ACTIVATION OF NEUTROPHIL FUNCTION BY RECOMBINANT HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND MODULATION OF ITS ACTION BY ALPHA-2-MACROGLOBULIN

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DEDICATION

To my dear parents,

in appreciation of

their unending love and support.

獻給我

親愛的父母

以感謝他們

無盡的愛與支持

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ABSTRACT

Neutrophils play an important role in inflammation as they are the predominant cell type at the sites of infection. The function of these cells can be activated _in vitro_ by a number of agents. Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are known to have an influence on neutrophil function. G-CSF and GM-CSF belong to a family of cytokines known as colony-stimulating factors (CSFs). CSFs are a group of cell-derived products responsible for the proliferation and differentiation of progenitor cells. These factors are available in large amounts through recombinant DNA techniques.

Superoxide species are important for oxygen-dependent mechanisms by which neutrophils can kill certain microorganisms. In this research recombinant human G-CSF (rhG-CSF) and recombinant human GM-CSF (rhGM-CSF) were used to activate human neutrophils to release superoxide anion. Recombinant human GM-CSF at 5 to 500 U/ml greatly enhanced the production of superoxide anion by neutrophils stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP). However, rhG-CSF at 50 U/ml did not show this enhancement. In addition, rhGM-CSF alone at 50 U/ml stimulated superoxide production by neutrophils in a time-dependent manner.

The modulation of neutrophil function by alpha-2-macroglobulin (alpha-2M), which is the only plasma inhibitor of a number of proteases, was also studied. The binding of some cytokines to alpha-2M has been shown by previous studies to regulate certain immunological functions. Recombinant human GM-CSF and native alpha-2M alone and in combination stimulated a significant increase of superoxide production. In contrast, the fast form of alpha-2M, modified by either trypsin or ammonium sulphate, inhibited the release of superoxide anion by neutrophils.

These findings reflect that rhGM-CSF could activate neutrophil function relevent to resistance to infection and that alpha-2M may modulate the action of neutrophils.

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CHAPTER I

INTRODUCTION

Approximately 60% of the circulating leukocytes in human blood are neutrophils. Mature neutrophils have five-lobed nuclei and are therefore designated polymorphonuclear neutrophils (PMNs). Neutrophils arise in the bone marrow and are continuously discharged in vast numbers, about 10^{11} per day, into the blood. These cells only live for a few days, and the daily loss is balanced by entry of replacements into the blood from the bone marrow. During the early stages of inflammation, neutrophils are the most prominent phagocytes. At an inflammatory site, neutrophils bind, ingest, or lyse foreign or aberrant cells (Ackerman et al., 1982; Olsson & Venge, 1980).

Neutrophils generally carry out their functions after leaving the blood-stream, but under certain circumstances they can adhere to the endothelium of small blood vessels, especially in the lungs, and act as "fixed" phagocytes. They are specifically capable of movement. One type of movement displayed is chemokinesis, which is non-directed in all planes, and to some extent this could bring the cells to the site of infection. Another characteristic type of neutrophil movement is chemotaxis. Chemotaxis is a

directed movement of the cell in response to chemical gradients formed by the release of chemotactic materials such as soluble bacterial products (formyl peptide) and activated components of complement (C5a). Because of this chemotactic response, neutrophils are able to rapidly mobilize when chemotactic stimuli are produced at the site of infection and they are then able to carry out their most important function, phagocytosis (Zakhireh et al., 1979; Klebanoff, 1975).

Before phagocytosis can take place, attachment of the object to be ingested to the phagocytic cell surface must occur. Eukaryotic cells have a negative surface charge, resulting in net electrostatic forces that oppose cell-to-cell adherence (Harlan, 1985). Therefore, divalent cations such as Ca(2+) or Mg(2+) are required for the initial attachment to occur. The firm attachment and ingestion of particles is facilitated by serum substances called opsonins, which include immunoglobulins and complement. On the surface of neutrophils, there are receptors for formyl peptide, C3b and C5a complement, and the Fc portion of antibodies (Wright, 1985). When appropriate ligand binds these receptors, neutrophils are attracted to the site of infection and once they arrive they are able to interact with the opsonin-coated microorganisms (Wright, 1985).

As soon as neutrophils encounter foreign cells or opsonized particles, the plasma membrane infolds due to the

contraction of actin and myosin filaments anchored to a skeleton of microtubules in the cytoplasm. As a result of the infolding of the plasma membrane, the attached particles are engulfed and a phagocytic vacuole is formed (Wright, 1985).

Two to three types of granules are present in the cytoplasm of neutrophils: regular lysosomes, specific granules, and azurophil granules. These granules move towards the phagocytic vacuole, fuse with its plasma membrane to form a phagolysosome, and the contents of granules are then discharged into the vacuole. This process initiates the intracellular killing and the digestion of the invading microorganisms (Gallin, 1985; Wright et al., 1977).

The biochemical basis of the killing mechanism is not fully understood. Nevertheless, some specific microbicidal activities have been identified. Within minutes after phagocytosis the pH within the phagosome falls to about 3.5-4.0. The low pH is toxic to the entrapped microbe (McRipley & Sbarra, 1967). The granules also discharge myeloperoxidase, lactoferrin, lysozyme, a vitamin B12-binding protein, a variety of cationic proteins, and acid hydrolysases, all of which have been shown to be toxic to certain microorganisms (Gallin et al., 1982).

In addition to the contents of granules, a brief burst of respiratory activity that accompanies phagocytosis is needed for optimal killing to occur. When the neutrophil

membrane is perturbed and exposed to soluble stimuli during phagocytosis, a 10- to 20-fold increase in respiratory activity occurs and what has come to be known as the oxygen-dependent antimicrobial mechanism is initiated (Babitor, 1984). Many agents, both particulate and soluble, are able to activate the respiratory burst (Cheson et al, 1977). Particulate activating agents include opsonized bacteria, zymosan, which is a preparation of yeast cell walls, and latex spherules. Latex spherules are unique in that they do not require opsonization for activation of neutrophils to occur. Among the soluble activating agents are phorbol myristate acetate, a complex plant product distantly related to steroids, a variety of ionophores, the complement pepteide C5a, and fluoride ion (F-) (Cheson et al., 1977).

When a neutrophil membrane is exposed to these stimuli, an enzyme which was dormant in the resting cell is rapidly activated. This enzyme has been identified as a reduced nicotinamide-adenine-dinucleotide-phosphate (NADPH) oxidase and it is responsible for the primary oxygen-consuming reaction of the respiratory burst. NADPH oxidase catalyzes the reduction of oxygen to superoxide anion (O_2^-) through the reaction of oxygen with NADPH, which itself is generated by the hexose monophosphate shunt (Umei et al., 1986).

Hydrogen peroxide production and hexose monophosphate shunt activation are the secondary products when this

system becomes activated. Hydrogen peroxide is formed either by the spontaneous interaction of two superoxide molecules or with the action of the enzyme superoxide dismutase (SOD) on superoxide (Root and Metcalf, 1977). The hydrogen peroxide and superoxide in turn react to generate hydroxyl radical, which may have antimicrobial activity (Beauchamp et al., 1970; Rotrosen and Gallin, 1987). The antimicrobial activity of hydrogen peroxide is also greatly enhanced by its conversion to hypochlorous acid. This transformation occurs in the presence of myeloperoxidase and chloride (McRipley and Sbarra, 1967). Hypochlorous acid not only disrupts bacterial cell walls by halogeneration, but also reacts with hydrogen peroxide to form singlet oxygen, which also has been shown to possess microbicidal activity (Krinsky, 1974).

Superoxide produced in this system is thought to have little direct toxicity. However, it is able to cross cell membranes via anion channels or by penetration of the lipid bilayer and thus reach sites where its highly reactive products can exert their toxic effects (lynch et al., 1978; Rotrosen and Gallin, 1987). The production of free hydroxyl and superoxide radicals, hydrogen peroxide, hypochlorous acid and singlet oxygen occur in the membrane of the phagosome, mostly by means of electron transport chain (Gallin et al., 1983).

It has been found that neutrophils from patients with chronic granulomatous disease (CGD) can ingest bacteria but

cannot kill them, probably due to diminished superoxide generation during phagocytosis. As a result, severe bacterial infections plague CGD patients (Johnson et al., 1975). The generation of superoxide species, therefore is thought to be a very important step in the acquisition by neutrophils of functions that are important in resistance to infection.

On the other hand, site of infection quickly becomes relatively anaerobic due to tissue destruction, disruption of the blood supply and the neutrophils which accumulate in the tissues often need to operate at low oxygen tension (Elsbach, 1980). Therefore, the fusion of granules with the phagocyttic vacuole and their contents are an important component of the neutrophil's arsenal for the killing of invading microorganisms.

While low oxygen concentration may pose a problem for the oxygen-dependent killing mechanism, the importance of the toxic oxygen products generated under normal conditions cannot be overlooked. In fact, the identification of agents which are able to increase the level of neutrophil activation would theoretically aid in the immune system's response to bacterial infection. One such group of agents able to activate mature neutrophils are colony-stimulating factors (CSFs), which were initially identified as growth factors able to increase the production of neutrophils in the bone marrow.

Mature neutrophils and their immediate precursors are

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referred to as myeloid cells and are derived from a small population of undifferentiated stem cells which have extensive capacity for self renewal. The in vitro viability and proliferation of bone marrow progenitor cells to form colonies of mature cells in a semisolid medium requires the presence of one or more structurally heterogeneous glycoproteins termed colony-stimulating factors (CSF) (Sieff, 1987; Clark & Kamen, 1987).

Four major human CSFs whose cDNAs have been cloned and whose respective genes have been localized are interleukin-3 (IL-3), granulocyte-macrophage CSF (GM-CSF), granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF). Through recombinant DNA techniques, large amounts of these cytokines have become available in purified form for the investigation of the effects of CSFs on bone marrow progenitor cells as well as on these cells' mature progeny.

IL-3 is capable of stimulating the growth of early multipotent progenitor cells as well as inducing the proliferation of later, unipotent and bipotent progenitors (Sieff et al., 1987). Consistently, the administration of recombinant human IL-3 to mice has been found to elevate the levels of progenitor cells, maturing granulocytes and monocytes in mice (Metcalf et al., 1986).

Human recombinant M-CSF selectively stimulates the growth of macrophages in colonies formed from normal human bone marrow in vitro (Clark and Kamen, 1987). In vivo studies have shown that intravenous administration of

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murine M-CSF to mice pretreated with cyclophosphamide increases the fraction of bone marrow progenitor cells in the active cell cycle (Broxmeyer et al., 1987).

Human granulocyte-macrophage-CSF (GM-CSF), which stimulates the maturation of granulocytes and macrophages is a glycoprotein with a molecular weight of 22,000 daltons and its sources include activated T lymphocytes, endothelial cells, and fibroblast (Sieff et al., 1987; Zueali et al., 1986; Griffin et al., 1984). In addition to stimulating the maturation of granulocytes and macrophages, GM-CSF is capable of inducing the growth of multipotent, unipotent and bipotent progenitors as well, although it may not be as effective as IL-3 (Sieff et al., 1985). Donahue et al. (1986) have reported that the continuous infusion of recombinant human GM-CSF to Macaque monkeys resulted in rapid, dose-dependent, sustained increase in neutrophils, bands, monocytes, and eosinophils within three days after cessation of treatment.

Granulocyte-CSF (G-CSF), which has a molecular weight of 19,600 daltons, selectively promotes the growth of granulocyte colony formation <u>in vitro</u> and induces the differentiation of certain bone marrow progenitor cells (Souza et al., 1986). Continuous infusion of recombinant human G-CSF to hamsters has been shown to selectively increase the peripheral blood granulocyte count (Cohen et al., 1987).

The presence of human CSF (CSF-H) at 500 U/ml during

the 30-minute period of neutrophil incubation with Trypanosoma Cruzi markedly enhances parasite internalization. Neutrophil treatment with CSF-H for 60 minutes causes a much greater enhancement. When subsequently incubated with CSF-H, neutrophils which have internalized T. cruzi kill the parasites at a faster rate (Villalta and Kierszenbaum, 1986). A subcutaneous rhG-CSF (60 mg/kg/day, two doses) administration to normal monkeys twice daily increases the number of circulating T cells but does not increase the number of circulating neutrophils. Neutrophils from the G-CSF treated monkeys demonstrate an enhanced ability to kill and to phagocytose bacteria in vitro, which might reflect a maturational change in the circulating neutrophil population (Steinbeck and roth, 1989).

The mechanism by which CSFs can enhance the ability of neutrophils to kill ingested microorganisms is not clear. However, reports have shown that rhGM-CSF can enhance the capability of neutrophils against invading microorganisms. Important functions such as chemotaxis, which bring neutrophils to infected sites, and the increase of superoxide anion production are enhanced after the treatment of rhGM-CSF and/or rhG-CSF. The first effect of rhGM-CSF on neutrophil migration identified was its ability to inhibit random migration (Gasson et al., 1984). GM-CSF alone has also been found to have chemotactic activity as well as to enhance the chemotactic response of the cells to

N-formyl-leucyl-methionyl-phenylalanine (FMLP), which is a member of a family of synthetic formyl peptides known to be potent chemoattractants for neutrophils and macrophages (Weisbart et al., 1986; Wang, et al., 1987).

Atkinson et al (1988) reported that preincubation of neutrophils with 10^{-11} M rhGM-CSF does not stimulate superoxide anion production directly but that the growth factor enhances the subsequent release of superoxide anion in response to stimulation with FMLP. GM-CSF also affects the binding of FMLP to purified human neutrophils as the affinity of the FMLP receptors increases following treatment. However, the total number of receptors does not significantly change following stimulation with 2 X 10^{-11} M rhGM-CSF. It has also been found that treatment with rhGM-CSF alone at 10 U/ml to 3000 U/ml can induce neutrophil degranulation <u>in vitro</u> (Richter et al., 1989).

In contrast to the number of studies demonstrating the interaction of rhGM-CSF with mature neutrophils, relatively few reports on the influence of the other CSFs on neutrophil function have appeared. However, treatment with G-CSF has been found to have some effects on neutrophil function similar to that of GM-CSF. Neutrophils isolated from hamsters four hours following a single subcutaneous injection of rhG-CSF (30 ug/kg) exhibit significant increases in superoxide generation and specific binding of FMLP. Moreover, resistance to an otherwise lethal infection is gained when neutropenic animals treated daily

with rhG-CSF are challenged with an injection of Staphylococcus aureus (Cohen et al., 1988).

It has been shown that rhG-CSF by itself was not an effective stimulus for inducing the release of superoxide anion in human granulocytes. However, like rhGM-CSF, rhG-CSF when present at 25 ng/ml is able to prime human granulocytes and enhance their release of superoxide when stimulated by FMLP (Kitagawa, 1987). The enhancement of superoxide release by rhG-CSF was observed over the complete range of effective concentrations of FMLP (10^{-8} to 10^{-6} M).

Mice which have undergone burn injury and seeding of the burn wound site with the bacterial pathogen Pseudomonas aeruginosa demonstrate an improved mean survival time and an increase in myelopoietic response when rhG-CSF is administered subcutaneously (Mooney et al., 1988). Bacterial growth in the infected thigh muscle of mice has been found to be suppressed by rhG-CSF treatment, probably due to an increase in the number of neutrophils present in the host as a result of the treatment (Yasuda et al., 1990).

Recently both rhGM-CSF and rhG-CSF have been used in immunotherapy to prevent infectious diseases in immunocompromised patients. The results of clinical trials have shown the application of rhGM-CSF is valuable in clinical therapy (Gianni et al., 1989; Groopman, 1990). However, there is still some controversy concerning the

results observed in previous studies of rhGM-CSF and rhG-CSF. Buescher et al. (1988) reported no effect of rhGM-CSF therapy on either neutrophil chemotaxis or oxygen metabolism, despite excellent proliferative effects on the doses used. Moreover, Woodman et al.(1988) have shown that rhG-CSF does not increase the production of superoxide anion by neutrophils stimulated with FMLP. One of the goals of this research is to reexamine the effects of rhGM-CSF or rhG-CSF alone and in combination with FMLP on the release of superoxide anion.

Cytokines which are released into the blood have been shown to interact with alpha-2-macroglobulin (alpha-2M) (Borth and Luger, 1989). By binding to cytokines alpha-2M is able to protect the cytokines from destruction by proteases and regulate the activity of the cytokines which are bound.

Alpha-2M is one of the major plasma proteins in humans and is the only plasma inhibitor of a number of proteinases used by pathogens and parasites in attacking the body. Moreover, it is the "acute phase" protein in many species. Before antibodies can be produced by lymphocytes, the level of alpha-2M increases within a short time to response the infection. This may reflect that alpha-2M may protect the host from further damage. In addition, there is evidence that the protein has an evolutionary relationship to some of the components of the complement system (Barrett, 1981). Therefore, it has been thought to represent an ancient

defensive system of the body (Barrett, 1981). Alpha-2M is synthesized _in vivo_ by mononuclear phagocytes and by fibroblasts (Forrester et al., 1983). Alpha-2M has a molecular weight of 725 kDa and the concentrations in healthy individuals vary between 2000 and 4000 mg/l, depending on age and sex.

The interaction between native alpha-2M in peripheral blood and proteolytic enzymes results in conformational changes in the alpha-2M molecule from a tetraplanar to a tetrahedral form. This conversion results in a change in electrophoretic mobility from the slow native form to the fast converted form. These changes can also be brought about by ammonium salts and methylamine (Barrett et al., 1979). Conversion of alpha-2M results in the exposure of a hydrophobic region on the molecule that can bind to receptors present on the surface of macrophages, fibroblasts and hepatocytes (Debanne et al., 1976). As a consequence of the interaction of alpha-2M fast form with its cellular receptors, it is rapidly removed from the circulation (James, 1990).

In recent years, studies have revealed that alpha-2M can bind to a wide range of physiologically important cytokines. The biological latency of serum transforming growth factor-β (TGF-β) has been shown to be due to the interaction of TGF-β with a specific serum binding protein which has now been identified as alpha-2M (O'Connor-McCourt and Wakefield, 1987; Huang et al., 1988). This suggests

that alpha-2M may serve an important multifunctional role at sites of inflammation by scavenging both active peptides and proteases that are released by platelets at the site of injury.

Interleukin-6 (IL-6) has been found to bind alpha-2M (Matsuda et al., 1989). After binding to alpha-2M, IL-6 retains its biologic activity and becomes resistant to treatment with proteases. These findings indicate that alpha-2M plays an important role as a carrier protein for IL-6 in serum and makes IL-6 produced at the local inflammatory site available to lymphocytes, hepatocytes, and hematopoietic stem cells.

Alpha-2M rapidly loses functional and structural integrity in the course of a short-term incubation with either activated neutrophils or eosinophils. Analysis of the mechanism by which alpha-2M is inactivated by neutrophils has revealed that the process is dependent on the generation of hypochlorous acid, an oxidant generated by hydrogen peroxide-myeloperoxidase-chloride system.

In addition to the important role in the development, maintenance and functioning of the immune system, modified alpha-2M has been found to inhibit superoxide production by activated macrophages (Hoffman et al., 1983) and to enhance neutrophil and monocyte locomotion and monocyte chemotaxis.

No studies have been done up to present to determine if rhGM-CSF is bound by alpha-2M and whether or not alpha-2M affects the interaction of rhGM-CSF with

neutrophils. In this research neutrophils were also preincubated with native alpha-2M or the fast form of alpha-2M alone and/or in combination of rhGM-CSF to determine whether or not alpha-2M slow form and fast form would affect the interaction of rhGM-CSF with neutrophils.

This research shows that rhGM-CSF greatly enhanced the superoxide production by neutrophils in response to bacterial product, FMLP. Also native alpha-2M alone could augment the superoxide production; but the fast form of alpha-2M, on the other hand, inhibited all the production of superoxide anion by neutrophils. This study shows that rhGM-CSF plays an important role in the activation of neutrophil function and that the interaction of alpha-2M with proteases may modulate neutrophil action.

CHAPTER II

MATERIALS AND METHODS

Immunomodulators The immunomodulators used in this study were recombinant human granulocyte colony-stimulating factor (rhG-CSF; Lot No.1003C-1; AMGEN Biologicals, Thousand Oaks, CA) and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; Lots No.1317-1 and No.1317-4; AMGEN Biologicals, Thousand Oaks, CA), and human alpha-2-macroglobulin (alpha-2M; Lot No.49F9313; Sigma Chemical Co.; St.Louis, MO).

Recombinant hGM-CSF and rhG-CSF were obtained in liquid form and stored at 2 to 4°C. The stability of the proteins was stated to be four months by the manufacturer. Alpha-2M was obtained in powder form, aliquoted and stored at -20°C before use.

Fast form of alpha-2M was prepared from trypsin-treated alpha-2M which had been dialyzed for 48 hours at 4°C using a Spectra/Por membrane (mw. cut off : 25,000, Spectrum; Houston, TX) against two changes of 200 ml of Hank's balance salt solution (HBSS) (without carbonate and phenol red).

Preparation of Neutrophils Peripheral neutrophils were isolated by the sedimentation of heparinized blood from normal human donors as described by Johnson et al. (1975). Six ml of blood was mixed with 3 ml of 6% Dextran 70 (Travenol Laboratories Inc., Deerfield, IL). An hour was allowed for sedimentation of erythrocytes at room temperature.

The neutrophil-rich plasma was washed with Hank's balanced salt solution ([HBSS] without calcium, magnesium, and sodium bicarbonate, GIBCO Laboratories; Grand Island, NY) buffered with 10 mM HEPES (United States Biochemical Co.; Cleveland, OH). An aliquot of neutrophil mixture was diluted 1:10 with a gentian violet solution (3% acetic acid in saline) and neutrophils were enumerated using a hemocytometer (Improved Newbauer, 0.1 mm deep, AO Brand; Buffalo, NY).

The cell mixture was centrifuged at 200X g for 7 min and resuspended with HBSS (without NaCO3 and phenol red; GIBCO Laboratories, Grand Island, NY) buffered with 10 mM Hepes and containing 0.1% gelatin (Type B; Sigma Chemical Co.; St.Louis,MO). The cell concentration was adjusted to 5 X 10^{6} cells/ml.

Preparation of Opsonized Zymosan Zymosan (Sigma Chemical Co.; St.Louis, MO) was prepared as a 5 mg/ml stock solution in water and stored at 2 to 4°C. To prepare opsonized zymosan, 5 ml of the stock was centrifuged for 7 minutes at 1425 X g and then resuspended with 1.5 ml serum. Serum was prepared by allowing normal human blood to clot at room temperature for 20 min followed by centrifugation at 1425 X g (Dynac Centrifuge, Clay Adams, Parisippany, NJ) to remove cells.

The mixture was incubated at 37°C for 30 minutes on a rocking table (model 4651, Miles Scientific; Naperville, IL). The opsonized zymosan was washed with [HBSS] without ions and resuspended with HBSS (without sodium bicarbonate and phenol red). The concentration was adjusted to 10mg/ml.

Assay of Superoxide Anion Production by PMNs Production of superoxide anion was measured spectrophotometrically by a superoxide dismutase-inhibitable reduction of cytochrome C assay (Type III, Sigma Chemical Co.; St.Louis, MO; Fujita et al., 1984). In each of triplicate tubes (12X75 mm polyethlene culture tube; VWR Scientific Inc.; Philadelphia, PA) 1 ml of the cell mixture (5×10^6 cells per ml) was preincubated with rocking at 37°C in the presence or absence of 100 microliters of the immunomodulator(s) (rhGM-CSF, rhG-CSF, or alpha-2M) to be tested.

One hundred microliters of cytochrome C (10 mg/ml) and 100 microliters of a neutrophil activator, either opsonized zymosan or N-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma Chemical Co.; St. Louis, MO) prepared from a 10^{-3} M stock (dissolved in dimethyl-sulfoxide (DMSO)), was added to the cell mixture. In initial experiments, 100 ul of superoxide dismutase (SOD; Lot No.029F9317; Sigma Chemical Co.; St.Louis, MO) was added to the cell mixture stimulated by opsonized zymosan to demonstrate that the superoxide production could be inhibited by this classical inhibitor.

HBSS (without sodium carbonate and phenol red) was added to bring the final volume of the cell mixture to 1.5 ml. The reaction mixture was then incubated on rocker at 37°C for 10 minutes. Five minutes incubation in an ice bath was used to stop the reduction of cytochrome C by superoxide anion produced by the neutrophils. Tubes were then centrifuged at 2000X g (GPR Centrifuge, Beckman Instruments Inc., Palo Alto, CA) at 4°C for 10 minutes. The supernatant was collected and absorbance was determined spectrophotometrically (DU-64, Beckman Instruments, Inc.;Palo Alto, CA) at 550 nm.

<u>Alpha-2-Macroglobulin Analysis</u> Two distict types of assays were used to determine which form of alpha-2M, slow or fast, was present in the commercial preparations.

The thermolysin-hide powder assay (Barrett, 1981) is based on alpha-2M's inhibition of the activity of the enzyme thermolysin (Sigma Chemical Co.; St.Louis, MO) on dyed hide powder. In each of triplicate assay tubes (12X75 mm polyethylene culture tube; VWR Scientific Inc.; Philadephia, PA) 0.1 ml alpha-2M (50 ug/ml) diluted in 0.1% Brij35 (Sigma Chemical Co.; St.Louis, MO) was mixed with 0.5 ml of 0.4M Tris-HCl buffer (containing 40 mM CaCl2 - 0.1% Brij 35 - 0.1% sodium azide) and incubated for 10 min at room temperature with 0.1 ml of thermolysin (2 ug/ml in Tris-HCl buffer containing 1 mg/ml bovine serum albumine). Five tenths ml of water was then added to the tube followed by 0.8 ml of substrate suspension (12.5 mg of hide powder azure/ml, Sigma Chemical Co.; St.Louis, MO, in 0.6 M sucrose - 0.1% Brij 35 - 0.1% Sodium azide).

The mixture was incubated on a rocker for 20 minutes at 37°C. The reaction was stopped with 1.0 ml of 100 mM disodium EDTA (Sigma Chemical Co.; St.Louis, MO). After centrifugation (600X g for 3 minutes), the absorbance of the supernatant at 595 nm was determined. A standard curve was generated using concentrations at 0 to 0.2 ug/ml of thermolysin. Standard curve tubes were run in parallel for each experiment and results were represented as the amount of thermolysin inhibited per unit weight of alpha-2M.

Demonstration of alpha-2M by agarose gel electrophoresis was accomplished using the TITAN GEL High-Resolution Protein Kit (Helena Laboratories; Beaumont,

TX). Two mg/ml of alpha-2M in 50 mM TrisHCl buffer (pH 8.2) was mixed in an equal volume with either 400 microgram/ml of trypsin for 10 minutes at 37°C or with 200 mM ammonium sulfate for 30 minutes at room temperature. Two microliters of each sample were loaded on the template slits of the gel. The gel was composed of agarose in barbital buffer with sodium azide added as a preservative.

Electrophoresis was carried out on a flat bed electrophoresis system (Flat Bed Apparatus FBE-Immuno, Pharmacia LKB Biotechnology; Uppsala, Sweden). Each chamber in the electrophoresis system was filled with 150 ml of buffer composed of 25.9g mixed powder of barbital sodium barbital, calcium lactate and sodium azide dissolved in 1500 ml purified water and electrophoresis proceeded at 100 volts for one hour.

For protein visualization, the gel was stained in 0.2% Coomassie blue in fixative/destain solution, 50% methanol and 10% acetic acid. After 15 minutes of staining, the gel was destained in fixative/destaining solution until the background staining was removed. The gel was then dried in an incubator at 65°C for 10 minutes or until dry.

<u>Calculations and Data Analysis</u> All absorbance data collected were converted to nanomoles of superoxide anion produced using the following formula:

$$A = E\ L\ C$$

where A is the absorbance difference from blanks which contained only buffer and cytochrome C and were incubated as described above, E is the extinction coefficient (E550 nm = 2.1×10^{4} /M·cm; Massey, 1959), L is the length of cuvette in cm, and C is the molar concentration of superoxide anion produced (M).

All the experiments were carried out in triplicate except where the results from multiple experiments representing a number of neutrophil donors were used. Data are presented as mean ± standard deviation . Significance was determined by standard Student's t test.

CHAPTER III

RESULTS

<u>Effects of rhG-CSF and rhGM-CSF on Superoxide Production by Neutrophils</u> It has been established that several agents are able to stimulate neutrophils to produce superoxide. Opsonized zymosan, when added to human neutrophils, is one such agent that strongly stimulates the production of superoxide anion. The experiments summarized in Table 1 show that superoxide production by neutrophils was greatly increased when they were stimulated by opsonized-zymosan (22.4 nmole/10^6 PMNs, compared with 0.6 nmole/10^6 PMNs in control ($P < 0.001$).

To demonstrate that the assay was in fact measuring superoxide production in our hands, superoxide dismutase (SOD) was added to one group of stimulated cells as SOD catalyzes the degradation of the superoxide molecule to hydrogen peroxide. Superoxide production was practically abolished in the cells treated with opsonized zymosan and SOD (2.7 nmole/10^6 PMNs). Thus, absorbance measured in our experiments was in fact demonstrating the production of superoxide anion.

However, opsonized zymosan was not used in this research to activate neutrophils as its effect was difficult to quantify. In contrast, N-formyl-methionyl-leucyl-phenylalanine (FMLP) could be quantified; therefore, it was used in this research to stimulate neutrophils. To determine the optimal concentration , FMLP was diluted to 10^{-9} to 10^{-6} M to stimulate neutrophils. In Figure 1 the increase of superoxide production stimulated by FMLP at 10^{-8} M was statistically significant (0.9 nmole/10^{6} PMNs, compared with 0.3 nmole/10^{6} PMNs in control). The maximum level of superoxide production was reached at 10^{7} M (2.3 nmole/10^{6} PMNs).

To determine if GM-CSF alone would stimulate neutrophils to release superoxide, neutrophils were incubated with rhGM-CSF for varying lengths of time before cytochrome C was added to the cell suspension (Figure 2). Superoxide production was significantly increased after a 60-minute preincubation of neutrophils with rhGM-CSF (1.1 nmole/10^{6} PMNs, compared with 0.3 nmole/10^{6} PMNs in control). When the preincubation was increased to 120 minutes, two-fold more superoxide was released than at 60 minutes. These results demonstrated that rhGM-CSF alone was able to stimulate neutrophils to release superoxide, although two hours of interaction were required before a significant increase was observed.

The effect of rhGM-CSF (50 U/ml) on the superoxide

production by neutrophils in response to FMLP was determined and the results were summarized in Table 2. Preincubation of neutrophils with rhGM-CSF (50 U/ml) alone for 60 minutes did not cause a statistically significant increase in superoxide production (1.0 nmole/10^6 PMNs) when compared to controls (0.5 nmole/10^6 PMNs). The amount of superoxide production brought about by FMLP was similar to that resulting from rhGM-CSF pretreatment but the level of superoxide production elicited by FMLP was statistically different from the control (1.4 nmole/10^6 PMNs). However, after a 60-minute pretreatment with rhGM-CSF, the superoxide release was greatly increased by neutrophils stimulated with FMLP (10.5 nmole/10^6 PMNs).

To determine the optimum concentration of GM-CSF needed to act synergistically with FMLP, concentrations of 5 U/ml, 50 U/ml, and 500 U/ml of GM-CSF were tested. Following a 1-hour preincubation with rhGM-CSF, FMLP was added to the neutrophil suspension. Synergism was evident between rhGM-CSF and FMLP at all of the concentrations of rhGM-CSF tested (Table 3) and pretreated with all three rhGM-CSF concentrations prepared the neutrophils to release similar concentrations of superoxide following the addition of FMLP.

Experiments summarized in Table 4 were designed to determine if the effect of rhGM-CSF on neutrophils' subsequent response to FMLP is time-dependent. Preincubating neutrophils for as little as 10 minutes with

50 U/ml rhGM-CSF enhanced the superoxide production to the same degree as did a 60-minute preincubation.

Since rhG-CSF also selectively stimulates granulocyte maturation, the ability of rhG-CSF to activate neutrophils alone or in combination with FMLP was determined (Table 5). The increase in superoxide production brought about by rhG-CSF alone (50 U/ml) following a 60 minute incubation was not statistically significantly different from controls. The addition of FMLP to the neutrophils pretreated with rhG-CSF did not even result in an additive increase over the levels of superoxide production brought about by FMLP and rhG-CSF alone. As in experiments using rhGM-CSF, FMLP alone was able to bring about a significant increase in superoxide production when compared with controls (1.4 nmole/10^6 PMNs v. 0.5 nmole/10^6 PMNs).

<u>Effect of Alpha-2-Macroglobulin on rhGM-CSF's Ability to Stimulate Release of Superoxide by Neutrophils</u> If rhGM-CSF , like other cytokines, is able to bind to alpha-2M, one might expect that increased levels of superoxide production normally induced by rhGM-CSF might be affected. On the other hand, to determine if alpha-2M and CSF do interact, neutrophils were preincubated with rhGM-CSF (50 U/ml) and alpha-2M (500U/ml) alone and in combination for 30 minutes at 37°C. Results from the two experiments are shown in Table 6.

No significant increase in superoxide production was

observed in neutrophils treated with either alpha-2M alone or rhGM-CSF. However, the combination treatment of rhGM-CSF and alpha-2M resulted in a three-fold increase in superoxide production. However, no synergism was observed with the combination treatment of alpha-2M and FMLP and the effect of the combind treatment was additive. This demonstrated that alpha-2M did not suppress the ability of rhGM-CSF to stimulate superoxide release. On the contrary, it actually facilitated the ability of rhGM-CSF to activate neutrophils.

However, the results shown in Table 6 were not obtained in every trial. Results from two additional experiments using blood from two different donors are summarized in Table 7. In both experiments response of the neutrophils to rhGM-CSF alone was much stronger than that in previous cases (1.8 nmole/10^6 PMNs v. 0.7 nmole/10^6 PMNs) and was significantly higher than that in control (1.8 nmole/10^6 PMNs v. 0.5 nmole/10^6 PMNs). However, when neutrophils were preincubated with the combination of rhGM-CSF and alpha-2M for 30 minutes, no difference was seen in the levels of superoxide production induced by rhGM-CSF alone and the combination of rhGM-CSF and alpha-2M (1.8 nmole/10^6 PMNs v. 1.9 nmole/^{6} PMNs).

In all previous experiments the concentration of alpha-2M used was 500 ug/ml. However, human blood levels of alpha-2M range from 2000 ug/ml to 4000 ug/ml. To determine the effect of alpha-2M on neutrophil superoxide production

at physiological concentrations, increased concentrations of alpha-2M were used in the experiment summarized in Table 8.

The increased superoxide production by neutrophils incubated with alpha-2M at 500 ug/ml and 1000 ug/ml were not statistically different from controls. Nevertheless, when the concentration of alpha-2M was elevated to a physiological concentration, 2000 ug/ml, the enhancement of superoxide production was statistically significant (1.9 nmole/10^6 PMNs v. 0.3 nmole/10^6 PMNs in control). The combination of rhGM-CSF and alpha-2M brought about an additive rather than synergistic effect on neutrophils. To determine if the fast form of alpha-2M could still enhance the action of rhGM-CSF on neutrophils, neutrophils were preincubated with trypsin-treated alpha-2M which had been dialyzed against HBSS for 48 hours with four changes.

After 30-minute preincubation with the combination of rhGM-CSF (50 U/ml) and native alpha-2M (200 ug/ml) enhanced the production of superoxide anion occured as in previous experiments (Table 9). However, preincubation of neutrophils with alpha-2M-trypsin complex (modified from 2000 ug/ml of native alpha-2M) alone and in combination with rhGM-CSF did not stimulate superoxide production (0.1 and 0.0 nmole/10^6 PMNs, respectively). When preincubated with trypsin (3 mg/ml) alone and in combination with rhGM-CSF (50 U/ml), neutrophils released 0.5 and 1.3 nmole/10^6 PMNs, which were not significantly different

from control (0.8 nmole/10^6 PMNs).

The fast form of alpha-2M was also obtained from ammonium sulphate treatment (see the result below) (Figure 4b). Alpha-2M treated with ammonium sulphate (0.4 M) was dialyzed against HBSS (without sodium carbonate and phenol red) for 48 hours with 4 changes. Since the ammonium sulphate could be completely removed by dialysis, the fast form of alpha-2M would be purer than the trypsin treated alpha-2M. While shows that the combination of rhGM-CSF (50 U/ml) with native alpha-2M (500 ug/ml) stimulated the release of superoxide anion as usual (Table 10), the fast form of alpha-2M alone once again ablated the production of superoxide and the alpha-2M/rhGM-CSF combination could not stimulate the neutrophils.

Determination of the Form of alpha-2-Macroglobulin In order to verify that the alpha-2M preparation being used was in fact the fast or slow form, the thermolysin-hide powder assay was used. The data in Figure 3 show that alpha-2M did not inhibit the reaction of thermolysin with the substrate (dyed hide powder azure). Alpha-2M purified from peripheral blood normally is the slow, intact form and is thus able to bind thermolysin and inhibit the enzyme's interaction with the hide powder substrate (Barrett et al., 1979). Since these results from the thermolysin - hide

powder assay indicated that the alpha-2M was not the slow form, agarose gel electrophoresis was used as an alternative means to identify the form of alpha-2M which was used in the previous experiments. When alpha-2M was saturated with trypsin the protein did migrate faster (Figure 4a Lane 2) than did the native alpha-2M (Figure 4a Lane 1).

When the native alpha-2M (5 mg/ml) was treated with equal volume on 0.4 M ammonium sulphate for 2 hours at room temperature, the fast form was observed also (Figure 4 Lane 2). Thus, native alpha-2M was present in the previous studies as the native alpha-2M slow form was completely converted to fast form after being saturated with trypsin or treated with ammonium sulphate.

CHAPTER IV

DISCUSSION

When N-Formylated chemotactic peptides like FMLP bind to their receptor on the neutrophil's surface, a complex, coordinated sequence of metabolic events is initiated within the cell which culminates with activation of the oxidative burst (Fletcher et al., 1982). Studies using neutrophils from patients with chronic granulomatous disease have shown that the oxidative burst is required for neutrophils to function effectively against invading microorganisms (Rotrosen & Gallin, 1987). Neutrophil functions have also been found to be impaired following thermal and traumatic injury. The defects include decreased neutrophil chemotaxis and bactericidal activity, decreased serum colony-stimulating activity, and a decreased monocyte release of colony-stimulating factor (Mooney et al., 1988). Due to impaired immunological function burn patients, and individuals with cancer and AIDS (acquired immune deficiency syndrome), are more susceptible to infection. In addition, delays in wound healing are also often observed (Wolfe et al., 1982).

Several efforts have been made to restore the immune system of immunocompromised or immunosuppressed patients.

Evidence is accumulating to indicate that rhGM-CSF, which is able to stimulate production of both granulocytes and macrophages, and rhG-CSF, which controls production of granulocytes, are potent neutrophil activators. Therefore, they may become important agents to increase the resistance of impaired patients who are prone to bacterial infection. Thus, the major purpose of this study was to elucidate the precise effects of rhGM-CSF and rhG-CSF on the superoxide production by mature neutrophils as several investigators have reported contradictory results. In addition, the role of alpha-2M as a modulator of immunological activity has been established due to its ability to interact with cytokines and alter their activity. It therefore become important to determine if alpha-2M could interact with CSFs and alter the effect of CSFs on the mautre neutrophils.

Previous studies on the effect of rhGM-CSF and rhG-CSF on superoxide production by neutrophils have shown that rhGM-CSF (Atkinson et al., 1988; Lopez et al., 1986) and rhG-CSF (Kitagawa et al., 1987) do not stimulate superoxide production by themselves but greatly enhance the production of superoxide anion by neutrophils stimulated with FMLP. Recombinant human GM-CSF at 2000 pg/ml enhances FMLP-stimulated superoxide production following preincubation with neutrophils for two hours at 37°C (Lopez et al., 1986). A significant increase in superoxide release appears after 15 minutes and reaches the maximum at 60 minutes when neutrophils are preincubated with 10^{-11} M

rhGM-CSF (Atkinson et al., 1988).

Similar results were obtained in several of the experiments in this study (Tables 2, 3, and 4). Ten-minute priming of neutrophils with rhGM-CSF at 50 U/ml was able to enhance the superoxide production in response to FMLP stimulation. The effect of 5 U/ml rhGM-CSF was the same as those of 50 U/ml and 500 U/ml rhGM-CSF. However, rhGM-CSF alone at 50 U/ml was able to stimulate the production of superoxide anion in a time-dependent manner (Figure 2). This result is different from those previously described. If the specific activity of rhGM-CSF used in previous studies was the same as that in this study, which is 5 X 10^{7} U/mg protein, the 10^{-11} M and 2000 pg/ml would be equivalent to 0.4 U/ml and 8 U/ml, respectively. Those concentrations are lower than 50 U/ml. The difference in the concentration of rhGM-CSF may account for the different results obtained from previous studies and this research.

Recombinant human G-CSF at 50 U/ml did not enhance superoxide production in neutrophils stimulated by an optimal concentration of FMLP (10^{-6} M) (Table 5). Woodman et al. (1988) have reported similar results (rhG-CSF at 1000 U/ml). However, Kitagawa et al. (1987) reported that rhG-CSF at 25 ng/ml, like rhGM-CSF, is able to provoke the release of superoxide anion by neutrophils in response to FMLP (10^{-8} M - 10^{-6} M). If the specific activity of the rhG-CSF used in Kitagawa's study was equivalent to that used in these experiments, which was 2 X 10^{8} U/mg

protein, then 25 ng/ml of rhGM-CSF would be equivalent to 5000 U/ml. This high concentration may explain why rhG-CSF could augment superoxide release by neutrophils in response to FMLP in Kitagawa's study.

After infecting mice with Listeria monocytogenes, elevated levels of CSFs in the serum are observed (Cheers et al., 1988) with M-CSF, which supports the production of macrophages, and G-CSF being the major CSFs detected. GM-CSF was present but at lower levels. Forty-eight hours after infection almost 3000 U/ml of both M-CSF and G-CSF can be detected. Forty-two U/ml of GM-CSF was found to be the maximum concentration and this level was observed 24 hours post-infection. These in vivo results plus previous in vitro studies imply that during infection neutrophils may be produced in great numbers due to the presence of significant amounts of CSFs in serum. Therefore, it is possible that G-CSF and GM-CSF may also interact with circulating and marginatal neutrophils and thus play an important role in the activation of mature neutrophils.

The mechanism by which GM-CSF activates neutrophils to release superoxide anion in response to FMLP has been suggested to be via regulation of the expression of FMLP receptors on the surface of neutrophiles. Two receptor affinities have been identified. Enhanced superoxide production is accompanied by a decrease in the high-affinity receptors and an increase in the lower-affinity FMLP receptor population (Atkinson et al.,

1988). Previous studies have been shown that the chemotactic response is most sensitive to low formyl peptide concentrations, whereas the generation of superoxide anion requires higher concentration of formyl peptide (Sklar, 1986). Those findings suggest that the high-affinity formyl peptide receptors may mediate neutrophil chemotaxis, whereas the lower-affinity receptors mediate superoxide anion generation. After preincubation of neutrophils with rhGM-CSF, the number of lower-affinity increases (Atkinson et al., 1988). This finding may explain the augmentation of superoxide production by neutrophils in response to FMLP stimulation after the priming of neutrophils by rhGM-CSF.

Such a proposal is in agreement with the conditions neutrophils encounter *in vivo*. When neutrophils are far from the site of invading bacteria, they would encounter very low concentrations of FMLP which could only be detected by the high affinity receptors. Upon migrating towards the source of infection (i.e., up the FMLP gradient), the low affinity receptors would become reponsive and superoxide production would be induced. CSFs, produced locally by activated T lymphocytes during infection (Gasson et al., 1984), would assist in maintaining the neutrophils at the site of inflammation and optimize superoxide production where it is required most.

Besides regulating the expression of FMLP receptor, rhGM-CSF also primes neutrophils for a host of other

activities stimulated by FMLP. Within minutes after rhGM-CSF contacts the granulocyte's surface, arachidonic acid is released from membrane phospholipids (Sullivan et al., 1987). Arachidonic acid is a precursor of eicosanoids, which are local hormones able to alter the activities of the cells in which they are synthesized as well as the activity of adjoining cells. Thus, rhGM-CSF regulates the amount of local hormones which is produced and the hormones in turn directly regulate the expression of FMLP receptors on neutrophil's surface. The subquent binding of FMLP to neutrophils then triggers the production of superoxide anion.

Sullivan et al. (1988) suggested that the binding of rhGM-CSF to the neutrophil's surface is likely to be coupled to activation of a membrane phospholipase, which participates in enzymatic cascades that generate highly active lipids or transduce signals. Since the oxygen-dependent antimicrobial mechanism occurs in the plasma membrane, rhGM-CSF may activate the membrane phospholipase which in turn influences transduce signals, resulting in the production of superoxide anion.

This activation of neutrophils by rhGM-CSF may be modulated by alpha-2-macroglobulin (alpha-2M) (Tables 6, 8, 9 and 10). Alpha-2M, one of the major proteins in serum, is the only protease inhibitor which can inhibit almost all endogenous proteinases and those used by pathogens and parasites in attacking the host (Barrett, 1981). Due to

its inhibitory profile, it is not surprising that alpha-2M may modulate the physiological activity of hormones found in blood. It has been reported that alpha-2M has a wide range of effects on the immune system (James, 1990).

The interactions between alpha-2M and cytokines which are physiologically important are particularly interesting. The binding of alpha-2M to cytokines such as fibroblast growth factor (Dennis et al., 1989), transforming growth factor-beta (O'Connor-McCourt & Wakefield, 1987) and interleukin-6 (Masuda et al., 1989) may offer a plausible explanation for many of the immunological properties previously ascribed to alpha-2M.

To determine if alpha-2M would influence the effect of rhGM-CSF on the activation of neutrophil function, several experiments were carried out. Alpha-2M did not ablate the ability of rhGM-CSF to stimulate neutrophil function but was able to enhance the production of superoxide anion by neutrophils activated by rhGM-CSF (Tables 6 and 8). This interaction between alpha-2M and rhGM-CSF may be similar to that of IL-6 where binding to alpha-2M occurs but the original fuction of the cytokine isn't altered. However, the type of results shown in Tables 6 and 8 was not obtained when the neutrophils were obtained from other donors. This phenomenon occurs in other studies even within the same laboratory, particularly with respect to repopulation studies in irradiated animals (James, 1990).

Identification of a role for alpha-2M in the activation of neutrophils is not too surprising as the precursor polypeptide chains of C3 and C4 are of similar molecular weight to the alpha-2M subunit. In addition, the locations of their proteolysis-sensitive and covalent-linking/autolysis sites correspond closely to those in alpha-2M. Thus, it is suggested that there is a strong possibility of evolutionary homology between alpha-2M, C3, C4 and possibly C5 (Barrett, 1981). Perhaps because of this homology, alpha-2M could trigger the superoxide release through the complement receptors on the surface of neutrophils. The evidence that serum-opsonized zymosan can strongly trigger the production of superoxide anion (Table 1; Cheson et al, 1977) may explain the enhancement of superoxide production by neutrophils preincubated with alpha-2M, the structural and functional analog of these complement components (Barrett, 1981).

Clearance of alpha-2M is completed by the Kupffer cells of the liver and is greatly accelerated when the inhibitor is complexed with protease (Forrester et al., 1983). In this study the results summarized in Tables 9 and 10 demonstrated that the fast form of alpha-2M inhibited the production of superoxide anion by neutrophils. This may imply that the clearance of alpha-2M-protease complexes from sites of inflammation may play a role in the feedback regulation of the activation of neutrophil function by CSFs. However, little is known

about the mechanism by which alpha-2M modulates neutrophil function.

In conclusion, rhGM-CSF may play an important role in the activation of a neutrophil funtion which is very relevent to resistance to infection. While stimulation of superoxide production is a result of <u>in vitro</u> rhGM-CSF treatments, more study is needed to determine if rhGM-CSF may have a possible role in immunotherapy. Moreover, the activation of neutrophils by rhGM-CSF may be modulated by the interaction of the cytokine and alpha-2M.

Table 1. Production of superoxide in neutrophils stimulated by opsonized zymosan.

Treatment	$O_2^{(-)}$ release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.6 ± 0.3 (3)*
opsonized zymosan	22.4 ± 0.2 (3)
opsonized zymosan +SOD	2.7 ± 0.3 (3)

Opsonized zymosan (10 mg/ml) and/or superoxide dismutase (SOD) (30 ug/ml) were added to the cell mixture at the same time as was cytochrome C.
* Number of determinations.

Table 2. Effect of rhGM-CSF on the production of superoxide by neutrophils stimulated by FMLP.

Treatment	$O_2(-)$ release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.5 ± 0.3 (9)*
FMLP	1.4 ± 0.7 (9)**
rhGM-CSF	1.0 ± 0.4 (9)
rhGM-CSF + FMLP	10.5 ± 0.7 (9)***

PMNs were preincubated with rhGM-CSF (50 U/ml) or buffer for 60 minutes at 37°C. Cytochrome C was then added to the cell mixture along with FMLP (10^{-6} M). Data were obtained from three separate experiments.
* Number of determinations.
** $P < 0.01$ vs. control as determined by Student's t test.
*** $P < 0.001$ vs. control as determined by Student's t test.

Table 3. Effect of differing concentrations of rhGM-CSF on superoxide production by neutrophils activated by FMLP.

Treatment	$O_2(-)$ release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.5 ± 0.3 (3)*
FMLP	1.6 ± 0.3 (3)**
rhGm-CSF at 5 U/ml	0.8 ± 0.8 (3)
rhGM-CSF at 50 U/ml	1.0 ± 0.4 (3)
rhGM-CSF at 500 U/ml	1.4 ± 0.4 (3)
rhGM-CSF (5 U/ml) + FMLP	10.8 ± 0.7 (3)***
rhGM-CSF (50 U/ml) + FMLP	11.1 ± 0.4 (3)***
rhGM-CSF (500 U/ml) + FMLP	11.5 ± 0.2 (3)***

PMNs were preincubated with rhGM-CSF 5 U/ml, 50 U/ml, 500 U/ml, or buffer for 60 minutes at 37°C. Cytochrome C was then added to the cell mixture along with FMLP (10[illegible] M).
* Number of determinations.
** $P < 0.02$ vs. control as determined by Student's t test.
*** $P < 0.001$ vs. control as determined by Student's t test.

Table 4. Effect of different preincubation with rhGM-CSF on superoxide production by neutrophils stimulated with FMLP.

Treatment	O2(-) release (nmole/10^6 PMNs) (mean ± standard deviation)
preincubation for 10 minutes	
control	0.7 ± 0.2 (3)*
rhGM-CSF	1.1 ± 0.6 (3)
FMLP	3.2 ± 0.8 (3)***
rhGM-CSF + FMLP	11.5 ± 0.1 (3)****
preincubation for 30 minutes	
control	0.5 ± 0.3 (3)
rhGM-CSF	1.8 ± 0.6 (3)**
rhGM-CSF + FMLP	11.5 ± 0.8 (3)****
preincubation for 60 minutes	
control	0.3 ± 0.3 (3)
rhGM-CSF	1.3 ± 0.3 (3)**
FMLP	1.9 ± 1.0 (3)**
rhGM-CSF + FMLP	11.1 ± 0.4 (3)****

PMNs were preincubated in the absence or in the presence of rhG-CSF (50 U/ml) for 10, 30, or 60 minutes before cytochrome C with or without FMLP (10^{-6} M) was added to the cell mixture.
* Number of determinations.
** P < 0.05 vs. control as determined by Student's t test.
*** P < 0.01 vs. control as determined by Student's t test.
**** P < 0.001 vs. control as determined by Student's t test.

Table 5. Effect of rhG-CSF on superoxide production by neutrophils.

Treatment	$O_2(-)$ release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.5 ± 0.5 (9)*
rhG-CSF	1.0 ± 0.3 (9)
FMLP	1.5 ± 0.3 (9)**
rhG-CSF + FMLP	1.4 ± 0.2 (9)***

PMNs were preincubated in the absence or in the presence of rhG-CSF (50 U/ml) for 60 minutes at 37°C. FMLP (10^{-6} M) and cytochrome C were then added to the cell mixture. Data were pooled from three separate experiments.

* Number of determinations.

** P < 0.1 vs. control as determined by Student's t test.

*** P < 0.01 vs. control as determined by Student's t test.

Table 6. Effect of the combination of alpha-2M with rhGM-CSF on the production of superoxide anion by neutrophils.

Treatment	O2(-) release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.6 ± 0.2 (8)*
alpha-2M	1.0 ± 0.3 (8)
rhGM-CSF	0.7 ± 0.3 (8)
FMLP	1.5 ± 0.3 (4)**
alpha-2M + rhGM-CSF	3.5 ± 0.3 (8)***
alpha-2M + FMLP	2.4 ± 0.1 (4)**

PMNs were preincubated in the absence or in the presence of alpha-2M (500 ug/ml) and /or rhGM-CSF (50 ug/ml) for 30 minutes before cytochrome C with or without FMLP (10^{-6} M) was added to the cell mixture. Data were obtained from two separate experiments using cells from the same donor.
* Number of determination.
** $P < 0.01$ vs. control as determined by Student's t test.
*** $P < 0.001$ vs. control as determined by Student's t test.

Table 7. Effect of alpha-2M on superoxide production by neutrophils.

Treatment	O2(-) release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.5 ± 0.3 (8)*
alpha-2M	0.9 ± 0.6 (8)
rhGM-CSF	1.8 ± 0.5 (8)***
alpha-2M + rhGM-CSF	1.9 ± 0.9 (8)**

PMNs were preincubated in the absence or in the presence of alpha-2M (500 ug/ml) and/or rhGM-CSF (50 U/ml) for 30 minutes. Cytochrome C was then added to the cell miture. Data were obtained from two separate experiments and different donors.
* Number of determinations.
** P < 0.01 vs. control as determined by Student's t test.
*** P < 0.001 vs. control as determined by Student's t test.

Table 8. Effect of alpha-2M concentrations on superoxide production by neutrophils.

Treatment	O2(-) release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.3 ± 0.4 (4)*
alpha-2M (500 ug/ml)	0.5 ± 0.4 (4)
alpha-2M (1000 ug/ml)	1.4 ± 0.4 (4)**
alpha-2M (2000 ug/ml)	1.9 ± 0.2 (4)****
rhGM-CSF	0.7 ± 0.2 (4)
alpha-2M (500 ug/ml) +rhGM-CSF	1.8 ± 0.4 (4)***
alpha-2M (1000 ug/ml) + rhGM-CSF	2.5 ± 0.8 (4)***
alpha-2M (2000 ug/ml) + rhGM-CSF	2.7 ± 0.3 (4)****

PMNs were preincubated in the absence or in the presence of alpha-2M at 500 ug/ml, 1000 ug/ml, or 2000 ug/ml and/or rhGM-CSF (50 U/ml) for 30 minutes. Cytochrome C was then added to the cell mixture.
* Number of determinations.
** $P < 0.1$ vs. control as determined by Student's t test.
*** $P < 0.01$ vs. control as determined by Student's t test.
**** $P < 0.001$ vs. control as determined by Student's t test.

Table 9. Effect of alpha-2M-trypsin complex on superoxide production by neutrophils.

Treatment	O2(-) release (nmole/10^6 PMNs) (mean ± standard deviation)
control	0.8 ± 0.3 (4)*
rhGM-CSF	0.7 ± 0.3 (4)
alpha-2M	1.1 ± 0.0 (4)
trypsin	0.5 ± 0.1 (4)
alpha-2M-trypsin	0.1 ± 0.0 (4)
rhGM-CSF + alpha-2M	2.7 ± 0.0 (4)**
alpha-2M-trypsin + rhGM-CSF	0.0 ± 0.2 (4)
trypsin + rhGM-CSF	1.3 ± 0.2 (4)

PMNs were preincubated in the absence or presence of rhGM-CSF (50 U/ml), native alpha-2M (2000ug/ml), and/or alpha-2M-trypsin for 30 minutes before cytochrome c was added to the cell mixture.
* Number of determinations.
** $P < 0.01$ vs. control as determined by Student's t test.

Table 10. Effect of the fast form of alpha-2M modified by ammonium sulphate on the production of superoxide anion by neutrophils.

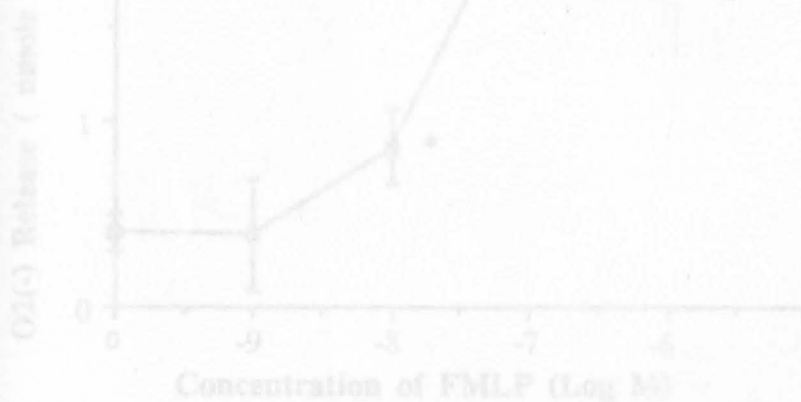
Treatment	$O_2(-)$ release ($nmole/10^6$ PMNs) (mean ± standard deviation)
control	0.1 ± 0.3 (4)*
alpha-2M	0.9 ± 0.2 (3)
alpha-2M fast form	0.0 ± 0.7 (4)
rhGM-CSF	0.7 ± 0.7 (3)
rhGM-CSF + alpha-2M	1.7 ± 0.1 (4)**
rhGM-CSF + alpha-2M fast form	0.1 ± 0.3 (4)

PMNs were preincubated in the absence or presence of rhGM-CSF (50 U/ml), native alpha-2M (500 ug/ml), and/or the fast form of alpha-2M (500 ug/ml) modified by ammonium sulphate (0.4M) for 30 minutes before cytochrome C was added to the cell mixture.
* Number of determinations.
** $P < 0.01$ vs. control as determined by Student's t test.

Figure 1. Superoxide production by neutrophils stimulated with FMLP. Neutrophils were incubated with FMLP along with cytochrome C for 10 minutes at 37°C. The experiment was done in triplicate.

* $P < 0.05$ vs. control as determined by Student's t test.

** $P < 0.01$ vs. control as determined by Student's t test.



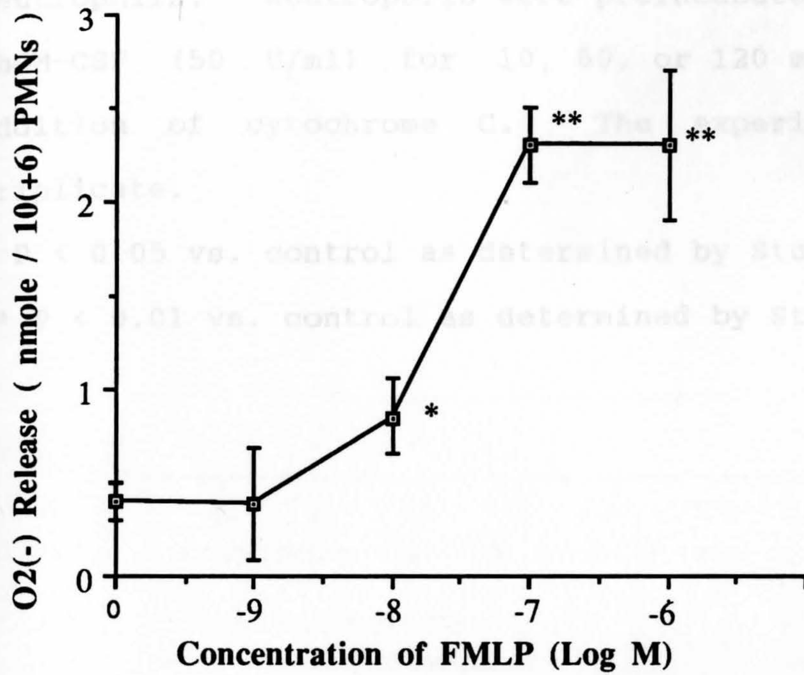
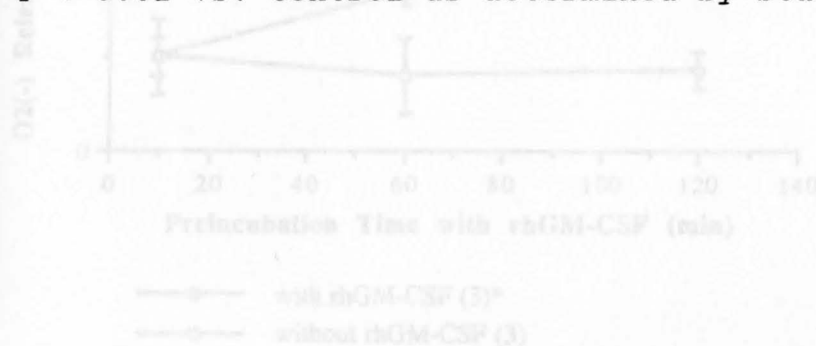
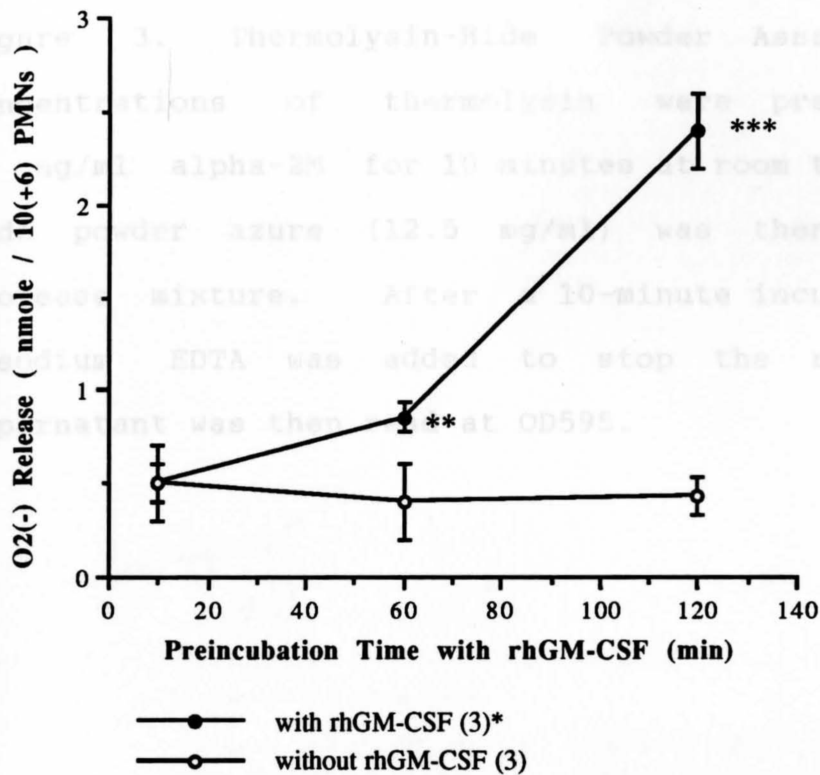

O2(-) Release (nmole / 10(+6) PMNs)
3
2
1
0
0
-9
-8
-7
-6
Concentration of FMLP (Log M)
*
**
**

Figure 2. Effect of rhGM-CSF on superoxide production by neutrophils. Neutrophils were preincubated with or without rhGM-CSF (50 U/ml) for 10, 60, or 120 minutes before the addition of cytochrome C. The experiment was done in triplicate.

* $P < 0.05$ vs. control as determined by Student's t test.

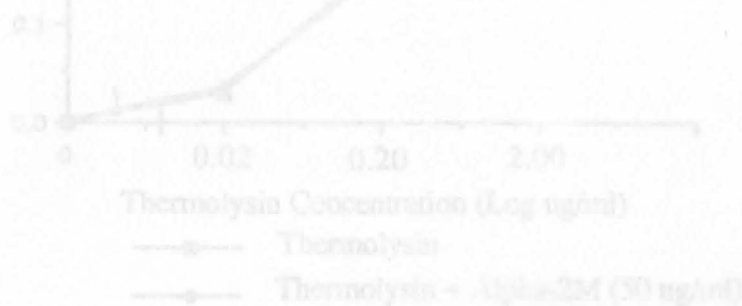
** $P < 0.01$ vs. control as determined by Student's t test.





O2(-) Release (nmole / 10(+6) PMNs)
3
2
1
0
0
20
40
60
80
100
120
140
Preincubation Time with rhGM-CSF (min)
***
**
with rhGM-CSF (3)*
without rhGM-CSF (3)

Figure 3. Thermolysin-Hide Powder Assay. Differing concentrations of thermolysin were preincubated with 50 ug/ml alpha-2M for 10 minutes at room temperature, and hide powder azure (12.5 mg/ml) was then added to the protease mixture. After a 10-minute incubation at 37°C, disodium EDTA was added to stop the reaction. The supernatant was then read at OD595.



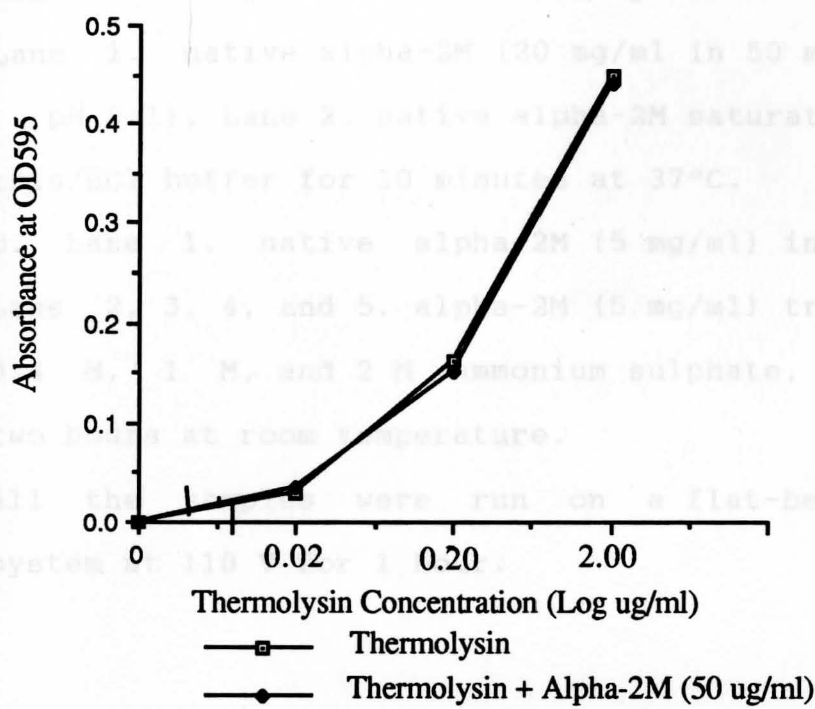
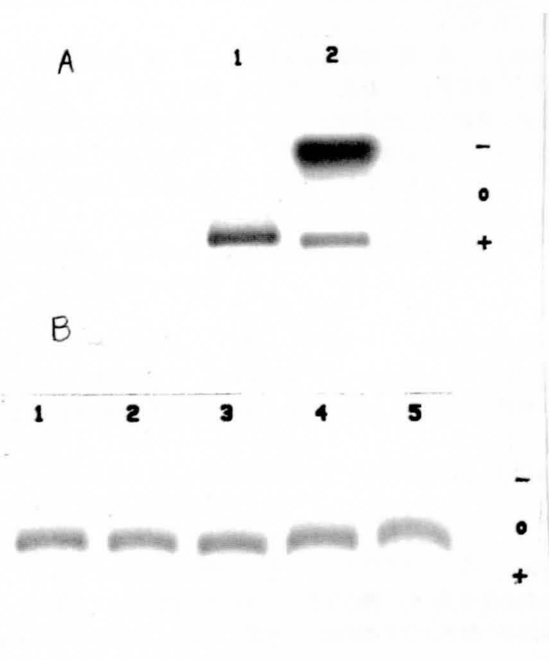

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at OD595
0
0.02
0.20
2.00
Thermolysin Concentration (Log ug/ml)
Thermolysin
Thermolysin + Alpha-2M (50 ug/ml)

Figure 4. Agarose Gel Electrophoresis of alpha-2M. a. Lane 1. native alpha-2M (20 mg/ml in 50 mM tris/HCl buffer , pH 8.1), Lane 2. native alpha-2M saturated with trysin in tris/HCl buffer for 10 minutes at 37°C.

b. Lane 1. native alpha-2M (5 mg/ml) in tris/HCl buffer, Lane 2, 3, 4, and 5. alpha-2M (5 mg/ml) treated with 0.2 M, 0.4 M, 1 M, and 2 M ammonium sulphate, respectively, for two hours at room temperature.

All the samples were run on a flat-bed electrophoresis system at 110 V for 1 hour.


A
1
2
−
o
+
B
1
2
3
4
5
−
o
+

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