of N-(fluorenyl-9-methoxycarbonyl) amino acids, collectively called leumedins. These compounds have been described by Burch et al.[23] as having anti-inflammatory properties.

Although leumedins inhibit T-lymphocyte and neutrophil infiltration in animal models of inflammation, they do not depress the numbers of circulating leukocytes or bone-marrow progenitor cells.[23] These characteristics make leumedins an attractive non-steroidal agent for the treatment of inflammatory diseases such as asthma which are potentiated by the products and actions of recruited effector cells. Although the ability of leumedins to block the upregulation of Mac-1 integrin molecules on stimulated neutrophils has been described, no studies have been published to date focusing on the effects of this new class of drugs on the activation of human eosinophils.

**Steroidal Antiinflammatory Agents**

Profound airway inflammation is a characteristic feature of fatal asthma. The aims of asthma treatment are to eliminate symptoms, maintain normal activity levels, achieve normal long-term pulmonary function, reduce the risk of severe attacks, and prevent recurrent exacerbations. Corticosteroids have potent antiinflammatory effects and are the most effective agents available for controlling chronic asthma, although they do not cure the disease. After regularly taking steroids over one to three months, many patients show fewer eosinophils, mast cells, and lymphocytes; in patients with very mild inflammation of the airways, there may be complete resolution.[5]

Although steroids are highly effective, many patients are reluctant to take them because they fear the side effects. Local effects of inhaled steroids include hoarse voice, oropharyngeal candidiasis, and throat irritation due to oropharyngeal deposition. These side effects can be markedly reduced by the use of a large volume

spacer device and, in the case of dry powder inhalers, by mouth washing.[5] These procedures remove residual steroids from the throat and mouth.

Of greater concern are the systemic effects of inhaled steroids absorbed from the gastointestinal tract of the swallowed fraction, "by mouth" fraction, and from the lungs of the inhaled fraction. Although effective, systemically active steroids have a number of side effects including cataract formation, depression, bone demineralization, hyperglycemia, hypertension, sodium and water retention, and weight gain secondary to appetite stimulation.[5]

Adrenal suppression occurs promptly during administration of exogenous corticosteroids due to the feedback effect on the output of corticotrophin-stimulating hormone and adrenocorticotrophin from the hypothalamus and pituitary gland, respectively. This suppression of the hypothalamic-pituitary-adrenal complex is temporary, with rapid return of normal function within days after discontinuation of short-term systemic corticosteroids. The longer systemic corticosteroids are maintained or the more frequent the short courses, the greater the likelihood of sustained suppression of normal adrenal output.[5]

## Flow Cytometry

In flow cytometry studies, cells are labeled with fluorescently labeled antibodies that specifically bind to the markers to be measured. Markers are commonly labeled with monoclonal antibodies conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Carried by a microscopic

jet of fluid, the cells pass one by one through an intense beam of excitation light (argon laser) in the measuring region of the flow cytometer. Each cell thereby produces a short flash of fluorescence, the intensity of which is proportional to the cellular content of the fluorescently labeled marker. These flashes of fluorescence are collected by appropriate photomultiplier tubes (PMT) which focus the light into electrical pulses, which are measured and recorded. Each cell also causes scattering of the excitation light. The intensity of this scattering is a function of the size, shape and structural complexity of the cell. The resulting flash of scattered light is recorded by separate detectors. Thus, the cellular content of several markers, labeled with dyes fluorescing at different wavelengths as well as size and shape or structure, may be recorded for each individual cell.

Flow cytometry techniques can be applied to eosinophil activation assays. Mac-1 integrin molecules are upregulated on both eosinophils and neutrophils during activation.[2] Fluorescently labeled mouse monoclonal antibodies which recognize a variety of granulocyte surface markers, including Mac-1, are commercially available. The integrin VLA-4 is present on eosinophils but is not present on neutrophils.[2] Both fluorescein isothiocyanate (FITC)-labeled mouse monoclonal antibody directed against CD11b (Mac-1 component), and phycoerythrin (PE)-labeled mouse monoclonal antibody directed against CD49d (VLA-4 component) are commercially available. The use of these two antibodies with mixtures of eosinophils and neutrophils would allow the monitoring of Mac-1 upregulation as well as provide a means of

differentiating the two cell types. The increase in intensity of green fluorescence over base line would correspond to upregulation of Mac-1 molecules (both eosinophils and neutrophils) while only the eosinophils would display red fluorescence.

In this study, eosinophil and neutrophil activation was assessed using flow cytometry analysis of PAF-induced upregulation of CD11b in the presence of FMOC-leucine. Additionally, PAF-induced chemotaxis was assessed using polycarbonate filters in the presence of FMOC-leucine. The effect of leumedins on neutrophils have been described. However, no such studies are available regarding the effect of leumedins on the function of human eosinophils. The effects of FMOC-leucine on neutrophils were used to compare with the eosinophil results. This step was made necessary due the lack of published reports regarding the effect of FMOC-leucine on human eosinophils. This study focused on determining the *in vitro* effect of FMOC-leucine on the activation and chemotaxis of human eosinophils.

# MATERIALS AND METHODS

## Reagents

Pyrogen-free Histopaque, pyrogen-free percoll, bovine serum albumin (BSA), eosin, N-(fluorenyl-9-methoxycarbonyl)-leucine, pyrogen-free Hank's balanced salt solution (HBSS), hepes buffer, phosphate buffered saline (PBS), and PAF (C18:1, 1-O-octodecyl-9-enyl-2-acetyl-N-glyceryl-3-phosphocholine) were purchased from Sigma Chemical Company (St. Louis, MO). Transwell tissue culture plates were purchased from Costar Corp.(Cambridge, MA). Modified Wright's stain and pyrogen-free 1.6% saline were purchased from Baxter Healthcare Corp., Fenwal Division (Deerfield, IL). Ethyl alcohol (200 proof) was purchased from Publicker Chemical Co. (Linfield, PA). Sephadex density marker beads and dextran T500 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Sodium azide and paraformaldehyde were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Pyrogen-free water was purchased from McGaw Inc. (Irvine, CA). PE-labeled mouse monoclonal anti-CD49d ($IgG_2$), FITC-labeled mouse monoclonal anti-CD11b ($IgG_1$), unlabeled mouse monoclonal anti-CD16, goat anti-mouse IgG coated magnetic beads, and CaliBRITE beads were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Mouse monoclonal $IgG_2$ phycoerythrin conjugate control antibody and mouse monoclonal $IgG_1$ fluorescein conjugate control antibody were purchased from Biosource International (Westbrook, ME).

## Blood Donors

Eosinophils were isolated from adult therapeutic blood donors. This group of patients were suffering from the effects of polycythemia vera. The therapeutic removal of blood is a common treatment for this disorder. Blood was collected from donors into standard 800 ml blood bags which contained 63 ml of citrate as anticoagulant. Approximately 450 ml of whole blood was bled from each donor. These therapeutically donated units are normally discarded. Freshly collected specimens of opportunity were taken from this pool of donors and used in this study.

## Isolation of Granulocytes

Leukocytes were isolated from citrate anticoagulated whole blood by aliquoting 25 ml of well mixed fresh whole blood (8 aliquots total or 200 ml whole blood) into 50 ml conical tubes followed by the addition of an equal volume of 4° C 3% dextran in PBS. The conical tubes were gently inverted several times and allowed to remain undisturbed at 4° C for 30 minutes. The cloudy supernatant was harvested from the tubes by pipet and transferred into clean 50 ml conical tubes. The supernatant was diluted 1:2 with 4°C HBSS and centrifuged at 500 x *g* for 10 minutes at 4°C. The supernatant was discarded and the resulting pellet of cells was mixed thoroughly. The remaining erythrocytes in the cell pellet were hypotonically lysed by adding 20 ml of 4° C pyrogen-free water and mixing for 20 seconds followed by the addition of 20 ml of 4°C pyrogen-free 1.6% NaCl to restore isotonicity. This cell

suspension was then centrifuged at 500 x *g* for 10 minutes at 4° C. The hemolyzed supernatant was decanted and the pellet was washed once with 45 ml of 4° C HBSS and centrifuged at 500 x *g* for 10 minutes at 4° C. This procedure produced a leukocyte concentrate.

Granulocytes were isolated from the leukocyte concentrate by Histopaque (1.077 specific gravity) density gradient centrifugation. Three ml aliquots of the leukocyte concentrate were placed in 15 ml conical test tubes, underlayed with 9 ml of Histopaque (1.077 specific gravity) and centrifuged at 400 x *g* for 30 minutes at 22° C. All resulting layers of cells were discarded leaving the granulocyte button in the bottom of the tube. The granulocyte button was resuspended to a concentration of 2 x $10^6$ cells per ml in HBSS with 2% BSA and placed in a wet ice bath.

**Isolation of Eosinophils**

The granulocyte suspensions were prepared for eosinophil enrichment by adding 15 µl of unlabeled mouse monoclonal anti-CD16 (IgG) and incubating for 30 minutes at 4° C in a refrigerator. After the 30 minute incubation, the granulocytes were washed by adding 3 ml of 4° C HBSS with 2% BSA and centrifuging at 500 x *g* for 10 minutes at 4° C. The supernatant was decanted and the wash procedure repeated. After the second wash, the granulocyte button was resuspended in 500 µl of HBSS with 2% BSA. After resuspension of the granulocytes, 15 µl of goat anti-mouse IgG labeled magnetic beads were added to the suspension and allowed to

incubate at 4° C in a refrigerator for 30 minutes with occasional gentle mixing. Two ml of 4° C HBSS with 2% BSA was then added to the test tubes. The test tubes were then placed on a magnetic holder and refrigerated at 4° C for 5 minutes to remove CD16 positive cells. The eosinophil enriched cell suspensions were then decanted into test tubes and placed in an ice bath. Eosinophil suspensions were analyzed for eosinophil content and purity using eosin and a Neubauer hemocytometer. Eosinophils were washed once with 4° C HBSS followed by resuspension and adjustment to 1,000 cells/μl in HBSS. Eosinophil preparations with purity ≥50% were used for the experiments.

**PAF-induced Eosinophil Chemotaxis**

PAF-induced migration of eosinophils through polycarbonate filters were investigated with Transwell plates. Transwell plates have 24 wells that are separated into upper and lower chambers by polycarbonate filters that have a 6.5 mm diameter and 3.0 μm pore size. A total of 100,000 eosinophils in a volume of 100 μl HBSS were placed in the upper chambers (above the filter). PAF (1 μM) in 500 μl PBS were placed in the lower chambers (below the filter). PBS alone was placed in the lower chambers as a negative control in each experiment. A chemokinesis control was included for each cell source which consisted of PAF (100 μl, 3.0 μM) added to both upper and lower chambers prior to the addition of the eosinophils. A FMOC-leucine diluent (vehicle) control was included for each cell source which consisted of 100 μl of

3% ethanol (ETOH) placed in the upper well with the test cells and 500 µl PAF in the lower well. The final concentration of ethanol in all experiments was 1%.

FMOC-leucine was used in three concentrations, 0.3µM, 3.3µM, and 33.3µM. All FMOC-leucine dilutions were made with 3% ethanol in PBS. Both FMOC-leucine and PAF were added to the lower chambers prior to the addition of the eosinophils to the test system (Table 1). The plates were then incubated at 37° C in 5% $CO_2$ and 100% humidity for 180 minutes. All chemotaxis assays were conducted in duplicate. After incubation, the filters were immediately removed to stop the migration assay. The lower chamber contents were then collected and the cells were stained with modified Wright's stain and counted on a hemocytometer.

**PAF-induced Eosinophil Activation**

Eosinophil and/or neutrophil activation studies were conducted with the use of three separate cell preparation techniques designated as procedures A, B, and C. Activation procedure A involved the activation of granulocytes with PAF in a whole blood suspension followed by the lysis of erythrocytes and staining with FITC-labeled anti-CD11b. Activation procedure B involved the activation of a purified granulocyte suspension with PAF followed by staining with FITC labeled anti-CD11b and PE-labeled anti-CD49d. Activation procedure C involved the activation of enriched eosinophil suspension with PAF followed by staining with FITC labeled anti-CD11b and PE- labeled anti-CD49d. All activation studies were analyzed by flow cytometry.

| | Well 1 | Well 2 | Well 3 | Well 4 | Well 5 | Well 6 | Well 7 |
|---|---|---|---|---|---|---|---|
| **Upper Well** | PAF, Cells | PBS, Cells | ETOH, Cells | PBS, Cells | 0.3 μM FMOC, Cells | 3.3 μM FMOC, Cells | 33.3 μM FMOC, Cells |
| **Lower Well** | PAF | PBS | PAF | PAF | PAF | PAF | PAF |

Table 1. Transwell Plate Chemotaxis Assay. The protocol used for the loading of Transwell plates is shown in this table. FMOC-leucine (FMOC) was used in three concentrations, 0.3μM, 3.3μM, and 33.3μM. All FMOC-leucine dilutions were made with 3% ethanol in PBS. Both FMOC-leucine and PAF were added to the lower chambers prior to the addition of cells to the test system. The plates were then incubated at 37°C in 5% $CO_2$ and 100% humidity for 180 minutes. All tests were conducted in duplicate. After incubation, the filters were immediately removed to stop the migration assay. The lower chamber contents were then collected and the cells were stained with modified Wright's stain and counted with a hemocytometer.

All three activation procedures were identical with the exception of cell suspension type. The activation of these cell suspension were conducted in the following manner: Tube 1 contained 1.4 ml of PBS and no PAF or FMOC-leucine. Tube 2 contained 0.9 ml of PBS, 0.5 ml of FMOC-leucine diluent (vehicle) and no PAF. Tube 3 contained 0.9 ml PBS, 0.5 ml of 3.0 μM PAF and no FMOC-leucine. Tube 4 contained 0.4 ml of PBS, 0.5 ml of 3.0 μM PAF and 0.5 ml of 1.0 μM FMOC-leucine. Tube 5 contained 0.4 ml of PBS, 0.5 ml of 3.0 μM PAF and 0.5 ml of 10 μM FMOC-leucine. Tube 6 contained 0.4 ml of PBS, 0.5 ml of 3.0 μM PAF and 0.5 ml of 100 μM FMOC-leucine (Table 2). Cell suspension volumes of 100 μl were used for all activation assays. In all assays the PAF, FMOC-leucine and diluents were added and thoroughly mixed prior to the addition of the test cell suspension. When present, the final concentration of PAF and ETOH were 1.0 μM and 1%, respectively due to assay dilutions. FMOC-leucine was also diluted by assay design and was present at 0.3μM, 3.3 μM, and 33.3 μM. All activation assays were conducted in duplicate. After the addition of cells, the suspensions were thoroughly mixed by gentle inversion and incubated at 37° C for 15 minutes. The activation studies were stopped by washing the cell suspensions with 3 ml of 0.1% sodium azide in PBS. The cell suspensions were then incubated with 5 μl of FITC-labeled mouse monoclonal anti-CD11b and 5 μl of PE-labeled mouse monoclonal anti-CD49d for 15 minutes at 4° C in a darkened refrigerator. After incubation, the eosinophil suspensions were washed

| Tube 1 | Tube 2 | Tube 3 | Tube 4 | Tube 5 | Tube 6 |
|---|---|---|---|---|---|
| PBS and Cells | ETOH, PAF and Cells | PAF and Cells | 0.3 μM FMOC, PAF and Cells | 3.3 μM FMOC, PAF and Cells | 33.3 μM FMOC, PAF and Cells |

Table 2. Activation Assay. The protocol used for the activation of cells is shown in this table. Tube 1 contained 1.4 ml of PBS and no PAF or FMOC-leucine. Tube 2 contained 0.9 ml of PBS, 0.5 ml of FMOC-leucine diluent and no PAF. Tube 3 contained 0.9 ml PBS, 0.5 ml of 3.0 μM PAF and no FMOC-leucine. Tube 4 contained 0.4 ml of PBS, 0.5 ml of 3.0 μM PAF and 0.5 ml of 1.0 μM FMOC-leucine. Tube 5 contained 0.4 ml of PBS, 0.5 ml of 3.0 μM PAF and 0.5 ml of 10 μM FMOC-leucine. Tube 6 contained 0.4 ml of PBS, 0.5 ml of 3.0 μM PAF and 0.5 ml of 100 μM FMOC-leucine. Cell suspension were added in volumes of 100 μl. When present, the final concentration of PAF and ETOH were 1.0 μM and 1%, respectively due to assay dilutions. FMOC-leucine was also diluted by assay design and was present at 0.3μM, 3.3 μM, and 33.3 μM.

once with 0.1% sodium azide in PBS and fixed with 1% paraformaldehyde in PBS. CD11b expression was then determined by flow cytometry analysis.

**Flow Cytometry Analysis**

A FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used for flow cytometry analysis. The flow cytometer was calibrated on each day of use with CaliBRITE calibration beads and AutoCOMP software, version 2.2 (December 1992) (Becton Dickinson Immunocytometry Systems, San Jose, CA). The calibration beads used were a collection of three different types of plastic microspheres (unlabeled, FITC-labeled, and PE-labeled). Each bead was approximately 6.6 μm in diameter. The unlabeled beads simulated the autofluorescent behavior of unstained cells. FITC-labeled beads simulated FITC-stained cells and PE-labeled beads simulated PE-stained cells. Each fluorochrome emitted light at a different wavelength when excited at 488 nm by an argon-ion laser. The FITC-labeled particles emitted yellow-green light (emission maximum approximately 515 nm) while the PE-labeled particles emitted red-orange light (emission maximum approximately 580 nm). The flow cytometer detected the yellow-green fluorescent FITC signal in the fluorescence-1 (FL1) detector, which was then displayed on the x-axis of a two parameter display. The red-orange PE signal was detected in the fluorescence-2 (FL2) detector and displayed on the y-axis of the display. The position of the fluorescence signals along the x-axis or the y-axis correlated with the intensity of their respective signals. Even though the emission

peaks were separated by 65 nm, FITC and PE had overlapping emission spectra between 540 nm and 600 nm. Because FL1 and FL2 channels did not detect FITC and PE exclusively, the amount of spectral overlap observed in these channels was corrected using an electronic compensation network.

Unlabeled beads were used to adjust the voltage settings. Sufficient data were gathered (software controlled function) so that a forward scatter (FSC) gate was set to isolate single events from debris and multiple-bead events. The FSC threshold was adjusted to a level that eliminated background signals. The voltages were then adjusted by setting mean channel values for FL1, FL2 and side scatter (SSC) that corresponded with initial target values. Next, a mixture of unlabeled, PE-labeled and FITC-labeled beads were used to adjust fluorescence compensation. The fluorescence compensation adjustments were FL1 - % FL2 for PE-labeled beads and FL2 - % FL1 for FITC-labeled beads. The FL2 - % FL1 compensation level was adjusted so that the FL1-positive population was aligned along the y-axis with the unlabeled bead population. The FL1 - % FL2 compensation level was adjusted so that the FL2-positive population was aligned along the x-axis with the unlabeled bead population. This adjustment corrected for spectral overlap by shifting the labeled populations so that they were aligned with the corresponding autofluorescence signals. A sensitivity test was conducted by running the three bead suspension through the flow cytometer using the settings from the voltage and compensation adjustments. Sensitivity in the FL1 and FL2 channels was determined by the amount of separation between the

unlabeled bead population and the corresponding labeled bead population. The sensitivity in SSC and FSC was determined by the separation between the mean light-scatter signal for the mixed beads and background signal.

Cells prepared for flow cytometry analysis were stored in 1% paraformaldehyde and were stable for up to three days while held at 4° C in a darkened refrigerator. All flow cytometry data acquisition was performed within 48 hours of cell preparation on a FACScan™ using Lysis II software version 1.1 (February 1992) by Becton Dickinson Immunocytometry Systems (San Jose, CA). Gates were appropriately set on both FSC x SSC dotplots and histograms to capture fluorescence intensity of the cells and individual CD markers of interest.

## Calculations and Statistics

Percent net stimulated movement (NSM) and percent NSM inhibition were calculated for both eosinophils and neutrophils from two separate cell suspension types, granulocytes and eosinophil-enriched granulocytes (>50% eosinophils). Percent NSM was calculated in the following manner:

$$\frac{\text{\# cells collected in test well - \# cells collected in negative control}}{\text{\# cells added to test well}} \times 100$$

Percent inhibition of NSM was calculated in the following manner: 100% - % NSM.

In order to determine the percent inhibition of CD11b, a PAF positive control was included with all assays and was interpreted as 100% activation. A positive control was used for each cell source for each assay which allowed for pairing of the

data. The average fluorescence intensity of the positive control was used to calculate the degree of inhibition due to the effects of FMOC-leucine by using the following formulas: (Test MFI/Positive control MFI) x 100 = %Activation , 100% - %Activation = %Inhibition. A paired student's t-test was conducted on the data from both the activation and chemotaxis assays. Significance was defined as $p<0.05$. Additionally, standard error was calculated and included with summary mean data.

Both positive and negative controls were included with all assays which provided a means determining the effects of the test systems on the cells being tested. Negative controls contained the FMOC-leucine vehicle and no stimulant (PAF). The positive control contained the FMOC-leucine vehicle and stimulant. The negative control was considered "0" or 100% inhibition while the positive control was considered "100" or 0% inhibition. Additionally, chemokinesis control values were subtracted from the positive control values to compensate for random movement of cells. These control values were used to determine the degree of inhibition in both the migration and activation assays.

# RESULTS

## Percoll Separation of Eosinophils

Isolation of eosinophils from whole blood was attempted using the technique of discontinuous gradient of percoll centrifugation as described by Casale et al.[7,12] No cell bands were ever identified after centrifugation. However, the suspensions were withdrawn in 2 ml aliquots and each aliquot was stained and analyzed for eosinophil content. Very little enrichment of eosinophils was evident in any of the aliquots. It was determined that centrifugation at 4° C had altered the specific gravity of the percoll dilutions as the percoll had been diluted at room temperature. The procedure was repeated with centrifugation at 22° C. Additionally, Sephadex density standard beads were prepared for incorporation in the procedure which would ensure that specific gravity of the bands was maintained during the gradient preparation and centrifugation. Although the discontinuous gradient of percoll was evident as demonstrated by the Sephadex beads, the grams/ml dilutions did not correspond to specific gravity and there was no enrichment of eosinophil content in any of the bands. As described by Casale, et al.[7,12], the percoll dilutions were made on the basis of grams/ml. Weight verification of the specific gravity performed at 22° C revealed that the gram/ml dilutions did not correspond to specific gravity in a linear fashion (data not shown).

After centrifugation, each resulting band of cells was harvested and eosinophil

content was determined by staining with modified Wright's stain. Any band that contained more than 3% eosinophils was stained with eosin and a eosinophil count performed on a hemocytometer. No more than 10% eosinophils were ever harvested from any specimen. Pyrogen-free hypaque (prediluted) was later purchased for the separation of granulocytes and used for all experiments from which data were gathered.

**Immunomagnetic Bead Separation of Eosinophils**

The discontinuous gradient of percoll technique proved to have flaws as related to the isolation of eosinophils from a leukocyte preparation. The enrichment of granulocyte preparations with eosinophils by immunomagnetically removing neutrophils was the next procedure that was attempted. This procedure was accomplished by the addition of mouse monoclonal anti-CD16 followed by the addition of a secondary goat monoclonal anti-mouse IgG magnetic bead conjugate. Since neutrophils expressed CD16 while eosinophils did not, eosinophils were left suspended as the neutrophils were attracted to the magnetic strip which was placed adjacent to the test tube. Greater than 50% suspensions of eosinophils were obtained with this procedure. The technique of immunomagnetic removal of neutrophils was chosen as the eosinophil enrichment procedure for this study due to the ease of performance and the quality of results obtained with the procedure.

This procedure although considerably more expensive in terms of reagent cost, proved to be a far more effective method of preparing an eosinophil enriched

suspension of granulocytes for testing. The evolution of cell suspensions, i.e., whole blood (1% eosinophils), granulocytes (3% eosinophils), and magnetic bead enrichment of eosinophils (≥50% eosinophils), provided a set of data which was interesting to compare and contrast.

**Activation Studies**

Mean fluorescence intensity (MFI) of FITC-labeled mouse monoclonal anti-CD11b was captured on the flow cytometer for all assays. When possible, 10,000 events were collected inside of the defined gates. However, when analyzing eosinophils only, the events captured had to be lowered to 2,000 events. Representative dot plots and histograms of whole blood, leukocytes and eosinophil enriched suspensions were printed and included in this work (Figures 2-6). The activation studies were conducted on cells prepared by three separate techniques designated procedures A, B, and C (Figures 7-9). PE-labeled mouse monoclonal anti-CD49d was used as a counter stain which allowed for the detection of eosinophils in a granulocyte preparations. Eosinophils were also differentiated from CD49d positive mononuclear cells (lymphocytes) on the basis of FSC and SSC. Procedure A involved the activation of whole blood samples. MFI data were captured for the six different treatments of the cells. The negative control tests which were not exposed to either PAF or FMOC-leucine demonstrated an average CD11b MFI of 52 (32% of the positive control). The positive control tests which were exposed only to PAF demonstrated an average CD11b MFI of 155. This value was used as the maximal

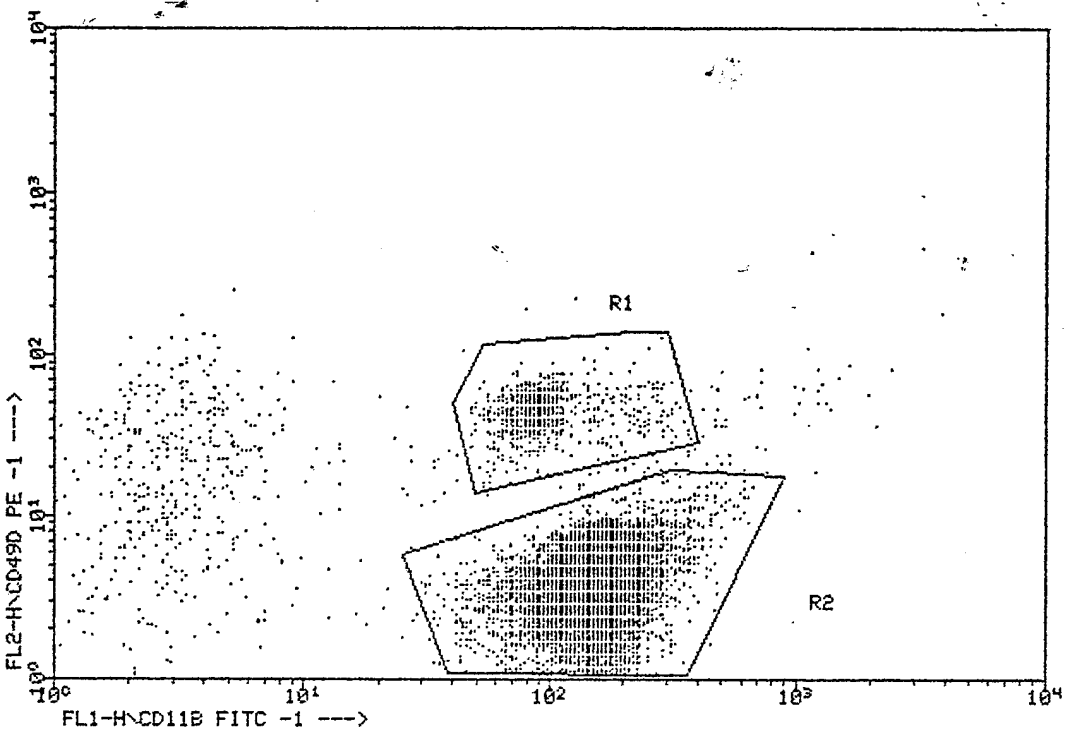


Figure 2. Flow Cytometry CD49d Gate. The x axis is flow channel one (FITC) fluorescence intensity and the y axis is flow channel two (PE) fluorescence intensity. The cells depicted have been marked with FITC-labeled mouse monoclonal anti-CD11b and PE-labeled mouse monoclonal anti-CD49d. The cells are separated on the dot plot on the basis of the type and intensity of fluorescence they emit after stimulation with an argon laser. The regions R1 and R2 represent two distinct populations of cells which are both positive for CD11b. Region R1 represents eosinophils as these cells are positive for CD49d and CD11b. Region R2 represents neutrophils as these cells are positive for CD11b but negative for CD49d.

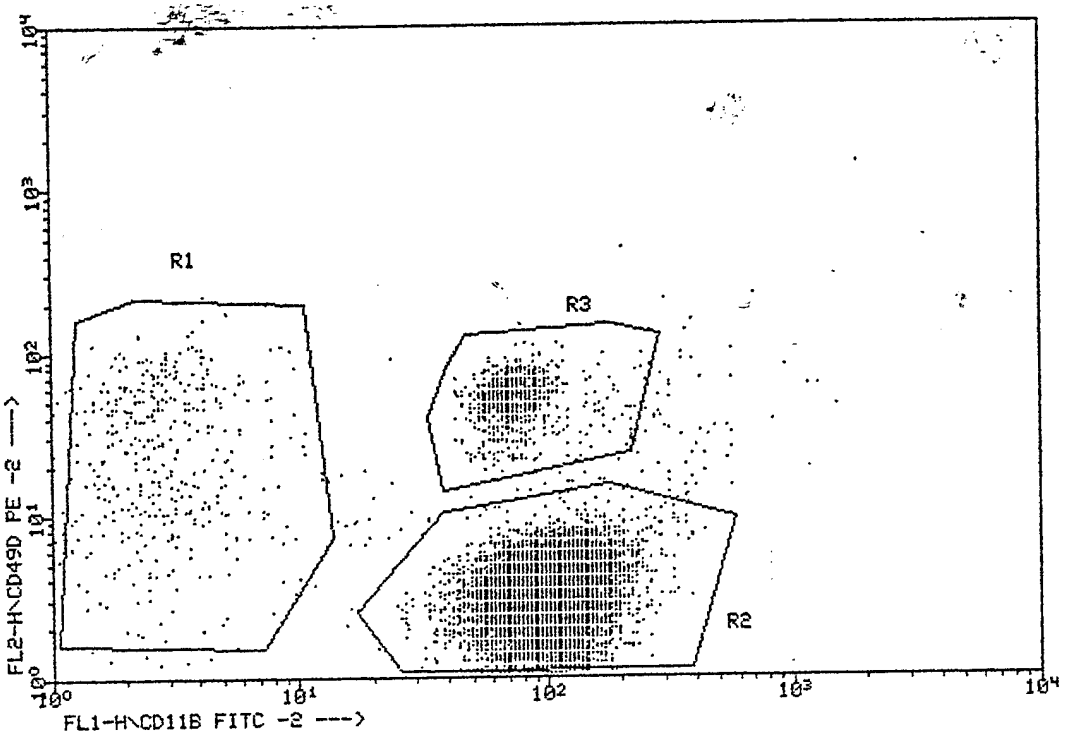


Figure 3. Flow Cytometry CD11b Gate. The x axis is flow channel one (FITC) fluorescence intensity and the y axis is flow channel two (PE) fluorescence intensity. The cells depicted have been marked with FITC-labeled mouse monoclonal anti-CD11b and PE-labeled mouse monoclonal anti-CD49d. The cells are separated on the dot plot on the basis of the type and intensity of fluorescence they emit after stimulation with an argon laser. The regions R1, R2 and R3 represent three distinct populations of cells. Region R1 represents mononuclear cells and debris. Region R1 was not of interest during granulocyte studies. Region R2 represents neutrophils as these cells are positive for CD11b but negative for CD49d. Region R3 represents eosinophils as these cells are positive for both CD11b and CD49d.

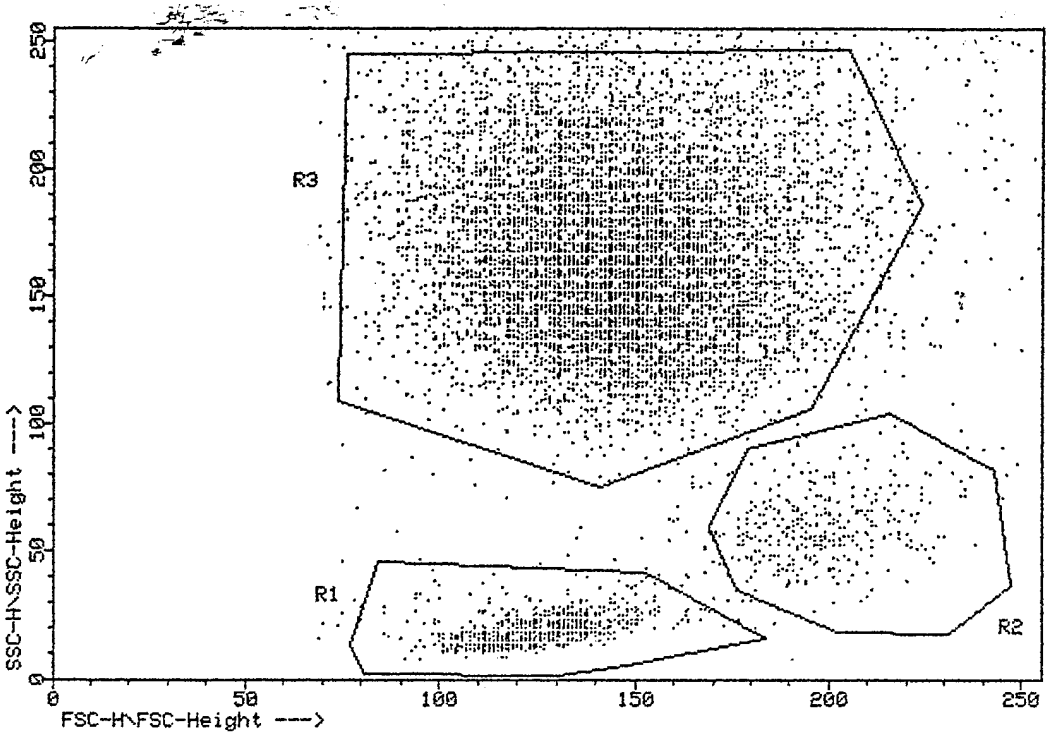


Figure 4. Whole Blood Leukocyte Flow Cytometry Dot Plot. The x axis is forward scattered light intensity and the y axis is side scattered light intensity. The cells depicted have not had antibodies added prior to analysis. The cells are separated on the dot plot on the basis of their size and structural complexity which is determined by the amount of light scattered by each cell as it passes through an argon laser beam. The regions R1, R2 and R3 represent three distinct populations of cells. Region R1 represent lymphocytes which are relatively small and structurally simple. Region R2 represents monocytes which are larger than lymphocytes and also relatively structurally simple. Region R3 represents granulocytes which are large and structurally complex.

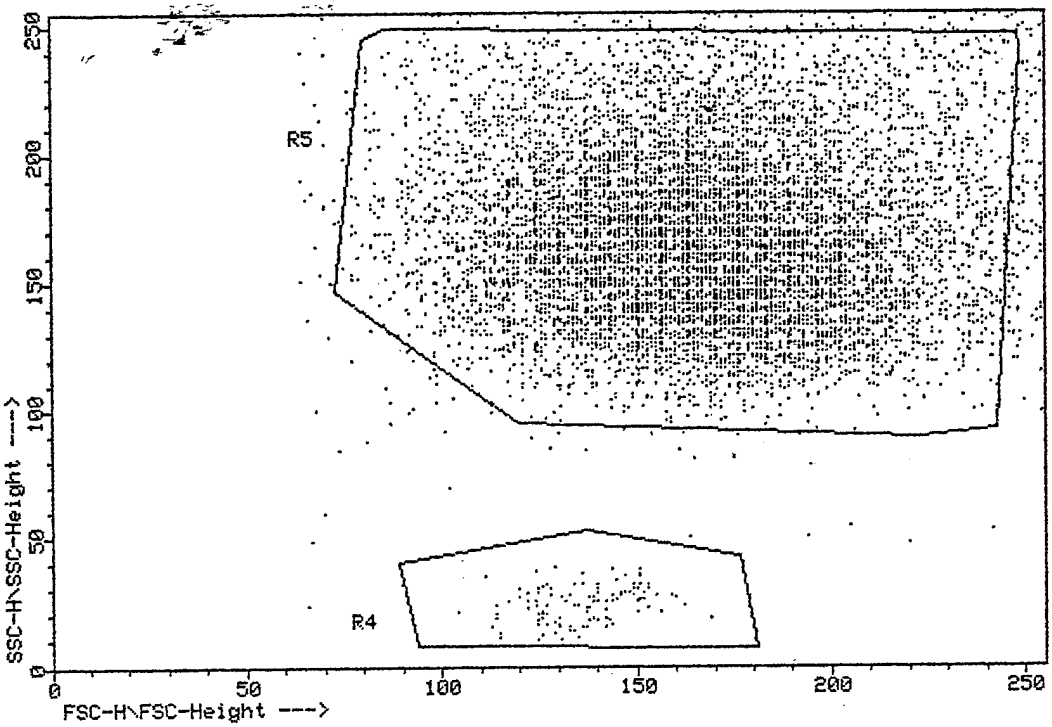


Figure 5. Neutrophil Flow Cytometry Dot Plot. The x axis is forward scattered light intensity and the y axis is side scattered light intensity. The cells depicted have not had antibodies added prior to analysis but have been subjected to mononuclear cell separation. The cells are separated on the dot plot on the basis of their size and structural complexity which is determined by the amount of light scattered by each cell as it passes through an argon laser beam. The regions R4, R5 represent two distinct populations of cells. Region R4 represent lymphocytes which are relatively small and structurally simple. Region R5 granulocytes which are large and structurally complex. Most monocytes were removed and thus are not shown in a region.

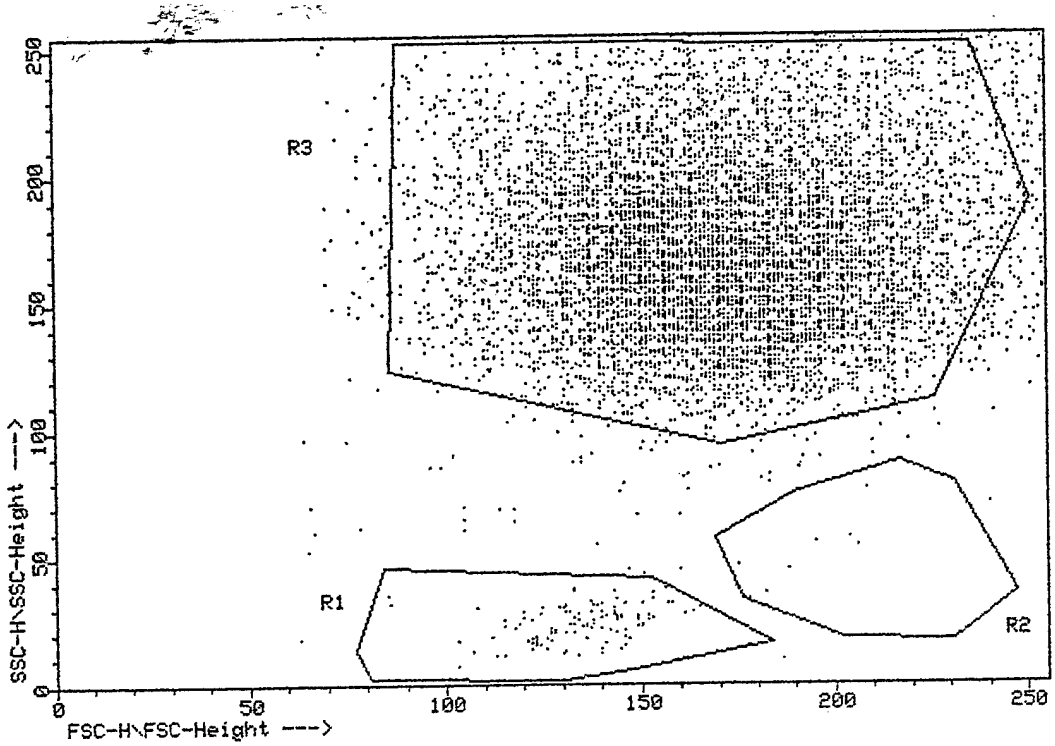


Figure 6. Eosinophil Flow Cytometry Dot Plot. The x axis is forward scattered light intensity and the y axis is side scattered light intensity. The cells depicted have not had antibodies added prior to analysis but have been subjected to mononuclear cell separation. The cells are separated on the dot plot on the basis of their size and structural complexity which is determined by the amount of light scattered by each cell as it passes through an argon laser beam. The regions R1, R2 and R3 represent three distinct populations of cells. Region R1 represents the remaining lymphocytes which are relatively small and structurally simple. Region R2 represents the remaining monocytes which are larger than lymphocytes and also relatively structurally simple. Region R3 represents eosinophils and neutrophils which are large and structurally complex.

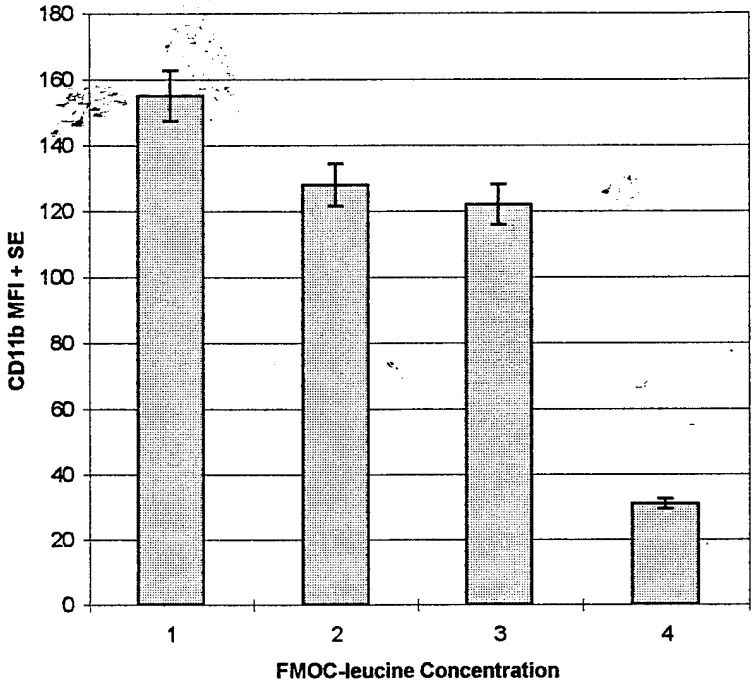


Figure 7: Whole Blood CD11b Fluorescence. CD11b mean fluorescence intensity (MFI) measurements plus standard error are shown in this figure. All MFI data shown were collected after 1.0 $\mu$M PAF activation of neutrophils in whole blood suspensions. FMOC-Leucine concentration are represented on the x axis and are as follows: (1) positive control with no FMOC-leucine (2) 0.3 $\mu$M, (3) 3.3 $\mu$M, and (4) 33.3 $\mu$M. These results are based on CD11b fluorescence studies conducted with a flow cytometer. The positive control demonstrated a CD11b MFI of 155. The 0.3 $\mu$M FMOC-Leucine assay demonstrated a CD11b MFI of 128. The 3.3 $\mu$M FMOC-Leucine assay demonstrated a CD11b MFI of 122 and the 33.3 $\mu$M FMOC-Leucine assay demonstrated a MFI of 31.

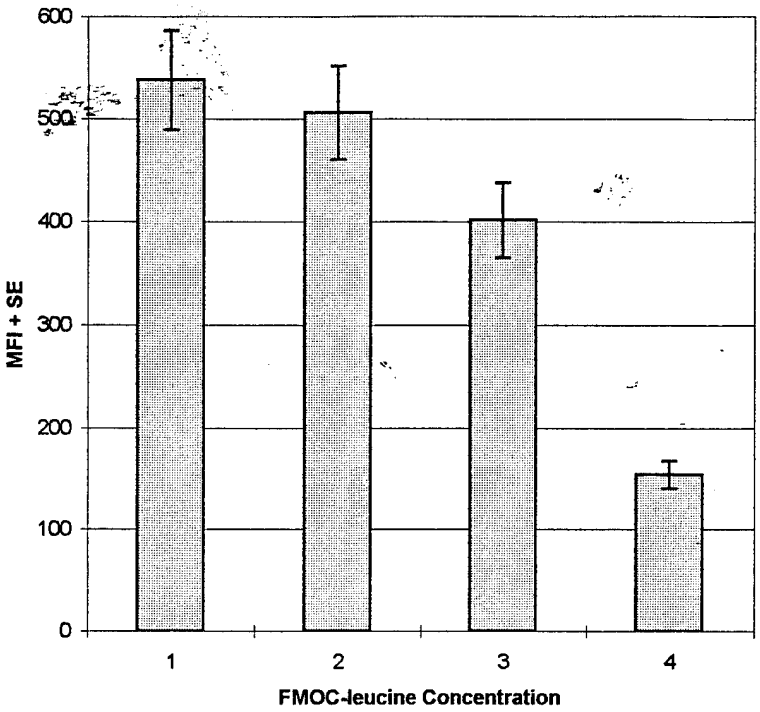


Figure 8: Neutrophil CD11b Fluorescence. CD11b mean fluorescence intensity (MFI) measurements plus standard error are shown in this figure. All MFI data shown were collected after 1.0 $\mu$M PAF activation of neutrophils in a purified suspension. FMOC-Leucine concentration are represented on the x axis and are as follows: (1) positive control with no FMOC-leucine (2) 0.3 $\mu$M, (3) 3.3 $\mu$M, and (4) 33.3 $\mu$M. These results are based on CD11b fluorescence studies conducted with a flow cytometer. The positive control demonstrated a CD11b MFI of 538. The 0.3 $\mu$M FMOC-Leucine assay demonstrated a CD11b MFI of 506. The 3.3 $\mu$M FMOC-Leucine assay demonstrated a CD11b MFI of 402 and the 33.3 $\mu$M FMOC-Leucine assay demonstrated a CD11b MFI of 154.

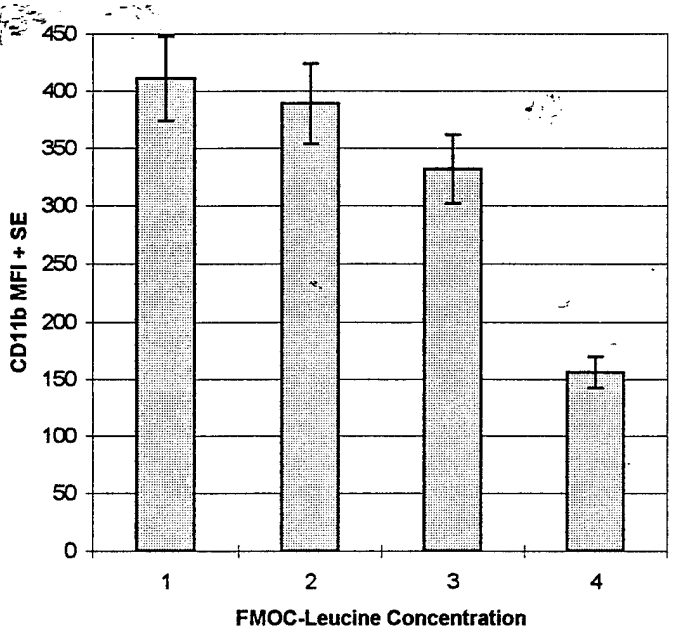


Figure 9: Eosinophil CD11b Fluorescence. CD11b mean fluorescence intensity (MFI) measurements plus standard error are shown in this figure. All data were collected after 1.0 $\mu$M PAF activation of eosinophils in an enriched suspension. FMOC-Leucine concentration are represented on the x axis and are as follows: (1) none present which is the positive control (2) 0.3$\mu$M, (3) 3.3$\mu$M, and (4) 33.3$\mu$M. These results are based on CD11b fluorescence studies conducted with a flow cytometer. The positive control demonstrated a MFI of 411. The 0.3$\mu$M FMOC-Leucine assay demonstrated a MFI of 389. The 3.3$\mu$M FMOC-Leucine assay demonstrated a MFI of 332 and the 33.3$\mu$M FMOC-Leucine assay demonstrated a MFI of 156.

stimulation in inhibition calculations (Table 3). The 0.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 0.3 μM FMOC-leucine demonstrated an average CD11b MFI of 128 which corresponded to a 17% inhibition of CD11b upregulation. The 3.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 3.3 μM FMOC-leucine demonstrated an average CD11b MFI of 122 which corresponded to a 21% inhibition of CD11b upregulation. The 33.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 33.3 μM FMOC-leucine demonstrated an average CD11b MFI of 31 which corresponded to a 80% inhibition of CD11b upregulation (Figure 10).

Procedure B involved the activation of granulocyte suspensions. MFI data were captured for the six different treatments of the cells. The negative control tests which were not exposed to either PAF or FMOC-leucine demonstrated an average CD11b MFI of 173 (32% of the positive control). The positive control tests which were exposed to only PAF demonstrated an average CD11b MFI of 538. This value of 538 was interpreted as the maximal stimulation in inhibition calculations. The 0.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 0.3 μM FMOC-leucine demonstrated an average CD11b MFI of 506 which corresponded to a 6% inhibition of CD11b upregulation. The 3.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 3.3 μM FMOC-leucine demonstrated an average CD11b MFI of 402 which corresponded to a 25% inhibition

| FMOC-leucine Concentration | 0.3 μM | 3.3 μM | 33.3 μM |
|---|---|---|---|
| **Whole Blood Neutrophils** | 17% (p=0.03) | 21% | 80% |
| **Neutrophils** | 6% | 25% (p<0.01) | 71% |
| **Eosinophils** | 7% | 19% (p=0.03) | 62% |

Table 3. CD11b Inhibition. Activation inhibition of CD11b data is shown in this table. These data were calculated from mean fluorescence intensity (MFI) readings collected by flow cytometry. Probability (p) values are shown for the lowest concentration of FMOC-leucine that demonstrated statistically significant inhibition. The percent inhibition is a reflection of the decrease in MFI of CD11b in the presence of FMOC-leucine as compared to a matched positive control which was exposed to the same stimulant (1 μM PAF) and no FMOC-leucine. FMOC-leucine at the 0.3 μM concentration inhibited the activation of neutrophils by 6% (p=0.07). The 3.3 μM FMOC-leucine assays showed an average activation inhibition of 25% decrease (p<0.01). As expected, the 33.3 μM FMOC-leucine assays demonstrated the greatest inhibition of CD11b upregulation with an average inhibition of 71% (p<0.01).

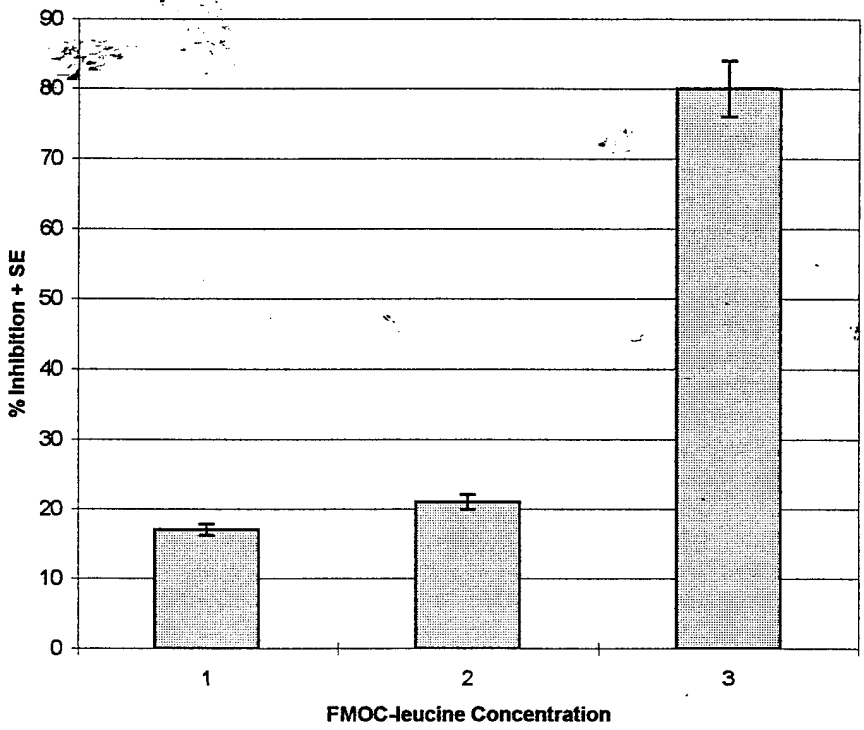


Figure 10. Whole Blood CD11b Inhibition. FMOC-leucine inhibition of PAF-induced CD11b activation of neutrophils from whole blood samples are shown in this figure. Data are presented as mean inhibition percentage plus standard error. FMOC-leucine concentrations are represented on the x axis and are as follows: (1) 0.3 $\mu$M, (2) 3.3 $\mu$M, and (3) 33.3 $\mu$M. These results are based on activation studies conducted on neutrophils and analyzed by flow cytometry. Percent inhibition was calculated by comparing the intensity of CD11b fluorescence of cells in the presence of 1.0 $\mu$M PAF (positive control) and in the presence of PAF and FMOC-leucine. The 0.3 $\mu$M FMOC-leucine assay demonstrated a 17% inhibition of CD11b expression, the 3.3 $\mu$M FMOC-leucine assay demonstrated a 21% inhibition of CD11b expression, and the 33.3 $\mu$M FMOC-leucine assay demonstrated an 80% inhibition of CD11b expression.

of CD11b upregulation. The 33.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 33.3 μM FMOC-leucine demonstrated an average CD11b MFI of 154 which corresponded to a 71% inhibition of CD11b upregulation (Figure 11).

Procedure C involved the activation of eosinophil-enriched granulocytes. MFI data were captured for the six different treatments of the cells. The negative control tests which were not exposed to either PAF or FMOC-leucine demonstrated an average CD11b MFI of 195 (47% of the positive control). The positive control tests which were exposed only to PAF demonstrated an average CD11b MFI of 411. This value of 411 was used as maximal stimulation in inhibition calculations. The 0.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 0.3 μM FMOC-leucine demonstrated an average CD11b MFI of 389 which corresponded to a 7% inhibition of CD11b upregulation. The 3.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 3.3 μM FMOC-leucine demonstrated an average CD11b MFI of 332 which corresponded to a 21% inhibition of CD11b upregulation. The 33.3 μM FMOC-leucine tests which were activated with 1 μM PAF in the presence of 33.3 μM FMOC-leucine demonstrated an average CD11b MFI of 156 which corresponded to a 67% inhibition of CD11b upregulation (Figure 12).

## Chemotaxis Studies

Net stimulated movement (NSM) percentages were calculated for all

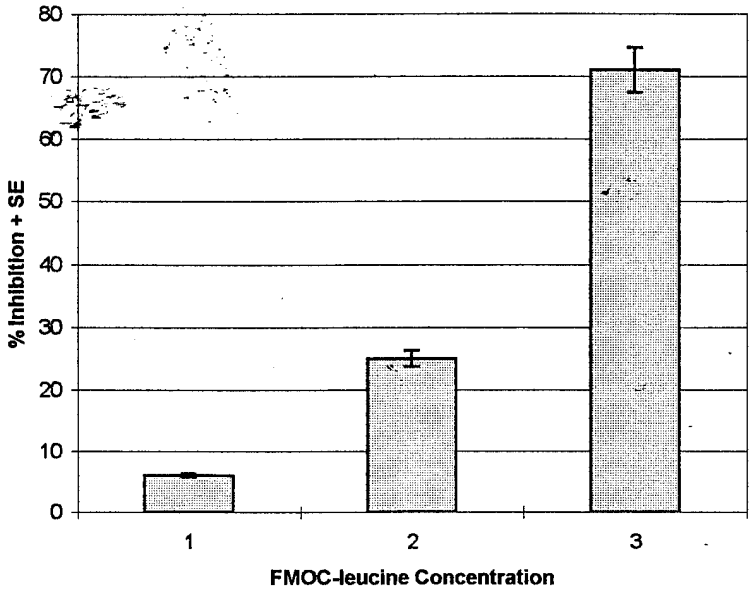


Figure 11. Neutrophil CD11b Inhibition. FMOC-leucine inhibition of PAF-induced CD11b activation of purified neutrophils are shown in this figure. Data are presented as mean inhibition percentage plus standard error. FMOC-leucine concentrations are represented on the x axis and are as follows: (1) 0.3 $\mu$M, (2) 3.3 $\mu$M, and (3) 33.3 $\mu$M. These results are based on activation studies conducted on purified neutrophil suspensions and analyzed by flow cytometry. Percent inhibition was calculated by comparing the intensity of CD11b fluorescence in the presence of 1.0 $\mu$M PAF (positive control) and in the presence PAF and FMOC-leucine. The 0.3 $\mu$M FMOC-leucine assay demonstrated a 6% inhibition of CD11b expression, the 3.3 $\mu$M FMOC-leucine assay demonstrated a 25% inhibition of CD11b expression , and the 33.3 $\mu$M assay demonstrated a 71% inhibition of CD11b expression.

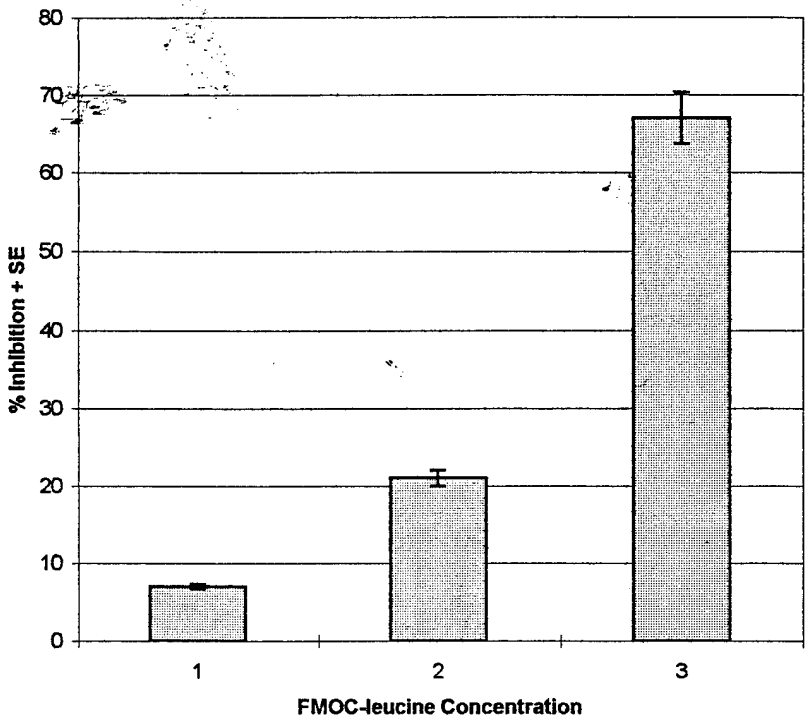


Figure 12. Eosinophil CD11b Inhibition. FMOC-leucine inhibition of PAF-induced CD11b activation of Eosinophils are shown in this figure. Data are presented as mean inhibition percentage plus standard error. FMOC-leucine concentrations are represented on the x axis and are as follows: (1) 0.3 $\mu$M, (2) 3.3 $\mu$M, and (3) 33.3 $\mu$M. These results are based on activation studies conducted on eosinophil suspensions and analyzed by flow cytometry. Percent inhibition was calculated by comparing the intensity of CD11b fluorescence of cells in the presence of 1.0 $\mu$M PAF (positive control) and in the presence of PAF and FMOC-leucine. The 0.3 $\mu$M FMOC-leucine assay demonstrated a 7% inhibition of CD11b expression, the 3.3 $\mu$M FMOC-leucine assay demonstrated a 21% inhibition of CD11b expression, and the 33.3 $\mu$M FMOC-leucine assay demonstrated a 67% inhibition of CD11b expression.

chemotaxis assays as previously described. The chemotaxis studies were conducted on cells prepared in one of two separate manners designated procedures B and C. Cells collected in the bottom wells were stained with modified Wright's stain and differentiated via slide microscopy. Additionally, eosin stained preparations were used for quantitating granulocytes on a hemocytometer which provided a means of easily distinguishing eosinophils from neutrophils.

Procedure B involved granulocyte suspensions which were then placed on the migration plates. Percent NSM values were calculated for each of seven different treatments of the cells. Each well had 100,000 cells added to the upper well at the beginning of the assay. The number of cells that were collected in the bottom well after the assay was used to determine the extent of chemotaxis. The negative control tests which were not exposed to PAF demonstrated an average of 0 cells in the bottom well which corresponded to a 0% NSM. The chemokinesis control which had PAF in both the upper and lower wells at the same concentration demonstrated an average of 3,000 cells in the bottom well which corresponded to a 3% NSM due to chemokinesis. This random movement was compensated for in the % NSM calculations by subtracting the chemokinesis control value from the positive control value. The positive control which contained the FMOC-leucine vehicle and a gradient of PAF demonstrated an average of 70,000 cells in the bottom well and was used as a 100% NSM benchmark. A value of 67,000 cells (70,000 positive control minus 3,000 random movement) was used as a maximal chemotaxis benchmark (100% NSM) for

calculation purposes. A second positive control which contained PBS and a gradient of PAF demonstrated an average of 80,000 cells in the bottom well. The 0.3 μM FMOC-leucine tests which were stimulated with PAF in the presence of 0.3 μM FMOC-leucine demonstrated an average of 54,000 cells in the bottom wells which corresponded to a 77% NSM (23% inhibition) (Figure 13). The 0.3 μM FMOC-leucine tests which were stimulated with PAF in the presence of 0.3 μM FMOC-leucine demonstrated an average of 30,000 cells in the bottom wells which corresponded to a 43% NSM (57% inhibition). The 33.3 μM FMOC-leucine tests which were stimulated with PAF in the presence of 33.3 μM FMOC-leucine demonstrated an average of 0 cells in the bottom wells which corresponded to a 0% NSM (100% inhibition).

Procedure C involved eosinophil-enriched granulocyte suspensions which were placed on the migration plates. Percent NSM values were calculated for each of seven different treatments of the cells. Each well had 100,000 cells added to the upper well at the beginning of the assay. The number of cells collected in the bottom well at the completion of the assay was used to determine the extent of chemotaxis. The negative control tests which were not exposed to PAF demonstrated an average of 0 cells in the bottom well which corresponded to a 0% NSM. The chemokinesis control which had PAF in both the upper and lower wells at the same concentration demonstrated an average of 0 cells in the bottom well which corresponded to a 0%

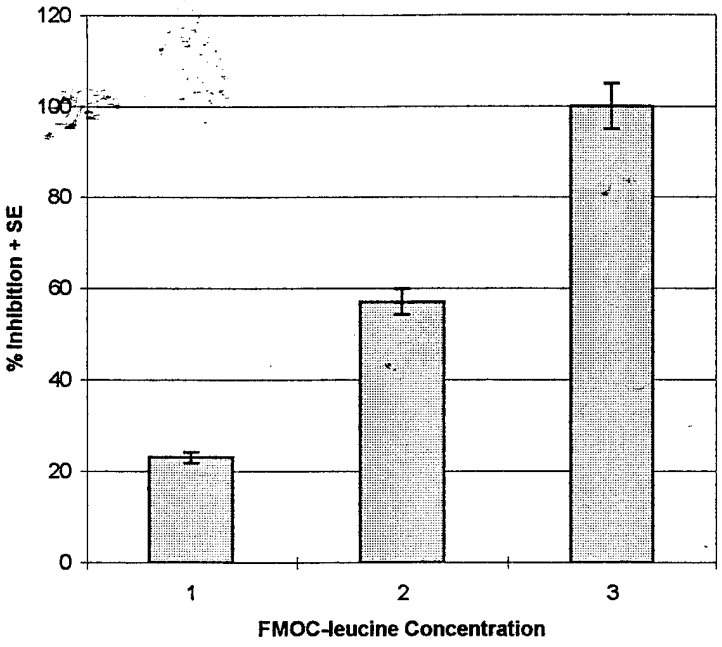


Figure13: Neutrophil Chemotaxis Inhibition. FMOC-Leucine inhibition of PAF-induced chemotaxis of neutrophils in a purified suspension are shown in this figure. FMOC-Leucine concentration are represented on the x axis and are as follows: (1) 0.3 $\mu$M, (2) 3.3 $\mu$M, and (3) 33.3 $\mu$M. These results are based on chemotaxis studies conducted with Transwell migration plates (3 $\mu$m pores). Percent inhibition was calculated by comparing the migration of cells in the presence of 1.0 $\mu$M PAF (positive control) and in the presence of PAF and FMOC-Leucine. The 0.3 $\mu$M FMOC-Leucine assay demonstrated a 23% inhibition of chemotaxis, the 3.3 $\mu$M FMOC-Leucine assay demonstrated a 57% inhibition of chemotaxis, and the 33.3 $\mu$M FMOC-Leucine assay demonstrated 100% inhibition of

NSM also. The positive control which contained the FMOC-leucine vehicle and a gradient of PAF demonstrated an average of 47,000 cells in the bottom well which was used as a 100% NSM benchmark. This value of 47,000 cells was used as a maximal chemotaxis % NSM for calculation purposes (Table 4). A second positive control which contained PBS and a gradient of PAF demonstrated an average of 62,000 cells in the bottom well. The 0.3 μM FMOC-leucine tests which were stimulated with PAF in the presence of 0.3 μM FMOC-leucine demonstrated an average of 46,000 cells in the bottom wells which corresponded to a 97% NSM (3% inhibition) (Figure 14). The 3.3 μM FMOC-leucine tests which were stimulated with PAF in the presence of 3.3 μM FMOC-leucine demonstrated an average of 31,000 cells in the bottom wells which corresponded to a 66% NSM (34% inhibition). The 33.3 μM FMOC-leucine tests which were stimulated with PAF in the presence of 33.3 μM FMOC-leucine demonstrated an average of 0 cells in the bottom wells which corresponded to a 0% NSM (100% inhibition).

| FMOC-leucine Concentration | 0.3 μM | 3.3 μM | 33.3 μM |
|---|---|---|---|
| **Eosinophil Inhibition** | 3% | 34% ($p<0.01$) | 100% |
| **Neutrophil Inhibition** | 23% ($p<0.01$) | 57% | 100% |

Table 4. Net Stimulated Movement Inhibition. FMOC-leucine inhibition of PAF-induced eosinophil and neutrophil chemotaxis data are shown in this table. Probability (p) values are shown for the lowest concentration of FMOC-leucine that demonstrated statistically significant inhibition. The assays were conducted with Transwell plates that had 3 μm pores separating the wells. The chemotactic agent used was 1.0 μM PAF. The 0.3 μM concentration of FMOC-leucine inhibited neutrophil chemotaxis by 23% whereas eosinophils only showed a 3% inhibition of chemotaxis. FMOC-leucine at a concentration of 3.3 μM demonstrated a stronger inhibition of both eosinophil and neutrophil chemotaxis. Again, the neutrophils appeared to be inhibited to a greater degree than the eosinophils. The 3.3 μM FMOC-leucine assays demonstrated a 57% inhibition of neutrophil chemotaxis and a 34% inhibition of eosinophil chemotaxis. The 33.3 μM FMOC-leucine assays all demonstrated complete NSM inhibition (100%) of neutrophils and eosinophils. FMOC-leucine was shown to be less potent in eosinophils than neutrophils. However, efficacy was similar for both cell types.

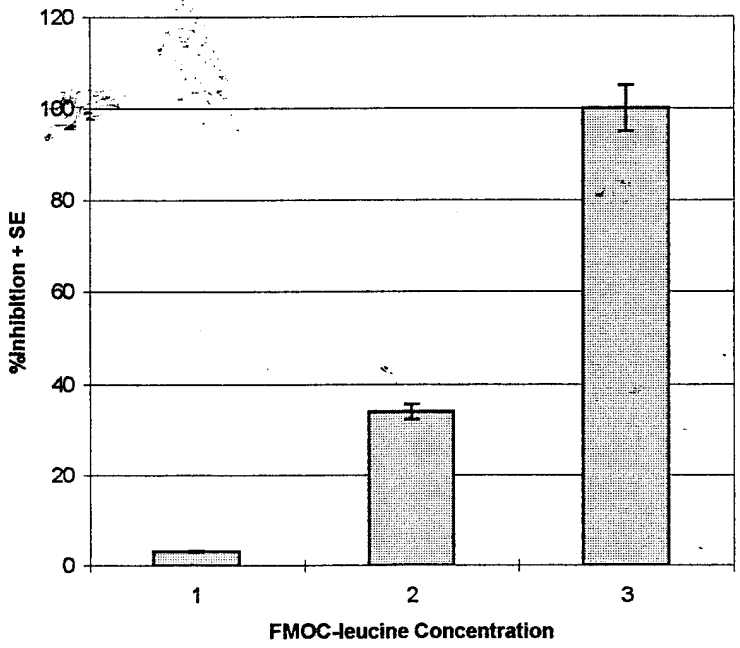


Figure14: Eosinophil Chemotaxis Inhibition.
FMOC-Leucine inhibition of PAF-induced chemotaxis of eosinophils in an enriched suspension are shown in this figure. FMOC-Leucine concentration are represented on the x axis and are as follows: (1) 0.3 $\mu$M, (2) 3.3 $\mu$M, and (3) 33.3 $\mu$M. These results are based on chemotaxis studies conducted with Transwell migration plates (3 $\mu$m pores). Percent inhibition was calculated by comparing the migration of cells in the presence of 1.0 $\mu$M PAF (positive control) and in the presence of PAF and FMOC-Leucine. The 0.3 $\mu$M FMOC-Leucine assay demonstrated a 3% inhibition of chemotaxis, the 3.3 $\mu$M FMOC-Leucine assay demonstrated a 34% inhibition of chemotaxis, and the 33.3 $\mu$M FMOC-Leucine assay demonstrated 100% inhibition of chemotaxis.

## DISCUSSION

This study focused on determining the effect of FMOC-leucine on PAF-induced activation and chemotaxis of human eosinophils. Chemotaxis studies were conducted with neutrophil and eosinophil suspensions. Eosinophil purification proved to be more difficult than was anticipated. The procedure as described by Casale et al.[7,12] did not produce purified eosinophils in this study. Production of eosinophil suspensions with >80% purity were reported in the cited studies.[7,12] However, the technique never yielded >10% eosinophils in this study. This difference may have been due to laboratory errors in maintaining the clarity of the density gradients during centrifugation. This problem was never clarified and the procedure was abandoned.

Eosinophils were purified using a negative selection immunomagnetic selection technique. Results obtained compared favorably with results reported by Kaneko et al.[28] who reported that eosinophil suspensions of 90% purity were obtained using this technique. Eosinophil suspensions of 50-60% were obtained in this study. The two techniques were not identical and the difference in results probably reflect the difference in technique. Although both techniques relied on the negative selection of neutrophils by a CD16-magnetic bead conjugate, neutrophils were extracted on a rotating magnetic holder in the study by Kaneko et al.[28] as opposed to a stationary magnetic holder in this study.

The chemotaxis assays were time consuming but relatively simple to perform. It was found the cell counts could be easily accomplished by staining with eosin and

enumerating the cells on a hemocytometer. Modified Wright's stain provided an excellent and simple method of differentiating cell populations. The activation assays were also quite time consuming but relatively simple to perform. However, a flow cytometer was required to perform analysis of the activated cell suspensions. Once appropriate gates were set on the flow cytometer for the cell population of interest, data were captured fairly rapidly and with ease (Figures (2-6).

Eosinophils chemotaxis studies conducted on Transwell plates were performed in accordance with the procedure described by Casale et al.[7,12] who published findings that no differences exist between 1 μM PAF-induced migration through endothelial cell lined polycarbonate filters and naked polycarbonate filters. This group found that 1 μM PAF induced >60% net stimulated movement of eosinophils which is very similar to the findings in this study of 62% net stimulated movement of eosinophils. The results of PAF-induced neutrophils chemotaxis was quite different. Casale et al.[3,18,22] reported 27% NSM in neutrophils while this study found 80% NSM. This vast difference is felt to be due to a lower concentration of PAF, 500 nM versus 1 μM in this study. The dose of PAF was maintained at 1 μM for both eosinophils and neutrophils in this study to simplify the comparison of results between the two groups.

The neutrophils in the migration assays appeared to be stimulated to a greater extent than the eosinophils with the same dose of PAF. A likely explanation for this observation is that the eosinophil suspensions were subjected to approximately 25% more processing time than the neutrophils. This could have led to inadvertent

activation, degranulation, and/or cell death. Cell viability was not assessed prior to performing the chemotaxis or activation assays. A great deal of attention was paid to avoiding the inadvertent activation of the cell suspensions during processing. This was accomplished by using only freshly collected blood and also by maintaining the cells at 4° C during all processing steps. However, hypaque density separation of mononuclear cells was conducted at 22° C in accordance with the recommendations of the manufacturer. Evidently, the precautions taken were not adequate to prevent the inadvertent stimulation of the cells.

The PAF did not show the same degree of activation potential in the whole blood as was demonstrated in the neutrophil and eosinophil assays of CD11b (Figures 7-9). The difference in PAF stimulation of CD11b may be attributable to the plasma proteins, erythrocytes, and/or other factors present in the whole blood which were not present in the leukocyte preparations. The presence of these additional components in the whole blood samples may have possibly absorbed or otherwise neutralized the PAF. Another factor that may explain the difference in the degree of PAF-induced activation is the time and processing required to prepare the different cell suspensions. Whole blood samples were not subjected to as many processing steps and was not subjected to the time delays associated with the additional processing steps. The additional processing steps provided an opportunity for the neutrophil and eosinophil suspensions to become activated.

The solution of 3% ETOH which was used as the diluting vehicle for FMOC-

leucine appeared to have mild inhibitory effects (1%) on both neutrophils and eosinophils in the CD11b activation assay (data not shown). Three percent ETOH was used as the diluting vehicle for FMOC-leucine without inhibitory effects in a mouse model by Burch et al.[23] In an *in vivo* study utilizing NPC 15669 in New Zealand white rabbits, Navab et al.[29] used dimethylsulfoxide as the diluting vehicle. In an *in vivo* study utilizing NPC 17923 in rats, McCafferty et al.[30] used 1% carboxymethylcellulose as the diluting vehicle. Neither of these two studies reported inhibition of neutrophil CD11b by the diluting vehicle. However, these studies were measuring the extravastation of neutrophils and were performed *in vivo* as opposed to the assay in this study which measured CD11b after *in vitro* stimulation. Although the issue was not specifically addressed, dimethylsulfoxide and carboxymethylcellulose were not reported to demonstrate inhibitory effects on the activation of neutrophils.[23,29] This may have been due to the fact that potential toxicity could have been negated by the livers of the test animals used in the studies. Compensation for the inhibition of CD11b by ETOH was accomplished by maintaining the concentration of vehicle constant (1%) in all assays including the controls.

A thorough review of the literature revealed that the *in vitro* effects of FMOC-leucine have not been published. However, the *in vivo* effects of FMOC-leucine were described by Miller et al.[19] in a guinea pig model. FMOC-leucine doses of 10 mg/Kg were found to inhibit ileitis induction by trinitrobenzenesulfonic acid with complete inhibition of neutrophils at the induction site. Although drug distribution and tissue

concentrations were not discussed, these data compares favorably with the *in vitro* assays that demonstrated complete inhibition of neutrophil and eosinophil chemotaxis in the presence of 33.3 μM FMOC-leucine.

Neutrophil and eosinophil CD11b upregulation was found to be significantly inhibited by FMOC-leucine in a dose dependent manner; a 10-fold increase in drug increased inhibition by a factor of 3 (Figures 10-12). These data are consistent with previously published data demonstrating the dose dependent actions of leumedins by Burch et al.[23] who reported that the antiinflammatory actions of FMOC-leucine on neutrophils occurred in a dose dependent manner. A 10 μM dose of FMOC-leucine demonstrated a 40% inhibition of CD11b in neutrophils which is approximately twice the inhibition demonstrated in this study. However, neutrophils were pretreated with FMOC-leucine in the Burch et al.[23] study prior to stimulation. This difference in drug administration may account for the two-fold difference noted in potency of the drug.

FMOC-leucine was shown by this study to decrease both PAF-induced upregulation of CD11b and chemotaxis of eosinophils (Figures 10-14). Furthermore, significant inhibition was shown to occur at pharmacologically achievable levels (Tables 3,4). Inhibition appeared to work in a dose dependent fashion which compared favorably with inhibition data reported by Burch et al.[23] for *in vivo* studies using mice and rats (Figures 10-14). These properties make FMOC-leucine an interesting drug to evaluate as it offers definite possibilities as a potential treatment

modality for inflammatory diseases. Certainly, more study of this exciting new class of drugs is in order.

## BIBLIOGRAPHY

1. Benjamini E, Leskowitz S. *Immunology: A Short Course*. New York, NY: Wiley-Liss Inc., 1993.

2. Abbas A, Lichtman A, Pober J. *Cellular and Molecular Immunology*. Philadelphia, PA: W.B. Saunders Co.,1994.

3. Casale T, Smart S. Interleukin-8-induced transcellular neutrophil migration is facilitated by endothelial and pulmonary epithelial cells. *American Journal of Respiratory Cell Molecular Biology*, 1993; 9: 489-495.

4. Bochner B, Luscinskas F, Gimbrone M, Newman W, Sterbinsky S, Anthony C, Klunk D, Schleimer R. Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. *Journal of Experimental Medicine*, 1993; 173: 1553-1556.

5. Agrawal D, Townley R. *Inflammatory cells and mediators in bronchial asthma*. Boca Raton, FL: CRC Press, 1991.

6. Walsh G, Mermod J, Hartnell A, Kay A, Wardlaw A. Human eosinophil, but not neutrophil, adherence to IL-1-stimulated human umbilical vascular endothelial cells is alpha-4, beta-1 dependent. *Journal of Immunology*, 1991; 146: 3419-3423.

7. Casale T, Erger R, Little M. Platelet-activating factor-induced human eosinophil transendothelial migration: evidence for a dynamic role of the endothelium. *American Journal of Respiratory Cell Molecular Biology*, 1993; 8: 77-82.

8. Parkos C, Colgan S, Delp C, Arnaout M, Madara J. Neutrophil migration across a cultured epithelial monolayer elicits a biphasic resistance response representing sequential effects on transcellular and paracellular pathways. *Journal of Cell Biology*, 1992; 117: 757-764.

9. Kimani G, Tonnesen M, Henson P. Stimulation of eosinophil adherence to human vascular endothelial cells in vitro by platelet-activating factor. *Journal of Immunology*, 1988; 140: 3161-3166.

10. Cheng J, Pillar J, Shirley J, Showell H, Watson J, Cohan V. Antigen-mediated pulmonary eosinophilia in immunoglobulin G1-sensitized guinea pigs: eosinophil peroxidase as a simple specific marker for detecting eosinophils in broncoalveolar lavage fluid. *American Journal of Pharmacology and Experimental Therapeutics*, 1993; 204: 922-929.

11. Bozeman P, Learn D, Thomas E. Inhibition of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase by dapsone. *Journal of Chemical Pharmacology*, 1992; 44: 553-563.

12. Casale T, Little M. Comparison of platelet-activating factor-induced chemotaxis of normodense and hypodense eosinophils. *Journal of Allergy and Clinical Immunology*, 1991; 88: 187-192.

13. Horie S, Kita H. CD11b/CD18 is required for degranulation of human eosinophils induced by human recombinant GM-CSF and platelet activating factor. *Journal of Immunology*, 1994; 152: 5457-5467.

14. Menegazzi R, Zabucchi G, Knowles A, Cramer R, Patriarca P. A new, one-step assay on whole cell suspensions for peroxidase secretion by human neutrophils and eosinophils. *Journal of Leukocyte Biology*, 1992; 52: 619-624.

15. Egesten A, Gullberg U, Olson I, Richter J. Phorbol ester-induced degranulation in adherent human eosinophil granulocytes is dependent on CD11/CD18 leukocyte integrins. *Journal of Leukocyte Biology*, 1993; 53: 287-293.

16. Fukuda T, Makino S. *Eosinophil heterogeneity*. New York, NY: Morley Academic Press, 1989.

17. Hiratsu Y, Hirashima M, Hayashi H. The mediation of tissue eosinophilia in hypersensitivity reaction. *Journal of Immunology*, 1983; 48: 59-67.

18. Casale T, Abbas M, Carolan E. Degree of neutrophil chemotaxis is dependent upon the chemoattractant and barrier. *American Journal of Respiratory Cell Molecular Biology*, 1992; 7: 112-117.

19. Miller M, Chotinaruemol S, Krowikca H, Zhang X, McIntyre J, Clark D. Guinea pig ileitis is attenuated by the leumedin N-(fluorenyl-9-methoxycarbonyl)-leucine (NPC 15199). *Journal of Pharmacology and Experimental Therapeutics*, 1993; 266: 468-472.

20. Motojima S, Loegering D, Frigas E, Dleich G. Toxic effects of eosinophil cationic proteins on respiratory epithelium. *Federation Proceedings*, 1986; 45: 945-952.

21. Page P. *Immunopharmacology of Neutrophils*. New York, NY: Academic Press, 1994.

22. Casale T, Carolan E. Effects of neuropeptides on neutrophil migration through noncellular and endothelial barriers. *Journal of Allergy and Clinical Immunology*, 1993; 92: 589-598.

23. Burch R, Weitzberg M, Blok N, Muhlhauser R, Martin D, Farmer S, Bator J, Conner J, Ko C, Kuhn W, McMillan B, Raynor M, Shearer B, Tiffany C, Wilkins D. N-(fluorenyl-9-methoxycarbonyl) amino acids, a class of anti-inflammatory agents with a different mechanism of action. *Proceedings of the National Academy of Sciences*, 1991; 88: 355-359.

24. Blob L, Lowe V, Muhlhauser R, Burch R. NPC 15669, an inhibitor of neutrophil recruitment, is efficacious in acetic acid-induced colitis in rats. *Gastroenterology*, 1993; 104: 1021-1029.

25. Burch R, Blob L, Bator J, Lowe V, Sullivan J. Mice treated with a leumedin or antibody to Mac-1 to inhibit leukocyte sequestration and survive endotoxin challenge. *Journal of Immunology*, 1993; 150: 3397-3403.

26. Hughes B, Hollers J, Torabi E, Smith C. Recruitment of CD11b/CD18 to the neutrophil surface and adherence-dependent cell locomotion. *Journal of Clinical Investigation*, 1992; 90: 1687-1696.

27. McCafferty D, Kubes P, Wallace J. Inhibition of platelet-activating factor-induced leukocyte adhesion in vivo by a leumedin. *European Journal of Pharmacology*, 1993; 232: 169-172.

28. Kaneko T, Jorens P, Richman-Eisenstat J, Dazin P, Nadel J. Leumedin NPC 15669 inhibits antigen-induced recruitment of inflammatory cells into the canine airways. *American Journal of Physiology*, 1994; 267: 250-255.

29. Navab M, Hama S, Lenten B, Drinkwater D, Laks H, Fogelman A. A new antiinflammatory compound, leumedin, inhibits modification of low density lipoprotein and the resulting monocyte transmigration into the subendothelial space of cocultures of human aortic wall cells. *Journal of Clinical Investigation*, 1993; 91: 1225-1230.

30. McCafferty D, Kubes P, Wallace J. Inhibition of platelet-activating factor-induced leukocyte adhesion in vivo by a leumedin. *European Journal of Pharmacology*, 1993; 232: 169-172.