MODULATION OF MATURE HUMAN NEUTROPHIL FUNCTION BY COLONY-STIMULATING FACTORS

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ABSTRACT

MODULATION OF HUMAN NEUTROPHIL FUNCTION
BY COLONY-STIMULATING FACTORS

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Master of Science

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Colony-stimulating factors (CSFs) are glycoproteins which have been identified as being involved in the regulation of hematopoietic pathways. While all the cells of the blood are formed from the pluripotent stem cell, the various CSFs direct the proliferation and differentiation of committed cells in the macrophage-granulocyte cell series. These factors have been chemically characterized and they have recently been synthesized using recombinant DNA technology. While the CSFs were originally defined as stimulators of blood cell production, the availability of these factors in pure quantities has now made it posssible to address the possible role of the CSFs in the regulation of the function of mature cells.

In this study mature human polymorphonuclear leukocytes (PMNs) were treated with either recombinant human granulocyte colony-stimulating factor (rhG-CSF) or recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF).

Functions of the PMNs which were relevant to resistance to bacterial infection were then studied in order to determine the potential value of the CSFs as biological response modifiers.

While rhG-CSF and rhGM-CSF did not affect the random motility, or chemokinesis, of the mature PMNs, they did bring about changes in the directed movement, or chemotaxis, of the cells. The hormones were able to act as chemoattractants and they also were able to inhibit the movement of the cells toward another chemoattractant agent. These properties of the hormones may be important in attracting PMNs to the site of infection and keeping them localized once they have arrived. No effects on the ability of the PMNs to degranulate or kill ingested Pseudomonas aeruginosa were observed. Evidence that the CSFs were interacting with the cells was also provided by microspectrophotometric analysis of deoxyribonucleic acid (DNA) template activity of treated cells. The increase in template activity is related to an increase in gene expression.

These results provide further evidence of a possible role for the CSFs in the modulation of the function of mature neutrophils.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
A	Area
C	Correction factor for unoccupied space $C = (2-Q)^{-1} \ln(Q-1)^{-1}$
E	Extinction
I_o	Transmitted flux
I_s	Flux of photons on chromophore
k	Specific absorptivity constant of the chromophore at a defined wavelength
L	Parameter equivalent to one minus the transmission (1-T) at a defined wavelength
M	Chromophore mass in the measured field
Q	Ratio of L_2/L_1
T	Transmission of the field
λ	Wavelength in nanometers

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CHAPTER I

INTRODUCTION

The polymorphonuclear leukocyte (PMN) is one of the two cells that are responsible for the engulfment and digestion of bacteria which may invade the body. The PMN is the predominant white blood cell in the bloodstream and it shares a common hematopoietic stem cell with the other formed members of the blood. The PMN is a non-dividing, short-lived cell with a multilobed nucleus and an array of granules which are difficult to stain using common histological dyes such as heamatoxylin and eosin. These granules are of three types: the primary azurophilic granule containing myeloperoxidase, some lysozyme and a group of cationic proteins, the secondary "specific" granules holding lactoferrin, lysozyme and a B-12 binding protein and tertiary granules which are like the conventional lysosomes and contain the acid hydrolases. The cell also contains abundant glycogen stores which are utilized during glycolysis, thus enabling the cell to operate under anaerobic conditions (Roitt,1988). The PMN is the primary cell involved in defense against bacteria which cause pyogenic (pus-forming) infections and has been called the "professional phagocyte" since its primary function is the

ingestion and destuction of those pathogens.

The macrophage, which is the other cell involved in host defense, is a rather long-lived cell that resides in the tissues or attached to them. This cell primarily combats those bacteria, viruses or protozoa which are capable of living within the cells of the host.

The bone marrow has the capability to produce approximately as many neutrophils each day (estimated at 120 billion) as it does erythrocytes, but the PMN has a much shorter lifespan (about 6 - 7 hours) in the circulation than does the erythrocyte (approximately 30 days). Under normal cicumstances there is only one PMN circulating in the blood for every 2,000 erythrocytes. The mature PMNs also collect along the walls of the capillaries and many more are held in reserve in the bone marrow. All of these populations are ready to be instantly mobilized should a bacterial invasion of the tissues occur. Under most normal conditions the PMNs migrate at a steady rate through the spaces between the endothelial cells into the tissues and body spaces. This rate of migration increases dramatically if bacterial invasion has occured. The PMNs are attracted to the site by the release of chemotactic stimuli by the bacteria and the generation of chemotactic factors following tissue injury (Craddock, 1972).

Normal neutrophils are capable of unidirectional movement towards a chemoattractant such as N-formyl-methionyl-leucyl-phenylalanine (FMLP) <u>in vitro</u> (Gallin et

al.,1978). In vivo the PMNs are attracted to the site of infection by chemotactic factors. The chemotactic factors which are involved in the attraction of leukocytes include the complement components C3a, C5a, and C567 (Wong and Wilson,1975; Ehlenberger and Nussenzweig,1977; Hart et al., 1987) and factors released from disrupted leukocytes and tissue cells (Gallin,1981). Bacteria and fungi also release potent chemotactic substances as they invade tissue in their attempt to colonize tissues in the host. The gradient formed by these chemotactic substances, which is at the highest concentration at the site of the bacterial invasion or tissue damage, starts the unidirectional locomotion of the PMNs towards the site. This unidirectional movement is known as chemotaxis (Gallin et al.,1978; Gallin,1981).

Once the neutrophils are attracted to the site of inflammation, phagocytosis of the invading microbe occurs. However, before phagocytosis can occur the PMN must adhere to the surface of the bacteria. This is accomplished by a number of mechanisms. First, complement adheres to the cell surface of the bacteria through a rather complex cascade system. The neutrophil has receptors on its surface for the C3b fragment of the cascade which enables binding of the bacteria to the PMN's surface (Stossel,1975; Stossel,1976). Once this binding has occurred the PMN extends pseudopods around the microbe through an actin-myosin contractile system until it has completely engulfed the bacteria in a vacuole called the phagosome (Horwitz,1982). Once ingested the

phagosome quickly fuses with the cytoplasmic granules which then dump their contents into the sac containing the bacteria. The antibacterial contents of the lysosomes act in concert with an oxygen-dependent killing mechanism to bring about destruction of the ingested microbes (Kavet and Brain,1980).

After the phagocytic process has started there is a dramatic increase in the activity of the hexose monophosphate shunt which generates large amounts of NADPH. This product is utilized to reduce oxygen bound to a plasma membrane cytochrome (cyt b-245) which causes a burst of oxygen consumption. The oxygen is then converted to a number of powerful antimicrobial agents such as superoxide anion, hydrogen peroxide, singlet 0_2, and hydroxyl radicals. The combination of peroxide, myeloperoxidase and halide ions constitutes a potent halogenating system capable of destroying both bacteria and viruses (Weening et al.,1974; Root et al.,1975).

The dismutation of superoxide consumes hydrogen ions, thus raising the pH slightly which enables the group of cationic proteins, which are found in the cytoplasmic granules, to operate optimally. These proteins damage the bacterial membrane both by neutral proteinase (cathepsin G) action and by direct transfer to the bacterial surface. However, the mechanism of action of these proteins is not fully understood at this time. The oxygen independent factors are low pH, lysozyme and lactoferrin. All of which

constitute bactericidal or bacteriostatic factors which can function under anaerobic conditions. The killed organisms are finally digested by the hydrolytic enzymes found in the lysosomes and the degradation products are then deposited outside of the cell (Elsbach,1973; Root et al.,1975; Anderson et al.,1978; Root and Cohen,1981).

One of the most important aspects of the host response to an infection is the increased production and delivery of granulocytes and monocytes (in some cases) to the site of infection. The bone marrow is capable of increasing its production of white blood cells ten-fold during stressful situations, such as an infection. The response of the bone marrow is also quite specific in its response to infection, whereby it will produce more granulocytes in response to bacterial infections or increase its production of monocytes in response to fungal disease or tuberculosis (Cannistra and Griffin,1988).

The agent or agents responsible for the reaction of the bone marrow have been the focus of much attention. In the late 1960's it was proposed that the bone marrow's response (myeloproliferation) involved factors which caused the proliferation of the pluripotent stem cells. In 1970 methods were devised utilizing mouse bone marrow and these techniques made it possible to grow human leukocytes rather easily through refinement of the colony-culture system. This system, as devised by Robinson, Iscove, and Chervenick, involved the use of feeder layers of human leukocytes or

medium, conditioned by exposure to the same types of cells (Golde and Gasson,1988).

In this assay, "feeder" layers consisting of various types of mature white blood cells (WBCs) in a semi-solid media were placed into a small tissue culture plate. Bone marrow cells (which included stem cells) were added over the "feeder" layer. After incubation, colonies of various types of mature WBCs would form in the upper layer. This led to the hypothesis that a soluble factor released by cells could bring about this differentiation of the immature precursor cells. A search then ensued to find this mystical "colony stimulating cell" in the peripheral blood. A number of researchers at about the same time discovered that the monocyte was the cell primarily responsible for the release of these factors, now known as colony-stimulating factors (CSFs) (Metcalf,1985). Another discovery was that cells of the monocyte-macrophage lineage also release substances which cause other cells to release CSFs, including interleukin-1 and tumor necrosis factor (Wing and Shadduck,1985; Hermann et al.,1988; Lindemann et al.,1988; Golde and Gasson,1988).

A number of CSFs have been discovered and through the use of recombinant DNA technology it is now possible to obtain large quantities of the pure hormones. These hormones have been named for the groups of cells whose differentiation they bring about from the stem cell. Four CSFs have been discovered to date. The first of these is a glycoprotein that induces formation of colonies of the monocyte-macrophage

lineage, hence the name macrophage colony stimulating factor (M-CSF). It has a molecular weight of 70,000 and a carbohydrate content of 50 - 70% (Wong et al.,1987).

Secondly, granulocyte colony-stimulating factor (G-CSF) is the hormone that induces the formation of granulocyte colonies. It has a molecular weight of 24,500 and its carbohydrate content has not yet been deduced. G-CSF was first isolated from media prepared from endotoxin-stimulated mouse lung (Souza et al.,1986).

The third of these hormones is granulocyte macrophage colony stimulating factor (GM-CSF). It is a glycoprotein with a molecular weight of 23,000 and a carbohydrate composition of 10 - 20% . This hormone promotes the proliferation of granulocyte and macrophage colonies (Wong et al.,1985). It is the most widely studied of the CSFs. The effects that this agent has upon the hematopoietic system, the immune system and upon the mature PMN have been studied.

The fourth of these factors is called muti-CSF or interleukin-1 (IL-1). It is a glycoprotein of molecular weight 28,000 with an unknown carbohydrate content. This hormone induces the formation of all cell lines, including the myeloid and erythroid cell lines (Yang et al.,1986).

It was discovered that CSFs are produced by a variety of organs such as murine lung, heart, placenta, submaxillary gland, embryonic tissue, and a variety of other tissues. Analysis of various cell types has also shown that CSFs are produced by fibroblasts, macrophages and endothelial cells

(Gasson et al.,1984; Metcalf,1985; Clark and Kamen,1987).

Research has recently focused on assessing the effect that the CSFs have upon both the hematopoietic system and the immune system. For example, Vadhan-Raj et al.(1988) discovered that recombinant human GM-CSF (rhGM-CSF) is effective in stimulating myelopoiesis in patients with severe aplastic anemia. Multi-CSF enhances the resistance of mice to bacterial infection by Pseudomonas aeruginosa or Klebsiella pneumoniae (Ozaki et al.,1987). Recombinant human GM-CSF enhances phagocytosis by murine peritoneal cells of Listeria monocytogenes. However, it does not increase the bactericidal activity of PMNs (Cheers et al.,1989). These examples show that the CSFs have varied effects upon the hematopoietic system and the mature cells of the immune system. As a result of these findings and others, much attention has been given to understanding the possible role of CSFs as immunomodulators.

It had been postulated that the mature PMN has a receptor which allows binding of G-CSF to the cell. This binding could help to explain how G-CSF brings about the reported changes in the cell's function. In 1988, Uzumaki et al. demonstrated that mature PMNs possess a receptor for rhG-CSF. They found that 560 high affinity binding sites are present on each cell. The receptor is a nondisulfide-bonded protein with a molecular weight of approximately 150 kDa. It is effectively demonstrated that a single G-CSF receptor type for human G-CSF exists on the circulating human PMN. The

discovery of the high affinity binding site is important because it shows that the PMN can readily bind with G-CSF which in turn could bring about some change in cellular function.

The recombinant human G-CSF (rhG-CSF) hormone is known to have an effect upon the PMN's oxygen dependent killing mechanism, particularly affecting the release of superoxide when the PMNs are stimulated with FMLP (Kitagawa et al.,1987). Kitagawa et al. indicate that rhG-CSF is not an effective stimulus by itself but rather it primes the PMN for a higher release of superoxide than the PMNs that are exposed to FMLP alone. This effect is seen with a concentration of 25 ng/ml of rhG-CSF and the effect occurs over a wide range of FMLP concentrations (10^{-8} to 10^{-6} M). Uzumaki et al. (1988) were also able to show that a result of the binding of rhG-CSF to PMNs is increased superoxide production in response to a FMLP stimulus.

Recombinant human G-CSF also induces the synthesis of alkaline phosphatase in the neutrophils of patients with chronic myelogenous leukemia. This is significant because the monocyte is responsible for the induction of alkaline phosphatase (NAP) synthesis in the PMNs of normal patients as well as in patients with chronic phase chronic myelogenous leukemia. When PMNs from these patients are incubated without monocytes, NAP synthesis does not occur. However, when these cells are incubated with rhG-CSF, NAP synthesis is induced in a dose-dependent manner. This indicates that NAP

is not prepackaged in the PMNs, and its synthesis occurs by sequential transcription at the DNA level and translation at the ribosomal level. Thus, binding of rhG-CSF to the neutrophil does bring about a profound change in the cellular activity in the same manner as other hormones do (Chikkappa et al.,1988).

As would be expected, rhG-CSF stimulates the production of peripheral blood PMNs when injected into neutropenic mice (Matsumoto et al.,1987). However, rhG-CSF does not increase the phagocytic abilities of PMNs in hamsters, but it does increase the production of superoxide and the binding of FMLP in the same animals following a single subcutaneous injection of the rhG-CSF. Recombinant human G-CSF also induces recovery from leukopenia in the hamster model (Cohen et al.,1988), similar to the effect noted when mice are treated in the same manner.

In their studies with <u>in vivo</u> administration of rhG-CSF into human subjects with small cell lung cancer, Bronchud et al. (1988) found that the rhG-CSF has two effects. The first effect is a drop in the peripheral PMNs within one hour of administration of the rhG-CSF followed by a rapid increase in mature PMNs in the peripheral circulation. <u>In vitro</u> testing of these PMNs showed that the rhG-CSF has no effect upon the motility and phagocytic ability of the PMNs. Their method for testing phagocytosis is a method utilizing the technique of luminol-dependent chemiluminescence using serum opsonized zymosan as the stimulating agent. Chemotaxis is measured by

using the under agarose method. However, rhG-CSF is responsible for the inducement of chemotaxis of human PMNs across polycarbonate or nitrocellulose filters (Wang et al.,1988). This discrepancy may be explained by the fact that two different methods to assay the PMN's motility were employed.

Recent work done with rhGM-CSF enhances our understanding of the multiple functions of this hormone. The in vivo administration of rhGM-CSF to patients in phase 1 clinical trials does not have any effect upon the activation of monocyte-mediated cytotoxicity but it does stimulate the bone marrow to produce more monocytes in a dose dependent manner. However, activation of the monocytes can be accomplished in vitro (Kleinermann et al.,1988). In vivo administration of rhGM-CSF in patients with refractory bone marrow failure or malignancy results in an increased proliferation of all white blood cells. However, this agent affects the in vitro chemotaxis of the PMNs in the under agarose method in an adverse manner. The PMNs from patients who are receiving intravenous administration of rhGM-CSF show a decrease in chemotactic motility towards the FMLP stimulus. There is no increase in production of hydrogen peroxide noted in the PMNs of the patients undergoing this therapy (Buescher et al.,1988). Similar results have been obtained by Kaplan et al. (1989) with the intravenous administration of rhGM-CSF to patients with refractory carcinoma. These findings, together with the marked stimulation of myelopoiesis,

suggests that the use of this immunomodulator may play an important role in the treatment of immunocompromised patients. Such treatments would serve to both increase the number of circulating WBCs and to enhance the defense capability of those WBCs.

Mitogenic responsiveness of HL-60 promyelocytic cells is enhanced by exposure to rhGM-CSF. The HL-60 cell line, which was derived from a patient with acute promyelocytic leukemia, proliferates continuosly in suspension culture. The predominant cell in these cultures is the promyelocyte. These cells can be induced to differentiate into morphologically and functionally mature granulocytes by incubation with a wide variety of compounds such as dimethyl sulfoxide (DMSO), butyrate or hypoxanthine (Breitman and Gallo,1981). HL-60 cells are also able to spontaneously form colonies on semi-solid media without the addition of any growth factors. When these cells are treated with DMSO and 6-thioguanine (TG), the proliferative capacity of the cells decreases dramatically. Treatment of these cells with rhGM-CSF returns the growth of these cells to normal (Schwartz and Mahar,1988). This study once again indicates the ability of rhGM-CSF to bring about proliferation of progenitor cells which in turn brings about an increase in the number of circulating white blood cells.

In addition to rhGM-CSF's ability to increase the proliferation of white blood cells, rhGM-CSF also appears to play a role in bringing about changes in the biochemical

functioning of the mature PMN. Once the rhGM-CSF binds to the surface of the cell, profound changes in the cell's biochemical processes occur. The rhGM-CSF hormone modulates some of the biochemical processes which occur in the PMN and it is capable of altering the concentration of free calcium in the cell and bringing about changes in the internal pH of the PMN. An increase in the free calcium level is thought to explain the potentiation of neutrophil functional responses, such as phagocytosis or superoxide production, when the PMN is pretreated with rhGM-CSF (Naccache et al.,1988).

It has been shown that the stimulation of some of the PMN's functions by rhGM-CSF may be mediated in part by the effects that GM-CSF has upon the production of guanylate cyclase or a reduction in the production of adenylate cyclase. Guanylate cyclase activity is stimulated by rhGM-CSF and treatment reduces adenylate cyclase activity in human PMNs. Increased levels of adenylate cyclase (cAMP) inhibits the PMN's ability to phagocytize, their ability to kill bacteria and to release lysosomal enzymes. Conversely, increased levels of guanylate cyclase (cGMP) are known to increase the phagocytic ability of PMNs as well as to increase the release of lysosomal enzymes. Chemotaxis is modulated by these nucleotides in that such movement is inhibited by increased levels of cAMP and enhanced by increases in the levels of cGMP within the cell. Antibody-mediated cytotoxicity is also inhibited by high levels of cAMP and enhanced by increased levels of cGMP. (Coffey et

al.,1988)

Leukotrienes play an important role in allergic and inflammatory responses and are highly active chemoattractants. Human PMNs which have been preincubated with rhGM-CSF and then stimulated with either of the chemoattractants, FMLP or C5a, produce substantial amounts of leukotrienes. The rhGM-CSF thus primes the PMNs for leukotriene production when exposed to a second stimulus because cells that are not preincubated with rhGM-CSF alone do not generate as much of the leukotrienes when they are then exposed to either FMLP or C5a. This stimulation of the leukotriene production may indeed act as an amplifying signal once the PMNs have been attracted to the site of infection by GM-CSF which is known to be released by cells at the site of inflammation. In addition, rhGM-CSF itself has been shown to be a chemoattractant. (Dahinden et al.,1988)

As discussed previously, neutropenia in animal models is reversed by administration of rhGM-CSF. However, the survival of neutropenic animal models when challenged by either bacteria or intracellular parasites gives widely varied results when the animals are treated prophylactically with rhGM-CSF. Mice treated with recombinant murine GM-CSF prior to a challenge by Leishmania major, an intracellular parasite which must be dealt with by macrophages, do not survive the infection. While there is an increase in the total number of both macrophages and PMNs, the macrophages are not activated because there is no increase in the

interferon level which is necessary for activation of macrophages (Greil et al.,1988). However, neutropenic mice which are prophylactically treated with rhGM-CSF are able to survive a challenge by Pseudomonas aeruginosa since the total number of circulating PMNs is increased. Neutrophils are the main defense that the host has against extracellular bacterial infections (Tanaka et al.,1989).

Phagocytosis of the bacteria Staphylococcus aureus is enhanced by treatment of the neutrophils by rhGM-CSF. However, intracellular killing of the microbe is not enhanced (Fleischmann et al.,1986). Enhancement of the phagocytosis of heat-killed baker's yeast and IgA opsonized latex beads has also been observed (Williamson and Brown,1987; Weisbart et al.,1988).

The rhGM-CSF hormone enhances superoxide anion production by neutrophils in response to FMLP, complement derived C5a and leukotriene. A two-hour preincubation period with rhGM-CSF prior to exposure to the chemoattractants is necessary for enhancement of the oxidative metabolism. Therefore, rhGM-CSF may be an endogenous regulator of neutrophil inflammatory responses induced by the major physiological chemoattractants (Weisbart et al.,1987). However, even though an increase in superoxide production would be expected to increase the intracellular killing of bacteria, this has not been observed (Williamson and Brown,1987; Weibart et al,1987).

Both Lopez et al. and Kapp et al. also demonstrate

that rhGM-CSF effectively activates the oxidative metabolism of human PMNs. Another effect which is been attributed to rhGM-CSF is its ability to enhance FMLP-stimulated degranulation of the PMNs pretreated with cytochalasin B (Lopez et al.,1986; Kapp et al.,1988). One other effect is that morphological changes are noted in the PMNs after treatment with rhGM-CSF in which the PMNs elongate and assume an irregular shape. These changes in morphology are correlated with an increase in the PMN's adherent ability. However, this increase in adherence did not affect the cell's motility.

One of the first functions attributed to the CSFs was in the inhibition of neutrophil chemotaxis (Neutrophil Inhibiting Factor [NIF]; Gasson et al.,1984). In order to determine if NIF and GM-CSF were identical, Gasson et al. compared the two by Ultrogel fractionation, fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fractionation by reversed-phase high-performance liqiud chromatography (HPLC). The NIF activity of the fractions was assayed by a variation of the under agarose method of chemotaxis (Nelson et al.,1975) and CSF activity was determined by exposing human bone marrow to the fractions and checking for the formation of mature differentiated cells in colonies. Neutrophil inhibition factor and CSF activities both reside in a single glycoprotein with an apparent molecular weight of 22,000, supporting the conclusion that a single human immunomodulator acts on progenitor cells in the

bone marrow and upon the mature PMNs in the peripheral blood stream. This also shows that rhGM-CSF can keep the PMNs localized at the place of inflammation. These mechanisms for the expression of NIF activity are partially explained by Atkinson et al. (1988). Preincubation of PMNs with rhGM-CSF reduces the binding affinity that the cells have for FMLP. In addition, PMNs have two affinity binding sites for FMLP. The first of these binding sites is a high affinity site with approximately 2000 of these sites per cell. The second site has a much lower affinity for the FMLP molecule than the first one does and there are thought to be about 40,000 sites per cell. The rhGM-CSF is thought to alter the affinity of both sites towards FMLP as FMLP's ability to bind to the PMN is reduced after the cells were pretreated with the rhGM-CSF. This is exemplified by a reduction of chemotatic ability as demonstrated using the under agarose technique of chemotaxis.

The aim of this study is to further the understanding of the action that these two immunomodulators, rhG-CSF and rhGM-CSF, have upon the mature human PMN. The human neutrophil possesses receptors for rhG-CSF (Uzumaki et al.,1988) and, with the binding of the hormone, profound changes are brought about in the cellular activity. As has been demonstrated, some of these changes are an increase in superoxide production and the inducement of alkaline phosphatase synthesis. However, some controversy does exist as to how this hormone affects the phagocytic and locomotive abilities of the PMN.

Similar changes in the functioning of PMNs are seen with rhGM-CSF. Unlike rhG-CSF, rhGM-CSF does not bring about changes in the oxidative killing mechanisms of the PMNs. This hormone is shown to precipitate changes in the biochemical functioning of the cell, but these processes have not been studied as extensively in cells treated with rhG-CSF. How rhGM-CSF affects the motility of PMNs is not clear at this point because of the inconsistency of the methods used in previous studies.

This study will seek to determine the effects rhG-CSF has upon the intracellular killing ability of the human PMN. A new approach to analyzing the effect of both agents on PMNs will be the use of microspectrophotometeric analysis of the DNA template activity. Changes in template activity or increased synthetic activity may account for the findings that the immunomodulators do stimulate the various functionings of the cell. The under agarose method for determining the chemotactic ability of these agents and their effect upon the chemotaxis of the PMNs in response to the chemoattractant FMLP will also be studied extensively. This method will provide the means to visualize both chemotaxis and chemokinesis. This same method will also be utilized to see whether these agents elicit a chemokinetic response which is the inducement of random motility of the PMNs. This study will also ascertain whether or not the agents, rhG-CSF or rhGM-CSF, have any effect upon the degranulation of PMNs when exposed to FMLP. It is hoped that these findings will

correlate with the study of the DNA template activity.

CHAPTER II

MATERIALS AND METHODS

IMMUNOMODULATORS

The immunomodulators utilized in this study were recombinant human granulocyte colony-stimulating factor (rhG-CSF) and recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) (AMGEN Biologicals, Thousand Oaks,CA). Lot numbers were 1003A and 1302-1 for the rhG-CSF and rhGM-CSF respectively. These same lots were used throughout the study. The agents were obtained in a liquid form and stored at 2 - 4 °C with a stated stability of greater than four months.

ANIMALS

Female mice from the Crl:CF-1 BR non-Swiss strain were employed for this study (Charles River, Wilmington,Mass.) and were older than 8 weeks old when used. All mice weighed between 22 - 24 g.

INTRAPERITONEAL ELICITATION

Mice were injected intraperitoneally with either 2.0 ml, 2.5 ml, or 5.0 ml of the agent being tested. Injections were made at various times to determine the maximum response.

Agents tested included normal strength and double strength nutrient broth (Difco laboratories, Detroit, Mi.), normal strength and triple strength brain heart infusion broth (Difco Laboratories), 3% proteose-peptone (Difco Laboratories), 0.2% glycogen - oyster Type II (Sigma Chemical Co., St. Louis, Mo.), and 12% sodium caseinate (Difco Laboratories). All the agents were dissolved in distilled water, and sterilized by autoclaving. The agents were stored at 2 - 4° C until needed, at which time they were warmed to room temperature prior to injection.

PERITONEAL EXUDATE CELL COLLECTION

Mice were sacrificed by cervical dislocation. The entire abdomen was disinfected with 70% ethanol and five milliliters of Hank's Balanced Salt Solution ([HBSS] without calcium, magnesium, and sodium bicarbonate; Gibco Laboratories, Chagrin Falls, Ohio) buffered with 10 mM Hepes (United States Biochemical Co., Cleveland, OH.) was injected into the peritoneal cavity using a 26-gauge needle. The abdomen was then massaged to maximize collection of the cells. The peritoneal skin was drawn back aseptically to expose the muscle layer, and the HBSS was then withdrawn. Cell counts were performed using a hemocytometer (improved Neubauer, 0.1 mm deep, AO Brand, Buffalo,N.Y.) A 1:100 dilution of the cell suspension was prepared using a gentian violet solution prior to counting the cells.

BACTERIA

Pseudomonas aeruginosa strain 6782 (obtained from Shriners Burns Institute, Cincinnati, Ohio) was stored in skim milk (Difco Laboratories, Detroit, Mi.) at -70° C. The stock cultures were prepared by mixing one part of skim milk with two parts of an overnight culture of P. aeruginosa which had been grown in nutrient broth (Difco Laboratories). Bacteria from frozen stock were grown on nutrient agar (Difco Laboratories) overnight at 37° C and then stored at 2° C. Fresh plates were made from the frozen stock culture each week. Bacteria utilized in this study were grown from these plates. The bacteria were grown in nutrient broth (Difco Laboratories) for 18 hours in a shaker incubator, after which the concentration of cells was adjusted to a Klett 95 (Klett MFG, Co.,Inc., N.Y.). Four milliliters of cells were inoculated into 50 mls. of nutrient broth and incubated for 3.5 to 4.5 hours in the shaker incubator. The cells were then harvested, washed two times in 0.9% saline, and resuspended to a final concentration of $1x10^7$ CFU/ml, which was obtained by adjusting the cells to a Klett 100 reading.

SERUM POOL PREPARATION

Blood was drawn from normal human volunteers into red top Vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, N.J.)without serum separators. The blood was allowed to clot at room temperature for 20 minutes. The

blood was then centrifuged at 1425xg (Dynac centrifuge, Clay Adams, Parisippany, N.J.) and the serum was collected, pooled, and aliquoted in 1ml portions into 2.0 ml cyrogenic vials (Corning Glass Works, Corning, N.Y.)The serum was then stored at -70° C and thawed just prior to use.

COLLECTION OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES (PMNs)

Blood was drawn from human volunteers, who were screened for antibiotic and other medication use, into green top Vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) containing 14.3 USP units heparin/ml. Six millliliters of the heparinized blood was mixed with three millliliters of 6% Dextran 75 in 0.9% NaCl (Abbott Laboratories, North Chicago, Ill.). The mixture was allowed to sediment (1xg) at room temperature for 1 hour, after which the leukocyte layer was collected and washed twice with HBSS. An aliquot of the leukocyte mixture was diluted into a gentian violet solution (3% acetic acid in saline) and the PMNs were counted in a hemocytometer. The gentian violet solution permits the visualization of the PMNs by staining the nucleus of these cells. The cells were resuspended in HBSS without sodium bicarbonate (Gibco Laboratories) in 0.1% gelatin (Sigma Chemical Co., St. Louis, Mo.) and buffered with 10mM Hepes (this will be referred to as HGS) to a final concentration of 5.55×10^{6} cells/ml.

NEUTROPHIL FUNCTION TEST - KILLING OF BACTERIA BY HUMAN NEUTROPHILS

The antibacterial capacity of rhG-CSF treated (15 min. preincubation at 37°C before the addition of bacteria and serum) and normal neutrophils was determined by a modification of the technique reported by Alexander, et al (1968). The reaction mixture contained 0.1 ml of P. aeruginosa 6782 (1×10^7 cfu/ml), 0.1 ml pooled normal human serum (diluted 1:5 prior to addition with HGS 0.5 ml Dextran-purified PMNs (5.55×10^6 PMNs/ml), and HGS in a final volume of 1.0 ml. The 12 x 75mm polypropylene tubes (Falcon, Cockeysville, MD) were incubated at 37°C for 1-2 hrs. on an aliquot mixer (Ames Division, Miles Laboratories, Elkhart, Ind.). Ten-fold serial dilutions were then made of the reaction mixture in sterile water to lyse the PMNs. Dilutions were then plated on nutrient agar plates and incubated overnight at 37°C. The number of surviving bacteria was determined by counting the isolated colonies and the log decrease was then calculated for all the groups;

$$\text{Log}_{10}\ \text{Decrease} = \log \frac{(\text{bacteria remaining in PMN only tubes})}{(\text{bacteria remaining in PMN plus serum tubes})}$$

CHEMOTAXIS DETERMINATION

Neutrophil chemotaxis and chemokinesis were determined using the migration under agarose technique (Nelson,et al. 1975). Agarose gel was prepared by combining boiled agarose (0.25g Agarose [ME], Marine Colloids,Inc., in 12.5 ml

distilled water) and culture medium (0.125g Type IV gelatin, Sigma Chemical Co., in 12.5 ml 2X HBSS, [Gibco Laboratories], supplemented with 10 ml/L of glutamine and 10mM HEPES, pH 7.2 - 7.4; heated at 56°C to dissolve the gelatin). The gel was cooled for 5 min to 56°C, 6 ml was pipetted into 60 x 15mm tissue culture plates (Falcon, Cockeysville,MD), and the agarose was allowed to harden. Following refrigeration, wells 2.5mm in diameter were cut in the agarose with the aid of a plexiglass template and punch. The wells in each group of three were separated from each other by a distance, edge-to-edge, approximating the hole diameter. Ten microliters of Dextran purified PMNs (5.55×10^7/ml) were placed into the center well. These cells had been pretreated with rhG-CSF, rhGM-CSF, N-formyl-methionyl-leucyl-phenylalanine (FMLP), or media depending upon which experiment was being performed. Into the outer well, 10ul of the chemotactic factor (FMLP, rhG-CSF, or rhGM-CSF) or control media was placed, once again depending upon which experiment was being performed. The inner well had 10 ul of control media placed into it. The plates were initially incubated for a variety of times at 37° C. After it was determined that two hours of incubation allowed for the ideal migration of the PMNs, subsequent experiments were incubated for that length of time. Chemotaxis was stopped by the addition of 2.5% gluteraldehyde (Sigma Chemical Co., diluted in phosphate buffered saline [PBS]) to each plate. The plates were refrigerated overnight at 4° C. On the following day the fixative was decanted and

the agarose, which was now firm, was removed from the plate. A sufficient quantity of Wright's stain (Sigma Chemical Co., St. Louis, MO.) was added to cover the bottom. After five minutes, Wright's stain phosphate buffer was added, and after 15 minutes the plate was rinsed twice with distilled water and allowed to air dry. Chemotaxis is the distance that the PMNs moved beyond the margin of the well and chemokinesis is the diameter of the area that the PMNs have randomly migrated. These distances were measured by projecting the migration patterns using a Ken-A-Vision microprojector (Raytown, MD.). The patterns were focused onto graph paper ruled in 1mm. increments and the migration of the PMNs was measured. The endpoint of the migration was determined by identifying those frontal cells which were separated from the main body of cells by no more than 1 mm. Results are expressed as the distance migrated. The distance figures are representative of the average of replicates on multiple plates.

MEASUREMENT OF DEOXYRIBONUCLEIC ACID (DNA) TEMPLATE ACTIVITY

The DNA template activity of the human PMNs was determined utilizing the Feulgen stain for localizing nuclear DNA. The stain binds stochiometrically to DNA and permits cytophotometeric measurements to be made. Analysis was made utilizing a Zeiss microspectrophotometer following the procedures described below.

Cytochemical Methods

Slides of nuclei to be measured were stained utilizing the Feulgen reaction. The basic fuchsin which makes up the Schiff's reagent had a certification number (CI) of 42510 and a dye content of 93% (Fisher Scientific). Basic fuchsins which have a CI number of 42510 have rosanalin (magenta I) as the major constituent with magenta II also being present (Conn,1977). Many of the basic fuchsins are fairly pure pararosanalin, whereas others may contain relatively large amounts of magenta II. Magenta II gives the dye a darker hue when used in staining procedures. This is due to the alkyl substitution on the dye molecule in which pararosanalin results in the lightest colored dye, rosanalin is darker, and magenta II is the darkest of the three (Penza,1985).

Hydrolysis of nuclear DNA is necessary in order to remove the purine bases from the DNA and therefore expose the reactive aldehyde groups which then permits stoichiometeric dye binding to DNA (DiStefano, 1948). Hydrolysis was carried out using 5N hydrochloric acid (HCl) at room temperature for 45 minutes. Hydrolysis at room temperature was done instead of the more conventional method whereby the hydrolysis is done at 60^{o}C for 15 minutes using 1N HCl. Room temperature hydrolysis provides an extended period for maximal Feulgen intensity (Itikawa and Ogura, 1953; Jordanov, 1963; and DeCosse and Aiello, 1966).

Feulgen Reaction

The Feulgen reaction (Feulgen and Rossenbeck, 1924; as modified by Bryant and Howard, 1969) was used to localize and quantify deoxyribonucleic acid (DNA) in the human PMNs. The reaction involves two basic steps:

1) Mild acid hydrolysis (using 5N HCl at room temperature) removes the purines at the level of the purine - deoxyribose glycosidic bond of DNA, thereby unmasking the aldehyde groups of deoxyribose.
2) Schiff's reagent reacts with the free aldehyde groups to yield a colored complex.

Schiff's reagent is prepared from a mixture of pararosanilin, rosanalin, and magenta II (Conn, 1977), which is made colorless prior to use. This colorless compound is better known as Schiff's leucofuchsin reagent (DeRobertis and DeRobertis, 1980). The Schiff's reagent, upon complexing with the free aldehyde groups of the deoxyribose sugar, forms a deep magenta color which can be measured by absorption spectrophotometry. The quantitative basis for the Feulgen reaction is due to the stable bond formed in a stoichiometric ratio between the Schiff's reagent and the polyaldehyde nature of hydrolyzed DNA. The bond between the dye and the deoxyribose sugar has been shown to be of an alkyl-sulfonic acid nature (Nauman,West, et al., 1960). This bond stability allows for the quantitative measurement of DNA content in individual nuclei (Kasten, 1967; Ris and Mirsky, 1949).

Staining Procedure

Prior to preparing the slides for staining, 5.55×10^7 cells/ml of human PMNs were incubated in the presence of the immunomodulator being tested. Control cells were incubated in HBSS. The concentration of the agents being tested ranged from 0.0 units/ml to 5000 units/ml. After incubating for 15 minutes a slide was prepared by placing an aliquot of the reaction mixture on to a pre-albuminized slide and smearing the mixture like a blood smear would be prepared. The slide was then allowed to air dry for 30 minutes.

All slides from a single experiment were stained simultaneously. The procedure used to stain the slides was as follows:

1) The smears were rinsed in distilled water prior to and after being hydrolyzed in 5N HCl for 45 minutes at room temperature.
2) The smears were stained one hour in Schiff's reagent (Lillie, 1951) freshly fortified with 10 % potassium metabisulfite in a ratio of 1 to 4 with the reagent (10 ml to 40 ml of Schiff's reagent) (Table 2).
3) The smears were rinsed three times for five minutes each in a sulfurous acid rinse solution (Table 2).
4) The smears were rinsed in distilled water and dehydrated in a graded ethanol series.
5) Finally, the smears were cleared in xylene and

TABLE 1

STAINING REAGENTS

1. Schiff's reagent - Add 2.0 g Basic Fuchsin and 4.4 g K-metabisulfite to 200 ml of 0.2 N HCL, stir mechanically for 2 hours, let stand overnight in a dark place, then mix with activated charcoal and filter. Use approximately 200 mg of activated charcoal (Norit A) for each 100 ml of Schiff's reagent. The Schiff's reagent must be water clear. If it is not water clear after the first filtering, add more Norit A and refilter. Store Schiff's reagent in a dark bottle at 5°C in a refrigerator. It is stable for approximately 2 weeks.

2. 10% K-metabisulfite - 10 g K-metabisulfite and bring volume to 100 ml with dH_2O.

3. Sulfurous Acid Rinse - 10 ml of 1N HCl
   10 ml of 10% K-metabisulfite solution
   200 ml of dH_2O

mounted in permount.

Cytophotometric Determinations

A Zeiss Type 01 microspectrophotometer was used for all cytophotometric determinations employing a Planachromat oil immersion objective, N.A. 1.30 x 100, at an optovar setting of 1.25 x. A Zeiss continuous interference filter monochromator (No. 47 43 10) was used for isolation of the chosen wavelengths of light. Instrument alignment and linearity of the phototube were checked prior to the instrument's use each time.

The two-wavelength method for quantitative DNA measurement (Patau, 1952; Ornstein, 1952; Mendelsohn, 1961) was used in these experiments. This method minimizes the distributional error in optical density measurements that come from heterogeneous staining within a specimen. Elimination of the need for direct measurement of the nuclear area is also provided for when employing this method (Mayall and Mendelsohn, 1970). The reliable estimation of the absorbing material is ensured by the selection of the two wavelengths. These wavelengths were chosen for each set of slides after determining its absorption curve for the Feulgen stained PMNs. The optical density (OD) at λ_2 = 2 x OD of λ_1. The wavelengths will give specific absorptivities at a 2:1 ratio since all hydrolysis and staining was done at the same time (Swift and Rasch, 1956). Heterogeneous dye distributions of interphase nuclei such as those found in

PMNs, can be measured once the proper wavelengths are chosen.

The two wavelengths (λ_1 and λ_2) chosen conformed to the extinction requirements for the method. The photometric field was adjusted by means of a specific aperature in order to circumscribe the nucleus to be measured. The absorbing molecules were measured by monochromatic transmitted light at the two pre-selected reference wavelengths. The amount of chromophore (M) within the measured area (A) was determined by using the equation $M = KAL_1Q$. The constant of absorption, K , was eliminated from these calculations since only arbitrary values of M, and not the absolute values of DNA content were used in this investigation. Transmissions (T_1 and T_2) were taken at wavelengths 1 and 2 for each nucleus. $T_1 = I_S/I_o$ and $T_2 = I_S/I_o$ where I_S is the transmitted flux and I_o is the flux of photons on the chromophore. From these values, L_1 and L_2 were calculated by the equations $L_1 = (1 - T_1)$ and $L_2 = (1 - T_2)$. The ratio, L_2/L_1, which corresponds to Q can be used to determine C, the correction factor for any distributional error (Swift and Rasch, 1956; Leuchtenberger, 1958), where $C = (2 - Q)^{-1} \ln (Q - 1)^{-1}$. The C value and corresponding Q value can be found in a table formulated by Patau (1952). In order to ensure the accuracy of the DNA measurements, all calculations, including the statistical analysis of the data, were carried out on the mainframe computer at Youngstown State University utilizing a program written by Dr. John J. Yemma (Department of Biological Sciences, Youngstown State University, Youngstown, Ohio).

EXOCYTOSIS ASSAY AND ENZYME ACTIVITY DETERMINATIONS

5×10^6 PMNs obtained by Dextran sedimentation of whole blood were incubated in 12 x 75mm polypropylene culture tubes (Falcon, Cockeysville, MD) in complete HBSS (Gibco, without indicator or sodium bicarbonate), containing 10 ug/ml Cytochalasin B (Sigma Chemical Co.,St. Louis, MO; frozen stock at 10 mg/ml in dimethyl sulfoxide[DMSO]), and HBSS (controls) or FMLP at a concentration of 10^{-6} molar in a final volume of 1.0 milliliter. The cells were preincubated for 15 - 20 minutes at 37° C in the presence of either rhG-CSF or rhGM-CSF (at a concentration of 50 units/ml) prior to the addition of the FMLP stimulus. Control cells were incubated in media without any of the agents. After a 45 minute incubation on an aliquot mixer (Ames) at 37° C, the mixtures were centrifuged in a Beckman Micro-fuge (Model E, Beckman Instruments, Palo Alto, CA) at 15,580 Xg and the supernatants were stored at -70° C in polypropylene tubes for analysis at a later time. Tubes which did not receive FMLP were used as controls to determine enzyme leakage induced by cytochalasin and rhG-CSF or rhGM-CSF alone. Duplicate tubes were also incubated with 0.1 ml 0.1% Triton X-100 (Sigma Chemical Co.) to determine total cellular enzyme content. Results were expressed as a percentage of the total cellular enzyme content (Henson et al., 1981).

Cytoplasmic lactate dehydrogenase (LDH) activity determination was based on the method of Wroblewski and LaDue (1955)(Sigma Technical Bulletin No. 340-UV) where the rate of

decrease in absorbancy at 340nm is measured as NADH is oxidized. A unit of activity is that which causes an initial rate of oxidation of one micromole of NADH per minute at 25°C.

B-glucuronidase acts on the substrate phenolphthalein mono-B-glucuronic acid, liberating free phenolphthalein, and the intensity of the resulting red color in alkali is measured at 550nm. The O.D. measured is proportional to the enzyme activity (Sigma Technical Bulletin No. 325).

The rate of lysis of Micrococcus lysodeikticus was used to determine the presence of lysozyme in the PMN supernatants (Shugar,1952). Lysozyme hydrolyzes B-1,4-glucosidic linkages in the mucopolysaccharide cell wall of the bacteria and one unit of activity is equal to a decrease in absorbancy, as determined at 450nm, of 0.001 per minute at pH 7.0 and 25°C.

All readings were made using a Beckman DU-64 spectrophotometer (Beckman Instruments, Palo Alto, CA).

ANALYSIS OF DATA

All tests were performed in triplicate except where noted, and results from multiple experiments representing a variety of PMN donors were pooled in a number of cases. Data is presented as means ± standard error of the mean. Levels of significance were determined by standard Student's t-test and one way analysis of variance (ANOVA).

CHAPTER III

RESULTS

CONCENTRATION OF IMMUNOMODULATORS USED

The stock concentration of the two CSFs was 50,000 units as obtained from Amgen. This is equivalent to 1.25 micrograms of the pure hormone. The range of concentrations of these agents used in this study was 0.05 to 5000 units/ml. This range was chosen because of the ease of diluting the stocks by ten-fold serial dilutions. This range was also chosen because of correlations with other work that is being done utilizing these hormones. For example, Golde (1989) demonstrated positive effects upon the hemopoietic system of AIDS patients utilizing a range of rhGM-CSF between 0.5 to 8 ug/kg of body weight in phase 1 clinical trials.

In a preliminary assay (Chemotaxis, Table 6), 100 and 1000 units/ml concentrations were utilized in order to conserve the stock solutions. If any effects had been observed, further testing would have been done utilizing the same range of the concentrations used in the other experiments.

ELICITATION OF MURINE POLYMORPHNUCLEAR LEUKOCYTES

The original premise of this investigation was to determine if the immunomodulators in question could return

normal function or enhance the function of the PMNs in the burned mouse model (BMM) (Holder, 1985). A variety of agents were tested that had been shown to elicit an inflammatory response in mice when injected intraperitoneally.(Baron and Proctor,1982; Hart et al.,1986) (Table 2) It should be noted that there was variability in the number of PMNs obtained by the stimulation of the various agents. For example, mice that had been injected with regular strength nutrient broth showed no increase in the total number of cells recovered at any of the post treatment times. However, if a comparison of the numbers of PMNs obtained is made, the large standard deviations demonstrate that there is a wide variation in the number of PMNs that were elicited. Also, the percentage of PMNs versus the total cell count was low. This is significant because of the large number of PMNs required to perform the various assays. For example, in the chemotaxis assays, 5.55 x 10^7 PMNs/ml are necessary to perform this assay and in the phagocytosis assays it is necessary to use 1 x 10^7 PMNs/ml. Since only 3.5 mls. of the HBSS which had been injected into the peritoneal cavities of the mice could be recovered, it would be necessary to use a large number of animals to obtain a sufficient quantity of PMNs to perform each experiment. In order to increase the percentage of PMNs in the preparations Percoll gradients were utilized. These attempts proved to be futile as no cells or very few cells were obtained from the purification process (data not shown). At this point the research shifted to utilizing PMNs from volunteer human

donors, which were much easier to obtain and purify.

PHAGOCYTIC KILLING ASSAY UTILIZING HUMAN PMNs

Believing that it would be difficult to demonstrate increased killing of P. aeruginosa by human PMNs treated with the CSFs, it was necessary to establish a system which would decrease the ability of the normal PMNs to kill the bacteria. Thus the suboptimal concentrations of normal human serum which would support opsonophagocytosis were determined (Table 3). When the serum was diluted 1:2 there was no reduction in the ability of the PMNs to kill. However, at the 1:5 and 1:10 dilutions there were 1/2 $\log_{10}$ reductions in the ability of the PMNs to kill the bacteria. At the 1:100 dilution of the serum there was one whole log reduction in the ability of the PMNs to phagocytize and kill the bacteria.

Serum diluted 1:5 was chosen as the suboptimal concentration of serum to use as the observed 1/2 $\log_{10}$ decrease when compared to controls still represented a significant reduction in bacterial number. Use of this dilution resulted in a final dilution of the serum of 1:50. The PMNs were then treated with varying concentrations of rhG-CSF (Table 4). The log reduction of the treated cells versus the untreated cells did not vary. Statistical analysis was not performed on the data because the means of the log reduction were virtually the same.

Testing of rhGM-CSF was not performed using this assay because no effect was noted when rhG-CSF was tested and

supplies of rhGM-CSF were limited.

CHEMOTAXIS AND CHEMOKINESIS

Determinations of the optimum concentration of FMLP and the optimum length of incubation of the chemotatic plates were necessary prior to any further experimentation utilizing the under-agarose method (Nelson, et al. 1975). The optimum concentration of the FMLP was found to be at 10^{-6} M at all times of incubation (Table 5). The optimum length of incubation was two hours, at which time the PMNs had migrated a projected distance of 4.9 $\pm$ 0.70 mm. from the edge of the well towards the chemoattractant FMLP (concentration of 10^{-6} M). When a comparison is made between the one hour and two hour incubation times, plates incubated two hours showed a three-fold increase in the distance that the PMNs migrated. As the length of time of incubation increased beyond the two hour incubation the distance migrated seemed to decrease. This phenomenon may be due to the PMNs migrating towards the highest concentration of FMLP. The gradient of the FMLP concentrations continues to develop during extended incubation and the highest concentration of the FMLP may be at the PMNs' point of origin. Hence the PMNs reverse their direction of travel back to their point of origin (Seligmann et al.,1982). For these reasons, the following experiments used an FMLP concentration of 10^{-6} M at an incubation time of two hours.

Once the optimal incubation times and concentration of

FMLP were determined, the effect that the immunomodulators would have on the response of PMNs to FMLP was studied (Table 6). Two concentrations, 100 and 1000 units/ml, of the immunomodulators were used and an untreated control was also included. Recombinant human G-CSF reduced the distance that the PMNs migrated towards the FMLP at both concentrations tested. In these experiments the distance migrated by the treated cells was about half the distance migrated by the untreated cells with a P value of <0.001 for both concentrations when compared to the untreated cells. In experiments with rhGM-CSF, a similar reduction in the distance migrated by the PMNs was seen. However, with this agent the reduction of the distance migrated was not as great as that seen with the rhG-CSF. The rhGM-CSF reduced the distance migrated by only one-third ($P < 0.02$, as compared to the untreated cells for both concentrations). These experiments were run simultaneously with both rhG-CSF and rhGM-CSF (i.e., 100 unit/ml of both agents with an untreated control on each plate) on the same plate to rule out any random error brought about by variances from day to day repetition of the assay. No difference was noted in the inhibition of chemotaxis by the cells pretreated with 100 or 1000 units/ml of either agent.

Chemokinesis is the random motility shown by PMNs in the presence of chemotactic agents (Gallin,1981). The immunomodulator rhG-CSF had no effect on increasing the random motility of the PMNs when compared to untreated PMNs

($P > 0.6$; Table 7) The distances shown on this table represent the diameters of the stained cell population which migrated outward from the well in projected millimeters. However, the cells treated with FMLP (a known chemoattractant) exhibited random motility when compared to the untreated cells ($P < 0.005$). Cells that were pretreated with rhGM-CSF did not exhibit any evidence of chemokinesis when compared to the untreated cells ($P > 0.5$; Table 8). Once again, chemokinesis was observed with cells that were treated with FMLP. ($P < 0.03$, when compared to the untreated cells)

Various concentrations of the two immunomodulators were tested to determine if they could act as chemotactic stimuli (Table 9). The distances in projected millimeters that the PMNs migrated towards the agent as compared to the distances that the PMNs migrated toward FMLP were similar. These data show that the immunomodulators do act as chemotactic stimuli, since a comparison of the agents (rhG-CSF and rhGM-CSF) with FMLP (a known chemoattractant) show similar results. These effects are seen at all concentrations of the agents tested (5.0 to 5000 units/ml) and all of the concentrations induced approximately the same amount of migration.

PEAK DNA TEMPLATE ACTIVITY AS DETERMINED BY MICROSPECTROPHOTOMETRIC ANALYSIS

The two wavelength method of microspectrophotometry requires the establishment of absorption curves for each time that the Feulgen stain is used (Swift and Rasch,1956). The

two wavelengths were determined for both sets of slides that were stained according to the calculations that were previously described. The wavelengths for the rhG-CSF treated cells were 550 nm for L_1 and 494 nm for L_2. The wavelengths for the rhGM-CSF treated cells were 562 nm for L_1 and 502 nm for L_2. The absorption curves from which these are determined from are shown in Figure 1.

The analysis of the nuclear DNA of the PMNs is presented in both tabular form and graphically as histograms. All measurements were limited to the nuclei of PMNs and were reported in arbitrary units as relative amounts of DNA.

The histograms were used because the DNA frequency distributions of the PMN cell population can be observed easily. The histograms also readily show any shifts of DNA content which may be due to an increase in DNA template activity or a change in ploidy level. Shifts in DNA template activity can also be seen as variations in the mean dye content as shown on the tables. Changes in DNA template activity are seen as changes in the relative DNA content which can be seen as a shift to the right or left on the histograms when compared to controls or as variations in the relative dye content as shown on the tabular form of data presentation. Changes in ploidy level are determined by looking at the control. For example, changes in ploidy level (i.e., haploid or diploid) are determined by comparing the means of the relative DNA content of the controls with the means of the treated cells. The nuclei that were measured

were plotted on the ordinate and the relative dye concentrations of the Feulgen stained DNA are plotted accordingly on the abscissa. One hundred nuclei were scored in order to give an adequate random representation of the cell population for each concentration of the immunomodulator being tested. The mean DNA values, standard deviations, and standard errors for each of the experiments are also noted on the appropriate figure or table.

There is a subtle increase in the relative DNA content at the 50 units/ml level of treatment when either immunomodulator is used as the treatment agent. (Tables 10 and 11) The histograms (Figures 3 and 4) show that there was no change in the ploidy level of the cells after treating the cells with the immunomodulators. Only histograms of the 0, 50 and 5000 units/ml treatment are included for comparison as there were no changes noted after any of the treatments (data not shown) except for a change in the means of the relative dye contents as shown in Tables 10 and 11.

EFFECT OF rhG-CSF AND rhGM-CSF ON NEUTROPHIL DEGRANULATION

Pretreatment of the PMNs with 50 units/ml of the immunomodulators was chosen because of the data obtained through the microspectrophotometric analysis that showed a change in DNA template activity at that concentration. It was also hoped that the changes in the DNA template activity at this concentration could also affect the degranulation of the PMNs since supplies of the immunomodulators were limited.

When comparisons were made between the cells treated with either rhG-CSF or rhGM-CSF and cells that were untreated but also stimulated with FMLP (Table 12), no differences in the release of B-glucuronidase and lysozyme by the PMNs were observed. Control studies, using the leakage of cytoplasmic lactic dehydrogenase (LDH) as an indicator of cellular damage, indicated that treatment of the PMNs with rhG-CSF, rhGM-CSF or FMLP did not result in cellular death (Table 13). Cellular death or damage would have been evidenced by increased levels of the enzyme LDH when the supernatants of the treated and control cells were assayed.

CHAPTER IV

DISCUSSION

Polymorphonuclear leukocytes are a major component of the first line of defense against microbial invasion or disposal of foreign bodies. In 1893, Metchnikoff first put forth the theory that phagocytes are essential for a good defense against microbial invasion by the host (Roitt,1988). Paubert-Braquet (1986) observed that the impairment of normal neutrophil function following thermal injury is the cause of increased susceptibility of the host to bacterial infection. A number of the impairments observed are diminished chemotactic responsiveness, diminished superoxide and hydrogen peroxide release, diminished arachidonate metabolite production, impairment of anti-bacterial activity, granulocytopenia, partial degranulation, and PMN leukocyte aggregation. He also observed that the impairment of normal neutrophil function usually does not occur for at least five days following the thermal injury and similar deficiencies have been seen in patients with other immunocompromising disease. It is this anergy which predisposes these patients to many life-threatening infections.

One goal of recent research is to identify those agents which will enhance or restore normal neutrophil function.

The agents which have come under close scrutiny are known as the colony-stimulating factors (CSFs). This general term refers to a group of glycoproteins which cause a proliferation and the differentiation of committed progenitor cells (Wing and Shadduck, 1985). CSFs were discovered in the late 1960's by two groups of scientists (Pluznik and Sachs in 1965; and Bradely and Metcalf in 1966) who developed techniques to grow colonies of the myeloid cell line in semi-solid media in vitro. The myeloid cell line is the lineage which includes erythrocytes, monocytes and granulocytes. Using these techniques it was found that it is necessary to have a feeder layer of cells (i.e., mature monocytes or macrophages) in order to induce the growth of colonies of a specific cell type such as the monocyte. From these experiments it was deduced that there is some sort of soluble growth factor or "colony stimulating cell" in the peripheral blood which releases these growth hormones. To date only a few cell types have been tested to determine whether or not they are capable of excreting these hormones. In situ hybridization studies using complementary DNA as probes are being utilized to identify those cells that have the messenger RNA necessary to produce CSFs. Because of the complexity of this method only a few cell types have been tested. To date only macrophages, T-lymphocytes, endothelial cells, fibroblasts and skin epithelial cells have been shown to synthesize one or more of the major CSFs (Metcalf,1985). The four CSFs that have been discovered to date are

macrophage CSF (M-CSF), granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), and multi-CSF or interleukin-3 (IL-3), which stimulates the development of mixed colonies of different cell types (Golde and Gasson,1988). This study centered around analyzing the effects that recombinant human G-CSF (rhG-CSF) and recombinant human GM-CSF (rhGM-CSF) could have upon enhancing or restoring neutrophil function.

Within the last decade research has shown that the CSFs do more than just bring about the differentiation of immature progenitor cells. These factors have been demonstrated by many authors to affect the locomotion of neutrophils (Gasson et al.,1984; Kitagawa et al.,1987; Buescher et al.,1988; Wang et al.,1988), the ability of neutrophils to phagocytize (both bacteria and latex particles)(Cohen et al.,1988; Greil et al.,1988; Weisbart et al.,1988; Tanaka et al,1989) and the degranulation of neutrophils (Weisbart et al.,1987; Kapp et al.,1988; Kaplan et al.,1989). It has also been shown that the factors themselves are chemoattractants (Gasson et al.,1984; Bronchud et al.,1988; Wang et al.,1988). As with any new avenue of research there is disagreement on the precise effects the CSFs do have on mature PMNs. Much of this controversy can be attributed to each experimentor using different assays to study the effects being attributed to the immunomodulators. For example, rhG-CSF does increase chemotaxis using the Boyden chamber technique (migration across polycarbonate or nitrocellulose filters) (Wang et al.,1988); while rhG-CSF decreases chemotaxis slightly

(Bronchud et al.,1988) when chemotaxis is assayed using the under agarose technique (Nelson et al.,1975). The purpose of this research is to elucidate some of the controversy surrounding the results achieved with rhG-CSF and rhGM-CSF. These agents were studied simultaneously to compare the effects that they will have upon a number of chemotactic functions. An attempt was made utilizing microspectrophotometric analysis of DNA template activity to determine if the agents increase the basal metabolic activity of the PMNs. It will also be determined if treating the PMNs with the agents will affect the amount of the enzymes, B-glucuronidase and lysozyme, that the PMNs will deliver upon degranulation. Future studies will have to be made in order to determine if rhG-CSF has any effect upon phagocytic intracellular killing of bacteria by PMNs pretreated with the hormone.

It was first necessary to determine the ideal model in which to test the hypothesis that these CSFs could restore normal neutrophil function or enhance the function of this mature cell type. The model that was chosen was the burned mouse model (BMM) (Holder, 1985). This model was ideal to test the hypothesis because, as has already been stated, following thermal injury neutrophil function is adversely affected and the animal becomes very susceptible to bacterial infection. Prior to doing the animal studies, <u>in vitro</u> testing had to be performed utilizing murine neutrophils so that later correlations could be made with any effects which

may occur during *in vivo* testing. *In vitro* testing would also allow screening of a number of agents in a short period of time. In order to proceed with *in vitro* testing of these agents, it was necessary to elicit PMNs from the mice by intraperitoneal injection of agents that were known to bring about an inflammatory response.

Baron and Proctor (1982) and Hart et al. (1986) demonstrated that it is possible to obtain PMNs through peritoneal injection of a variety of stimulatory agents such as nutrient broth, brain heart infusion broth or glycogen. However, as shown by the data (Table 2) this proved to be a rather formidable task in our laboratory. After testing a number of agents we realized that it would not be possible to obtain a sufficient number of PMNs for doing the *in vitro* assays. The percentage of PMNs obtained was not sufficient to provide enough cells for the assays as greater than 60% PMNs was rarely achieved. Further purification of these cell suspensions by Percoll gradients (Hart et al.,1986) did not prove to be beneficial because in most cases there were not any cells left after the purification process (data not shown). The number of cells necessary to perform the *in vitro* assays ranged from 5.55×10^7 to 1.0×10^7 . In order to obtain this number of cells it would require the use of an inordinarily large number of animals. Therefore, it was unreasonable to try to utilize the mouse for the *in vitro* testing of the agents, rhG-CSF and rhGM-CSF. One of the reasons for the difficulties in obtaining enough PMNs could

be that the blood volume of a mouse is only about 2cc and it would be difficult to obtain a sufficient number of PMNs for this study unless a large number of animals was used. Also, the strain of mouse we were using may have had some bearing on this due to some factors we were not aware of. At this point in the study it was decided to use normal human PMNs for the in vitro testing of the CSFs. This switch eliminated the previous difficulties in obtaining sufficient numbers of purified PMNs necessary to complete this study. The original plan to test the effect of the immunomodulators on murine PMNs in vitro before assessing their effectiveness in the burned mouse model could not be completed at this time. However, the interaction of rhG-CSF and rhGM-CSF with normal human PMNs utilizing in vitro methodologies would hopefully provide the preliminary results sought.

Early experiments were designed to test the effect of the immunomodulators upon neutrophil function. The first of these experiments was the phagocytic killing assay. In this assay it was hoped that it could be shown that the agent tested (rhG-CSF) could enhance the phagocytic and killing ability of the PMNs. When PMNs have been treated with CSFs, variable results were achieved showing the effect that these hormones have upon the phagocytic ability of the PMN. For example, rhG-CSF does not affect the phagocytic ability of hamster PMNs when the PMNs are pretreated with the immunomodulator (Cohen et al.,1988). However, Bronchud et al. (1988) were able to show that the phagocytosis of serum opsonized zymosan by human PMNs is increased two-fold.

Similar results have been obtained with rhGM-CSF (Lopez et al.,1986). However, while rhGM-CSF is able to enhance phagocytosis, it does not affect the intracellular killing of bacteria (Fleishmann et al.,1986).

In this particular part of the study it was shown that pretreatment of the PMNs with rhG-CSF had no effect upon the intracellular killing of P. aeruginosa by the mature human PMNs (Table 4). While this study had not addressed the discrepancy in the results of the phagocytosis studies, it has further confirmed the observations that pretreatment of the PMNs with rhG-CSF does not enhance the intracellular killing ability of the mature PMNs. An attempt was made to undermine the phagocytic ability of the PMNs by diluting the serum 1:5 (Table 3). Serum is necessary for proper opsonophagocytosis to occur (Ames et al.,1985) and it was hoped that by using suboptimal concentrations of serum it would be easier to demonstrate enhanced activity. We were not able to demonstrate increased antibacterial activity following treatment with rhG-CSF and our assay did not allow us to determine if the phagocytic ability of the PMNs was enhanced. While Bronchud et al. (1988) demonstrated that rhG-CSF increases phagocytosis, phagocytosis and intracellular killing of bacteria are two different functions of the phagocytic cell and involve entirely different mechanisms. Our results are somewhat suprising given the reported effects of rhG-CSF on chemiluminescence (Bronchud et al.,1988) and the generation of superoxide formation

(Kitagawa et al.,1987). Both of these observations would lead one to believe that the oxygen-dependent killing mechanisms of the PMNs would be stimulated, but no evidence for their ability to enable the PMNs to better kill ingested bacteria was found. However, if the treated cells were phagocytizing the bacteria more quickly than the controls, their ingested bacterial load could be greater. The fact that no differences were observed in the log decrease could be interpreted as providing evidence for some increase in killing if all the additional ingested bacteria were destroyed.

After the determination of ideal conditions (Table 5) for the study of the under agarose chemotaxis of mature human PMNs, it was shown that both the rhG-CSF and rhGM-CSF exhibited neutrophil inhibition factor (NIF) activity (Table 6) when the cells were pretreated with the agents. To assess the effect of the agents on chemotaxis PMNs were pretreated with either 100 or 1000 units/ml of rhG-CSF or rhGM-CSF. Both of these concentrations exhibited NIF activity. NIF activity is associated with rhGM-CSF (Gasson et al.,1984) and this study confirms these findings and demonstrates that NIF activity can also be attributed to rhG-CSF.

Once a cell has been exposed to these hormones, the locomotive ability of the cell is decreased. This effect is extremely important at the site of bacterial invasion and inflammation. Release of these hormones will localize the PMNs to the site of injury and prevent them from leaving. It

has been demonstrated that a number of cells are capable of producing and releasing CSFs (Metcalf,1985), and the NIF activity exhibited by both these agents is important for control of bacterial invasion or the repair of damaged tissue.

Chemokinesis is the random motility exhibited by neutrophils (Gallin,1981). This study showed that the agents, rhG-CSF and rhGM-CSF, did not have any effect upon the random motility of the PMNs as compared to cells that were treated with FMLP (Tables 7 and 8). A decrease in the spontaneous mobility (chemokinesis) is correlated with a lack of response to chemotactic factors (Nelson et al., 1975). This study does support this (Tables 6,7 and 8) because cells that were treated with either rhG-CSF or rhGM-CSF exhibited neither an increase in chemokinesis or chemotaxis. Testing was not performed to assess whether or not chemokinesis would have been affected if the cells had first been pretreated with the agents then incubated with FMLP. The two agents, rhG-CSF and rhGM-CSF, are both strong chemoattractants (Table 9) and they were able to induce chemotaxis at all of the concentrations tested from 5.0 to 5000 units/ml.

These results are important in understanding the role of these two agents in the immune response. It has been demonstrated that these two agents inhibit chemotaxis when the cells were preincubated with the agent (Table 6) and they also do not induce chemokinesis (Tables 7 and 8). It was demonstrated that the agents themselves act as a

chemoattractant for mature human PMNs (Table 9) at all ranges of the gradient of the agents tested. Since these agents exhibit NIF activity as well as being chemoattractants they may have an even more important role in the inflammatory response than originally believed.

This data explains how a peripheral PMN is brought to a site of infection or injury and how it is kept at the site once it has gotten there. Others have demonstrated that the CSFs are released by the cells of the phagocytic cell line (Golde and Gasson,1988). The "master" cell of the immune response is the T-lymphocyte which has been discovered to produce and release CSFs (Metcalf,1985). When these cells are exposed to specific antigens they produce CSFs which start the production of more white blood cells in the bone marrow (Matsumoto et al.,1987; Bronchud et al.,1988; Schwartz and Maher,1988; Vadhan-Raj et al.,1988) These same factors also attract the PMNs to the site of invasion as well as keeping the PMNs in the area. This is supported by the results obtained in this study. These results suggest that these agents would be an important addition to the therapy that immunocompromised patients receive. Research with animal models as well as Phase 1 clinical trials (Matsumoto et al.,1987; Bronchud et al.,1988; Kapp et al.,1988; Vadhan-Raj et al.,1988; Tanaka et al.,1989) show that these factors do reverse neutropenia and stimulate the proliferation of all white blood cells. Another study by Cheers et al. (1988) show that levels of all four CSFs (G-CSF, GM-CSF, M-CSF, and

multi-CSF) increase during bacterial infection, thus supporting the idea that these hormones play an important role in the immunune response.

Microspectrophotometric analysis of the PMNs treated with either rhG-CSF or rhGM-CSF showed an increase in the DNA template activity of these cells (Tables 10 and 11). In addition to testing the DNA template activity, the cells were also checked for any changes in ploidy level of the cell population. Figures 2 and 3 clearly show that there were no changes in the ploidy level of the cells as the histograms display an unimodal distribution of DNA content as expected. The ploidy level is a determination of the cell's chromosome content; for example, the cell may be haploid, diploid or polyploid. Since normal mature PMNs are believed to be incapable of any further cell division (Merrell,1975), it would not be expected that there would have been any changes in the ploidy level of these cells. The changes in DNA template activity are shown by the slight changes in the relative dye contents of the PMNs. (Tables 10 and 11) An interesting observation is that there was a change in the relative dye content at the 50 units/ml concentration of both the rhG-CSF and the rhGM-CSF. This change in template activity may affect the cell's production of enzymes or it may affect the cell in some other manner which we did not test for. An increase in template activity is indicative of an increase in DNA transcription. This increase in transcription could bring about a myriad of cellular changes

from a change in some biochemical process at the molecular level to a change in the locomotive ability of the cell. It was not possible to directly correlate this increase in activity at a concentration of 50 units/ml of the agents with any particular result obtained in this study. The increase in DNA template activity may be correlated with another cellular function if it would be possible to study whether or not this treatment brings about any changes in protein synthesis.

Since it was shown that a concentration of 50 units/ml had some effect upon the DNA template activity, PMNs were treated with this concentration of the agents in the exocytosis assays. Neither the rhG-CSF nor the rhGM-CSF had any effect upon the induced exocytosis of the PMNs as determined by quantitating the levels of B-glucuronidase and lysozyme released (Table 12). Another observation that should be noted is that the agents did not bring about any cellular damage or death as shown by the measurement of the cytoplasmic lactic dehydrogenase (LDH) (Table 13). These observations are in conflict with the work done by Coffey et al. (1988) in which they showed that rhGM-CSF stimulated guanylate cyclase (cGMP) activity but reduced adenylate cyclase (cAMP) activity in human neutrophils. It has been shown that increases in cellular levels of cAMP inhibit the ability of neutrophils to phagocytize, to kill pathogens and to release lysosomal enzymes. However, increased cellular levels of cGMP augment phagocytosis and the release of lysosomal enzymes. If these agents do stimulate the

production of cGMP as Coffey et al. have shown, this study should have shown that there was an increase in both phagocytosis and degranulation of the PMNs (Tables 4 and 12). It should be noted at this point that the agents were not tested by themselves to see if they would induce exocytosis without the presence of FMLP.

This study provides futher evidence that the two immunomodulators (rhG-CSF and rhGM-CSF) do affect mature human PMNs. While both rhG-CSF and rhGM-CSF act as chemoattractants, they both failed to induce chemokinesis in the normal PMNs. The fact that both of the agents showed NIF activity was significant since most of the research to date had shown that only rhGM-CSF had this ability. One avenue of future research should be to pursue testing the agents in some sort of model which would show if the agents could return normal function to impaired PMNs. In the animal studies cited in this study, no <u>in vitro</u> testing was done utilizing cells obtained from the animals. It would be possible to use the burned mouse model (Holder, 1985) in this fashion but it would be impossible to determine what effect if any the CSFs would have upon the murine PMNs unless <u>in vitro</u> testing utilizing PMNs from the mice were done. In the future it might be possible to devise micro-methods of the assays performed in this study to examine the effect of the CSFs upon the murine neutrophils. Since only healthy PMNs were employed in this study, it is not possible to determine if enhancement of normal neutrophil function is equivalent to restoration of dysfunctional PMNs.

Agent (a)	Time cells collected post treatment	Total cells recovered per ml	SD	Polymorphonuclear cells (PMN) recovered per ml	SD	% PMN
12% Sodium caseinate (2.5ml)	4 hrs	1.95×10^7(2)	0.56	4.94×10^6	0.005	25
12% Sodium caseinate (5.0ml)	4 hrs	4.42×10^6(3)	0.14	2.7×10^6	0.42	50
Regular strength NB (2ml)	overnight	1.22×10^7(6)	0.61	7.89×10^6	2.55	65
3% Proteose-peptone (2ml)	overnight	6.0×10^6(3)	1.18	3.0×10^6	0.43	50
Regular strength BHI (2ml)	overnight	4.96×10^7(7)	5.1	2.5×10^7	2.7	50
0.2% Glycogen (2.5ml)	overnight	5.34×10^6(4)	0.54	1.95×10^6	0.33	36
12% Sodium caseinate (2.5ml)	overnight	1.08×10^7(3)	0.32	5.16×10^6	0.84	48

a) All agents were tested in duplicate, except where noted.

b) Number of animals tested

c) Only one animal used due to similarity between BHI and NB.

Table 3

Determination of suboptimal serum dilution for the killing of Pseudomonas aeruginosa by polymorphonuclear leukocytes

Serum Dilution (prior to dilution in assay protocol)	Log_{10} Reduction (mean ± S.E.M.)[a]
NONE	2.04 ± 0.10 (8)[b]
1:2	1.92 ± 0.06 (8)*
1:5	1.46 ± 0.04 (4)**
1:10	1.23 ± 0.12 (8)***
1:100	0.94 ± 0.068 (4)****

a) Mean ± Standard error of the mean

b) number of determinations performed

* P > 0.3, compared to control

** P < 0.003, compared to control

*** P < 0.0002, compared to control

****P < 0.00004, compared to control

Table 4

Killing of Pseudomonas aeruginosa by human PMN treated with rhG-CSF.

Concentration of rhG-CSF (units/ml)	Log_{10} Reduction (mean ± S.E.M.)[a,b]
0	1.34 ± 0.06 (12)[c]
5.0	1.34 ± 0.04 (11)
50	1.31 ± 0.05 (12)
500	1.28 ± 0.05 (11)

a) Final dilution of serum was 1:50.

b) Mean ± Standard error of the mean

c) Number of determinations performed

Table 5

Determination of optimum concentration of FMLP and optimum length of incubation for chemotaxis and chemokinesis assays[a]

Molarity of FMLP	Length of incubation (Hrs.)	Distance migrated $\bar{x}$ ± SD
0	1	1.1 ± 0.00
10^{-5}		1.05 ± 0.05
10^{-6}		1.7 ± 0.10
10^{-7}		1.0 ± 0.00
0	2	0.0 ± 0.0
10^{-5}		2.05 ± 0.05
10^{-6}		4.9 ± 0.70
10^{-7}		2.0 ± 0.0
0	3	1.1 ± 1.10
10^{-5}		2.4 ± 0.40
10^{-6}		3.4 ± 0.20
10^{-7}		2.0 ± 0.0
0	4	1.0 ± 1.00
10^{-5}		2.0 ± 1.00
10^{-6}		2.5 ± 0.50
10^{-7}		1.0 ± 1.00

a) Run in duplicate

b) Distance migrated in projected millimeters.

Mean ± Standard deviation

Table 6

Effect of rhG-CSF and rhGM-CSF on polymorphonuclear leukocyte (PMN) chemotaxis

Pretreatment[a] agent	Concentration	Distance migrated $\bar{x}$ ± S.E.M.[b]
rhG-CSF	0 units/ml	4.27 ± 0.24 (15)[c]
	100 units/ml	2.21 ± 0.41 (14)*
	1000 units/ml	2.57 ± 0.39 (14)*
rhGM-CSF	0 units/ml	4.27 ± 0.24 (15)
	100 units/ml	3.20 ± 0.37 (10)**
	1000 units/ml	3.28 ± 0.26 (7) **

a) Pre-incubated with agent for 20 minutes

b) The chemotactic agent was FMLP (10^{-6}M) with the plates being incubated for 2 hours. The distance measured is in millimeters from the edge of the well.

c) Number of determinations; all experiments were run in duplicate

* $P < 0.001$, compared to untreated PMNs

** $P < 0.02$, compared to untreated PMNs

Table 7

Chemokinesis induced by rhG-CSF

Agent	Concentration	Distance migrated $\bar{x} \pm$ S.E.M.[a]
——	——	62.13 ± 0.59 (15)[b]
rhG-CSF	0.5 Units/ml	62.8 ± 0.99 (5)*
	5.0 Units/ml	62.2 ± 0.73 (10)
	50 Units/ml	62.12 ± 0.48 (8)
	500 Units/ml	62.5 ± 0.39 (6)
	5000 Units/ml	63.0 ± 0.61 (4)
FMLP	10^{-6}M	66.7 ± 1.48 (9)**

a) 2 hour incubation, distance is diameter of cells in projected millimeters

b) Number of determinations

* $P > 0.6$, compared to negative control

** $P < 0.005$, compared to negative control

Table 8

Chemokinesis induced by rhGM-CSF

Agent	Concentration	Distance migrated $\bar{x}$ ± S.E.M.[a]
----	----	63.0 ± 0.68 (8)[b]
rhGM-CSF	0.5 units/ml	63.25 ± 1.08 (4)*
	5.0 units/ml	62.0 ± 1.27 (4)
	50 units/ml	63.0 ± 0.93 (4)
	500 units/ml	62.75 ± 1.67 (4)
	5000 units/ml	63.25 ± 0.64 (4)
FMLP	10^{-6}M	65.75 ± 0.41 (4)**

a) 2 hour incubation, distance is diameter of cells in millimeters. Mean ± Standard error of the mean.

b) Number of determinations

* $P > 0.5$, compared to control

** $P < 0.03$, compared to control

Table 9

Induction of chemotaxis by rhG-CSF and rhGM-CSF

Chemotactic agent	Concentration	Distance migrated $\bar{x}$ ± S.E.M.[a]
———	———	0.0 ± 0.0 (63)[b]
rhG-CSF	5.0 Units/ml	4.0 ± 0.80 (5)*
	50 Units/ml	5.75 ± 1.08 (4)
	500 Units/ml	5.33 ± 0.69 (6)
	5000 Units/ml	4.83 ± 0.15 (6)
rhGM-CSF	5.0 Units/ml	6.4 ± 0.54 (5)*
	50 Units/ml	5.25 ± 0.82 (4)
	500 Units/ml	6.0 ± 0.56 (5)
	5000 Units/ml	4.6 ± 0.60 (5)
FMLP	10^{-6}M	4.0 ± 0.47 (16)

a) 2 hour incubation, distance in mm from well edge

b) Number of determinations, experiments run in duplicate

* $P < 10^{-6}$, compared to control. P value determined by one-way ANOVA.

Table 10

Determination of peak DNA template activity using rhG-CSF

Concentration of rhG-CSF (Units/ml)	Average dye concentration	SD[a]	S.E.M.[b]
0	6.89	0.576	0.0586
0.05	6.22	0.431	0.0431
0.50	6.54	0.453	0.0453
5.00	6.64	1.232	0.123
50.0	7.18	1.393	0.143
500	6.55	1.046	0.105
5000	6.39	0.477	0.0479

a) Standard deviation

b) Standard error of the mean

Table 11

Determination of peak DNA template activity using rhGM-CSF

Concentration of rhGM-CSF (Units/ml)	Average dye concentration	SD[a]	S.E.M.[b]
0	5.10	0.668	0.068
0.05	5.94	1.092	0.115
0.50	5.64	0.882	0.088
5.00	4.97	0.735	0.075
50.0	6.49	0.873	0.088
500	5.16	0.547	0.055
5000	5.49	0.580	0.058

a) Standard deviation

b) Standard error of the mean

Table 12

Effect of rhG-CSF and rhGM-CSF on
FMLP-induced exocytosis

Treatment[a]	B-Glucuronidase[b] ($\bar{x}$ ± S.E.M.)[c]	Lysozyme[b] ($\bar{x}$ ± S.E.M.)
PMNs only	14 ± 0.75	60 ± 4.52
PMNs + FMLP (10^{-6})	47 ± 2.80	84 ± 2.03
PMNs + FMLP (10^{-6}) + rhG-CSF (50 units/ml)	36 ± 2.13*	79 ± 3.26***
PMNs + FMLP (10^{-6}) + rhGM-CSF (50 units/ml)	42 ± 2.80**	87 ± 1.77****

a) culmination of two experiments, five determinations each experiment

b) percentage of total enzyme content

c) Mean ± Standard error of the mean

* P > 0.07, compared to cells treated with FMLP

** P > 0.2, compared to cells treated with FMLP

*** P > 0.5, compared to cells treated with FMLP

**** P > 0.3, compared to cells treated with FMLP

Table 13

Release of cytoplasmic LDH in the exocytosis assay

Treatment[a]	Percent of total content released ($\bar{x}$ ± S.E.M.)[b]
PMNs only	21 ± 2.74
PMNs + FMLP (10^{-6})	22 ± 0.92
PMNs + FMLP (10^{-6}) + rhG-CSF (50 Units/ml)	20 ± 0.97
PMNs + FMLP (10^{-6}) + rhGM-CSF (50 Units/ml)	20 ± 0.50

a) Culmination of two experiments, five determinations each experiment.

b) Mean ± Standard error of the mean

Figure 1.

Absorption spectrum of Feulgen stained polymorphonuclear leukocytes (PMNs). The graph represented by a broken line is the absorption spectrum for the PMNs treated with granulocyte-colony stimulating factor (G-CSF); where L_1= 550 nm and L_2= 494nm. The graph represented by the unbroken line is the absorption spectrum for the PMNs treated with granulocyte macrophage- colony stimulating factor (GM-CSF); where L_1= 562nm and L_2=502nm.

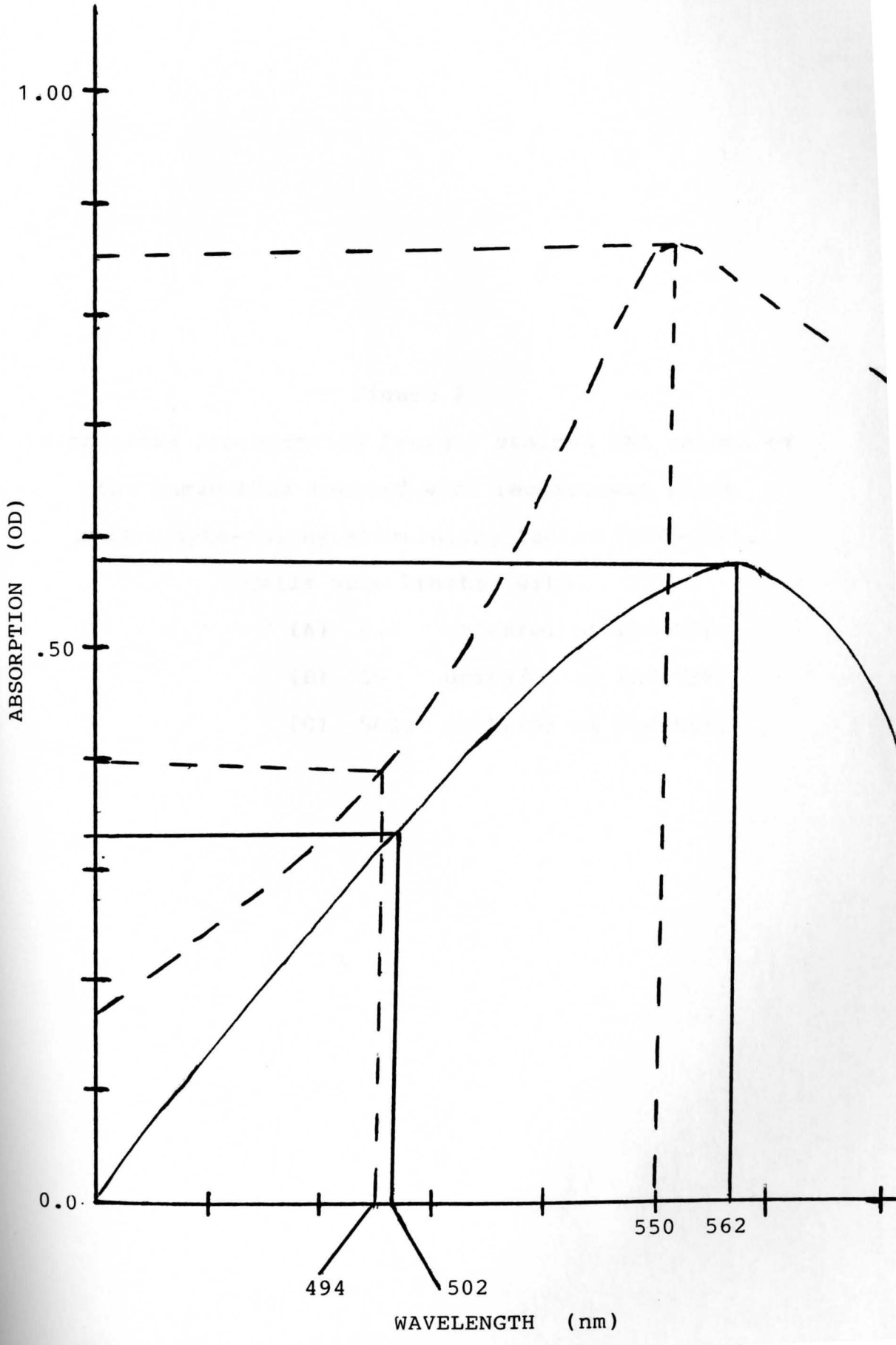

1.00
.50
0.0
ABSORPTION (OD)
494
502
550
562
WAVELENGTH (nm)

Figure 2.

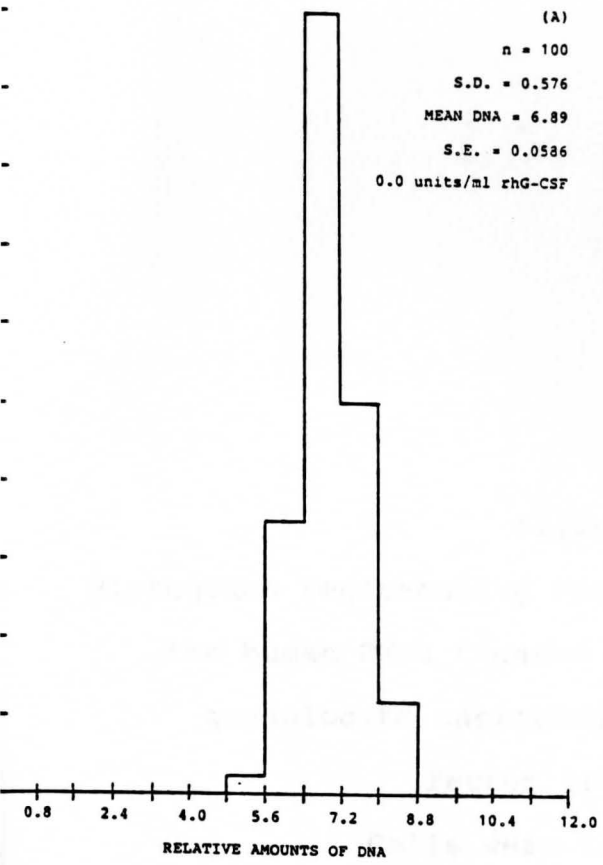
Histograms representing Feulgen stained DNA values of the human PMNs treated with recombinant human granulocyte-colony stimulating factor (rhG-CSF).

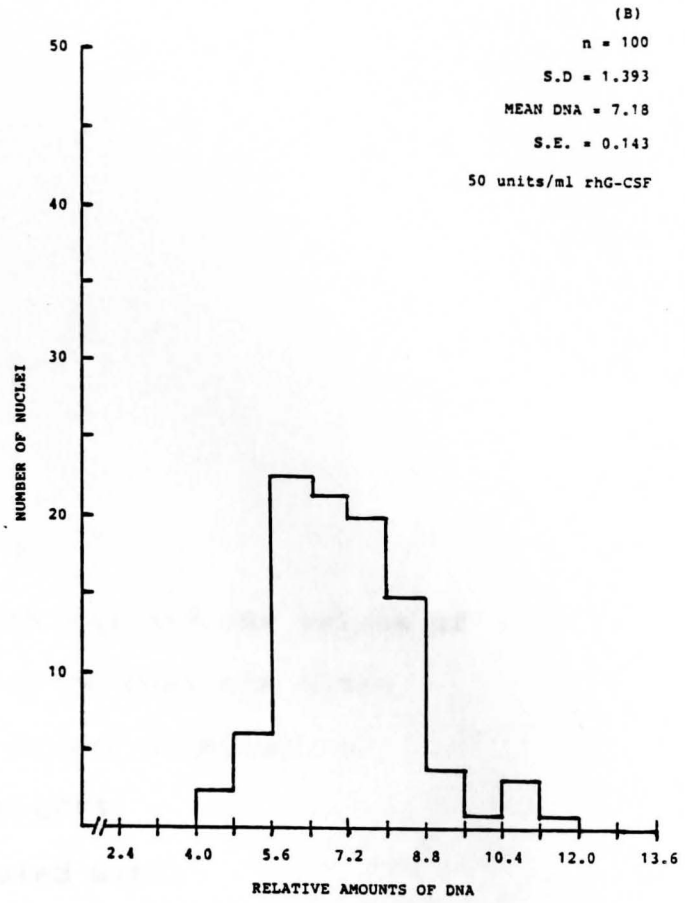
Cells were treated with:

(A) 0.0 units/ml of rhG-CSF

(B) 50 units/ml of rhG-CSF

(C) 5000 units/ml of rhG-CSF.


(A)
n = 100
S.D. = 0.576
MEAN DNA = 6.89
S.E. = 0.0586
0.0 units/ml rhG-CSF
0.8
2.4
4.0
5.6
7.2
8.8
10.4
12.0
RELATIVE AMOUNTS OF DNA


(B)
n = 100
S.D = 1.393
MEAN DNA = 7.18
S.E. = 0.143
50 units/ml rhG-CSF
NUMBER OF NUCLEI
50
40
30
20
10
2.4
4.0
5.6
7.2
8.8
10.4
12.0
13.6
RELATIVE AMOUNTS OF DNA

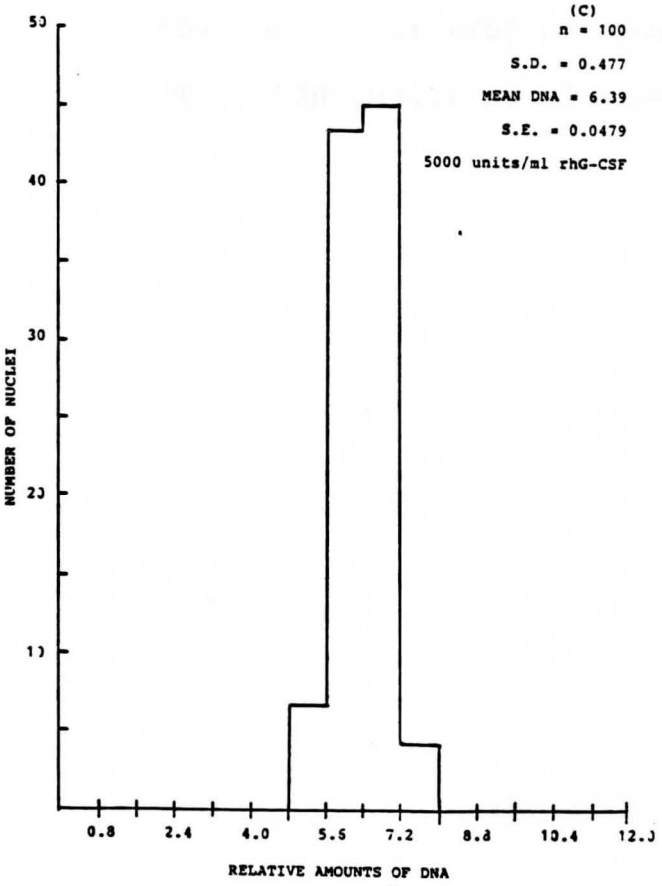

(C)
n = 100
S.D. = 0.477
MEAN DNA = 6.39
S.E. = 0.0479
5000 units/ml rhG-CSF
NUMBER OF NUCLEI
40
30
0.8
2.4
4.0
5.6
7.2
8.8
10.4
12.0
RELATIVE AMOUNTS OF DNA

Figure 3.

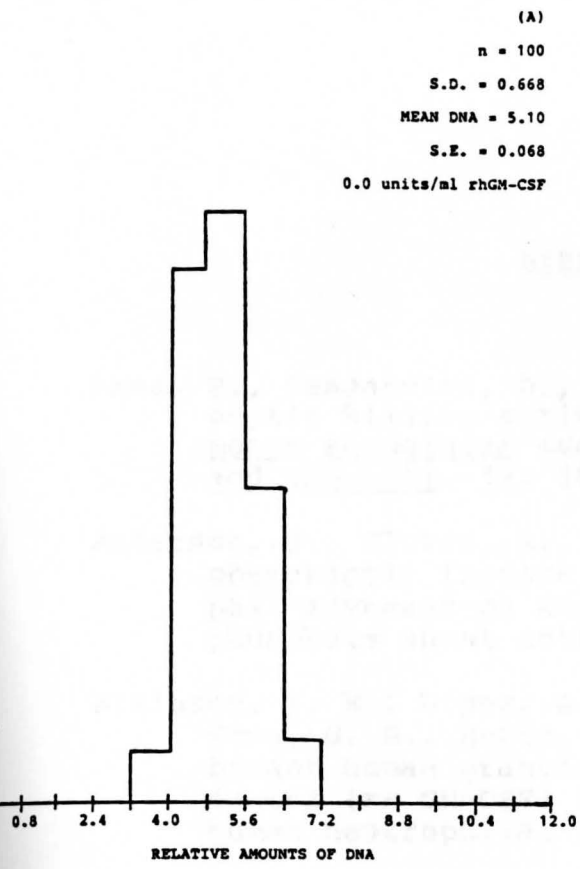
Histograms representing Feulgen stained DNA values of the human PMNs treated with recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF).

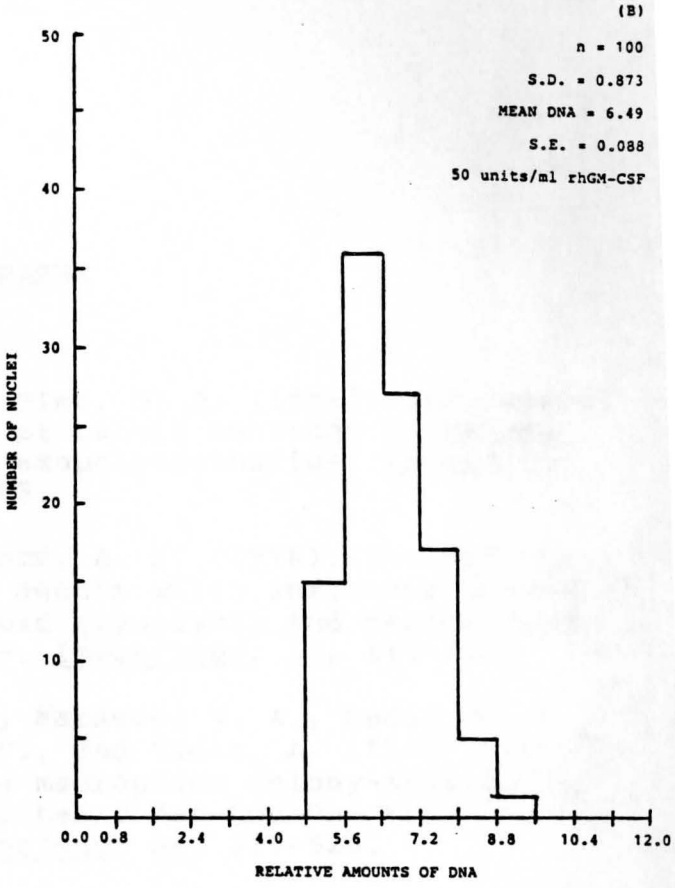
Cells were treated with:

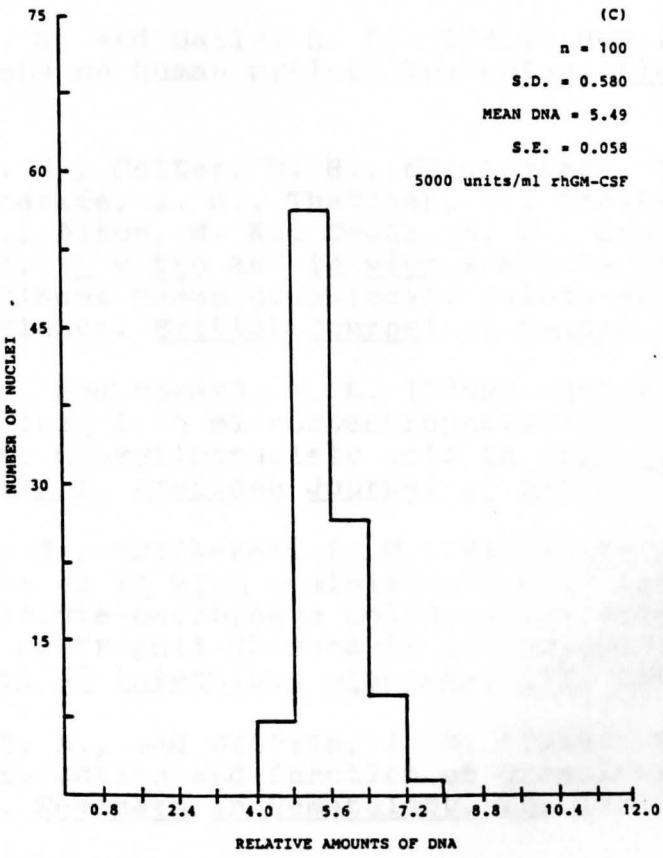
(A) 0.0 units/ml of rhGM-CSF

(B) 50 units/ml of rhGM-CSF

(C) 5000 units/ml of rhGM-CSF.


(A)
n = 100
S.D. = 0.668
MEAN DNA = 5.10
S.E. = 0.068
0.0 units/ml rhGM-CSF
0.8
2.4
4.0
5.6
7.2
8.8
10.4
12.0
RELATIVE AMOUNTS OF DNA


(B)
n = 100
S.D. = 0.873
MEAN DNA = 6.49
S.E. = 0.088
50 units/ml rhGM-CSF
NUMBER OF NUCLEI
50
40
30
20
10
0.0 0.8
2.4
4.0
5.6
7.2
8.8
10.4
12.0
RELATIVE AMOUNTS OF DNA


(C)
n = 100
S.D. = 0.580
MEAN DNA = 5.49
S.E. = 0.058
5000 units/ml rhGM-CSF
NUMBER OF NUCLEI
75
60
45
30
15
0.8
2.4
4.0
5.6
7.2
8.8
10.4
12.0
RELATIVE AMOUNTS OF DNA

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