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SYNTHETIC PEPTIDE EPITOPES OF HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND MACROPHAGE COLONY-STIMULATING FACTOR

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Robert Kreisberg

March 1988

DEDICATION

This work is dedicated to Mary, for allowing me to test her patience and her to test mine.

ACKNOWLEDGEMENTS

I would like to thank Dr. Robert N. Moore for his interest, patience, and unique sense of humor. I would also like to thank Dr. David A. Bemis for his time and suggestions, and would especially like to thank Dr. Barry T. Rouse for his photographic memory and inquisitiveness.

ABSTRACT

Colony stimulating factors (CSFs) are a functionally defined class of glycoprotein growth factors that stimulate the proliferation and differentiation of immature precursor cells to mature granulocytes and/or macrophages and have an additional role as central regulators of inflammatory and immune responses. Human granulocyte/macrophage colony-stimulating factor (GM-CSF) and human macrophage colony-stimulating factor (M-CSF) have recently been cloned and the nucleotide sequence determined. The predicted amino acid sequences were analyzed to detect potential antigenic determinants by contiguous hexameric hydrophilicity estimates. Two 18 amino acid peptides were produced, one representing the major predicted epitope of human GM-CSF and the other representing a potentially significant epitope of human M-CSF. The peptides were studied alone, and in combination with native CSFs to determine if they had any biological activity. The peptides were also used as antigens in the production of rabbit polyclonal antibodies.

Human GM-CSF peptide had no biological activity, as determined by tritiated thymidine (^{3}H-TdR) incorporation, alone or in combination with native CSFs. The rabbit anti-human GM-CSF peptide antibody was shown to: 1) react with GM-CSF peptide by enzyme linked immunoassay (ELISA), 2) detect human as well as murine GM-CSF by ELISA and, 3) possess limited neutralizing activity, as determined by ^{3}H-TdR incorporation and soft agar

assay, for murine GM-CSF. The human GM-CSF peptide, therefore, appears to have potential for the development of a heterogeneous ELISA for GM-CSF.

Human M-CSF peptide alone, like human GM-CSF peptide had no effect on the rate of ^{3}H-TdR incorporation by murine marrow cells. However, in combination with murine L cell M-CSF and recombinant human M-CSF the M-CSF peptide caused an increase in the rate of DNA synthesis. Additional experiments indicated the increase in the rate of DNA synthesis was due to the presence of human M-CSF peptide. M-CSF peptide in combination with recombinant human G-CSF or murine GM-CSF did not result in an increase in ^{3}H-TdR incorporation. The anti-human M-CSF peptide antibody did detect human M-CSF peptide by ELISA. However, the anti-human M-CSF did not detect, by ELISA, recombinant human M-CSF or L cell M-CSF. The M-CSF peptide appears to have potential for biological studies, but not for development of a heterogeneous ELISA for M-CSF.

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LIST OF ABBREVIATIONS

CSFs	Colony Stimulating Factors
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
M-CSF	Macrophage-Colony Stimulating Factor
RPMI 1640	RPMI 1640 growth medium
^{3}H-TdR	Tritiated-thymidine
ELISA	Enzyme-linked immunoassay
cpm	Radioactive counts per minute
hr	Hour
min	Minutes
Ci	Microcurie
ml	Milliliter
ul	Microliter
ng	Nanogram
ug	Microgram

CHAPTER 1

INTRODUCTION

Colony stimulating factors (CSFs) are a functionally defined class of glycoprotein growth factors that stimulate the proliferation and differentiation of immature precursor cells to mature granulocytes and/or macrophages (48). Moore has recently proposed that CSFs may have an additional role as central regulators of inflammatory and immune responses (31,32). CSFs existence as soluble factors was initially established through the replacement of a "feeder cell" requirement for colony formation by a cell free culture medium "conditioned" by feeder cells (42). However, not until the late seventies was it established that there were at least four subclasses of CSFs, with differing properties, including target cell specificity. Of these four subclasses one exclusively stimulates macrophage production (M-CSF) (48); the second, neutrophilic granulocyte and macrophage production (GM-CSF) (5); the third, neutrophilic granulocyte production (G-CSF) (10,39); and the fourth can stimulate production of neutrophilic granulocytes and macrophages, eosinophils, megakaryocytes, and erythroid cells (interleukin-3, IL-3; 8,29).

Evidence supporting the role of CSFs as proliferative and differentiation factors accumulated during the early to middle seventies. The first such evidence was presented in 1969 when Bradley, et al., (4) injected a crude preparation of murine M-CSF into adult mice eliciting a significant polymorphonuclear

leukocytosis within 24-48 hours. In 1971 Stanley and Metcalf (49) reported the same results after injecting partially purified human M-CSF into neonatal mice. Subsequently several communications reported that the level of CSFs in animal serum increased following the injection of microbial products (30,43) and during infection with gram negative and gram positive facultative intracellular pathogens. These elevated levels of serum CSFs along with observations that some of these treatments also stimulated increased levels of circulating CSFs and hyperplasia of monocyte-macrophage rich organs further supported the role of CSFs as proliferation and differentiation factors (1,28,52).

Evidence supporting the role of CSFs as regulators in inflammation and immune responses, in addition to proliferation and differentiation, began to accumulate between 1978 and 1980. The first support of such a role came from Burgess and Metcalf (6,7) who showed that exposure of bone marrow cells to M-CSF derived from mouse lung-conditioned medium resulted in a significant increase in ribonucleic acid (RNA) and protein synthesis without increased deoxyribonucleic acid (DNA) synthesis. This indicated that CSF may be able to stimulate biosynthetic processes unrelated to cell division. Within two years of this discovery other researchers found that M-CSF stimulated macrophages to produce enhanced levels of regulatory molecules including E prostaglandins (24), interleukin-1 (34), α /ß types of interferon (32,33,35), and plasminogen activator (17,27). M-CSF was found also to influence the functional state of macrophages. Macrophages

exposed to M-CSF expressed enhanced tumorcidal activity (55), augmented killing of Leishmania tropica (18), and enhanced responsiveness to bacterial lipopolysaccharide (LPS; 35).

During the past two years the four CSFs have been purified, sequenced, and obtained in recombinant form. These include M-CSF (21), G-CSF (38), GM-CSF (9,11,26), and IL-3 (13). All of these human CSF molecules are currently being produced, in recombinant form, by two different biotechnology firms. Amgen of Thousand Oaks, California is producing the human G-CSF molecule, and Genetics Institute of Cambridge Mass. is producing the human GM-CSF molecule. Cetus Corporation of Emeryville, California produces M-CSF while other companies including Immunex of Seattle, Washington as well as those listed above produce IL-3. All of these companies hope to find a large market for their CSFs. If results of two recent clinical tests are any indication, Amgen and Genetics Institute will probably have a large market.

In the first clinical test (23) human G-CSF was given to sixteen human patients receiving chemotherapeutic drugs, which are extremely toxic to the bone marrow, for cancers of the genital-urinary tract. The results of this clinical test showed a significant decrease in the time required for the depleted bone marrow to regenerate itself.

In the second clinical test (23) human GM-CSF was given to sixteen acquired immune deficiency (AIDS) patients, all of whom had very low white cell counts as a consequence of their AIDS

infection. The results indicated that human GM-CSF increased the white cell count in a dose dependent manner.

With all the new knowledge being acquired about CSFs, the development of a rapid and relatively inexpensive, assay for CSFs would have many applications (37). These applications include: 1) aiding in standardized measurement of CSFs within and between laboratories; 2) measuring the response to vaccination procedures designed to generate protective cell mediated immunity; 3) to quantitate lymphocyte function tests for the assessment of allergic and immunodeficient states; 4) to introduce quantitation in the control of immunosuppressive and immunosupportive therapy; and 5) as a basis for research into antagonists and promoters of CSF activity.

CSFs can be found in a variety of fluids including serum, urine, and medium conditioned by organs, tissues, and various cell types (3,39,41,44,45). Two bioassays are routinely used for the measurement and detection of CSFs. The first is a soft agar assay (36) in which bone marrow cells and the sample of interest are suspended in soft agar. Within seven days CSF stimulated bone marrow cells form discrete colonies which correlate directly to units of CSF activity in the sample. A second common assay is a blastogenesis assay based on incorporation of ^{3}H-TdR (36) which requires a total of three days. In this assay bone marrow cells incorporate ^{3}H-TdR in proportion to the concentration of CSFs in the sample. A radioimmunoassay (RIA) is also available (48). However, this assay is not as commonly used as the previously

mentioned assays and is specific for M-CSF. Use of this RIA yields results in approximately twenty four hours.

These three assays have particular drawbacks which include, expense, time, technical expertise, the requirement for tissue culture facilities, and the use of radioactive materials. It is known also that some of the biotechnology firms including Amgen and Cetus have assays available for measurement of specific CSFs. However, even though other assays do exist it appears as if they will not be available commercially for use in the near future.

Although the human CSFs have been purified, sequenced, and cloned their availability to scientific laboratories is quite limited which hinders the development of new assays and research. One way around the problem of limited availability is to determine the major antigenic determinants (epitopes) of these molecules and synthesize peptides representing these determinants.

Presently there are many different methods available for determining the epitopes of proteins. These include hydropathy (25), structural parameters (40), three dimensional analysis (51), and hydrophilicity (20). In this study the epitopes of human GM-CSF and human M-CSF were determined using the hydrophilocity technique of Hopp and Wood and secondary structure analysis (14).

Statement of Purpose

The primary purpose of this investigation was to produce epitopes of human GM-CSF and M-CSF. The peptide epitopes would be used to attempt to generate specific polyclonal antibodies with the

intention of: 1) developing specific detection assays for the parent molecules and 2) neutralizing the biological activity of the parent molecules. Next, the peptide would be investigated to determine whether or not they had any biological activities. First, the peptides would be studied alone to see if they could mimic the activities of the parent molecules. Second, the peptides would be investigated to see if they could affect the activity of the parent CSFs. Some of the biological effects that could be exerted by the peptides with the parent molecules include enhancement, inhibition, or no effect at all.

CHAPTER 2

MATERIALS AND METHODS

Materials

M-CSF had been purified previously from serum-free culture medium conditioned by murine L929 cells as described by Waheed and Shadduck (54). The specific activity of the preparation was 5×10^7 units/mg protein and the CSF was stored as a stock solution of 4×10^5 units/ml of 0.01M phosphate buffered saline, pH 7.2, (PBS) containing polyethylene glycol (M.W.= 3350; Sigma).

Recombinant human GM-CSF and recombinant human G-CSF (Amgen, Thousand Oaks, CA.) was obtained from Dr. Lothrop (University of Tennessee College of Veterinary Medicine, Knoxville, TN). The G-CSF was packaged at a concentration of 530 ug/ml 10mM Na Acetate buffer (pH4.1) containing 1% heat inactivated canine serum. The GM-CSF was packaged at a concentration of 100 ug/ml 25mM HEPES containing 1% heat inactivated canine serum.

Recombinant human M-CSF was obtained from Cetus Corporation (Emeryville, CA). It was received at a concentration of 1×10^5 units/ml. The carrier protein was bovine serum albumin (BSA, Sigma), 1.0 mg/ml final volume.

Murine GM-CSF was purchased from Genzyme (Boston, MA). The murine GM-CSF had an activity of 5,000 units/ml PBS containing 1% BSA.

Methods

Protein Determination

Protein concentrations of sodium sulfate immunoglobulin fractions were determined using the Bio-Rad Protein Assay System (Bio-Rad, Richmond, CA). This system is based on Bradford's method (2).

Epitope Determination

The method of Hopp and Woods (20) was used to predict the epitopes of human GM-CSF and M-CSF. This method involved assigning each amino acid in the proteins a hydrophilicity value. These values were then averaged in groups of six down the length of the proteins and plotted. The plots were then scanned to locate the most significant hydrophilic peak. The 18 amino acids within, and immediately adjacent to, the highest peak was predicted to contain a significant epitope of that CSF. To increase the chance of correctly predicting the epitopes of human GM-CSF and M-CSF a computerized secondary structure analysis (Microgenie, Beckman, Palo Alto, CA) was also run.

Two 18-Mer peptide epitopes were synthesized by Analytical Services at the University of Tennessee.

Antibody Production

Two female New Zealand white rabbits (Myrtles Rabbitry Thompson Station, Tn) were used to produce epitope antisera. One rabbit was used to produce GM-CSF peptide antisera, the other to produce M-CSF peptide antisera. The protocol for the production of both antisera was the same. One ml of the appropriate peptide at a concentration of 1.0 mg/ml PBS, was completely mixed with an equal volume of Freund's complete adjuvant (Sigma). The emulsion was injected subcutaneously (sc) as 0.25 ml at each of four sites, one above each appendage. After one month the rabbit was injected sc over each of its four appendages with 0.25 ml of peptide, without Freund's, at a concentration of 0.5 mg peptide/ml PBS. Seven days later the rabbit was bled from the central ear artery. The injection and bleeding procedure for peptide in PBS was continued at 30 day intervals.

Precipitation of Immunoglobulins

Generally 20 to 40 ml of blood was obtained from the central ear artery. The blood was placed in a 37° C incubator for one hr, rimmed, and then placed in a 5°C refrigerator overnight. The blood was then centrifuged (IEC HN-SII Centrifuge, Needham HTS., MA) at 600 x g for 20 min at room temperature. The serum was carefully removed. With continuous stirring an equal volume of 36% sodium sulfate (Na_2SO_4, Sigma) was slowly added to the serum and stirred at room temperature for 2 hr. The heavily precipitated suspension

was centrifuged at 500 x g for 15 min at room temperature. The resulting supernatant was discarded and the pellet resuspended in a volume of PBS equal to the original volume of serum. The Na_2SO_4 precipitation was repeated and the resulting pellet was suspended in a volume of PBS equal to half the original volume of serum and dialyzed at constant volume against several changes of PBS for 3 days at 5°C. The Na_2SO_4 precipitated serum was filtered through a 0.2 μm filter (Gelman, Ann Arbor, MI), aliquoted, and frozen at - 20°C until use (19,22).

Non-Competitive Enzyme-Linked Immunoassays

ELISA's were run in 96 well Immulon One plates (Dynatech, Chantily. VA). All washes were carried out using 100 uls of PBS-.05% Tween (Tween20; Sigma) per well. O-phenylenediamine (OPD; American Qualex, La Mirada, CA) was prepared 5 min prior to use at a concentration of 2mg/5mls carbonate buffer (pH9.6). The appropriate antigen was made up in .01M carbonate buffer (pH9.6). Fifty ul of the antigen was then placed into each well and allowed to adsorb to the at 5°C overnight. The plate was washed, then 50 ul of PBS-3% BSA was added to each well and the plate placed on a shaker (Scientific Products Rotor V, Miami, FL) for 30 min at 37°C. The plate was washed and 50 ul of the primary antibody in PBS-Tween was added to each well. The plate was placed on a shaker for 60 min and then washed. Next, 50 ul of peroxidase conjugated goat anti-rabbit IgG (Cooper Biomedical, West Chester, Pa), diluted 1:1000 in PBS-Tween, was added to each well. The plate was placed

on a shaker for 60 min at 37°C. The plate was washed and 50ul of OPD was added to each well. The reaction was stopped after 20 min with 2.5M H_2SO_4. The absorbance of individual wells was measured at 490nm or 540nm using an Automated Microplate Reader (Bio-Tek Instruments, Burlington, VA). The control wells received no primary antibody.

Tritiated Thymidine Assays

Tritiated thymidine assays were performed as described by Moore and Rouse (36). Briefly, femoral bone marrow cells (BalbC/ByJ; Breeding Facilities at Univ. of Tn. Memorial Rsch. Hospital, Knoxville, TN) in RPMI medium1640 (Gibco) containing 10% fetal bovine serum (Hyclone Sterile Systems, Logan, UT) and antibiotics were placed in 96-well flat bottom microtiter plates (Corning) at a concentration of 7.5 x 10^4cells/well. Next, the appropriate concentrations of peptide epitopes, antibodies and CSFs were added. The plate was then incubated for a total of 72 hr at 35° C in 7% CO_2. During the last 6 hr, 1μCi of ^{3}H-TdR (ICN, specific activity of 6.7Ci/mM) was added to each well. Plates were then frozen at -70° C until harvested. The cultures were harvested onto glass fiber filter strips (Cambridge Technology, Inc., Cambridge, MA) with an automated harvester (PHD Cell Harvester, Cambridge Technology Inc., Cambridge, MA). The ^{3}H-TdR incorporation was determined in liquid scintillation cocktail (Ecolume, ICN) in a Beckman LS 7000 Scintillation Counter. Results are the mean of triplicate samples plus or minus one standard error.

Soft Agar Assay

Soft agar assays were set up as described by Moore and Rouse (36). First, 10^5 femoral bone marrow cells per ml were suspended in warm (42°C) RPMI medium 1640 containing 15% fetal bovine serum, antibiotics, and 0.4% agar (Bacto-agar; Difco). The above mixture was then added to 35-mm petri-dishes containing the appropriate concentrations of peptide epitopes, antibodies and CSFs. The final volume per plate was 1.0 ml. After the agar hardened, the dishes were incubated for 5 days at 35°C in 7% CO_2. The dishes were examined using an Olympus CK inverted microscope (Olympus Corp. of America, New Hyde Park, N.Y.) at 40x magnification, and proliferative centers ($\geq$15 cells) scored. Results are the mean of triplicate samples plus or minus one standard error.

Protein A-Sepharose

Staphylococcal protein A, covalently linked to Sepharose CL-4B (protein A-Sepharose) was obtained from Pharmacia (Pharmacia Inc., Piscataway, N.J.), swollen in .01M PBS (pH8.4), washed, and placed in a 1.5 ml microfuge tube (American Scientific Products, Stone Mountain, GA). The column bed volume was approximately 0.5 ml. One milliliter of the appropriate antibody was added to the bed volume and placed on a mixer (Sarstedt Safety Monovette Mixer, Princeton, N.J.) overnight at 5°C. The microfuge tube was then centrifuged (Biofuge B, American Scientific Products, Stone

Mountain, GA) at 100 x g for 1.0 min. The resulting supernatant was removed and protein content determined. Next, 1.0 ml of the appropriate CSF was added and mixed for 5 hr at 5°C. Following centrifugation at 100 x g for 1.0 min, the supernatant was removed, diluted in an equal volume of 10% RPMI and passed through a 0.2 μM filter (Gelman, Ann Arbor, MI). The filtered supernatants were then used in ^{3}H-TdR assays.

Statistical Analysis

Standard error was determined from the formula:

$$\text{Standard deviation}/\sqrt{n},$$

where n equals the number of samples.

Data from competitive and non-competitive ELISA's were analyzed using R^2 statistics (50).

CHAPTER 3

GM-CSF RESULTS

Epitope Determination

The active human GM-CSF molecule begins at amino acid 18 (101, 103). Therefore, the hydrophilicity calculations were begun at that amino acid. The most hydrophilic hexamer was hexapeptide sequence 4 (Figure 1). Thus, the sequence Trp-Glu-His-Val-Asn-Ala-Ile-Gln-Glu-Ala-Arg-Arg-Leu-Leu-Asn-Leu-Ser-Arg,comprising hexapeptide sequences 3 through 5 (residues 30 through 47), contains a predicted primary sequence antigenic determinant. Secondary structure analysis indicated that helices and random coils were the major structural conformation in this region of human GM-CSF (Figure 2). A synthetic peptide corresponding to hexapeptide sequences 3 through 5 was synthesized.

Non-Competitive ELISA's

To determine if the rabbit injected with human GM-CSF peptide had produced antibodies against the peptide, a non-competitive ELISA was run. The antigen in the ELISA was human GM-CSF peptide at a concentration of 1.0 ug/well. The negative control was pre-injection rabbit serum serially diluted in PBS-Tween. The antibody in the ELISA was post-injection rabbit serum serially diluted in PBS-Tween. The results of this ELISA indicated

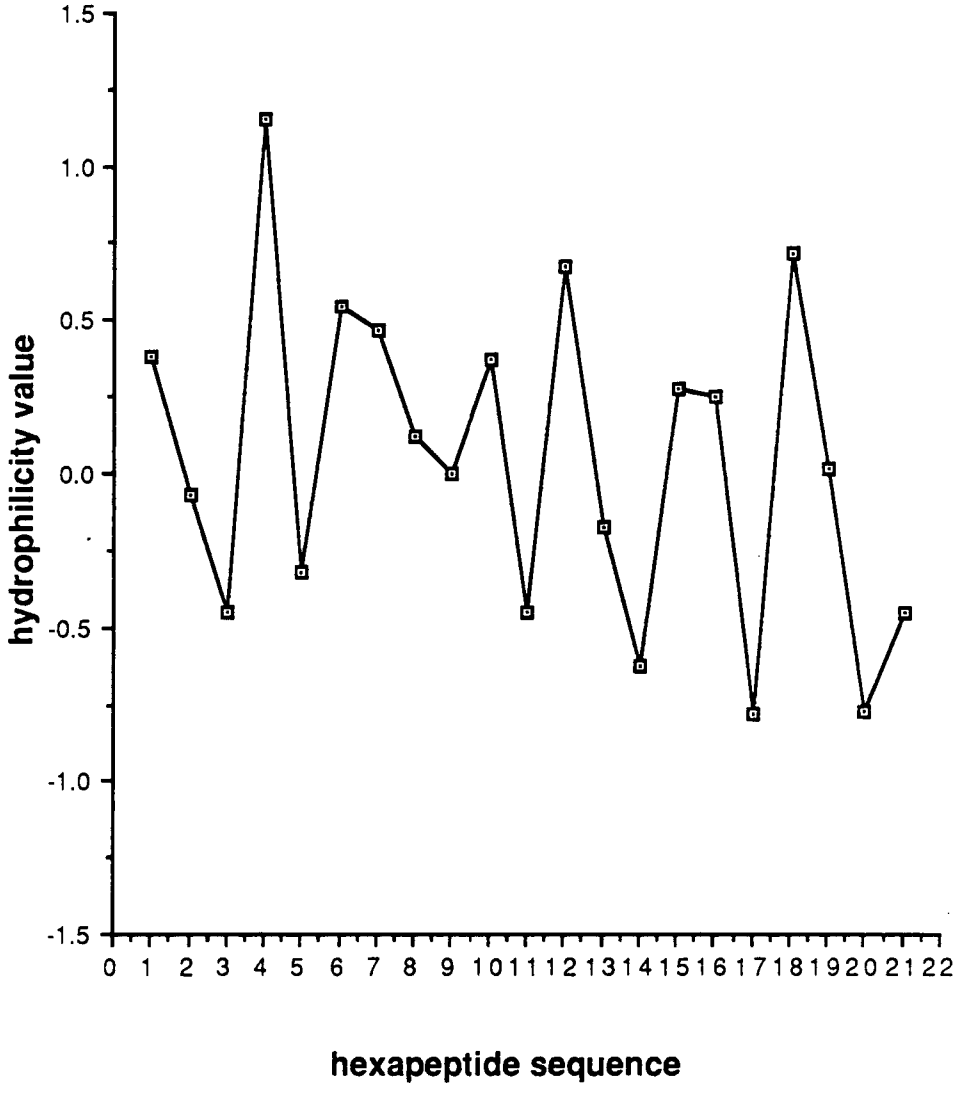

Figure 1: Hydrophilicity plot of human GM-CSF

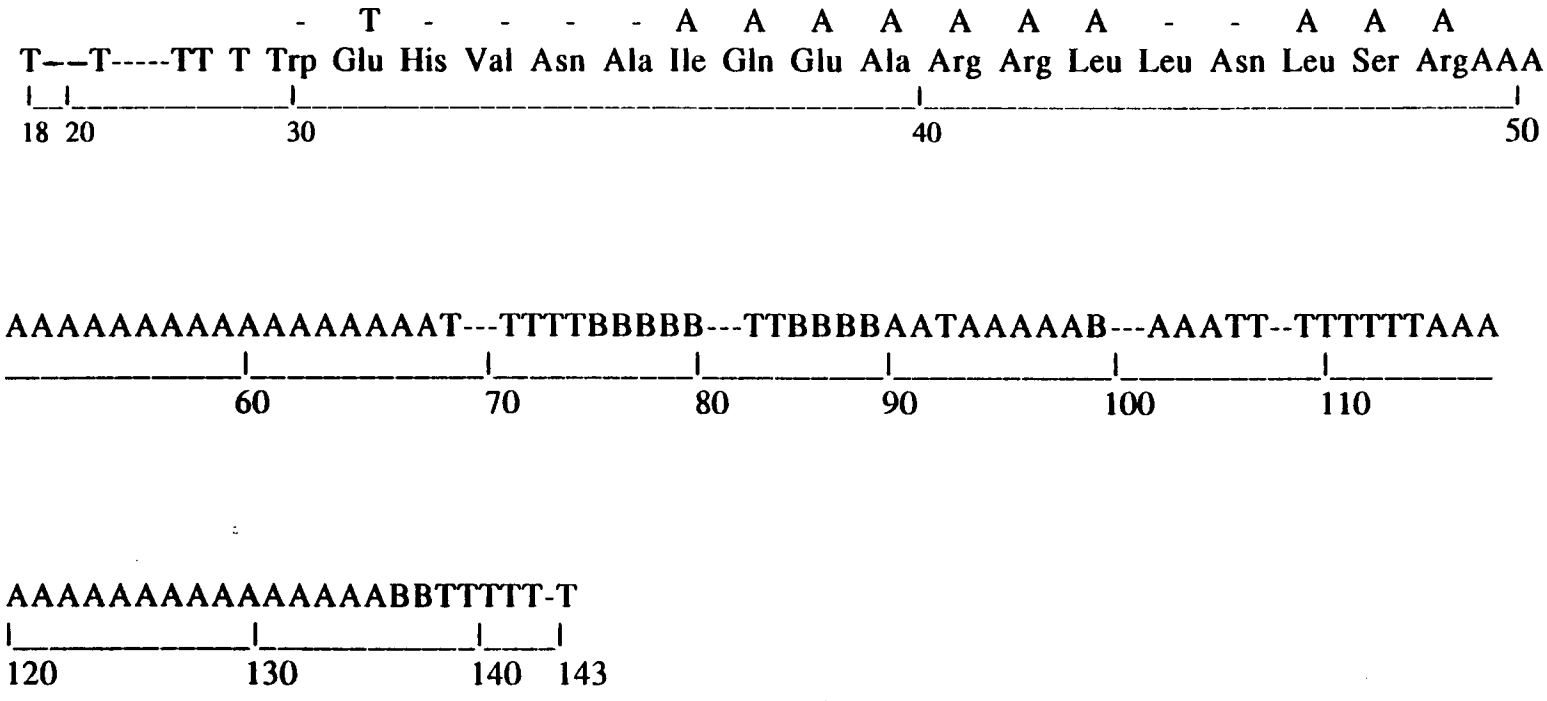

FIGURE 2: Secondary structure analysis of human GM-CSF. A, represents an α helix. B, represents a ß-sheet. T, represents a turn. -, represents a random coil.

the negative control serum contained no antibody against human GM-CSF peptide. However, the post injection rabbit serum did contain antibody against the human GM-CSF peptide to a dilution of 1:256 (Figure 3).

Immunoglobulins were precipitated from post-injection rabbit sera using 18% Na_2SO_4, and the total protein concentration in the immunoglobulin fraction was determined. The human GM-CSF peptide antibody in the immunoglobulin fractions were then used as primary antibodies in non-competitive ELISA's containing 1.0 ug human GM-CSF peptide/well. The ELISA results were analyzed using linear regression and the titration values were determined at an absorbance (540nm) endpoint of 0.100 (Figure 4). The titration values for immunoglobulins from bleedings performed on 12/2/86, 5/1/87, and 7/7/87, were 15.6 ug/ml, 20.1 ug/ml and 9.5 ug/ml, respectively.

The next step was to determine, using a non-competitive ELISA, if anti-peptide antibody would detect recombinant human GM-CSF, recombinant human G-CSF, or murine GM-CSF. As shown in figure 5, the antibody was capable of detecting recombinant human GM-CSF down to a concentration of 160 ng growth factor/ml assay volume. However, the antibody did not detect native murine GM-CSF, recombinant human G-CSF (Figure 5) or recombinant human M-CSF. That the antibody did not detect human G-CSF or human M-CSF was encouraging because this indicated that the antibody did not cross react with other CSFs. However, since the amino terminus of the murine GM-CSF is almost identical to the

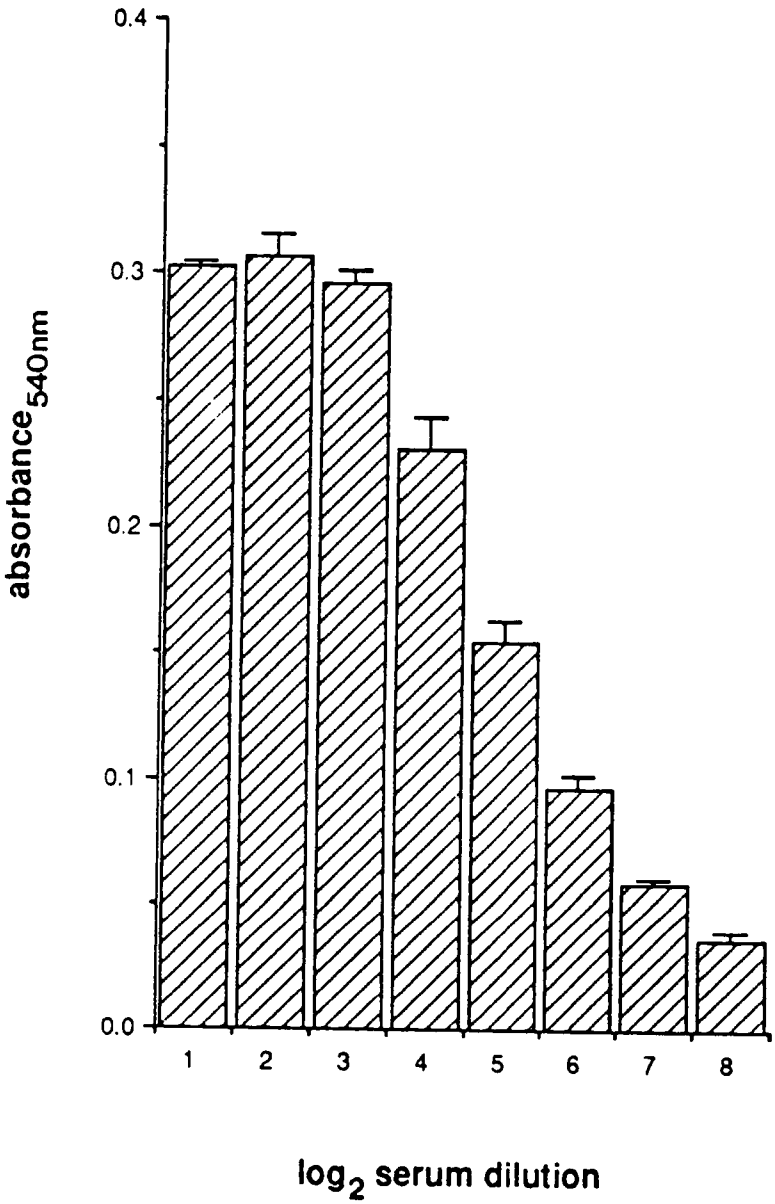

Figure 3: Detection of human GM-CSF peptide using post-injection rabbit serum. The individual wells were coated with 1.0 ug human GM-CSF peptide.

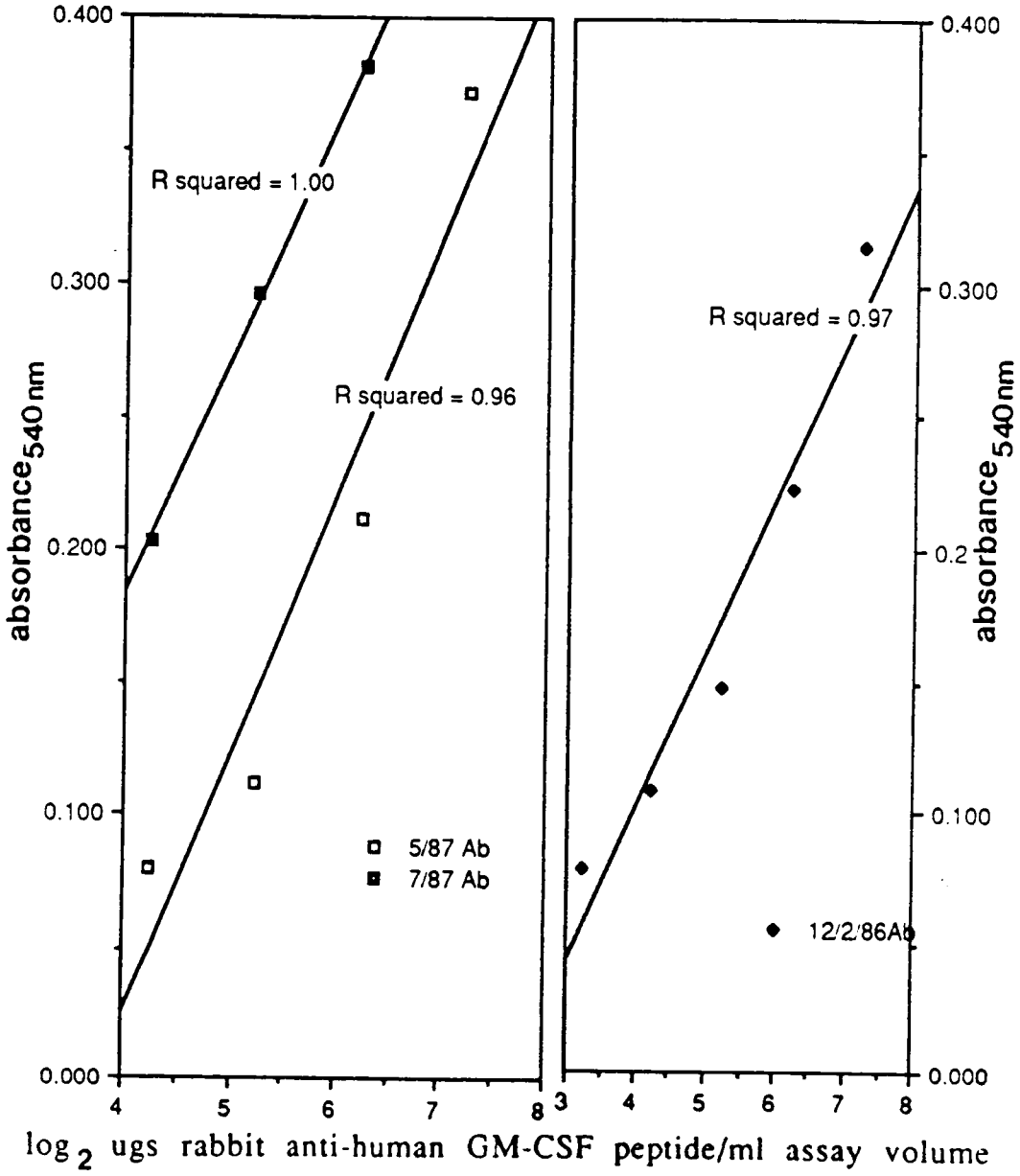

Figure 4: Detection of human GM-CSF peptide using non-competitive ELISA's. The individual wells were coated with 1.0 ug human GM-CSF peptide.

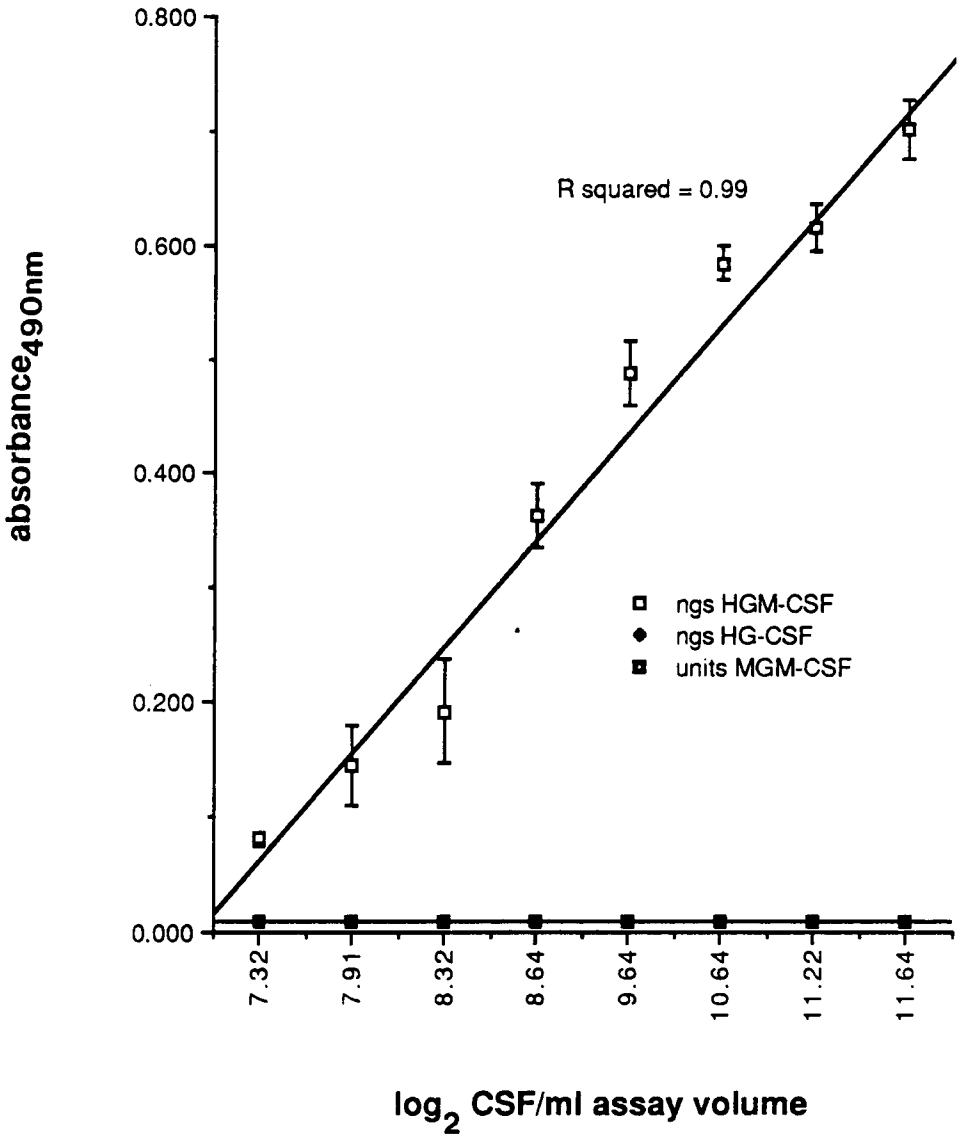

Figure 5: Detection of CSFs using non-competitive ELISA's. Rabbit anti-human GM-CSF peptide was used at a concentration of 1.23 mg/ml.

amino terminus of the human GM-CSF (9,26) the antibody should be capable of detecting murine as well as human GM-CSF.

Non-competitive ELISA's were then run against murine GM-CSF, recombinant human GM-CSF, and recombinant human G-CSF that had been denatured while being adsorbed to the ELISA wells. The denaturation was carried out by placing the appropriate CSF in ELISA wells containing carbonate buffer, pH 9.6, plus 6M urea (Bio-Rad). The CSFs were then allowed to adsorb to the plate at 5°C overnight. The results of the non-competitive ELISA's indicated the antibody could detect denatured murine GM-CSF down to a concentration of 400 units/ml assay volume (Figure 6). However, the antibody did not detect denatured recombinant human GM-CSF or recombinant human G-CSF (Figure 6).

Competitive ELISA's

A competitive ELISA was designed in an attempt to increase the sensitivity for detection of GM-CSF by the anti-human GM-CSF peptide antibody. Fifty ul of recombinant human GM-CSF (165 ng/ml carbonate buffer) was placed into each ELISA well and allowed to adsorb to the plate overnight at 5°C. The next day constant concentrations of antibody were incubated, for 150 min at 37°C, with increasing concentrations of antigen prior to being used in the ELISA as the primary antibody. The idea behind the competitive assay was that as the concentrations of antigen present during the preincubation increased, the amount of free antibody available to bind recombinant human GM-CSF on the plate would

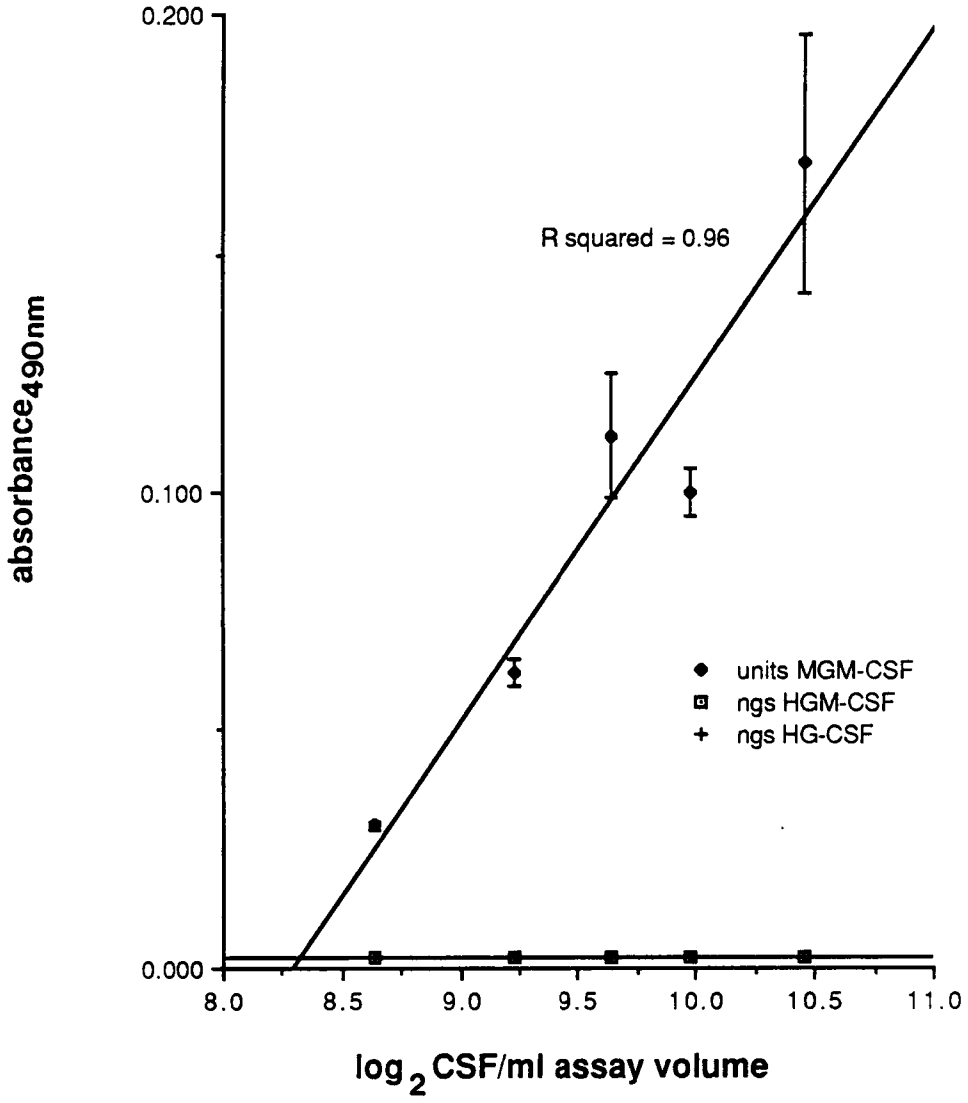

Figure 6: Detection of denatured CSFs using non-competitive ELISA'S. Rabbit anti-human GM-CSF peptide was used at a concentration of 1.23 mg/ml.

decrease. The absorbance of individual wells was measured at 490nm. A correlation should exist between the change in absorbance 490nm ($absorbance_{490nm}$ = $absorbance_{490nm}$ incubated without antigen - $absorbance_{490nm}$ of antibody incubated with antigen) and the amount of antigen present during the preincubation. A change in $absorbance_{490nm}$ was detected between 66 ng and 660 ng recombinant human GM-CSF/ml assay volume. A change in $absorbance_{490nm}$ was also detected between 160 ng and 3192 ng of human GM-CSF peptide/ml assay volume. No change in $absorbance_{490nm}$ was detected with murine GM-CSF, recombinant human G-CSF (Figure 7) or recombinant human M-CSF. The control wells received no anti-human GM-CSF peptide antibody.

The results indicate anti-human GM-CSF peptide antibody was capable of binding with human GM-CSF in solution. The competitive ELISA was approximately 2.5 times more sensitive than the non-competitive ELISA. For example, the competitive ELISA was capable of detecting recombinant human GM-CSF down to a concentration of 66 ng/ml assay volume while the non-competitive ELISA was capable of detecting recombinant human GM-CSF down to a concentration of 160 ng/ml assay volume. The competitive ELISA also indicated the antibody was reacting with the recombinant human GM-CSF at the predicted epitope. Since binding of human GM-CSF peptide to the ELISA plate is concentration dependent it was not possible to determine the sensitivity of the non-competitive ELISA to human GM-CSF peptide.

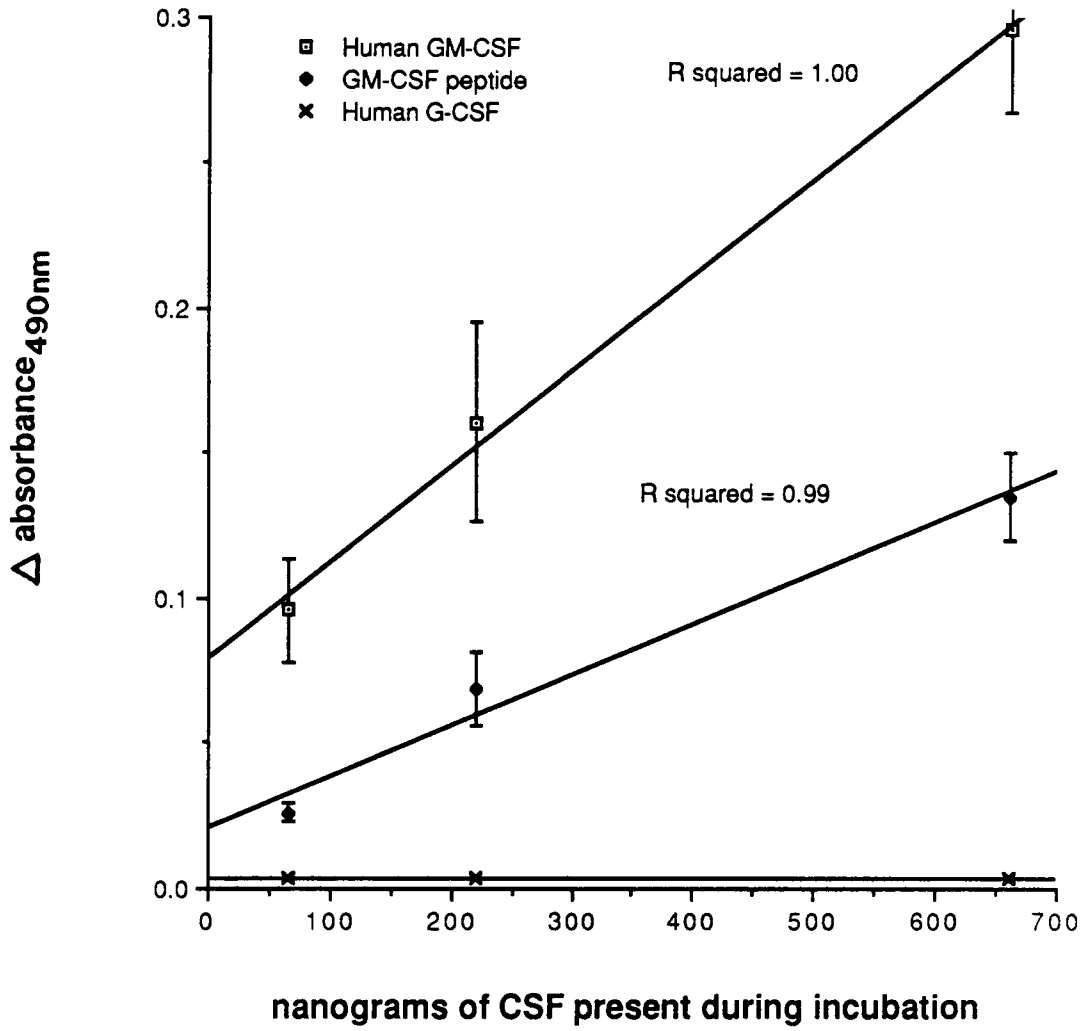

Figure 7: Detection of CSFs using competitive ELISA's. The individual wells were coated with 3.3 ng recombinant human GM-CSF protein. Rabbit anti-human GM-CSF peptide was used at a concentration of 7.2 mg/ml.

Biological Assays

DNA synthesis, as determined by ^{3}H-TdR incorporation, by murine marrow cells incubated with or without varying concentrations of human GM-CSF peptide was not appreciably different (Figure 8).

DNA synthesis was not affected by the addition of 10^{-5} M human GM-CSF peptide to marrow cells incubated with one of the following: 250 units of murine GM-CSF/ml assay volume, 250 units L cell M-CSF/ml assay volume, 2.5 ng recombinant human G-CSF/ml assay volume, or 250 units recombinant human M-CSF/ml assay volume (Table1). When the above experiment was repeated using the soft agar assay, no difference in the number of colonies was found between cells incubated with only CSF and cells incubated with both CSF and 10^{-5} M human GM-CSF peptide (Table 2)

The effects of rabbit anti-human GM-CSF peptide antibody on the biological effects of CSFs were determined by looking at DNA synthesis. A slight, but variable decrease (experiment to experiment) in the incorporation of ^{3}H-TdR was observed when cells which were incubated with 100 units murine GM-CSF/ml assay volume plus varying concentrations of antibody were compared to cells incubated with only 100 units murine GM-CSF/ml assay volume (Figure 9). Also, no appreciable decrease in ^{3}H-TdR incorporation was observed between cells incubated with one of the following: 250 units L cell M-CSF/ml assay volume, 2.5

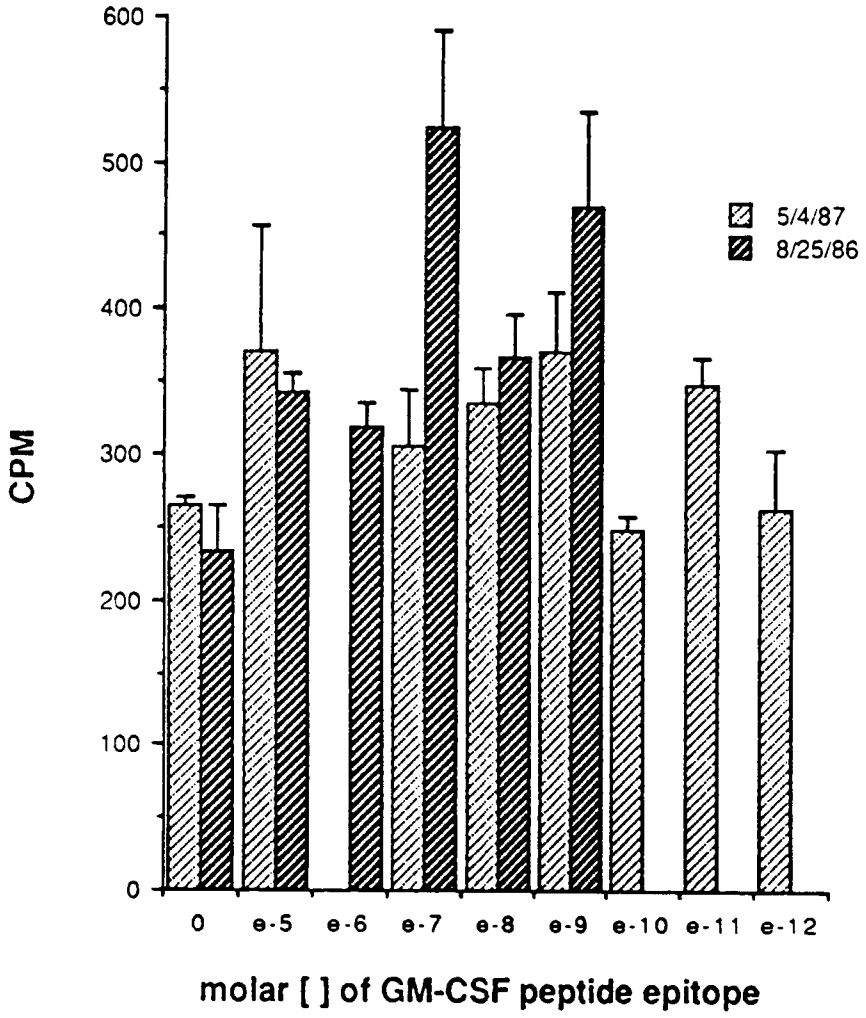

Figure 8: The biological effect of human GM-CSF peptide alone. The biological effect was assessed by ^{3}H-TdR incorporation.

Table 1: The biological effect of human GM-CSF peptide, as measured by ^{3}H-TdR incorporation, on CSFs other than human GM-CSF.

CSFs	ΔCPM of CSFs alone	ΔCPM using CSFs + 10^{-5} M human GM-CSF peptide
L cell M-CSF	1464 ± 136	1334 + 228
murine GM-CSF	12584 ±393	12380 ±191
recombinant human G-CSF	1331 ±42	1786 ±228
recombinant human M-CSF	3689 ±377	1050 ±368

The concentrations of the CSFs were as follows: 250U L cell M-CSF/ml assay vol., 100U murine GM-CSF/ml assay vol., 2.5 ng recombinant human G-CSF/ ml assay vol., and 250U recombinant human M-CSF/ml assay vol. Values were corrected for background (397±57).

Table 2: The biological effect of human GM-CSF peptide, as measured by the soft agar assay, on CSFs other than human GM-CSF.

CSFs	# of colonies using CSFs alone	# of colonies using CSFs + 10^{-5} M human GM-CSF peptide
Peptide alone	0 ±0	0±0
L cell M-CSF	91 ±2	93±2
murine GM-CSF	93±5	95±9
recombinant human G-CSF	65 ±1	53 ±5
recombinant human M-CSF	69 ±1	69 ±3

The concentrations of the CSFs were as follows: 250U L cell M-CSF/ml assay vol.,100U murine GM-CSF/ml assay vol., 2.5 ng recombinant human G-CSF/ ml assay vol., and 250U recombinant human M-CSF/ml assay vol.

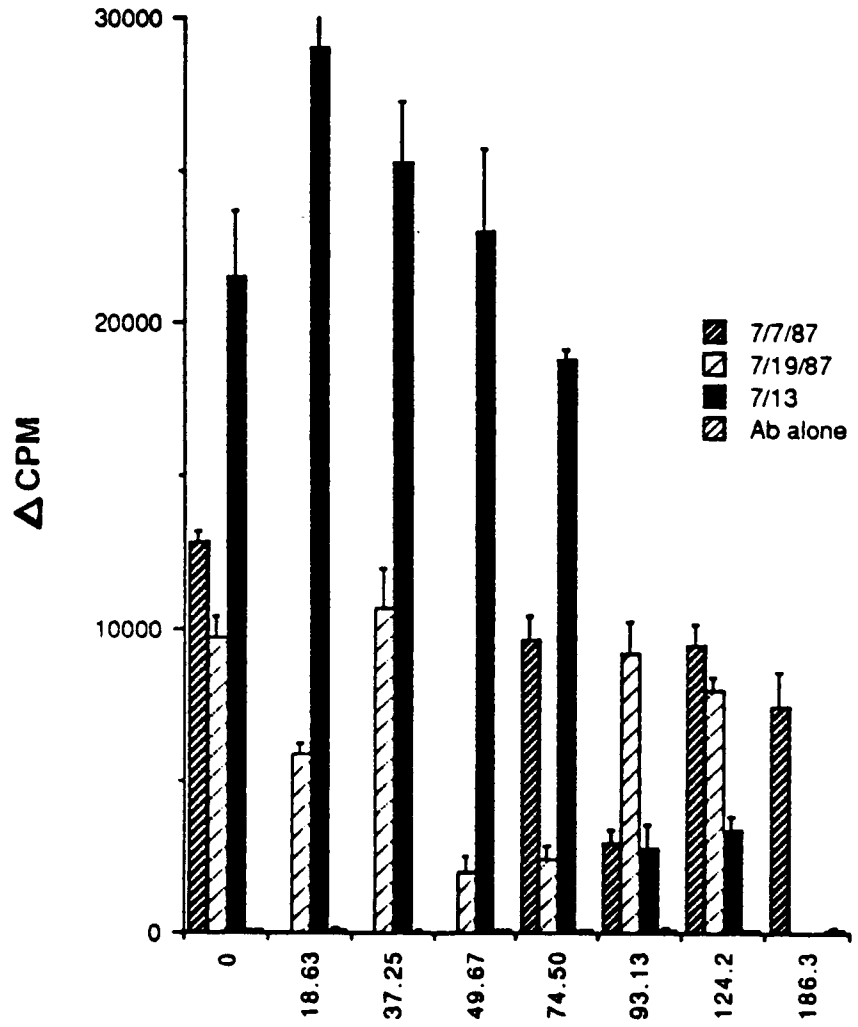

Figure 9: The biological effect of rabbit anti-human GM-CSF peptide on murine GM-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. The wells contained murine GM-CSF at a concentration of 100U/ml assay volume. Values were corrected for background (267 $\pm$ 33).

ng recombinant human M-CSF, 2.5 ng recombinant human G-CSF, and cells incubated with one of the former CSFs plus varying concentrations of antibody (Figure 10).

A slight decrease in colony formation, as determined using the soft agar assay, was found when cells incubated with 100 units murine GM-CSF/ml assay volume plus anti-human GM-CSF peptide antibody were compared to cells incubated only with 100 units murine GM-CSF/ml assay volume (Figure 11).

Protein A-Sepharose

Seven mg of 5/1/87 GM-CSF immunoglobulin fraction in 1.0 ml of PBS was added to a microfuge tube containing 0.5 ml staphylococcal protein A-Sepharose covalently linked to sepharose CL-4B and mixed overnight at 5°C. The suspension was centrifuged, and the resulting supernatant removed. Three thousand units of murine GM-CSF in 1.0 ml of 10% RPMI was added to the pelleted protein A-immunoglobulin and mixed for 5 hr at 5°C. Following centrifugation the supernatant was removed, diluted in an equal volume of 10% RPMI, and passed through a 0.2 M filter. Colony-stimulating activity remaining in the filtrate was assayed by the marrow ^{3}H-TdR incorporation assay. As shown in figure 12, ^{3}H-TdR incorporation by cells incubated with protein A-Sepharose treated murine GM-CSF did not differ from that of cells incubated with non-treated murine GM-CSF.

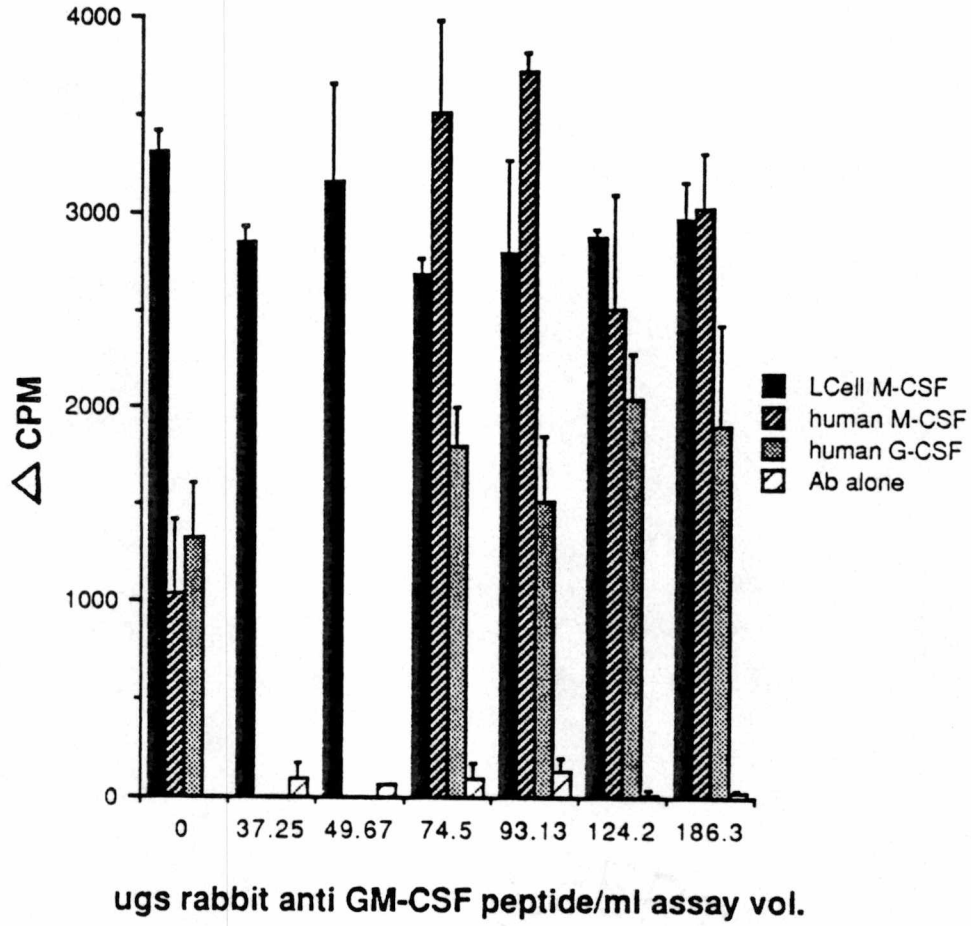

Figure 10: The biological effect of rabbit anti-human GM-CSF peptide on recombinant human G-CSF, recombinant human M-CSF, and L-cell M-CSF. The biological effect was assessed by H-TdR incorporation. The concentrations of the CSFs were as follows: 2.5 ng recombinant human G-CSF/ml assay volume, 250U recombinant human M-CSF/ml assay volume, and 250 units L-cell M-CSF/ml assay volume. Values were corrected for background (233 $\pm$ 27).

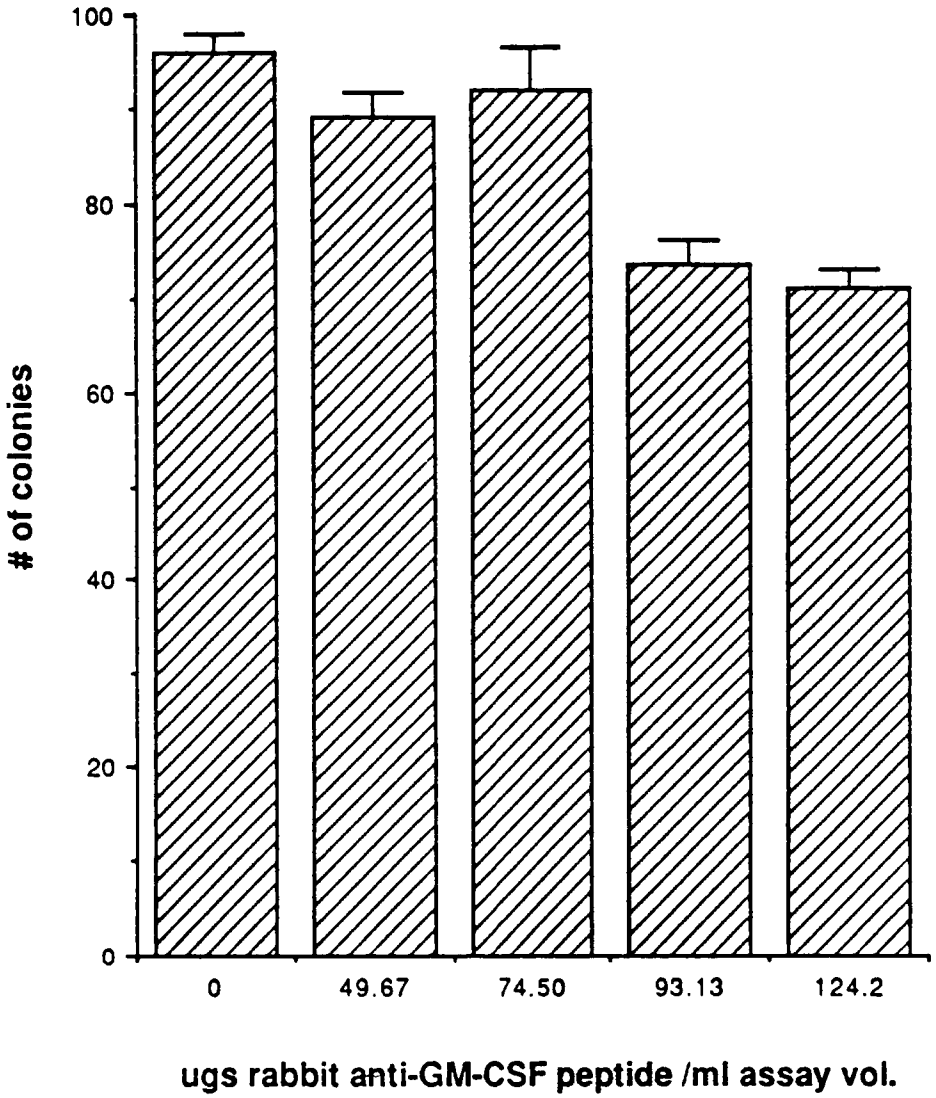

Figure 11: The biological effect of rabbit anti-human GM-CSF peptide on murine GM-CSF. The biological effect was assessed by using the soft agar assay. The plates contained murine GM-CSF at a concentration of 100U/ml assay volume.

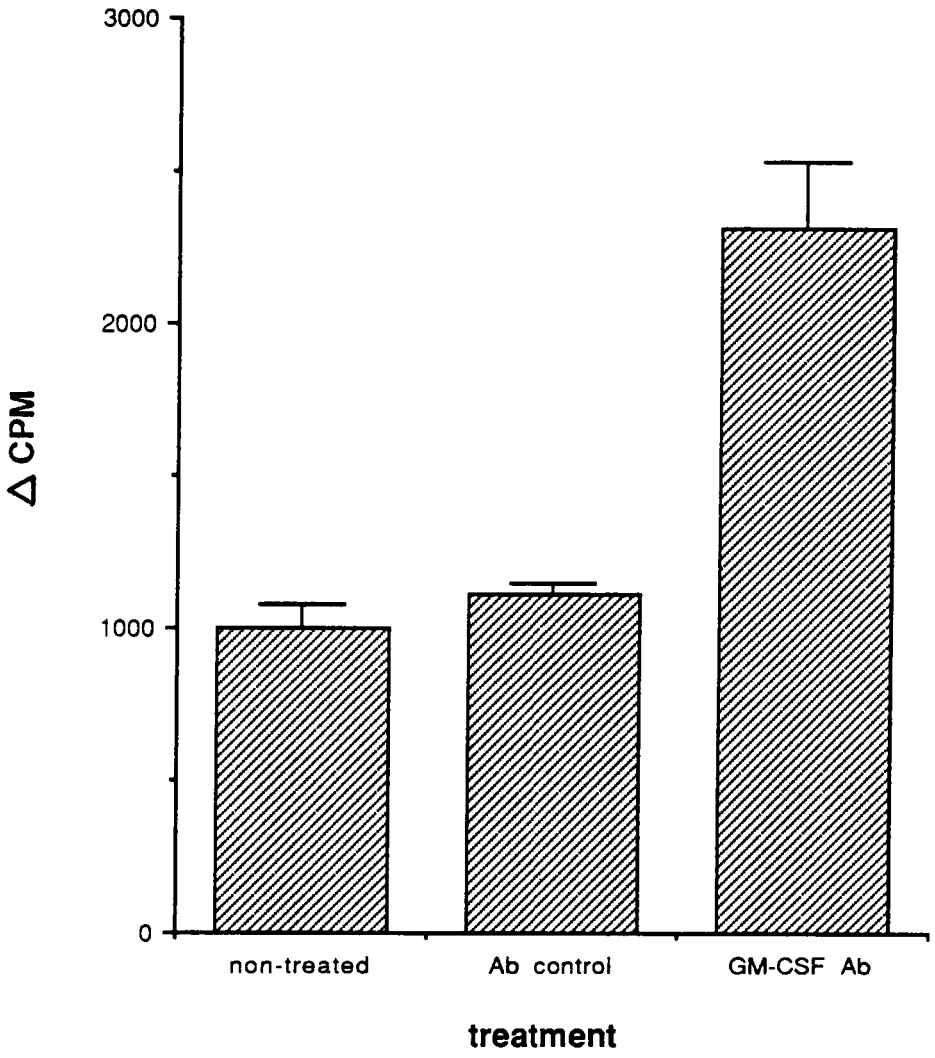

Figure 12: Treatment of murine GM-CSF with rabbit anti-human GM-CSF peptide bound to protein A-Sepharose. The biological effect was assessed by ^{3}H-TdR incorporation. The antibody control was rabbit gamma globulin bound to protein A-Sepharose. Values were corrected for background (729 ± 84).

CHAPTER 4

M-CSF RESULTS

Epitope Determination

Hydrophilicity calculations for human M-CSF were begun at amino acid 1. A significant hydrophilic peak was observed at hexapeptide sequence 34 (Figure 13). Thus, the sequence Arg-Ser-His-Gln-Glu-Pro-Gln-Arg-Ala-Asp-Ser-Pro-Leu-Glu-Gln-Pro-Glu-Glu, comprising hexapeptide sequences 33 through 35 (residues 193 through 210), contained a predicted primary sequence antigenic determinant. Secondary structure analysis indicated that turns and random coils were the major structural conformation in this region of human M-CSF (Figure 14). A synthetic peptide corresponding to hexapeptide sequences 33 through 35 was synthesized.

Non-Competitive ELISA's

To determine if the rabbit injected with human M-CSF peptide had produced antibodies against the peptide a non-competitive ELISA was run. The antigen in the ELISA was human M-CSF peptide at a concentration of 1.0 ug/well. The negative control was pre-injection rabbit serum serially diluted in PBS-Tween. The antibody in the ELISA was post-injection rabbit serum serially diluted in PBS-Tween. The results of this ELISA indicated the negative control serum contained no antibody against human M-CSF peptide.

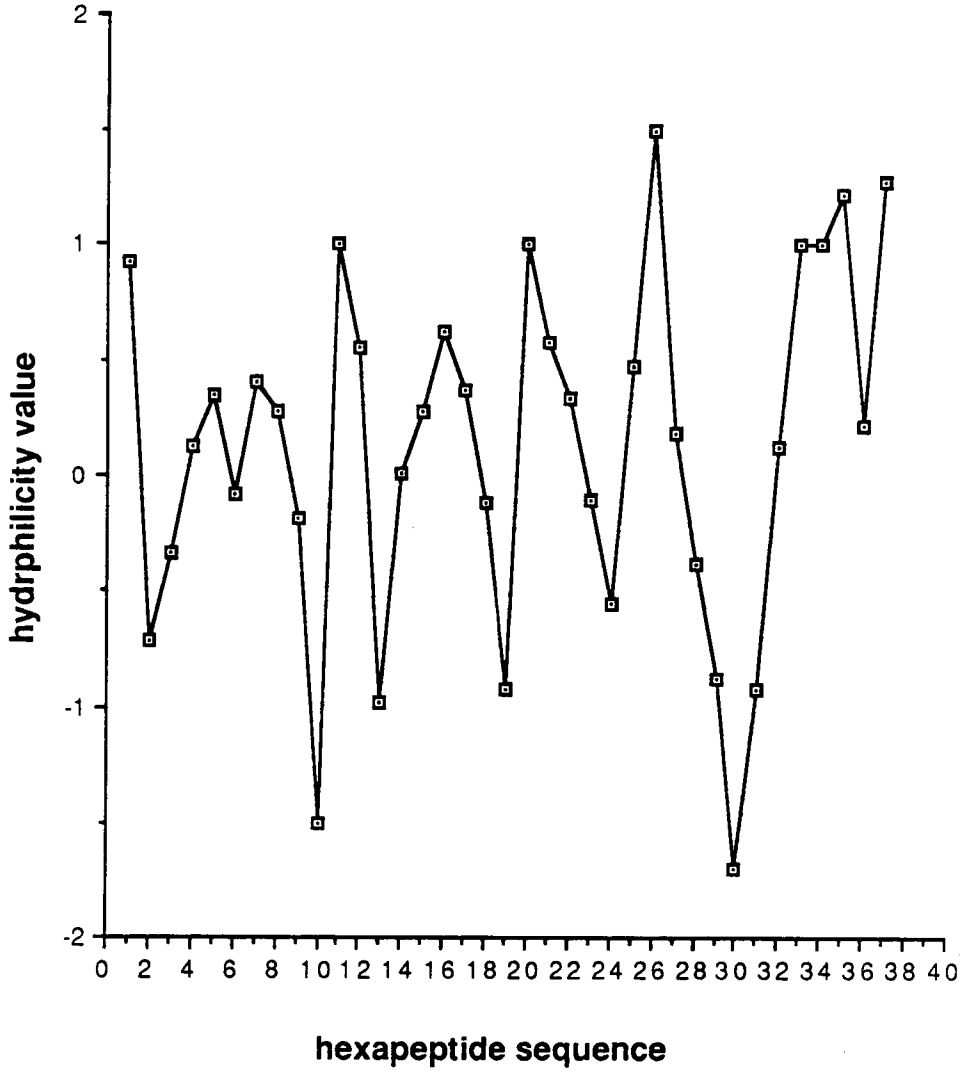

Figure 13: Hydrophilicity plot of human M-CSF.

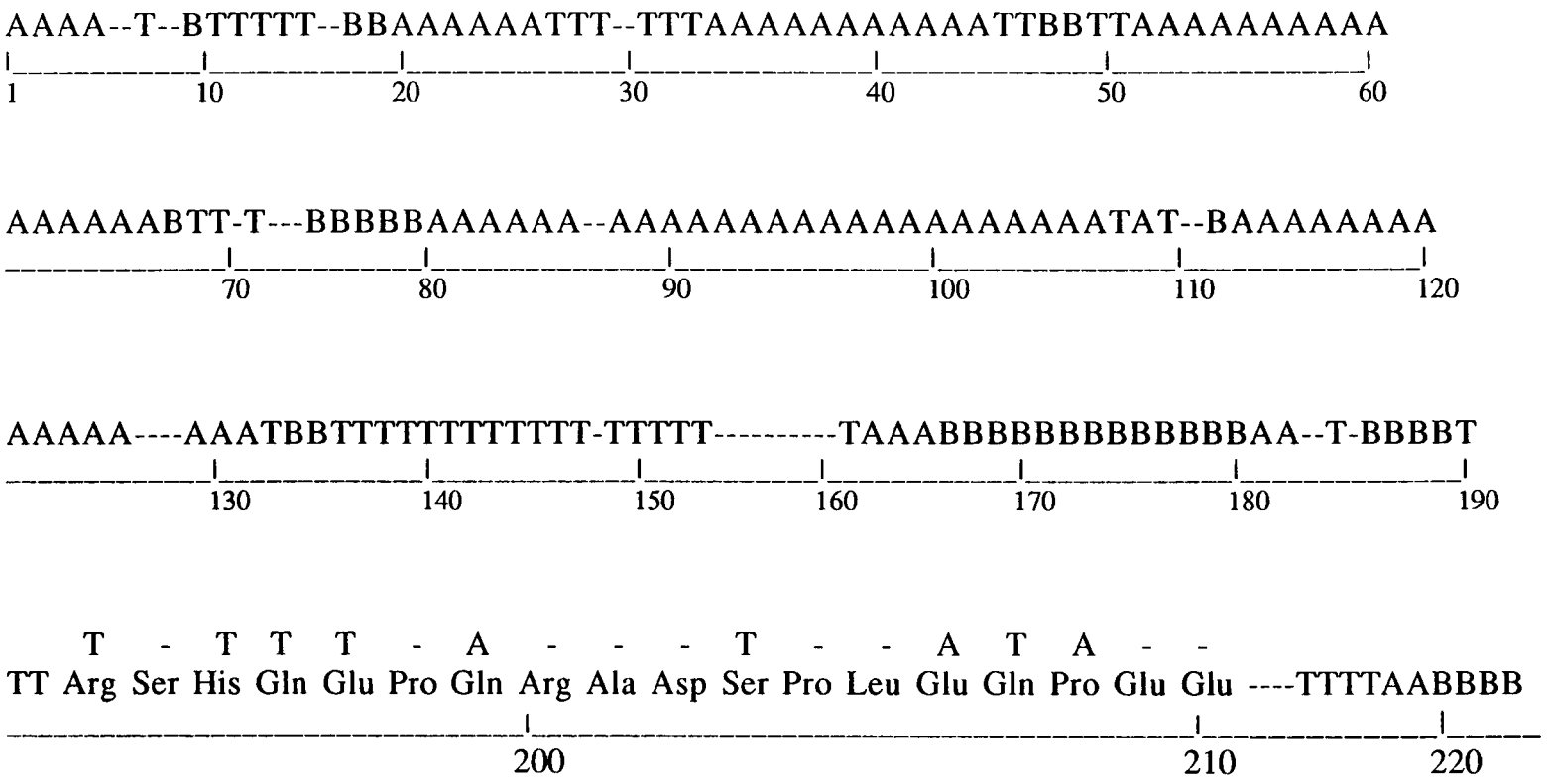

Figure 14 : Secondary structure analysis of human M-CSF. A, represents an α helix. B, represents a ß-sheet. T, represents a turn. -, represents a random coil.

However, the post-injection rabbit serum did contain antibody against the human M-CSF peptide to a dilution of 1:512 (Figure 15).

Immunoglobulins were precipitated from post-injection rabbit sera using 18% Na_2SO_4, and the total protein concentration in the immunoglobulin fraction was determined. The human M-CSF peptide antibody in the immunoglobulin fractions were then used as primary antibodies in non-competitive ELISA's containing 1.0 ug human M-CSF peptide/well. The ELISA results were analyzed using linear regression and the titration values were determined at an absorbance (540nm) endpoint of 0.100 (Figure16). The titration values for immunoglobulins from bleedings performed on 2/87, 5/87, and 7/87, were 64.0 ug/ml, 90.5 ug/ml and 32.0 ug/ml, respectively.

The next step was to determine, using a non-competitive ELISA, if the anti-peptide antibody would detect recombinant human M-CSF, recombinant human G-CSF, or L cell M-CSF. The antibody did not detect any of the above CSFs.

Non-competitive ELISA's were then run against recombinant human M-CSF, L cell M-CSF, and recombinant human G-CSF that had been urea denatured while being adsorbed to the ELISA wells. The denaturation was carried out by placing the appropriate CSF in ELISA wells containing carbonate buffer, pH 9.6, plus 6M urea. The CSFs were then allowed to adsorb to the plate at 5°C overnight. The results of the non-competitive ELISA's indicated that the antibody did not detect denatured recombinant human M-CSF, L cell M-CSF and recombinant human G-CSF.

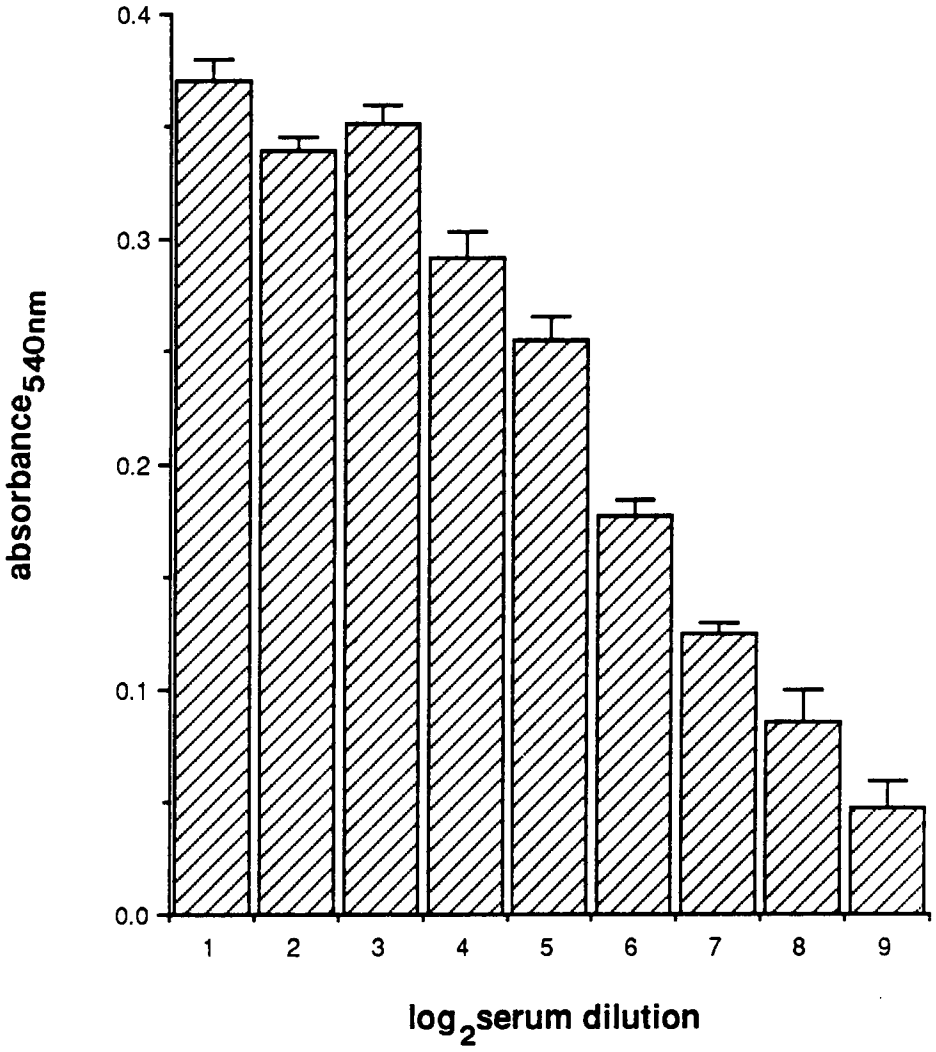

Figure 15: Detection of human M-CSF peptide using post injection rabbit serum. The individual wells were coated with 1.0 ug human M-CSF peptide.

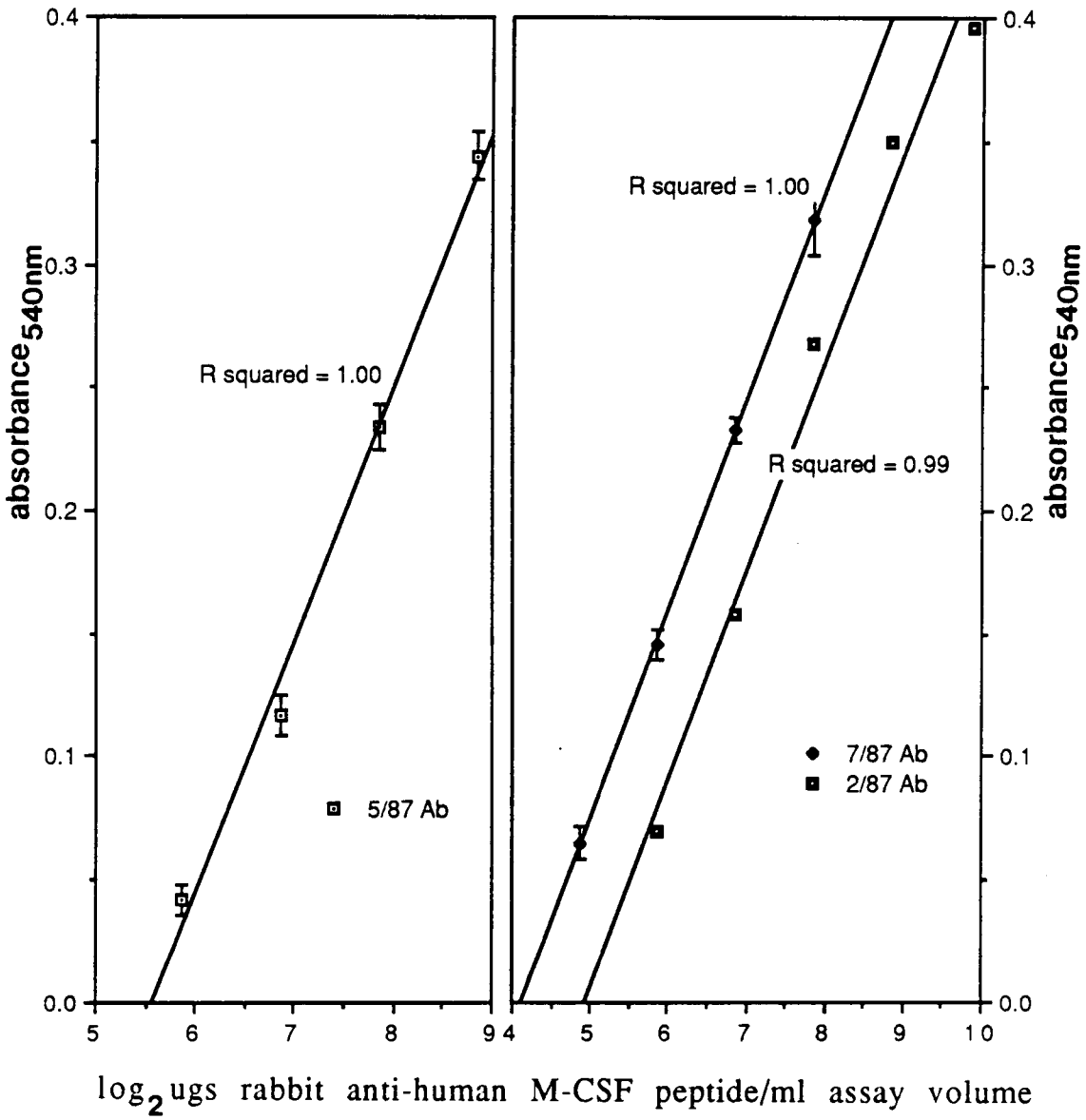

Figure 16: Detection of human M-CSF peptide using non-competitive ELISA's. The individual wells were coated with 1.0 ug human M-CSF peptide.

Competitive ELISA's

A competitive ELISA was designed in a final attempt to detect L cell M-CSF or recombinant human M-CSF by the anti-human M-CSF peptide antibody. Fifty ul of human M-CSF peptide (20 ug/ml carbonate buffer) was placed into each ELISA well and allowed to adsorb to the plate overnight at 5°C. The next day constant concentrations of antibody were incubated, for 150 min at 37°C, with increasing concentrations of antigen prior to being used in the ELISA as the primary antibody. The idea behind the competitive assay was that as the concentrations of antigen present during the preincubation increased, the amount of free antibody available to bind recombinant human M-CSF on the plate would decrease. The absorbance of individual wells was measured at 490nm. A correlation should exist between the change in $absorbance_{490nm}$ ($absorbance_{490nm}$ = $absorbance_{490nm}$ incubated without antigen - $absorbance_{490nm}$ of antibody incubated with antigen) and the amount of antigen present during the preincubation. No change in $absorbance_{490nm}$ was detected with recombinant human M-CSF, and L cell M-CSF. This indicated that anti-human M-CSF antibody could not detect either L cell M-CSF or recombinant human M-CSF.

Biological Assays

DNA synthesis as determined by ^{3}H-TdR incorporation for murine marrow cells incubated with or without varying

concentrations of human M-CSF peptide was not appreciably different (Figure 17).

Incorporation of ^{3}H-TdR was appreciably different between: 1) cells incubated with 250 units of recombinant human M-CSF plus varying concentrations of human M-CSF peptide when compared to cells incubated with only 250 units of recombinant human M-CSF (Figure 18), 2) cells incubated with either 1000 units or 3300 units of recombinant human M-CSF plus 10^{-5} M human M-CSF peptide when compared to cells incubated with only recombinant human M-CSF (Figure 19), and 3) cells incubated with 250 units L cell M-CSF plus 10^{-5} M human M-CSF peptide when compared to cells incubated with only L cell M-CSF (Table 3).

Incorporation of ^{3}H-TdR was not appreciably different between cells incubated with one of the following: 100 units of murine GM-CSF/ml assay volume, 2.5 ng recombinant human G-CSF/ml assay volume, and cells incubated with one of the former CSFs plus 10^{-5} M human M-CSF peptide (Table 3). When the above experiment was repeated using the soft agar assay, no difference in the number of colonies was found between cells incubated with only CSFs and cells incubated with both CSF and 10^{-5} M human M-CSF peptide (Table 4). No difference in the number of colonies was found between cells incubated with 250 units L cell M-CSF with or without 10^{-5} M peptide (Table 4).

The effects of rabbit anti-human M-CSF peptide antibody on the biological effects of CSFs were determined by looking at DNA synthesis. No appreciable decrease in the rate of DNA synthesis was

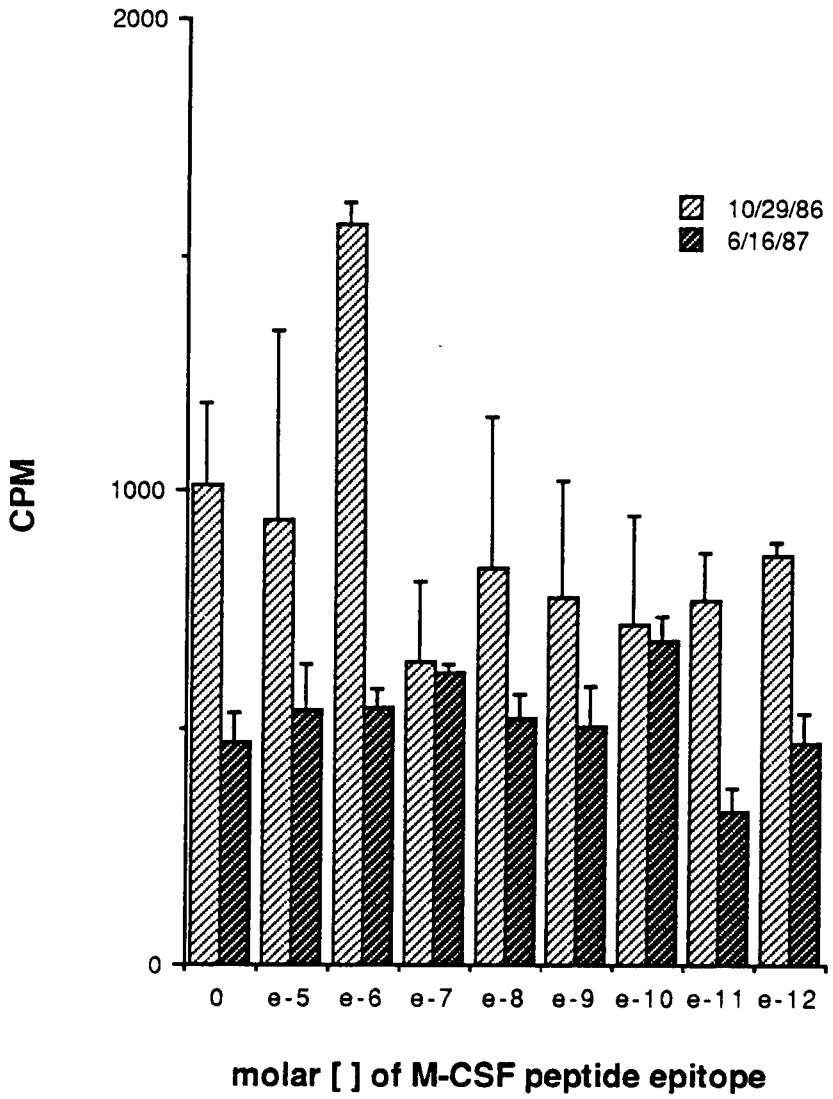

Figure 17: The biological effect of human M-CSF peptide alone. The biological effect was assessed by ^{3}H-TdR incorporation.

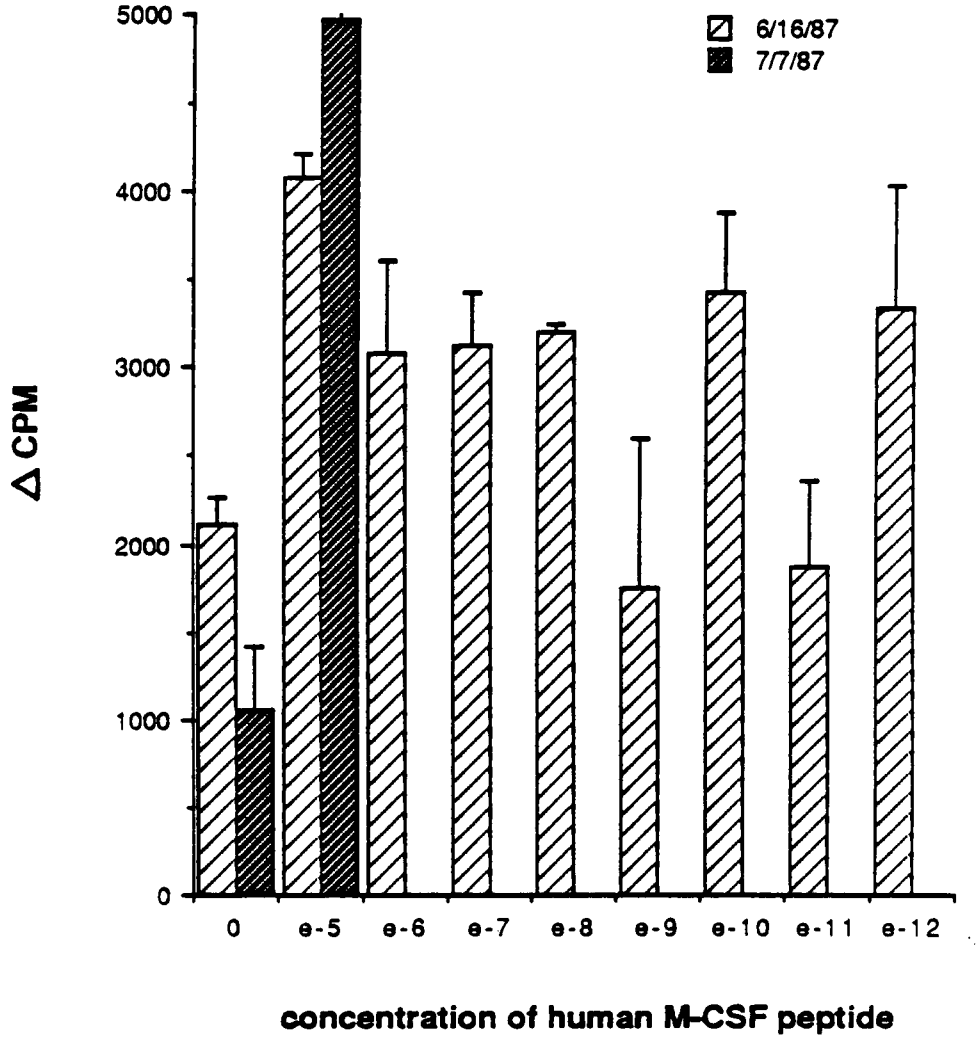

Figure 18: The biological effect of human M-CSF peptide on recombinant human M-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. The wells contained recombinant human M-CSF at a concentration of 250 units/ml assay volume. Values were corrected for background **(307 ±33).**

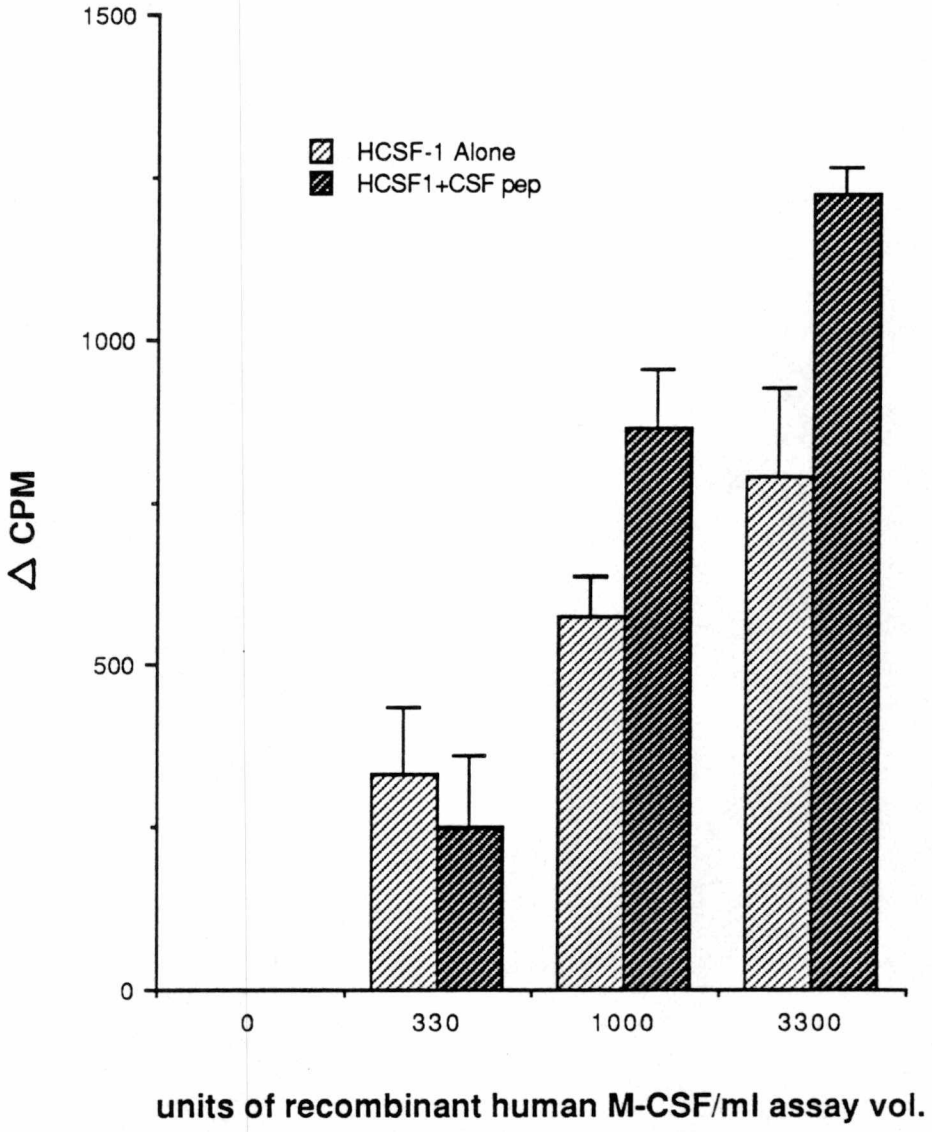

Figure 19: The biological effect of human M-CSF peptide on recombinant human M-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. Values were corrected for background (233 ± 57).

Table 3. The biological effect of human M-CSF peptide, as measured by ^{3}H-TdR incorporation, on CSFs other than human M-CSF.

CSFs	Δ CPM of CSFs alone	Δ CPM of CSFs + 10^{-5} M human M-CSF peptide
L cell M-CSF	1464 ±136	2176 ±11
murine GM-CSF	12548 ±393	10104 ± 1120
recombinant human G-CSF	1381 ±42	1218 ±135

The concentrations of the CSFs were as follows: 250U L cell M-CSF/ml assay vol., 100U murine GM-CSF/ml assay vol., and 2.5 ng recombinant human G-CSF/ml assay vol. Values were corrected for background (349 ± 61).

Table 4: The biological effect of human M-CSF peptide, as measured by using the soft agar assay, on CSFs other than human M-CSF.

CSFs	# of colonies using CSFs alone	# of colonies using CSFs + 10^{-5} M human M-CSF peptide
peptide alone	0±0	0±0
L cell M-CSF	69±0	68±1
murine GM-CSF	93±0	98±5
recombinant human G-CSF	65±1	68±1

The concentrations of the CSFs were as follows: 250U L cell M-CSF/ml assay vol., 100U murine GM-CSF/ml assay vol., and 2.5 ng recombinant G-CSF/ ml assay vol.

found when cells which were incubated with 250 units recombinant human M-CSF/ml assay volume plus varying concentrations of antibody were compared to cells incubated with only 250 units recombinant human M-CSF (Figure 20). Also, no appreciable decrease in DNA synthesis was observed between cells incubated with one of the following: 100 units murine GM-CSF/ml assay volume (Figure 21), 250 units L cell M-CSF/ml assay volume (Figure 21), varying concentrations of recombinant human G-CSF (Figure 22), and cells incubated with one of the former CSFs plus varying concentrations of antibody (Figures 21,22).

The effects of rabbit anti-human M-CSF antibody on the combined biological effects of recombinant human M-CSF or L cell M-CSF plus 10^{-5} M human M-CSF peptide was determined by looking at DNA synthesis. Incorporation of ^{3}H-TdR for cells incubated in the presence of recombinant human M-CSF or L cell M-CSF plus 10^{-5} M peptide and increasing concentrations of antibody was decreased to a level equal to that of cells incubated only in the presence of recombinant human M-CSF (Figures 23,24). This indicated the peptide was responsible for the increase in DNA synthesis seen in figures 18 and 19.

Protein A-Sepharose

Seven mg of 5/87 GM-CSF immunoglobulin fraction in 1.0 ml of PBS was added to a microfuge tube containing 0.5 ml staphylococcal protein A-Sepharose covalently linked to sepharose CL-4B and mixed overnight at 5°C. The suspension was centrifuged,

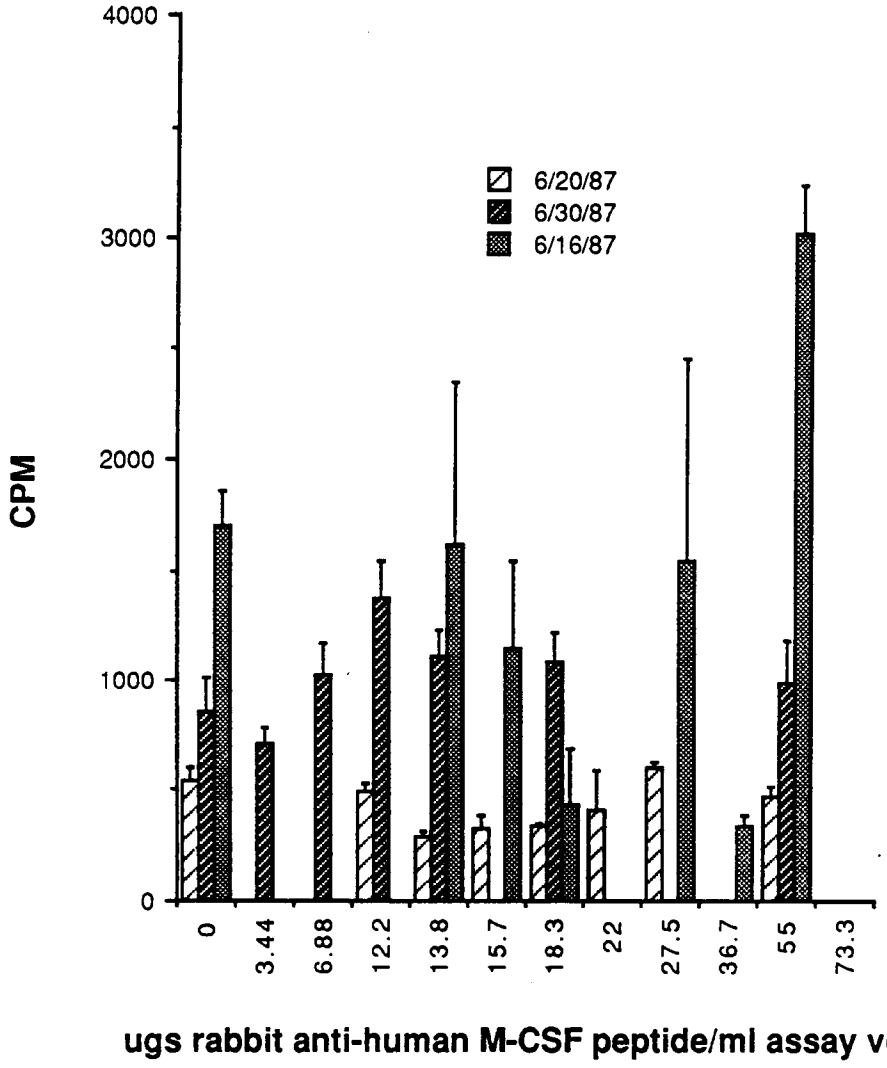

Figure 20: The biological effect of rabbit anti-human M-CSF peptide on recombinant human M-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. The wells contained recombinant human M-CSF at a concentration of 250U/ml assay volume. Values were corrected for background (411 $\pm$ 69).

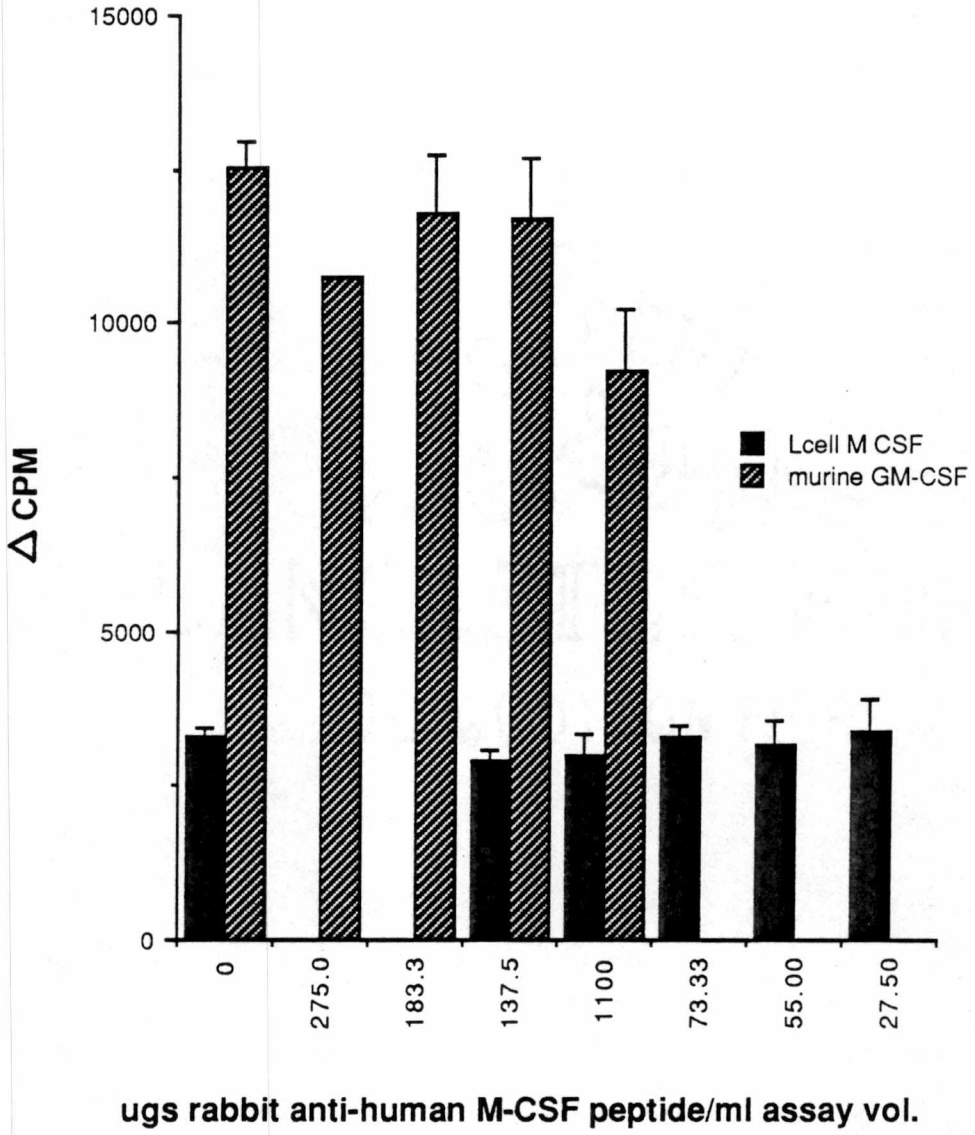

Figure 21: The biological effect of rabbit anti-human M-CSF peptide on murine GM-CSF, and L cell M-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. The concentrations of the CSFs were as follows: 100U murine GM-CSF/ml assay volume, and 250U L cell M-CSF/ml assay volume. Values were corrected for background (323 _ 51).

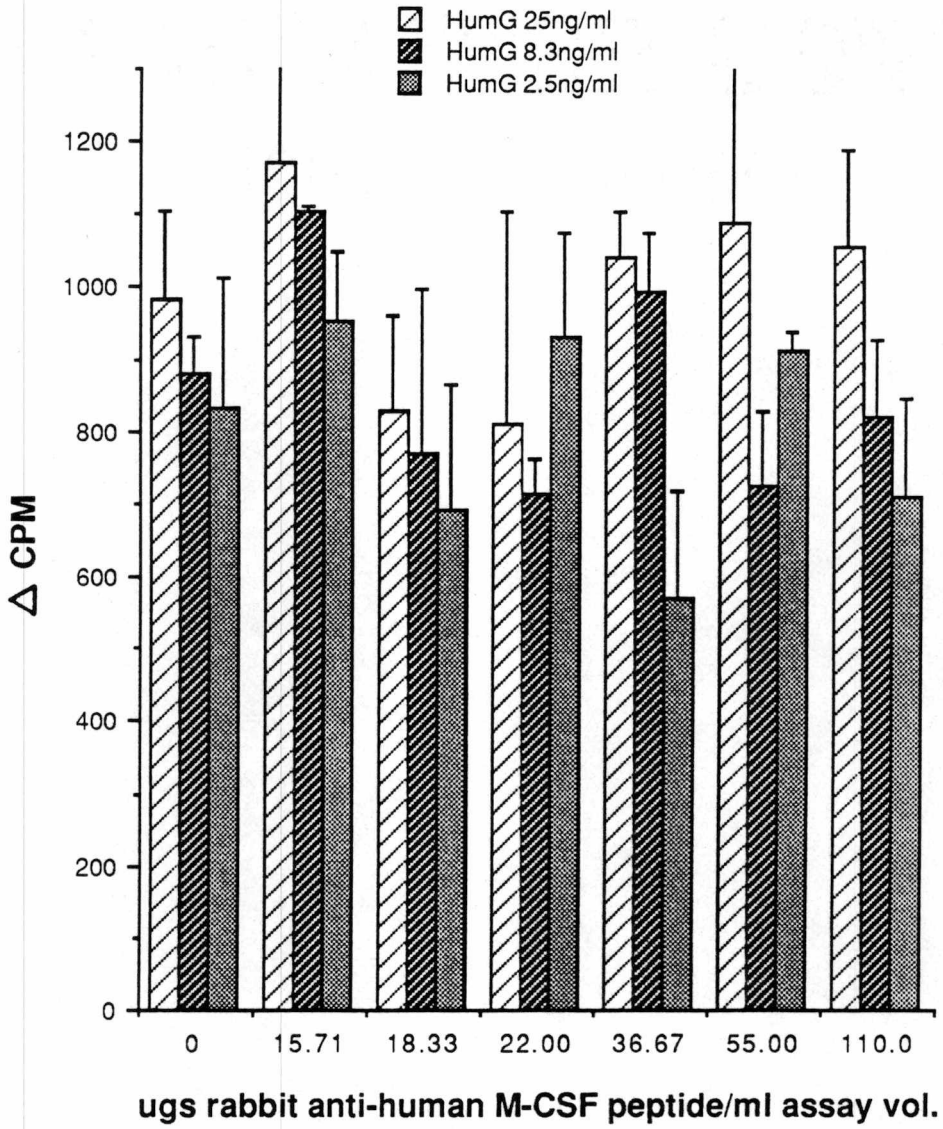

Figure 22: The biological effect of rabbit anti-human M-CSF peptide on varying concentrations of recombinant human G-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. Values were corrected for background (362 ± 97).

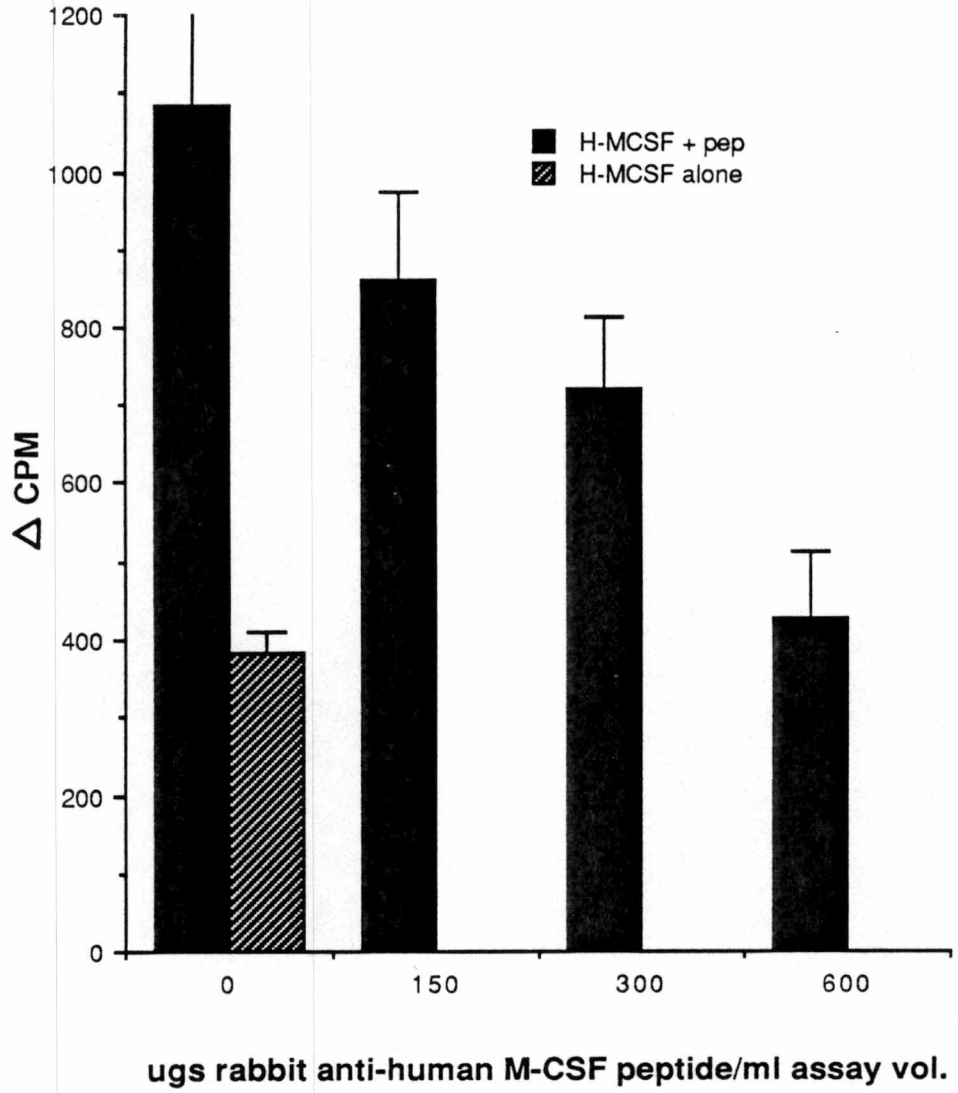

Figure 23: The biological effect of rabbit anti-human M-CSF peptide on human M-CSF peptide plus recombinant human M-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. The recombinant human M-CSF was used at a concentration of 250U/ml assay volume. Values were corrected for background (381 ± 67)

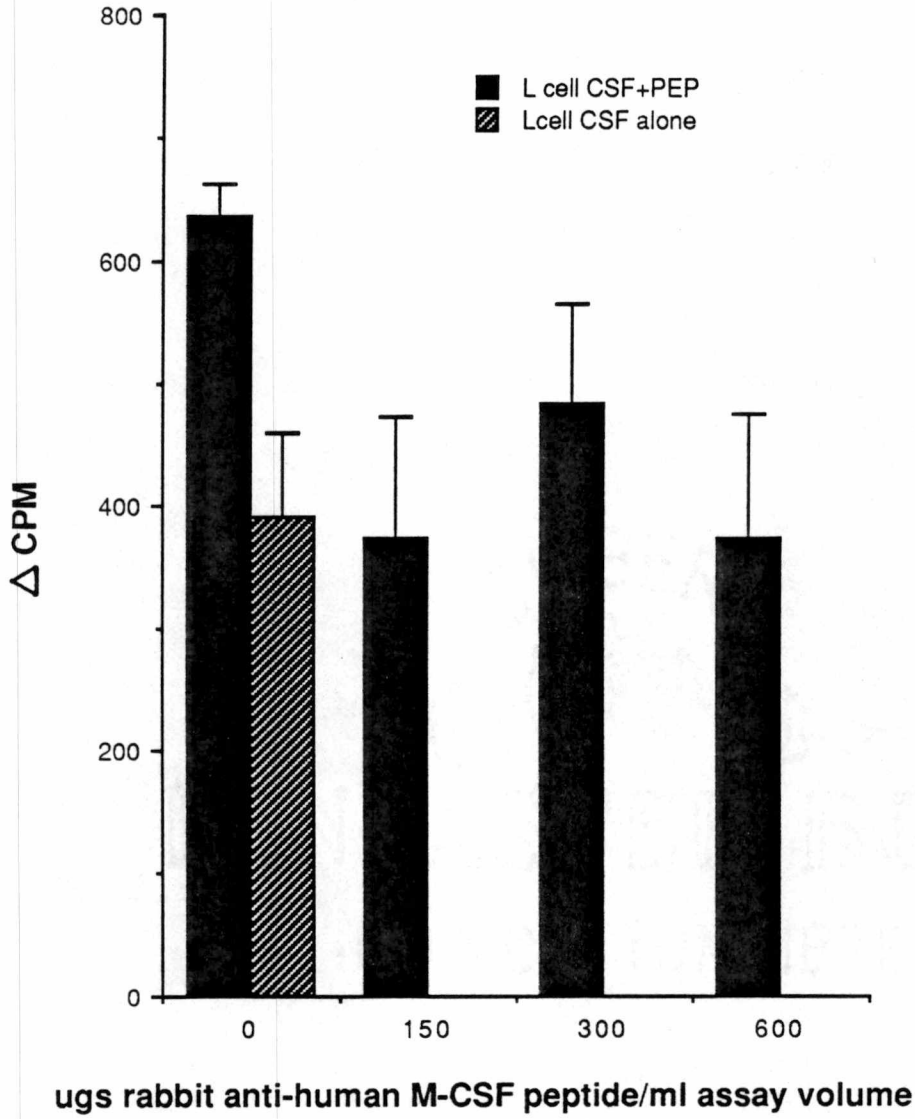

Figure 24: The biological effect of rabbit anti-human M-CSF peptide on human M-CSF peptide plus L cell M-CSF. The biological effect was assessed by ^{3}H-TdR incorporation. The L cell M-CSF was used at a concentration of 250U/ml assay volume. Values were corrected for background (271 ± 62)

and the resulting supernatant removed. Three thousand units of recombinant human M-CSF in 1.0 ml of 10% RPMI was added to the pelleted protein A-immunoglobulin and mixed for 5 hr at 5°C. Following centrifugation the supernatant was removed, diluted in an equal volume of 10% RPMI, and passed through a 0.2 M filter. Colony-stimulating activity remaining in the filtrate was assayed by the marrow ^{3}H-TdR incorporation assay. As shown in figure 25, DNA synthesis for cells incubated with protein A-Sepharose treated recombinant human M-CSF did not differ from that of cells incubated with non-treated recombinant human M-CSF.

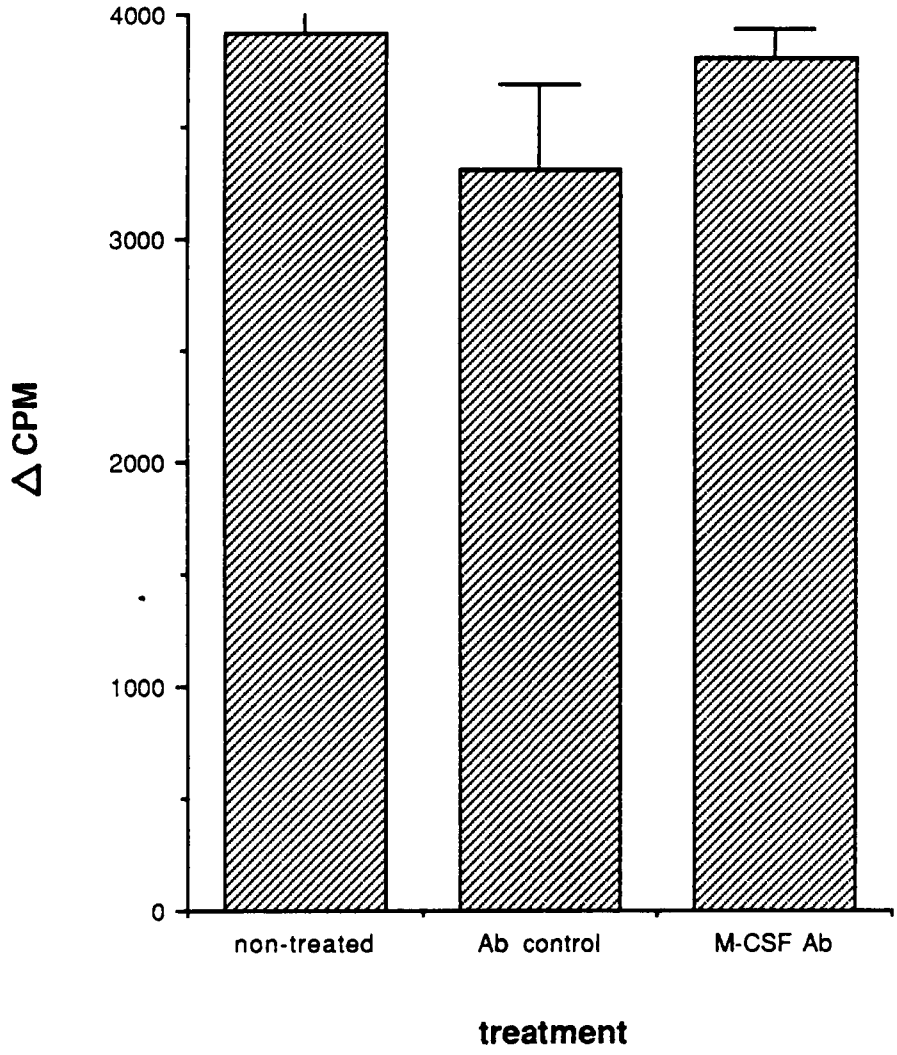

Figure 25: Treatment of recombinant human M-CSF with rabbit anti-human M-CSF peptide bound to protein A-Sepharose. The biological effect was assessed by ^{3}H-TdR incorporation. The antibody control was rabbit gamma globulin bound to protein A-Sepharose. Values were corrected for background (958 ± 44)

CHAPTER 5

DISCUSSION

Two peptides were synthesized, one representing a potential epitope of human M-CSF and the other representing a potential epitope of human GM-CSF. These peptides were used to determine if they had any biological activity alone, or in combination with native CSFs. The peptides were used also as antigens in the production of polyclonal antibodies with the intention of: 1) producing immunoassays for the detection of human M-CSF and human GM-CSF and, 2) determining if the antibodies could neutralize the biological activity of the appropriate CSF.

Rabbit anti-human M-CSF peptide antibody was capable of detecting, using a non-competitive ELISA, human M-CSF peptide (Figures 15, 16, pages 38, 39). However, the anti-human M-CSF peptide antibody did not detect, using both competitive and non-competitive ELISAs, recombinant human M-CSF or murine L cell M-CSF. This indicated that this antibody would not be a good choice for the development of a heterogeneous ELISA (46, 53) for human or L cell M-CSF.

M-CSF specifically stimulates survival, proliferation and differentiation of mononuclear phagocytes from precursor cell monoblast promonocyte monocyte macrophage (15). The biological responses of murine marrow cells to M-CSF are regulated by the M-CSF receptor, a tyrosine kinase of Mr 165,000, which induces phosphorylation of several membrane proteins (54, 56).

The receptor bound M-CSF is endocytosed and the M-CSF compartmentalized. The M-CSF is slowly degraded to tyrosine. Tyrosine is not reutilized for *de novo* protein synthesis and rapidly diffuses into the medium (16). Both binding and degradation of M-CSF somehow contribute to the survival, proliferation, and differentiation of mononuclear phagocytes.

The anti-human M-CSF peptide antibody had no effect on the biological activity, as determined by ^{3}H-TdR incorporation, of L cell M-CSF or recombinant human M-CSF (Figures 20, 21, pages 48, 49). Also, rabbit anti-human M-CSF peptide bound to protein A-sepharose was not capable of removing, as determined by DNA synthesis, human M-CSF from 10% RPMI (Figure 25, page 54).

Incorporation of ^{3}H-TdR by murine marrow cells incubated with or without varying concentrations of human M-CSF peptide was not appreciably different (Figure 17, page 42). ^{3}H-TdR incorporation and colony formation was not appreciably different between cells incubated with recombinant human G-CSF or murine GM-CSF, and cells incubated with one of the former CSFs plus human M-CSF peptide (Tables 3, 4, pages 45, 46). However, murine marrow cells incubated in the presence of L cell M-CSF or recombinant human M-CSF plus human M-CSF peptide had an appreciable increase in DNA synthesis over cells incubated only in the presence of L cell or recombinant human M-CSF (Figure 18, 19, pages 43, 44). No increase in colony formation was observed when the above experiment was repeated using the soft agar assay. Additional experiments involving L cell M-CSF or recombinant

human M-CSF plus human M-CSF peptide and anti-human M-CSF peptide antibody indicated the increase in the rate of DNA synthesis was due to the presence of M-CSF peptide (Figures 23, 24, pages 51, 52).

Perhaps, cells in the murine mononuclear phagocyte lineage require recombinant human M-CSF or L cell M-CSF to promote M-CSF uptake and start degradation. Once the process is started the receptors may be capable of binding, internalizing, and degrading M-CSF peptide. To determine if human M-CSF peptide is being internalized and degraded two more studies would be necessary. The first study would determine if M-CSF peptide is being internalized and would involve exposing murine marrow cells to M-CSF plus ^{125}I-human M-CSF peptide at 37°C for 2 hr. Intracellular radioactivity, indicating internalization of human M-CSF peptide, would be determined. If ^{125}I- human M-CSF peptide was being internalized a second study would involve determining if the peptide was being degraded to tyrosine. If human M-CSF peptide was being degraded antibody against whole human M-CSF would be utilized. The cells would be incubated in the presence of recombinant human M-CSF or L cell M-CSF plus ^{125}I-human M-CSF peptide. After a given time, the medium above the cells would contain either intact ^{125}I-human M-CSF peptide or ^{125}I-tyrosine. Intracellular degradation would be indicated if the supernatant contained ^{125}I not bound by anti M-CSF antibody.

The human GM-CSF peptide, like the M-CSF peptide, had no effect on the incorporation of ^{3}H-TdR by murine marrow cells

(Figure 8, page 26). However, unlike the human M-CSF peptide the human GM-CSF peptide showed no biological effect even in combination with murine GM-CSF (Tables 1, 2, pages 27, 28).

The rabbit anti-human GM-CSF peptide antibody possessed limited neutralizing activity, as determined by soft agar assay and ^{3}H-TdR incorporation, for murine GM-CSF (Figures 9,11, pages 29, 32). One possible explanation for the antibody possessing only limited neutralizing activity may be that 50% of the murine GM-CSF is comprised of carbohydrate (47). The carbohydrate may be masking the region of the GM-CSF molecule that the antibody is directed against. This theory is backed up by the: 1) necessity of having to denature the murine GM-CSF molecule, perhaps displacing the carbohydrate, for its detection by a non-competitive ELISA (Figure 6, page 22), 2) inability of anti-human GM-CSF peptide antibody bound to protein A-Sepharose to remove murine GM-CSF from 10% RPMI (Figure 12, page 34) and, 3) ability of anti-human GM-CSF peptide antibody to detect recombinant human GM-CSF without denaturation (Figure 5, 7, pages 20, 24). Recombinant human GM-CSF does not contain carbohydrate.

The rabbit anti-human GM-CSF peptide antibody was similar to the rabbit anti-human M-CSF peptide antibody in that both antibodies were capable of detecting, using non-competitive ELISA's, their appropriate peptide. Unlike the rabbit anti-human M-CSF peptide antibody, the GM-CSF peptide antibody was capable of detecting, using non-competitive and competitive ELISA's, recombinant human GM-CSF (Figures 5, 6, 7, pages 20, 22, 24). A

competitive ELISA in which recombinant human GM-CSF was adsorbed to the plate and the antibody incubated in the presence of human GM-CSF peptide was performed. Results showed that as peptide concentration increased less of the anti-human GM-CSF peptide antibody was available to detect recombinant human GM-CSF (Figure 7, page 24). This indicated that the antibody reacted with the predicted epitope of human GM-CSF. The GM-CSF peptide antibody was also capable of detecting murine GM-CSF ,using a non-competitive ELISA, but only after the murine GM-CSF had been denatured with 6M urea (Figure 6, page 22). The GM-CSF antibody was highly specific in that it did not cross react with recombinant human G-CSF (figures 5, 6, 7, pages 20, 22, 24) or recombinant human M-CSF. This indicated GM-CSF peptide antibody would be an excellent choice for a primary antibody in a heterogeneous ELISA(47, 53) for murine or human GM-CSF.

Future research will focus on locating another epitope of human GM-CSF. Once located a peptide representing this region will be synthesized and used in the production of antibody. This antibody will hopefully be used as a secondary antibody in a heterogeneous ELISA for human GM-CSF and murine GM-CSF. The biological effect of this antibody, alone and in combination with the above anti-human GM-CSF peptide, on murine GM-CSF will be investigated.

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VITA

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