ENHANCEMENT OF THE FREQUENCY OF TUMOR-ASSOCIATED MACROPHAGES CAPABLE OF *IN VITRO* PROLIFERATION BY COLONY-STIMULATING FACTORS AND HETEROLOGOUS ERYTHROCYTES

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PREFACE

Limiting dilution analysis was used to analyze the effects of colony-stimulating factors on the *in vitro* proliferation of tumor-associated macrophages and resident peritoneal macrophages. Specifically, macrophage colony-stimulating factor and granulocyte/macrophage colony-stimulating factor were examined as both crude natural and recombinant forms for enhancement of proliferative responses of these two populations. Sheep erythrocytes were also assayed for their ability to stimulate proliferation. Analysis of data showed little significant difference between proliferation of resident peritoneal macrophages and tumor-associated macrophages with the factors that were assayed. Further analysis of supernate isolated from cultured tumor cells indicated the production of colony-stimulating factors by the tumors. This suggests that some tumors may stimulate macrophage proliferation.

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CHAPTER I

INTRODUCTION

The presence of macrophages in rapidly growing tumors has long been a mystery to tumor researchers. Commonly called tumor-associated macrophages (TAM), these cells have been found in almost all solid tumors regardless of tissue origin or species and constitute from 2 to 80% of the tumor mass (7,13,14). Of particular significance is the point that the percentage of TAM found in a particular tumor remains relatively constant even during rapid tumor growth (7). The precise function of TAM in tumors is unknown; both cytotoxic functions and enhancement of tumor growth have been reported (7,13,14).

With the recent widespread acceptance of the concept that normal mature macrophages are capable of proliferation (26,30), it would be interesting to examine TAM to determine if their existence in large numbers is due to an influx of monocytes (immature macrophages) from the blood or from cell division of the macrophages present in the tumor. The possiblity of regulation of TAM growth by the tumor could be examined to determine if the tumor releases known macrophage colony stimulating factors and if TAM react to these factors in the same way as normal macrophages. An understanding of

this regulation and its effects on TAM could help lead to novel strategies in the treatment of tumors.

Limiting dilution analysis is one method that can be used to describe both proliferative function and its regulation. Application of Poisson statistics that enables one not only to be able to determine the frequencies of responding macrophages in an experiment but also to be able to infer these results to the entire population of macrophages (10).

Macrophages

Macrophages, also known as mononuclear phagocytes, comprise an important part of host defense against disease. Besides phagocytosis of invading microorganisms, they are capable of repairing tissue damage, processing and presenting antigen to T lymphocytes, and aiding in the fight against tumors through the production of tumor necrosis factor (25,28).

Macrophages originate in the bone marrow when a monoblast develops from a pluripotent stem cell (30,32). After 1 to 2 days, the monoblast divides and the daughter cells develop further into promonocytes (32). The promonocytes divide again and become monocytes within 24 hours, then exit the bone marrow and enter the blood stream (32). Monocytes circulate in the blood for 2 to 3 days and then migrate into the tissues, where they differentiate into mature resident macrophages and may survive for 1 to 5 weeks (30,32). Mature tissue macrophages are capable of carrying

out a number of functions, which appear to be determined by the development of the macrophage and its surrounding microenvironment (17). It is not certain if macrophages arise from a single precursor type and express functions determined by their age and environment or if the variety of subsets of macrophages arise from different precursors (26).

In suspension, most macrophages are spherical in shape and approximately 14-20 um in diameter (6). A single bean shaped nucleus, abundant cytoplasm, and a large number of lysosomes characterize these cells (6). Macrophages are noted for their ability to adhere to glass surfaces, and this is commonly used as a means of isolating them from heterogeneous cell populations (21,31). A variety of cell surface markers are expressed, including Fc receptors, MAC-1, lymphocyte function associated antigen-3 (LFA-3), interleukin-2 (IL-2) receptors, and major histocompatibility complex (Mhc) class I and class II molecules which can be complexed with processed antigen (2,5,6,8). Some of these markers are expressed constitutively by all macrophages, while others are expressed only at certain stages of development or in certain tissues (26). Macrophages are found throughout the body, including in all of the organs of the body, the connective tissues, and the serous cavities (31).

Macrophages are capable not only of direct cytotoxic activities on bacteria and tumor cells, but also activation of lymphocytes, induction of inflammation and fever, and

facilitation of tissue repair and reorganization (25,31). Macrophages are able to directly attack bacteria through phagocytosis and lysosomal destruction of the phagocytized particle by lysozyme, radical oxygen intermediates (ROI), acid hydrolases, and cationic proteins. Tumoricidal activity is achieved primarily by the secretion of the complement factors, ROI, various proteases, and the release of tumor necrosis factor (TNF) (25,28).

The ability of macrophages to actively phagocytize antigens makes them important antigen presenting cells (APC), particularly of particulate antigens such as bacteria. This, combined with the secretion of IL-1 (28), enables them to activate CD4+ T lymphocytes. CD4+ T lymphocytes comprise the 'helper' T cell population responsible for aiding in the activation of B lymphocytes and macrophages during an immune response (28).

Macrophages are also capable of causing the induction of inflammation and fever at the site of an infection (28). This is accomplished mainly by the production of known pyrogens, including IL-1 and TNF. They also release clotting factors, complement factors, and prostaglandins (28). Macrophages aid in tissue repair and reorganization through the production of a variety of growth factors, fibroblast stimulating factors, and factors which stimulate angiogenesis (28). Undesirable effects on the body can also be attributed to macrophages. Some factors secreted by macrophages in the host's defense against invasion are also capable of causing tissue damage, which can at times be considerable (28).

Macrophage Proliferation

It has only been in the last few years that mature macrophages are capable of proliferation *in vivo* as well as *in vitro* has gained general acceptance (11,22,25,26,30). The importance of the proliferative ability of macrophages lies in the suggestion that replacement of macrophages in the tissues can be carried out by cell division as well as by influx of monocytes from the blood. Van Furth, who had originally argued against macrophage proliferation, still contends that an influx of macrophages is the primary way in which replacement occurs and that fully mature macrophages are not capable of proliferation (32). Macrophage cell division has been shown to be stimulated by a number of cytokines, of which macrophage colony stimulating factor (M-CSF) is the most well known and characterized (1,9,24). Granulocyte macrophage colony stimulating factor (GM-CSF), and interleukin-3 (IL-3 or multi-CSF) are also known to upregulate macrophage growth (1,24,30). Phospholipids and sheep erythrocytes have also been shown to stimulate macrophage proliferation *in vitro* (33). The effect of interleukin-2 (IL-2), a T cell growth factor, is still uncertain; conflicting reports have labelled this factor as stimulatory, non-stimulatory, and suppressive in terms of macrophage proliferation (2,8).

Tumor-Associated Macrophages

The observation of macrophages within solid tumors was

first occurred in the 19th century, but the significance of this fact is unknown even today (7,13). One of the more fascinating aspects of TAM is that they may comprise a very large part of the tumor mass, and that this amount remains constant even during rapid tumor growth (7). The amount of TAM found seems to be tumor-dependent; the quantity of TAM varies between different tumors but remain relatively constant in tumors passaged from one animal to another (7,13,14). TAM also appear to have the ability to carry out all normal macrophage functions, including antigen presentation, production of cytokines such as IL-1, and cytotoxic functions (7,13).

TAM Characteristics And Functions

Characterization of TAM has been difficult since tumor cells can appear morphologically identical to normal macrophages when examined histologically (14). Two distinct subpopulations have been described in a number of tumors, based originally on the size of the macrophage by velocity sedimentation (7,14). One subpopulation consists of smaller, peroxidase-positive macrophages, and the other contains larger macrophages with higher levels of expressed Fc receptors and Mhc Class II (IA) molecules (7,14). It is thought that the population of smaller cells constitutes less mature macrophages and that they develop into the more mature larger population. Both populations appear to have intermediate levels of nucleotidase and acid phosphatase as

compared to resident peritoneal macrophages (high nucleotidase, low acid phosphatase) and *Corynebacterium parvum*-activated macrophages (low nucleotidase, high acid phosphatase) (7).

Functions of TAM, like the numbers of TAM found in tumors, appear to be tumor-dependent and not a part of a generalized host response. Many of the functions that TAM are known to be capable of seem to be beneficial to tumor growth, but cytotoxic functions such as TNF secretion have also been reported (7,13,14). All of these functions appear to be normal macrophage capablities, including lymphocyte activation, promotion of cell growth, and cytotoxic activities (7,13). Experiments have shown that TAM are capable of antigen presentation and activation of T lymphocytes (7,13). TAM have also been found to be potent secretors of a variety of cytokines and proteases, including IL-1, collagenase, and platelet-derived growth factor (13).

Tumoricidal activity has been demonstrated <u>in vitro</u> by TAM when exposed to activating agents, but the level of activity seen is much lower than that of normal tissue macrophages (13,14). Reactive oxygen intermediates (ROI) have also been found to be secreted by TAM, which could have not only have a potentially cytotoxic effect, but also a mutagenic effect. Mutagenesis might possibly increase tumor heterogeneity and resistance to host responses (7,13,14). TAM have also been described with procoagulant activity (PCA), which leads to fibrin deposition, which in turn modulates effector cell entry into the neoplastic tissue,

angiogenesis, and tumor cell motility (13). Experimentally it has been shown that tumor cells directly stimulate macrophages to express PCA (13). TAM have also been attributed with angiogenesis in the tumor site and in the invasion of distant sites by tumor cells (13,14).

Effects of TAM *in vivo*

Expression of the different functions outlined above by TAM would be expected to have very different effects on tumor growth, and metastasis (7,13,14). For instance, the injection of toxins, such as silica, which specifically block macrophage function, result in a decrease in the growth of the tumor but also enhance tumor metastasis (13,14). TAM could act to provide optimal conditions for neoplastic growth, by the production of growth factors and promoting blood vessel formation through PCA (7,13,14). Enhancement of tumor growth seems to function best when the TAM:tumor cell ratios are low *in vitro* (7,13). This same situation possibly occurs *in vivo*.

Cytotoxic TAM have also been described, although primarily in tumors that are in regression (7,13,14), and are commonly seen *in vitro* when the TAM:tumor cell ratio is high (7,13). Secretion of TNF, complement factors, and ROI could have cytotoxic effects on tumor cells, but can have other effects as well. Tumor necrosis factor can help select for TNF-resistant tumor cells by elimination of TNF-sensitive cells, thereby making it even harder for the host to

effectively respond to the tumor (7). ROI have been implicated with mutagenic capabilities, which could lead to an increase in tumor cell heterogeniety and, as with TNF, could give rise to a tumor much more resistant to host defense mechanisms (13,14).

Secretion of a wide range of proteases, including collagenase and elastase by TAM has also been described (13). These can contribute to invasion of the tumor into secondary sites. Ironically, TAM found at these secondary foci seem to act as restraints against metastasis and are frequently tumoricidal (13). Examination of these foci has revealed a higher TAM:tumor cell ratio than in the primary tumor, which can offer partial explanation of this phenomenon (13).

Proliferation of TAM

It is still uncertain whether the large quantities of TAM found in many tumors are due to influx of monocytes from the blood or from <u>in situ</u> proliferation of mature macrophages (13). The numbers of TAM are usually quite large, although, and it has been determined that in a solid tumor 1 cm in diameter with a TAM content of 30% or more would have a macrophage population larger than that of an entire normal mouse (14). This would indicate either an incredible influx of monocytes into the tumor coupled with increased generation of promonocytes in the bone marrow, proliferation of macrophages already in the tumor, or both (7,14). TAM have recently been shown to express receptors for M-CSF on their membranes, indicating that TAM may be capable of responding

to M-CSF (3). A better understanding of the capability of TAM to proliferate and the regulation of this growth by the tumor is needed, especially if TAM are ever to be considered as a potential immunotherapeutic strategy for cancer.

Cytokines

Cytokines are the messengers of the immune system, transferring signals from one cell to another and aiding in activation, differentiation, suppression, and proliferation of cells involved in the immune response as well as non-immune cells. These molecules comprise a broad and diverse group, varying widely in size, shape, sources, targets, and effective range; included in this group are the interleukins, interferons, and colony-stimulating factors.

Macrophage Colony-Stimulating Factor

Macrophage colony stimulating factor, also known as CSF-1 or M-CSF, is a potent stimulator of proliferation and activation in both mature and immature macrophages (19,20, 24). M-CSF has been found to be produced by fibroblasts, monocytes, macrophages, endothelial cells, and mitogen-stimulated lymphocytes (24). For study, it is commonly isolated from murine L-cell conditioned medium, although recombinant murine and human M-CSFs has recently become available (19,20,23,24). Studies have identified it as a glycoprotein that varies between 45 and 86kd in size with a carbohydrate content in excess of fifty percent (24). The

protein component is made up of 2 subunits of similar size and shape (24). It is believed, although not proven, that the quantity of carbohydrate causes the variation in the size of the molecule. M-CSF is inactivated by heating and gentle reduction methods, but resistant to most proteases (24). Besides proliferation, M-CSF has also been demonstrated to induce a number of differentiation functions in mature macrophages, including production of prostaglandin E, plasminogen activator, IL-1, interferons, myeloid growth factor, peroxide, and tumoricidal activity (19,20).

Granulocyte/Macrophage Colony-Stimulating Factor

Granulocyte/macrophage colony-stimulating factor, or GM-CSF, is a potent stimulator of macrophage and granulocyte proliferation and differentiation, and is found to be secreted by the same types of cells that secrete M-CSF as well as T lymphocytes. Natural GM-CSF can be obtained in quantity through <u>in vitro</u> incubation of murine lungs in medium, but is also available in a recombinant form (24). It is a sialic acid-containing glycoprotein approximately 25-40kd in size (24). Like M-CSF, GM-CSF has a large carbohydrate component, but in contrast to M-CSF, it is resistant to heating and gentle reduction and sensitive to proteases (24). GM-CSF,as well as M-CSF, has been demonstrated <u>in vitro</u> to stimulate tumoricidal activity in macrophages (20).

Limiting Dilution Analysis

Limiting dilution analysis (LDA) is based on a mathematical formula derived from binomial and Poisson distributions (10). The intention is that, with this formula, inferences can be made from cells in an experiment about colony formation of an entire population of cells. In other words, an experiment could be performed using resident peritoneal macrophages from a normal mouse and the results could be said to be true for the entire population of all resident peritoneal macrophages from that strain of mouse. LDA can be used to determine the frequency of cells in a responding cells in a population, even if the cells of interest are low in number, since the sensitivity of the assay is such that very low levels of proliferation can be detected (10). The use of LDA can also help determine whether cellular density can enhance or inhibit colony formation (10). Finally, LDA distinguishes between the frequency of responding cells and colony size, a pitfall of other commonly used methods (10). The purpose of this investigation is to examine and compare the ability of TAM and RPM to proliferate and the regulation of this growth by M-CSF, GM-CSF, and sheep erythrocytes using LDA.

CHAPTER II

MATERIALS AND METHODS

Animals

For all of the experiments, female C3H/Hen mice of approximately 6-8 weeks of age were obtained from Charles River of Wilmington, Massachusetts. Mice were kept in a facility at Oklahoma State University. In all experiments ether was used to euthanize the mice; severage of the spinal cord was not performed to insure that blood would not enter into the peritoneal cavity.

RPMI Culture Medium

For all of the experiments performed and the generation of crude supernatants for testing, cells were diluted in complete RPMI medium (cRPMI). Powdered RPMI obtained from Sigma Chemical Corp., St. Louis, MO was reconstituted in double distilled water with 2 grams of sodium bicarbonate added per liter of medium prepared. Complete RPMI contained 5% (vol/vol) heat inactivated (56°C water bath for 30 minutes) fetal bovine serum , sodium pyruvate (1.0 mM), nonessential amino acids (1.0 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), amphotericin B (2.5 ug/ml), and gentamycin sulfate (50 ug/ml). All of the

components of complete RPMI were obtained from Sigma. The pH was adjusted to 7.1, and the medium was sterilized by filtering the medium through 0.22 um sterile disposable filters into autoclaved 500ml bottles. Filtered medium was kept at 4° C until needed.

MEM Culture Medium

Alpha-MEM was used to maintain cultures of the tumor and L929 cell lines when supernatants were not being prepared. The medium was reconstituted from a powder (obtained from Hazleton Corp., Denver, PA) in double distilled water; 2.2 grams of sodium bicarbonate was added per liter of prepared medium. As with cRPMI, additional ingredients were added to insure cell growth, including L-glutamine (2mM), penicillin (10,000 U/ml), streptomycin (0.1 mg/ml), amphotericin B (2.5 ug/ml), gentamycin (50 ug/ml), and 10% heat-inactivated (56°C waterbath for 30 minutes) calf serum (all obtained from Sigma). The pH of the medium was measured and adjusted to 7.1. Sterilization was achieved by filtering the medium through 0.22 um sterile filters into 500 ml autoclaved bottles. Filtered medium was kept at 4° C until needed.

Maintenance Of Cultures

Cultures of L929 (obtained from American Type Culture Collection, Rockville, MD) and tumor cells were kept at 37° C in a 5% CO_2 (in air) atmosphere. For propagation and maintenance, cultures were replenished weekly with alpha-MEM.

Cultures were also examined weekly with an inverted phase-contrast microscope to assess cell growth. If the flask contained confluent monolayers, then the cells would be subcultured by the following procedure. The medium in the flask would be discarded and the flask washed once with approximately 10 ml of phosphate buffered saline (PBS) (0.05 M PO_4, pH 7.4). Two ml of trypsin-EDTA (.25% wt./vol. trypsin and 1 mM EDTA in PBS) were pipetted into the flask, and then the flask would be incubated for 10 minutes at 37° C. Approximately 10 ml of alpha-MEM would then be added to stop the enzyme reaction. The flask would be tapped several times to loosen any additional cells and then the medium would be discarded. The flask would then be washed once with 10 ml of PBS, and then 30 ml of fresh alpha-MEM would be added to the flask. The flask would then be returned to the 37° C incubator.

M-CSF

Crude natural M-CSF preparations were obtained by culturing 5 x 10^6 cells of the L929 cell line in 30 ml of cRPMI. This was allowed to incubate for 48 hours in a 37° C CO_2 incubator. The supernatant was harvested and then centrifuged at 650x g for 8 minutes to remove any cells floating free in the medium. This was then dispensed into sterile tubes in 3 ml aliquots and stored at -20° C until needed.

Recombinant human M-CSF (rM-CSF) was obtained frozen from Cetus Corporation at a concentration of 5 x 10^5

Units/ml. This solution was thawed and diluted to 1 x 10^4 Units/ml in PBS containing 0.1% bovine serum albumin. rM-CSF was aliquoted out in 1 ml aliquots into sterile test tubes and stored frozen at -20° C until needed.

GM-CSF

Crude natural GM-CSF preparations were obtained by the following method: lungs were removed from C3H/Hen mice, minced, and the fragments placed into cRPMI (12.5mls per pair of lungs) in a petri dish. This was sealed with wax film and incubated for 48 hours in a CO_2 incubator at 37° C. The medium was harvested and spun down at 650x g for 8 minutes to remove any cells. This supernatant fluid was dispensed in 1 ml aliquots into sterile tubes and stored at -20° C until needed.

Recombinant murine GM-CSF (rGM-CSF) was obtained from Immunex Corporation in Seattle, Washington This was diluted to 1 x 10^4 Units/ml in PBS containing 0.1% bovine serum albumin and dispensed in 1 ml aliquots into sterile tubes. This was then stored at -20° C until needed

Sheep Erythrocytes

Sheep erythrocytes (SRBCs) were obtained from the OSU College of Veterinary Medicine and from Organon Teknika in Durham, North Carolina. Prior to use in experiments, SRBC were stored at 4° C. SRBC were prepared for use in experiments by washing. PBS was added to the SRBC and mixed

gently, then centrifuged at 650x g for 8 minutes. The supernatant fluid was removed and replaced with an equal volume of PBS, and then the procedure was repeated at least three times or until the supernate was transparent in color. The packed SRBCs in the pellet were then diluted to 10% by volume in PBS for use in experimentation.

Isolation of Resident Peritoneal Macrophages

RPM were obtained from normal mice by peritoneal lavage with PBS (16). Mice were euthanized in an ether jar prior to peeling back the skin from around the peritoneal cavity. Three milliliters of cold PBS was then injected into the peritoneal cavity, and the cavity was gently massaged. PBS containing peritoneal cells was recovered by aspiration and transferred to a sterile test tube kept on ice. This procedure was repeated three additional times. The recovered fluid was then centrifuged at 650x g for 8 minutes. The supernate was discarded and the pellet resuspended in 2 mls cRPMI and kept on ice. Ten microliters of the cell suspension was removed and diluted 1:10 with 90ul of PBS, from which 10 ul was then loaded onto a hemacytometer and counted. Cell viability was determined by mixing 10ul of the cell suspension with 10ul of trypan blue and loading 10ul of this mixture onto a hemacytometer and examining under the microscope. Cells unable to exclude the stain were counted as dead. The cell suspension was then ready to use in limiting dilution analysis and dose response assays.

Generation and Isolation of Tumor-Associated Macrophages

For this portion of the study, two solid tumors derived from different sources were used to obtain TAM. The first, designated as 1X-11-6, was obtained from Dr. Jim Beeson at the University of Oklahoma at Tulsa Medical College. Tumor 1X-11-6 was a spontaneous tumor which arose from an *in vitro* culture of murine placental tissue of female C3H/Hen mice. The second tumor, designated as MC-4, was generated in Dr. Kim Burnham's lab following a single subcutaneous injection of 1 mg of methylcholanthrene (MCA) in 50ul of olive oil at the ventral surface of a female C3H/HeN mouse. Both tumors were passed in normal C3H/HeN mice by subcutaneous injection of tumor fragments. Two to three weeks following implantation of the tumors into the secondary hosts, the mice were euthanized and the tumor excised. The tumor was cut into fragments and dissociated into a single cell suspension enzymatically by incubation in 0.1% (weight/volume) collagenase and 0.1% (w/v) dispase with mechanical stirring in a 37°C waterbath for 1 hour. Following the incubation, the resulting cell suspension was removed and centrifuged at 650x g for 8 minutes. The pellet was harvested and resuspended in 2mls cRPMI. Cell concentration was determined with a hemacytometer and the cell suspension was diluted to 2 x 10^7cells/ml. The cell suspension was then mixed with an equal volume of 5% antibody-coated SRBC and incubated for 30 minutes at 37°C with vertical rotation. Following the

incubation the percentage of rosettes was determined with the aid of a hemacytometer. The suspension was then layered over 3 mls of ficoll hypaque (specific gravity 1.119, obtained from Sigma) and centrifuged at 1100x g for 20 minutes. The supernate was discarded and the pellet resuspended gently in 2 mls cRPMI. The cell suspension was again layered over ficoll hypaque and centrifuged at 1100x g for 20 minutes. The pellet was again harvested and resuspended in 2mls cRPMI. The percentage of rosettes was again determined by hemacytometer and the suspension was centrifuged at 650x g for 8 minutes. The SRBC were then lysed by resuspending the pellet in 1ml sterile double distilled water followed immediately by the addition of 5mls of cRPMI to prevent lysis of TAM. The cell suspension was again centrifuged at 650x g for 8 minutes and the pellet resuspended in 2mls cRPMI. Cells were counted as before and were then ready for use in limiting dilution analysis or dose response assays.

Culture Of Tumors and Generation of Supernatants

After the tumors had been excised and separated into a single cell suspension (see above), 1 ml of the suspension was inoculated into tissue culture flasks to observe tumor growth <u>in vitro</u> and to generate tumor supernatants that could be tested for effects on TAM and RPM growth. After four passages <u>in vitro</u>, 5 x 10^6 tumor cells were put into 30 mls cRPMI in a tissue culture flask. The flask was put into a 37°C incubator with 5% CO_2 for 48 hours, after which the

fluid was harvested. This fluid was centrifuged for 8 minutes at 650x g, and then the supernatant fluid was saved and dispensed into test tubes in 3ml amounts. This was stored at -20°C until needed.

Cytokine Dose Response Assays for Colony Formation

Dose response assays were performed to determine the optimal concentration of the colony stimulating factors and SRBC for RPM proliferation. Concentrations of 0%, 1%, 5%, 10%, and 20% (vol/vol) of the crude natural factors were tested in the first assay, while other concentrations were tested in following assays if needed. rM-CSF and rGM-CSF were tested at concentration ranges of units/ml similiar to those commonly used by other researchers (1,4,17,19). A single cell concentration of 1 x 10^5 RPM/ml was used in all dilutions of the dose response assays. The cells were dispensed in 0.1ml aliquots into wells on microtiter plates, with 60 wells being used for each dilution. The plates were incubated for 11 days at 37°C in 5% CO_2. Wells were scored for colony formation by adding 20ul of a 0.5% antibody-coated SRBC solution to each well, gently rocking the plates for 30 minutes, and then examining the wells with an inverted phase contrast microscope. Positive wells were designated as those with 6 or more rosetted cells in a colony. The data was then plotted on a linear graph as the fraction of responding wells on the y axis versus the concentrations of the factor on the

x axis. The optimal concentration of the factor determined by this assay was used as the concentration of the factor upon LDA of TAM and RPM.

Limiting Dilution Analysis

LDA was used to assess the proliferation of TAM and RPM in culture and the regulation of this growth by M-CSF, GM-CSF, and SRBC. This form of analysis is based on a formula derived from the Poisson and binomial distributions, and is stated as $F_o = e^{-u}$ with F_o = fraction of nonresponding cultures and u = the number of responding cells per culture (10). The mean number of responding cells per culture is therefore linearly proportional to the negative logarithm of the fraction of nonresponding wells ($u = -\ln F_o$), so that a plot of cell concentration versus the negative logarithm of F_o gives a straight line, passing through the origin (10). This is commonly known as a single-hit event When F_o is equal to 0.37 (37% of the cultures were nonresponding), u is equal to 1 (an average of 1 responding cell per culture)(10). Therefore, when the frequency of responding cells can be estimated as the inverse of the cell concentration at which 37% of the cultures fail to respond (10). The Poisson distribution can only be applied if the responding cell is truly limiting. If more than one cell is required for a response or if other cells modulate the response measured, the frequency of nonresponding cells will not obey Poissonian behavior and the data will not yield a straight line (10). This is a result of the fact that the probability of placing

2 rare cells in a given culture will be very small at low cell densities but would increase at higher densities (10).

In these experiments, the response examined was proliferation and individual wells on a microtiter plate were considered as cultures. Sixty wells were used for each concentration of cells to insure the reliability of the data obtained. In addition to diluting cells in cRPMI medium containing whatever factor was being tested, a negative control of cells diluted in cRPMI alone was a part of every experiment. The plates were incubated for 11 days at 37°C in a 5% CO_2 atmosphere, after which the wells would be scored for colony formation. This was accomplished by adding 20ul of 0.5% antibody-coated SRBC solution to each well, gently rocking for 30 minutes, and then examining the wells for colonies of rosetted cells with an inverted phase contrast microscope. Only those wells with colonies of 6 or more rosetted cells were considered positive. The data was then collated and plotted on a semilog graph as the concentration of cells on the X axis and the fraction of nonresponding wells on the Y axis. The line drawn through the data was then examined by linear regression analysis (Pearson's method)(35), and the coefficient derived from this analysis was tested at 95% confidence for linearity on Table 1.

Values of r at the 5% Level of Significance

Degrees of Freedom[a]	Value of r[b]
1	0.997
2	0.950
3	0.878
4	0.811
5	0.754
6	0.707
7	0.666
8	0.632
9	0.602
10	0.576

a. the degrees of freedom was determined as the number of data points in the line (not including the origin) minus 1.
b. r represents the Pearson's coefficient obtained through linear regression analysis of the data.

Table I. Confidence values for the Pearson's coefficient obtained from linear regression analysis of the data (95% confidence) (29).

CHAPTER III

RESULTS

The primary goals of this study were to analyze and compare the ability of TAM and RPM to proliferate, to analyze the enhancement of this ability by M-CSF, GM-CSF, and SRBC, and to examine the supernates from two tumors for the presence of M-CSF and GM-CSF activity. Limiting dilution analysis was utilized for these experiments because of the mathematics involved that allow detection of the proliferation of common as well as rare cell types. Unlike other methods of determining proliferation, limiting dilution can determine a frequency of proliferation which indicates not only the number of responding cells but also whether or not other cell types are inhibiting or aiding the response. The information provided is important because it provides detailed information on the ability of TAM to proliferate, compares TAM and a normal RPM population, (which could help determine the source of TAM), and sheds some light on the interaction between tumors and TAM.

Dose Response Assays of Crude Natural Cytokines and SRBC

In order to determine the maximum frequency of RPM and

TAM capable of proliferation in the presence of the natural cytokines (M-CSF and GM-CSF) and SRBC via limiting dilution analysis, it was necessary to first determine the optimal concentrations of the three factors M-CSF was tested initially, at concentrations of 5, 10, and 20% (vol/vol) in cRPMI, along with a negative control consisting of cRPMI alone. For each concentration of M-CSF, RPM were used at a single concentration of $1x10^4$ cells/well. In accordance with the protocol described in Chapter II, Materials and Methods, each concentration was pipetted in 100 ul aliquots into 60 wells of a microtiter plate, and placed into a 37°C CO_2 incubator. After 11 days of culture, optimal colony formation of FcR^+ colonies (as detected visually after the addition of opsonized SRBC) was observed in the wells containing medium with 5% M-CSF. Seventy-six percent of the wells with 5% M-CSF were positive for colony formation (see Figure 1.). At concentrations of M-CSF above 5% and in cRPMI alone, lower levels of colony formation were seen. GM-CSF, was also tested at concentrations of 5, 10, and 20% (vol/vol) in cRPMI for its effect on colony formation. A concentration of $1x10^4$ cells/well of RPM was used for each dilution of GM-CSF, as was done previously with M-CSF. A negative control consisting of RPM in cRPMI alone was also utilized in the experiment. Results obtained after 11 days of culture showed that 63% of the wells were positive for FcR^+ colony formation in the presence of 10% crude GM-CSF (see Figure 2.). Concentrations above and below 10% yielded lower responses, although not as significantly different as

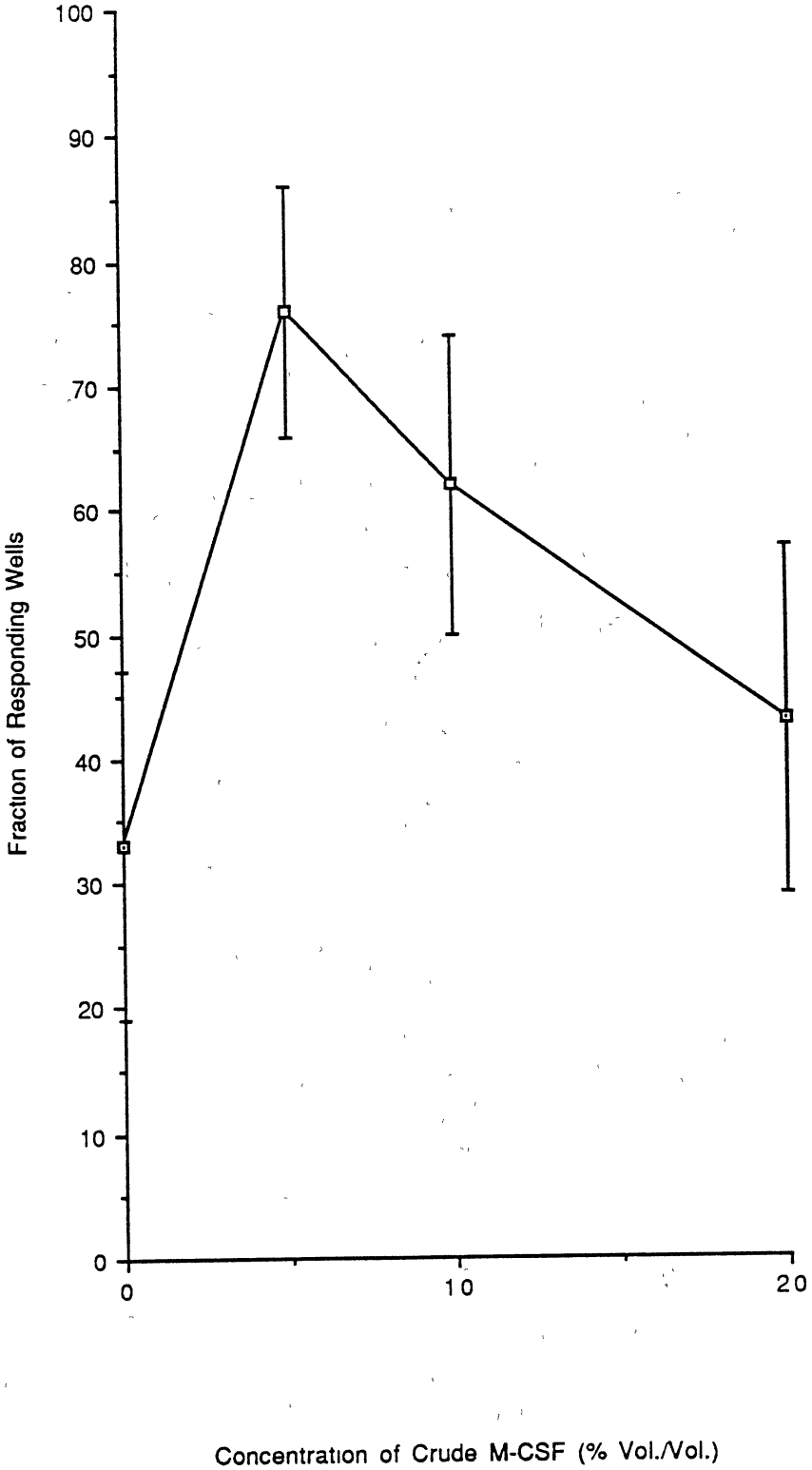

Figure 1 Effect of different concentrations of crude M-CSF on colony formation of RPM (10,000 cells/well).

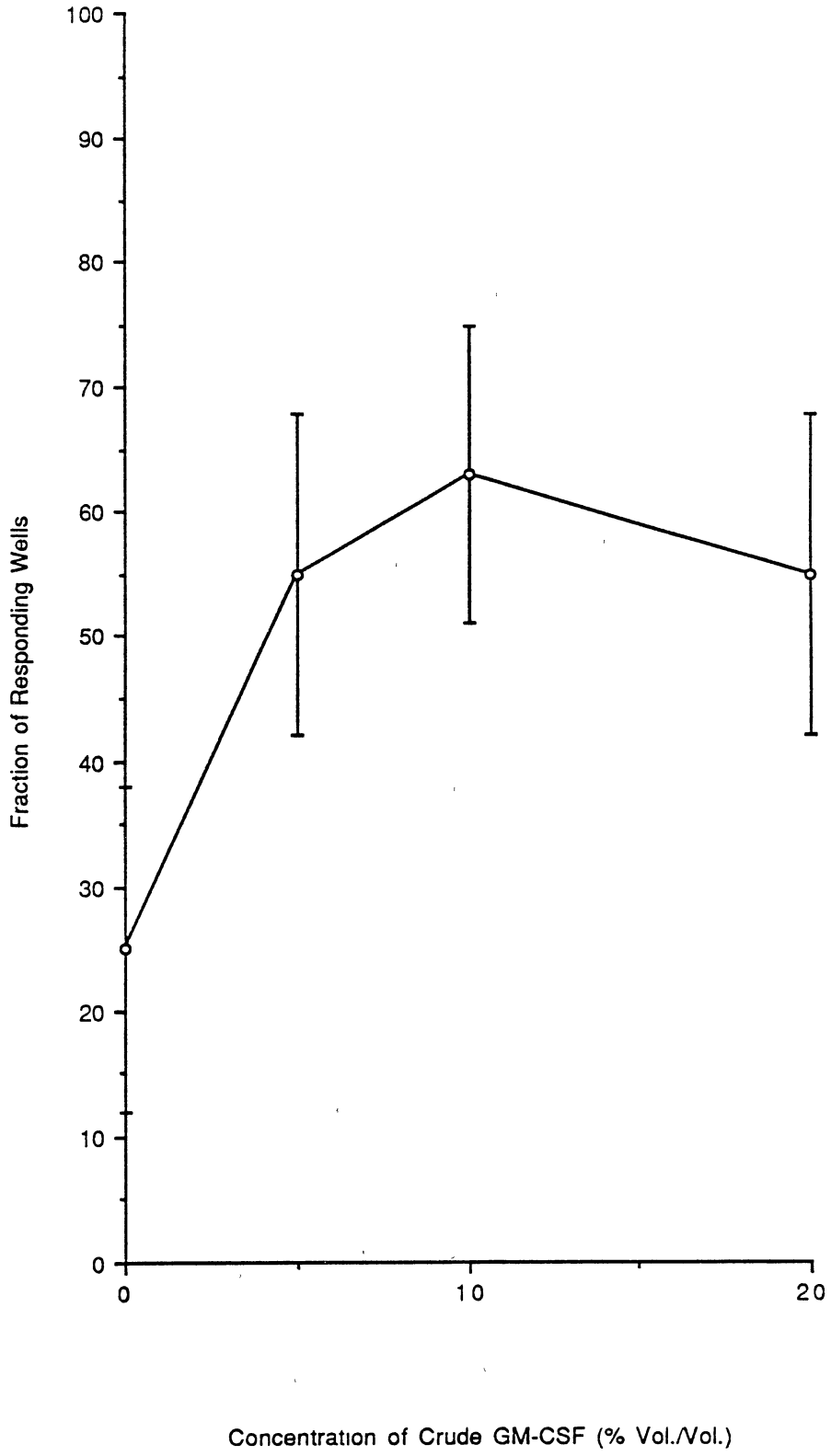

Figure 2. Effect of different concentrations of crude GM-CSF on colony formation of RPM (10,000 cells/well).

seen with M-CSF. For subsequent experimentation using limiting dilution analysis, crude GM-CSF was used at a concentration of ten percent.

Sheep erythrocytes (SRBC) were also tested for their effect on colony formation at concentrations of 0.25, 0.5, and 1% (vol/vol) in cRPMI. A negative control composed of cRPMI alone was also tested. As with previous experiments analyzing M-CSF and GM-CSF, a concentration of $1x10^4$ cells/ml of RPM was used. Care was exercised in scoring for colonies because of interference with rosetting by the SRBC already present in the well. The results showed a steady and linear increase in the percentage of wells positive for colony formation up through 1% (see Figure 3.). Therefore, a second experiment was performed testing SRBC concentrations of 1, 3, and 5% (vol/vol) in cRPMI. Results of this subsequent experiment showed that concentrations above 1% SRBC could not be used, because the numbers of SRBC in the well completely covered the bottom of the well and made it impossible to score for colony formation of RPM (results not shown). From the information provided by both experiments, then, the decision was made to use 1% SRBC in limiting dilution analysis experiments.

Response of RPM to Crude Cytokines and SRBC

Once the doses of the crude cytokines and the SRBC that produced optimal colony formation in RPM had been determined, the frequency of RPM capable of responding to these

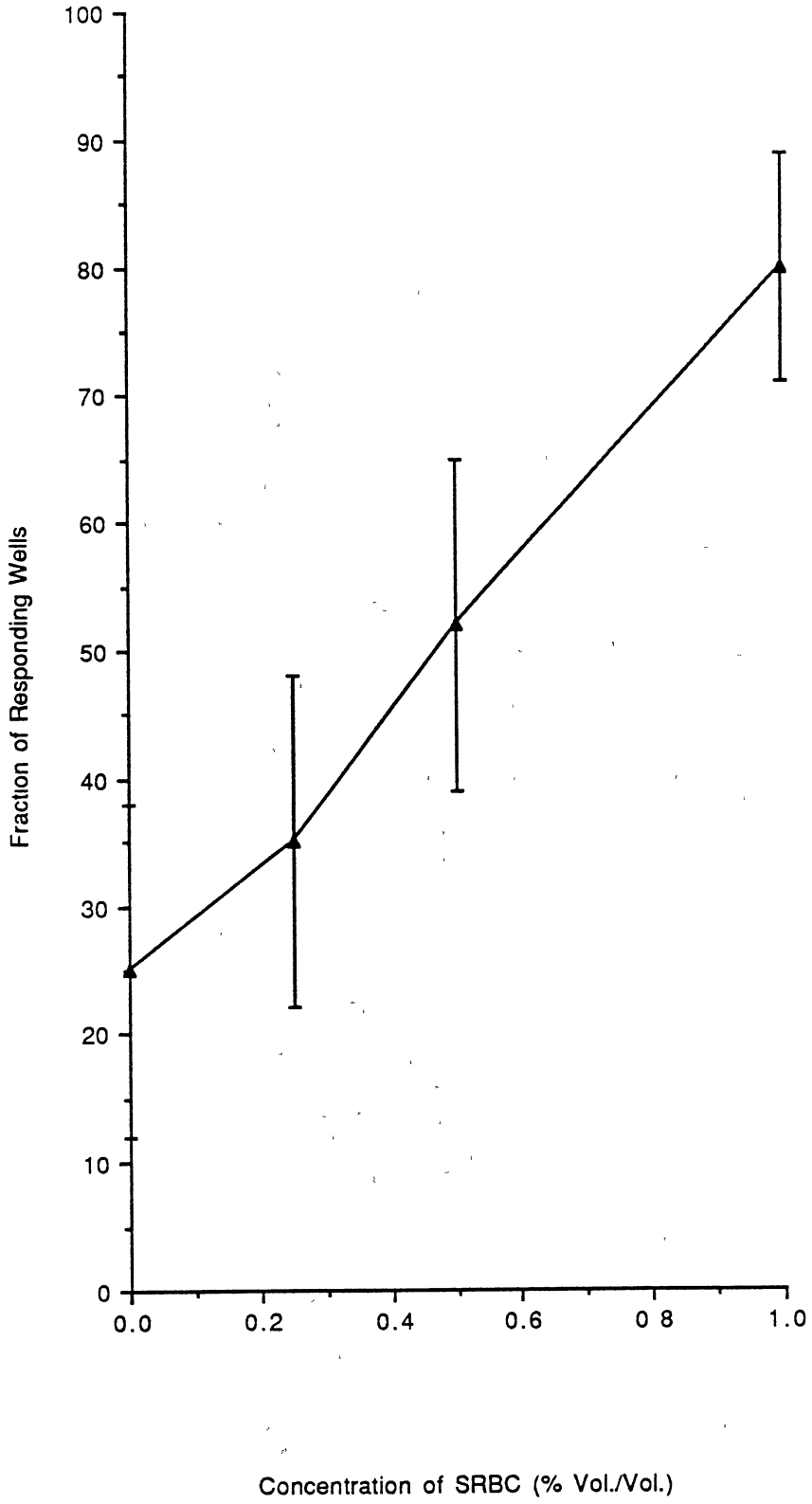

Figure 3. Effect of different concentrations of SRBC on colony formation of RPM (10,000 cells/well).

individual factors was determined. It was important to examine RPM first in order to employ the results obtained as a reference for comparison with TAM since RPM represent a population of normal non-inflammmatory tissue macrophages. M-CSF was examined first, at a concentration of 5% (vol/vol) Four different concentrations of RPM were used in the experiment, $1x10^3$, $2.5x10^3$, $5x10^3$, and $1x10^4$ per well. For a negative control, all 4 cell concentrations were tested in cRPMI alone. Data collected after 11 days of culture revealed that cRPMI containing 5% crude M-CSF supported a higher amount of colony formation than cRPMI alone (see Figure 4.). Linear regression analysis of the data by Pearson's method, yielded a coefficient between 0 and 1 (with 1 being a straight line), which for the data of the cultures containing M-CSF was 0.986. At 95% confidence with 2 degrees of freedom, this indicates that the data fits a straight line (see Table 1). The data must be in a straight line that crosses the point on the Y axis where .37 of the wells were nonresponding in order to determine the approximate frequency of responding cells. If the line also passes through the origin, this indicates a single hit event which would indicate that the cells being examined were proliferating without aid or hindrance from any other cells, and the exact frequency of responding cells can be determined. The line drawn through the data for the cultures containing M-CSF does not obey single hit kinetics, so a valid frequency cannot be determined. From the data in the experiment, the number

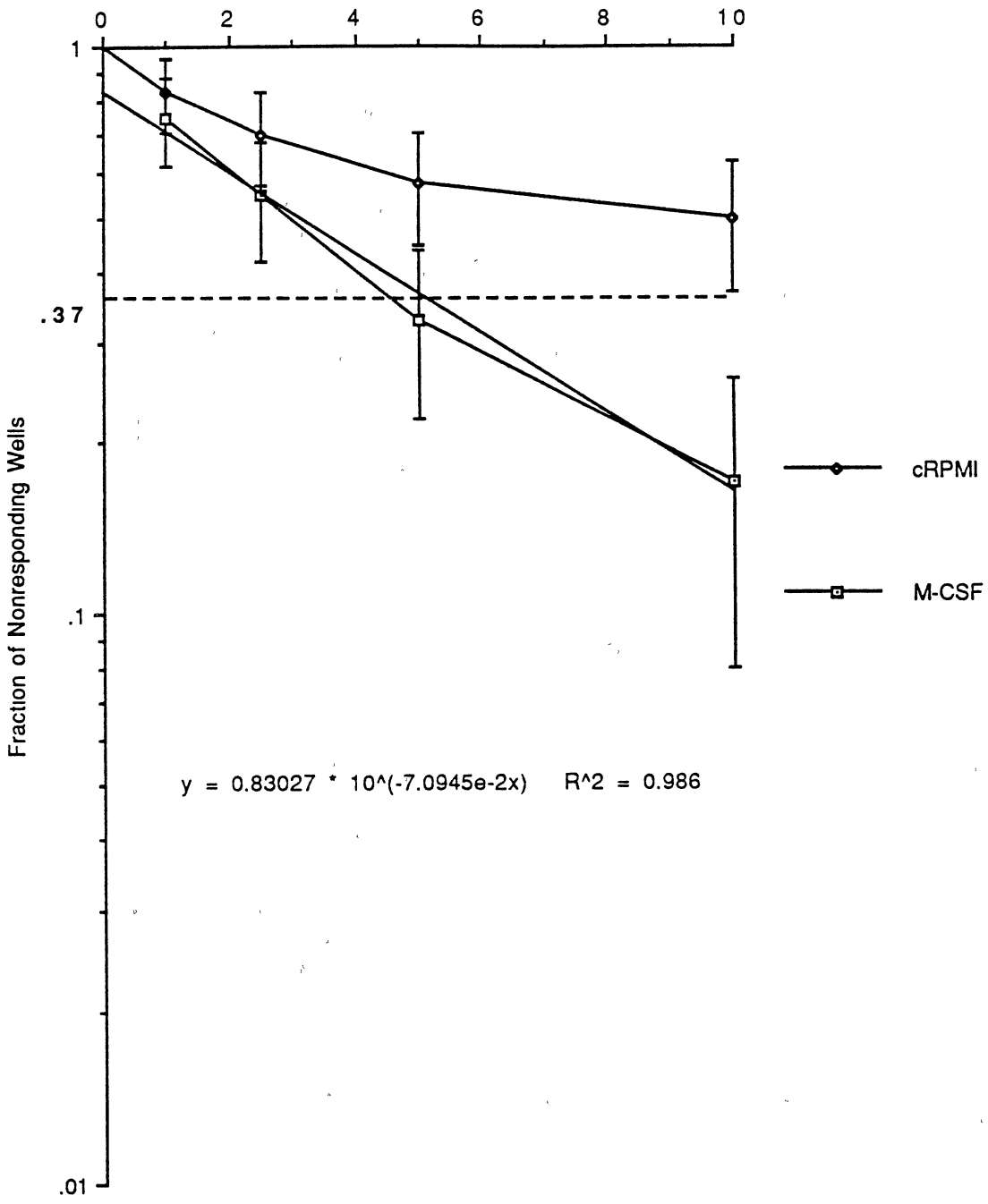

Figure 4. Limiting dilution analysis of the effect of 5% crude M-CSF on proliferation of RPM.

responding RPM was determined to be approximately 1 in 4500. This was determined by obtaining the reciprocal of the point on the X axis where the line crossed the point on the Y axis such that 37% of the wells were negative for colony formation.

GM-CSF was examined next, at a concentration of 10% (vol/vol) in cRPMI. Concentrations of $1x10^2$, $1x10^3$, and $1x10^4$ cells/well of RPM was tested in cRPMI alone and in cRPMI containing 10% crude GM-CSF. Results after incubation for 11 days showed that GM-CSF did enhance colony formation (see Figure 5.). A coefficent of 1.000 was obtained from linear regression analysis of the data points derived from the wells containing 10% GM-CSF in the medium. This value indicated greater than 95% confidence in linearity. This line, which did obey single hit kinetics, crossed the line at which 37% of the wells were nonresponding with an X value of 6000, indicating that 1 in 6000 RPM were responding to the 10% crude GM-CSF (see Figure 5.).

SRBC were also tested for their ability to enhance proliferation of RPM. Cell concentrations of $1x10^2$, $1x10^3$, and $1x10^4$ RPM/well were cultured in cRPMI alone and in cRPMI containing 1% SRBC (vol/vol). Results (see Figure 6.) yielded a straight line for both sets of data, which was demonstrated with linear regression analysis. For the data derived from cultures containing 1% SRBC, single hit kinetics were observed and the frequency of responding RPM was determined to be 1 in 4500 (see Figure 6.).

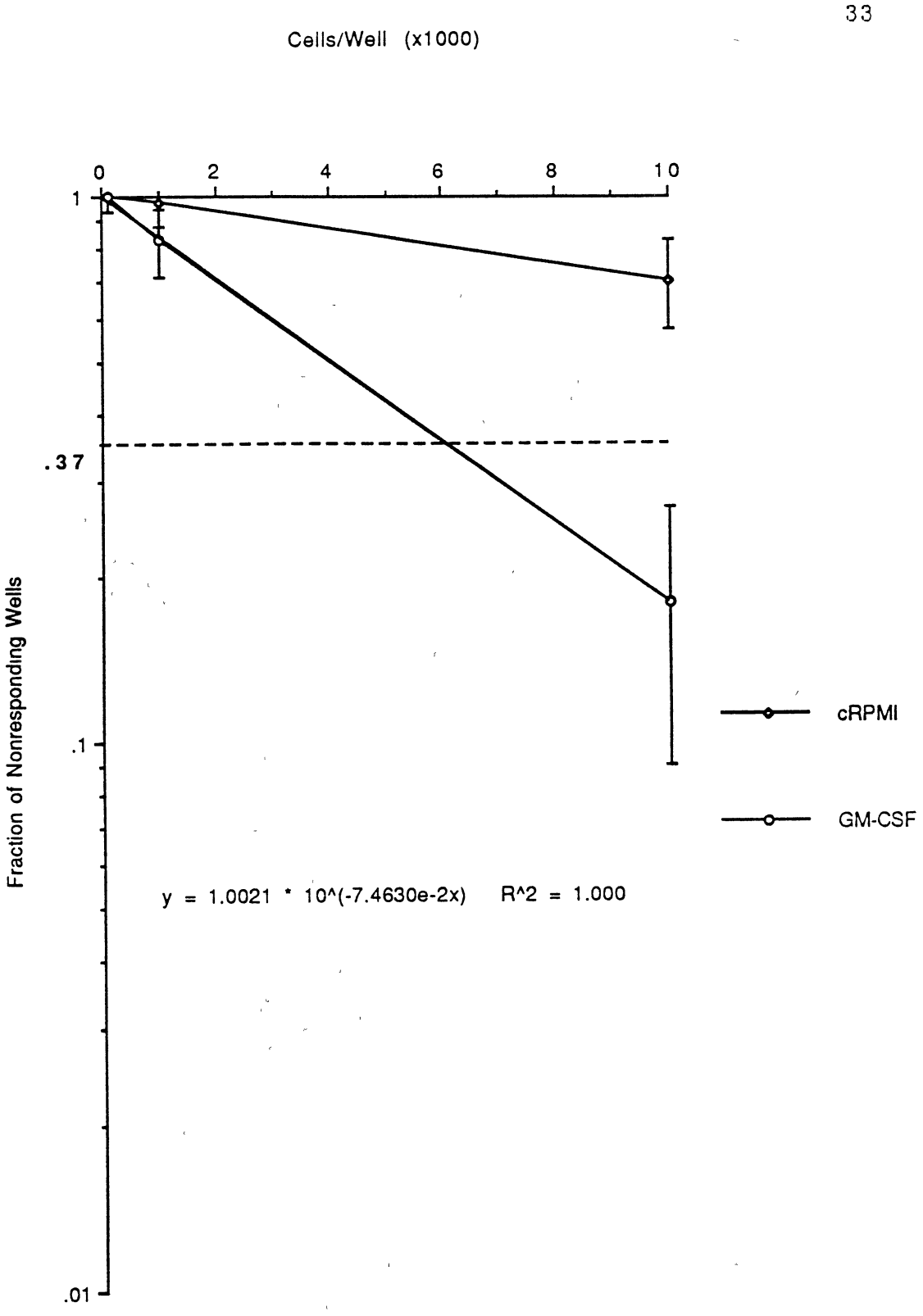

Figure 5. Limiting dilution analysis of the effect of 10% crude GM-CSF on colony formation of RPM

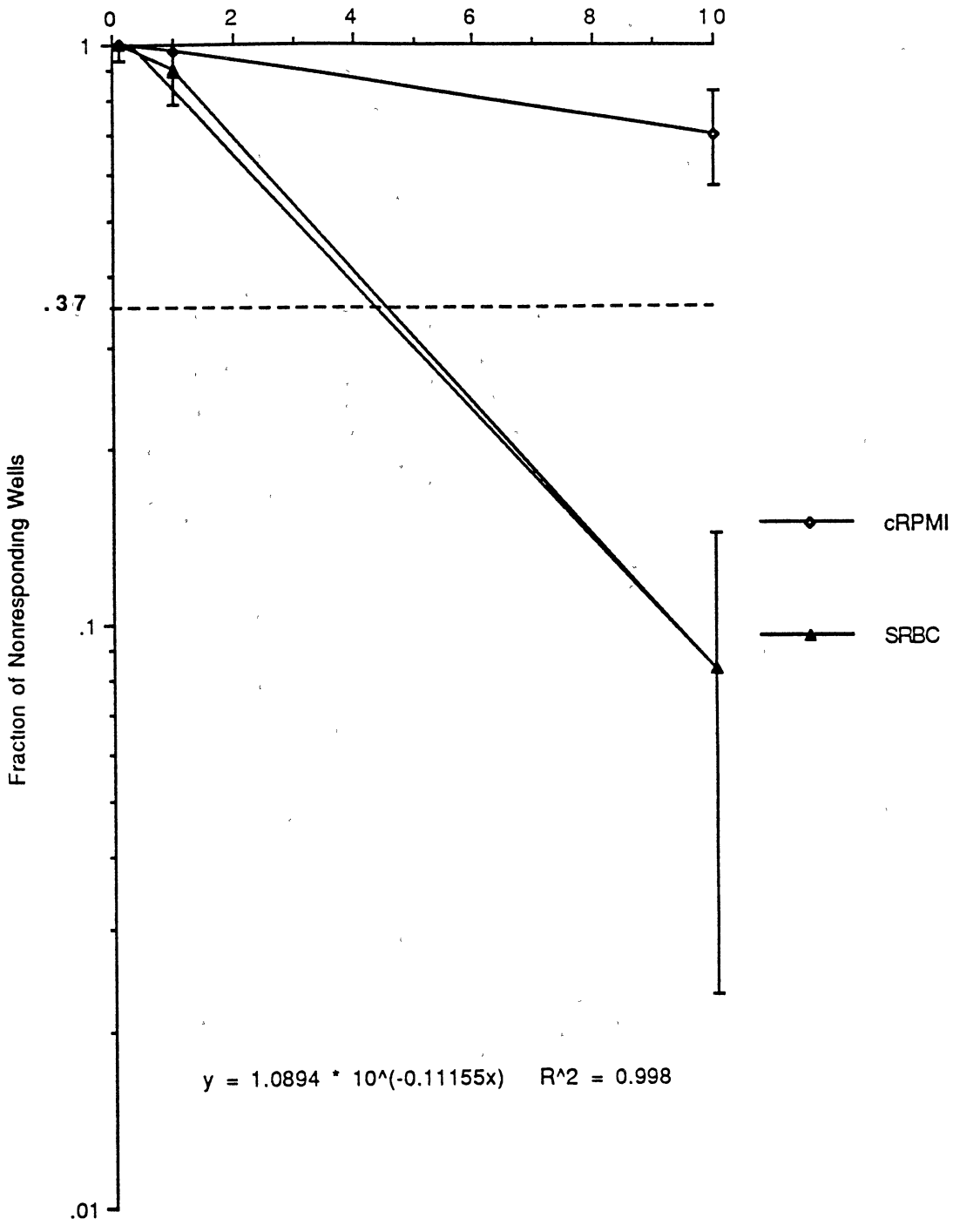

Figure 6. Limiting dilution analysis of the effect of 1% SRBC on colony formation of RPM.

Combinations of the three factors were then employed, to analyze the effect of two or more of the factors on proliferation of RPM. Five percent crude M-CSF and 10% crude GM-CSF were tested on cultures containing RPM at concentrations of $1x10^2$, $1x10^3$, and $1x10^4$ cells/well. Negative controls of each cell concentration in cRPMI alone and cRPMI + 5% M-CSF were also used. The data from this experiment was collected and plotted in Figure 7, and the results indicated that the combination of M-CSF and GM-CSF together stimulated colony formation in RPM better than M-CSF alone. From the graph it was determined that the frequency of RPM responding was 1 in 2750 when M-CSF and GM-CSF were combined.

The effect of a combination of all three factors on RPM proliferation was then tested. Concentrations of $1x10^3$, $5x10^3$, and $1x10^4$ RPM/well were prepared in cRPMI alone and cRPMI containing 5% crude M-CSF, 10% crude GM-CSF, and 1% SRBC. The three combinations involving the mixture of only two factors (5% M-CSF with 10% GM-CSF, 5% M-CSF with 1% SRBC, and 10% GM-CSF with 1% SRBC) were tested on cultures containing a single cell concentration of $1x10^4$ RPM/well. Also, the three factors were each tested individually on a single cell concentration of $1x10^3$ RPM/well. After 11 days the plates were scored for colony formation and the data graphed in Figure 8. The wells containing the combination of all three factors did show an increase in colony formation over the individual factors alone as well as the three combinations of two factors in this experiment. Linearity

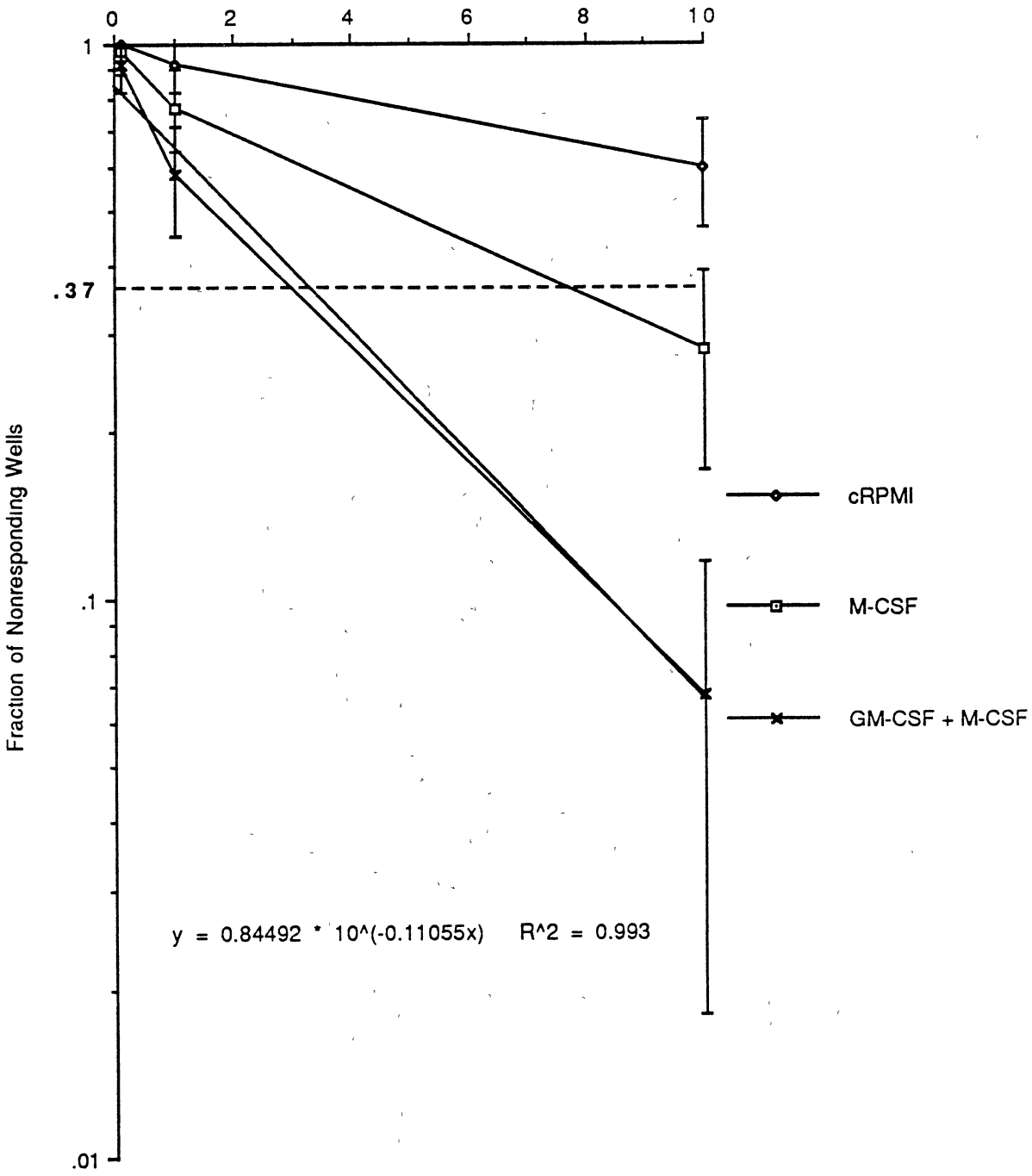

Figure 7. Limiting dilution analysis of the effect of crude 5% M-CSF and 10% GM-CSF on colony formation of RPM.

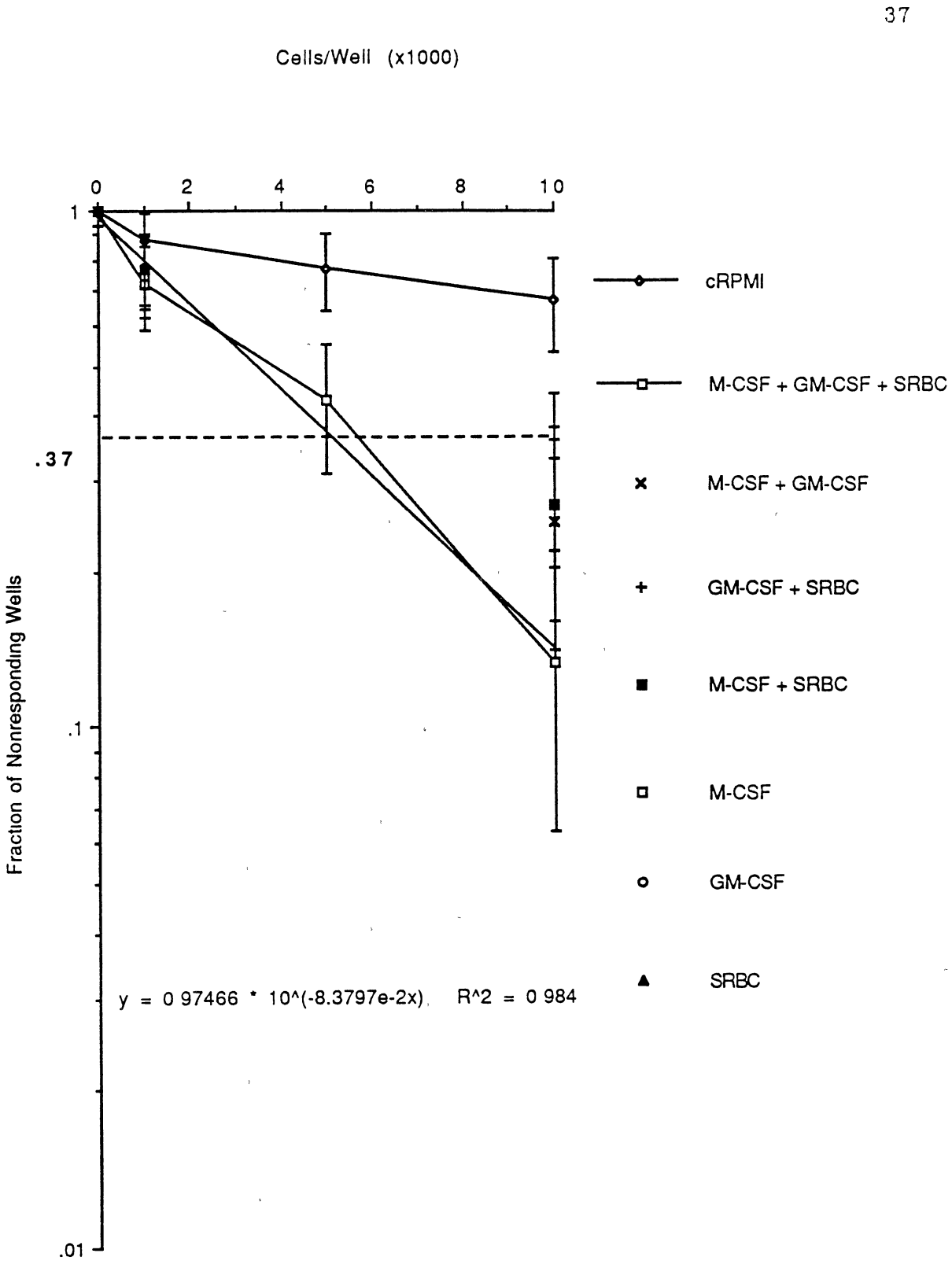

Figure 8. Limiting dilution analysis of the effect of crude 5% M-CSF, 10% GM-CSF, and SRBC on colony formation of RPM.

was confirmed through linear regression analysis of the data points for the three factors combined (see Figure 8.). The frequency of responding cells was determined to be 1 in 5000 RPM for the combination of all three factors.

This set of results indicates that M-CSF, GM-CSF and SRBC do enhance proliferation of RPM in vitro. It also shows that the effect of mixing M-CSF and GM-CSF is greater than either factor alone, and that the effect of combining all three factors further enhances this effect. From the data collected, the effect of the combination of the factors together would appear to be additive (the combinations of the factors produced frequencies that were very close to the result of adding together the frequencies from the individual factors). This could indicate that the same rare cell is responding to multiple signals for proliferation.

Response of TAM to Crude Cytokines and SRBC

After the data had been collected and analyzed for RPM, experiments on TAM could be performed and the results compared with those of RPM. These studies were done in exactly the same order as the experiments on RPM, using the same doses of crude M-CSF, GM-CSF, and SRBC that had been shown to produce optimal responses in RPM. For the first experiment, TAM were isolated from a 1X-11-6 tumor by rosetting with opsonized SRBC as described in the Materials & Methods section and examined for their ability to respond to 5% crude M-CSF. Cell concentrations of $1x10^2$, $1x10^3$, and

$1x10^4$ TAM/well were prepared in cRPMI alone and cRPMI containing 5% crude M-CSF. After 11 days of culture, the wells were examined and scored for colony formation and the resulting data was plotted in Figure 9. The graph clearly shows that crude M-CSF does enhance colony formation and that the line resulting from the plot of the data has a linear coefficient of 0.994 but does not pass through the origin, indicating that single hit kinetics do not apply. However, a frequency of approximately 1 in 4500 TAM would appear to be responding to the crude M-CSF.

Crude GM-CSF was then assayed for its ability to enhance proliferation of TAM. TAM were isolated from an MC-4 tumor and prepared in cRPMI containing 10% crude GM-CSF and cRPMI alone at cell concentrations of $1x10^2$, $1x10^3$, and $1x10^4$ TAM/well. Results obtained after 11 days of culture showed that GM-CSF did enhance colony formation, although not as greatly as M-CSF (see Figure 10.), with the line plotted through the data having a coefficient of 0.983 from linear regression analysis. A single hit event was not seen, but a frequency of 1 in 7000 TAM proliferating in the presence of 10% GM-CSF was estimated.

As with the earlier experiments involving RPM, SRBC were tested next for their ability to enhance colony formation of TAM. Macrophages from a 1X-11-6 tumor were isolated and used at concentrations of $1x10^2$, $1x10^3$, and $1x10^4$ TAM/well in cRPMI containing 1% SRBC and in cRPMI alone. Examination of the results showed that 1 in 3500 TAM were proliferating in response to the SRBC, with the linearity demonstrated by

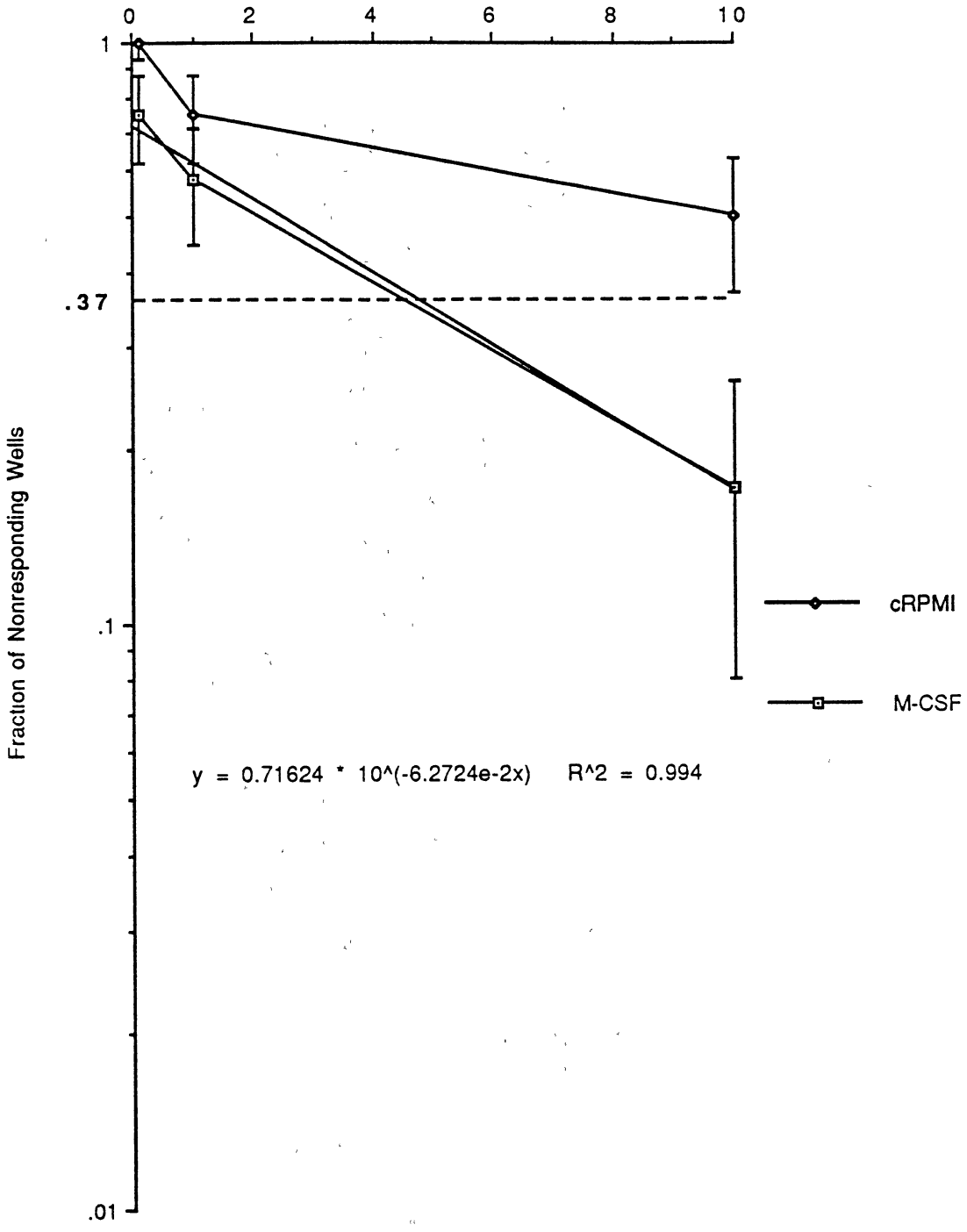

Figure 9 Limiting dilution analysis of the effect of 5% crude M-CSF on colony formation of 1X-11-6 TAM.

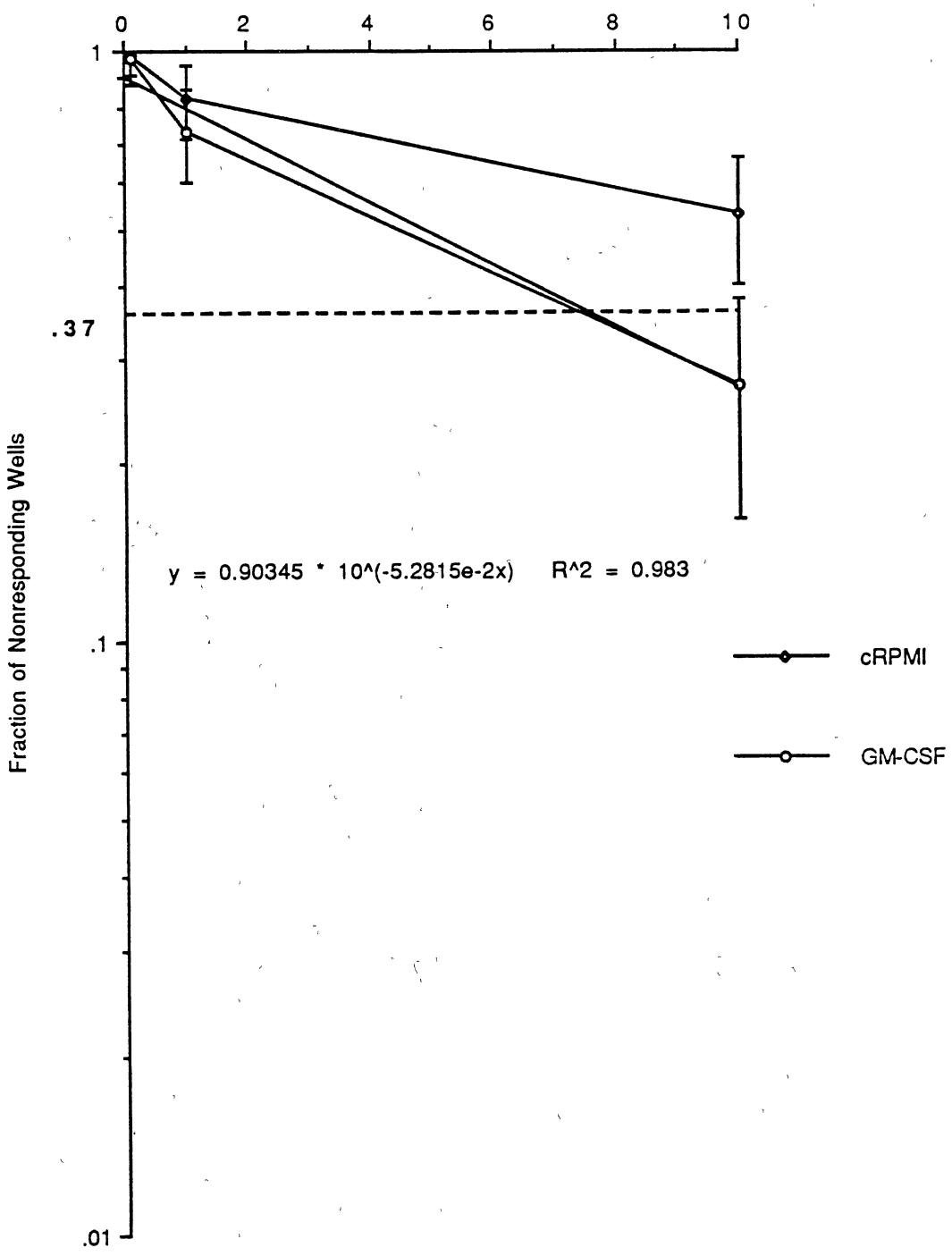

Figure 10. Limiting dilution analysis of the effect of 10% crude GM-CSF on colony formation of MC-4 TAM.

linear regression analysis (see Figure 11) Again, single hit kinetics were not seen.

Combinations of the three factors were subsequently examined, beginning with a combination of M-CSF and GM-CSF. TAM were harvested from a 1X-11-6 tumor and tested at concentrations of $1x10^2$, $1x10^3$, and $1x10^4$ TAM/well in cRPMI alone and cRPMI containing 5% crude M-CSF and 10% crude GM-CSF. M-CSF and GM-CSF were also analyzed individually at a single cell concentration of $1x10^4$ TAM/well. Results obtained upon scoring for colony formation indicated that the combination of the two factors did enhance proliferation of TAM greater than either one alone, with the line plotted through the data yielding a coefficient of 1.000 (see Figure 12.). Also from this line, it was determined that 1 in 3500 TAM were proliferating when both factors were present, and that a single hit event was indicated because the line passed through the origin.

The effect of M-CSF, GM-CSF, and SRBC combined on colony formation was examined next. Macrophages were isolated from an MC-4 tumor and cultured at concentrations of $1x10^3$, $5x10^3$, and $1x10^4$ TAM/well. These were prepared in two series, one with cRPMI medium alone and another with cRPMI containing 5% crude M-CSF, 10% crude GM-CSF, and 1% SRBC. The three possible combinations of only two of the factors (GM-CSF with M-CSF, M-CSF with SRBC, and GM-CSF with SRBC) were tested only at a cell concentration of $1x10^4$ TAM/well. Results showed an increase in proliferation for the combination of

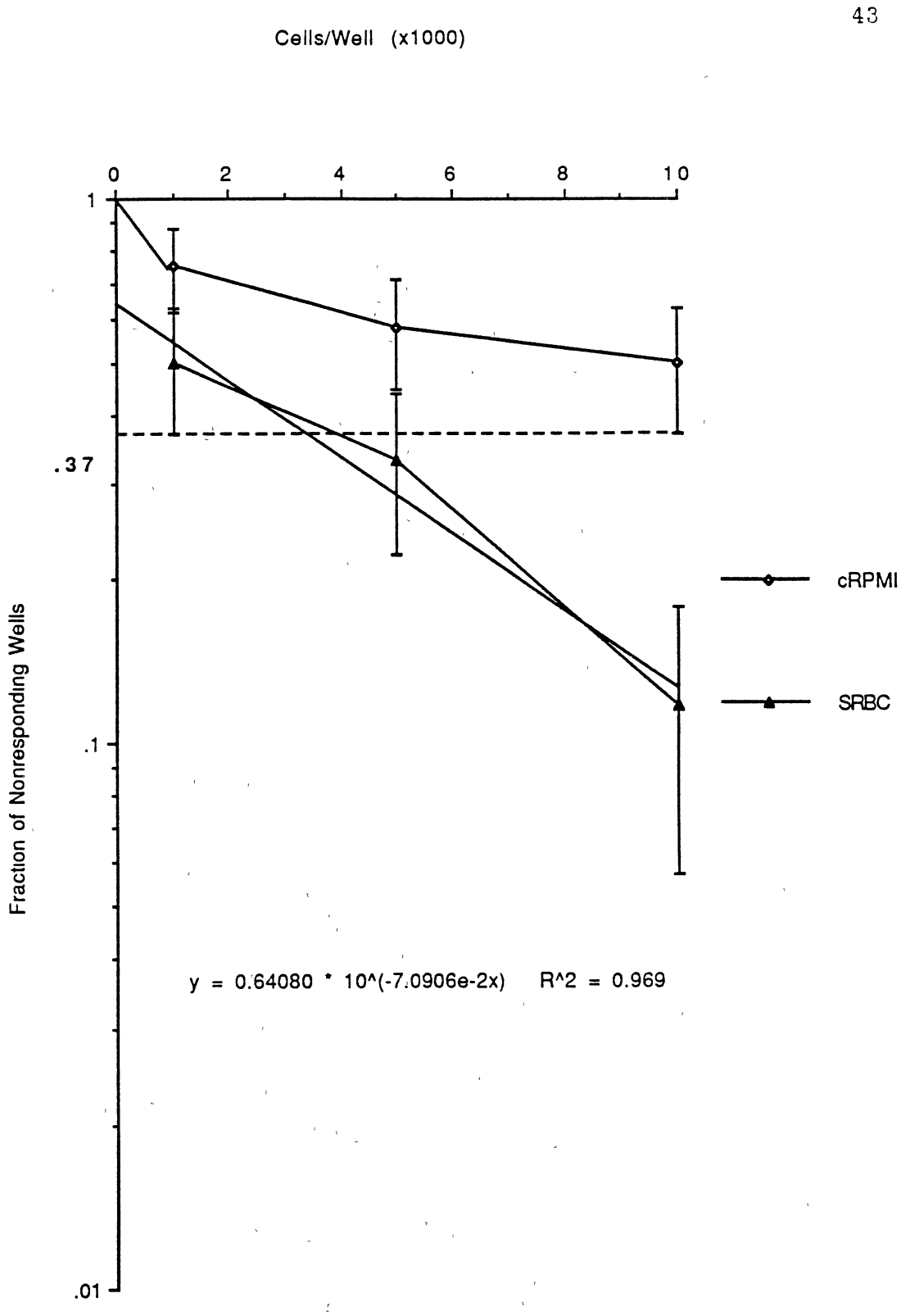

Figure 11. Limiting dilution analysis of the effect of 1% SRBC on colony formation of 1X-11-6 TAM.

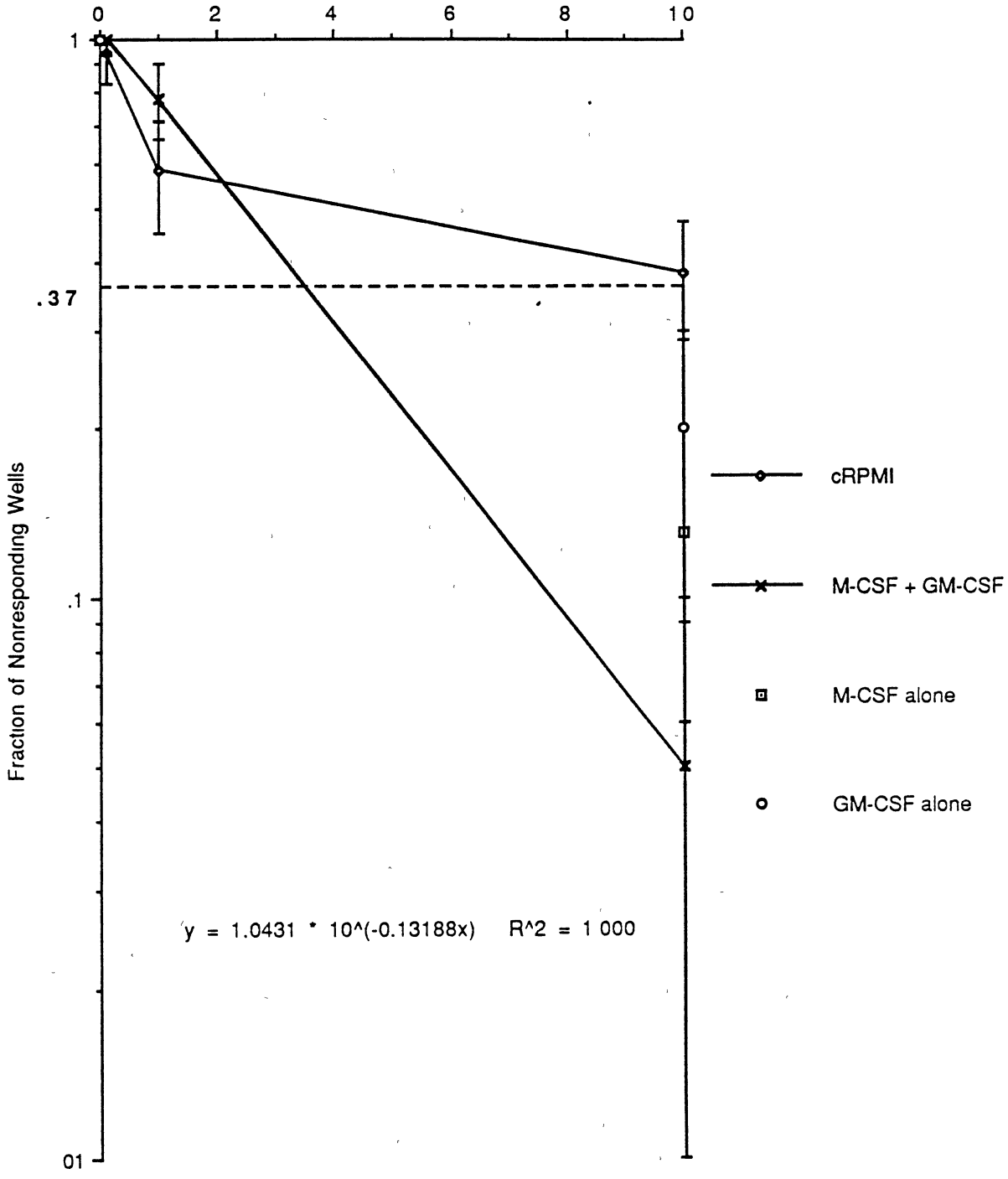

Figure 12. Limiting dilution analysis on the effect of crude 5% M-CSF and 10% GM-CSF on colony formation of 1X-11-6 TAM

all three factors over the combinations of two factors (see Figure 13.). Linear regression analysis of the line showed a coefficient of 1.000, with the line crossing the Y coordinate of .37 with an X coordinate of 4400 but not indicating single hit kinetics. In the presence of all three factors combined, approximately 1 in 4400 TAM from an MC-4 tumor were responding and proliferating.

The results of this section indicate that TAM are indeed capable of proliferation and of responding to M-CSF, GM-CSF and SRBC. As with the RPM, addition of any of the three factors significantly enhanced proliferation of TAM. The combination of crude natural M-CSF and crude natural GM-CSF produced greater proliferation than either alone, and the combination of all three factors showed a further enhancement of proliferation. As with the RPM, the effect of combining the three factors would appear to be additive and not synergistic.

Dose Response Assays of Recombinant Cytokines

Because the experiments above utilized crude preparations of M-CSF and GM-CSF, it was necessary to demonstrate with pure recombinant forms of the two factors that the two cytokines were the sole effectors of the response instead of some other factors in the crude natural preparations. As with the crude cytokines and SRBC, it was first necessary to determine the concentrations of the recombinant cytokines that would produce maximum proliferation in RPM. Recombinant M-CSF (rM-CSF) was tested

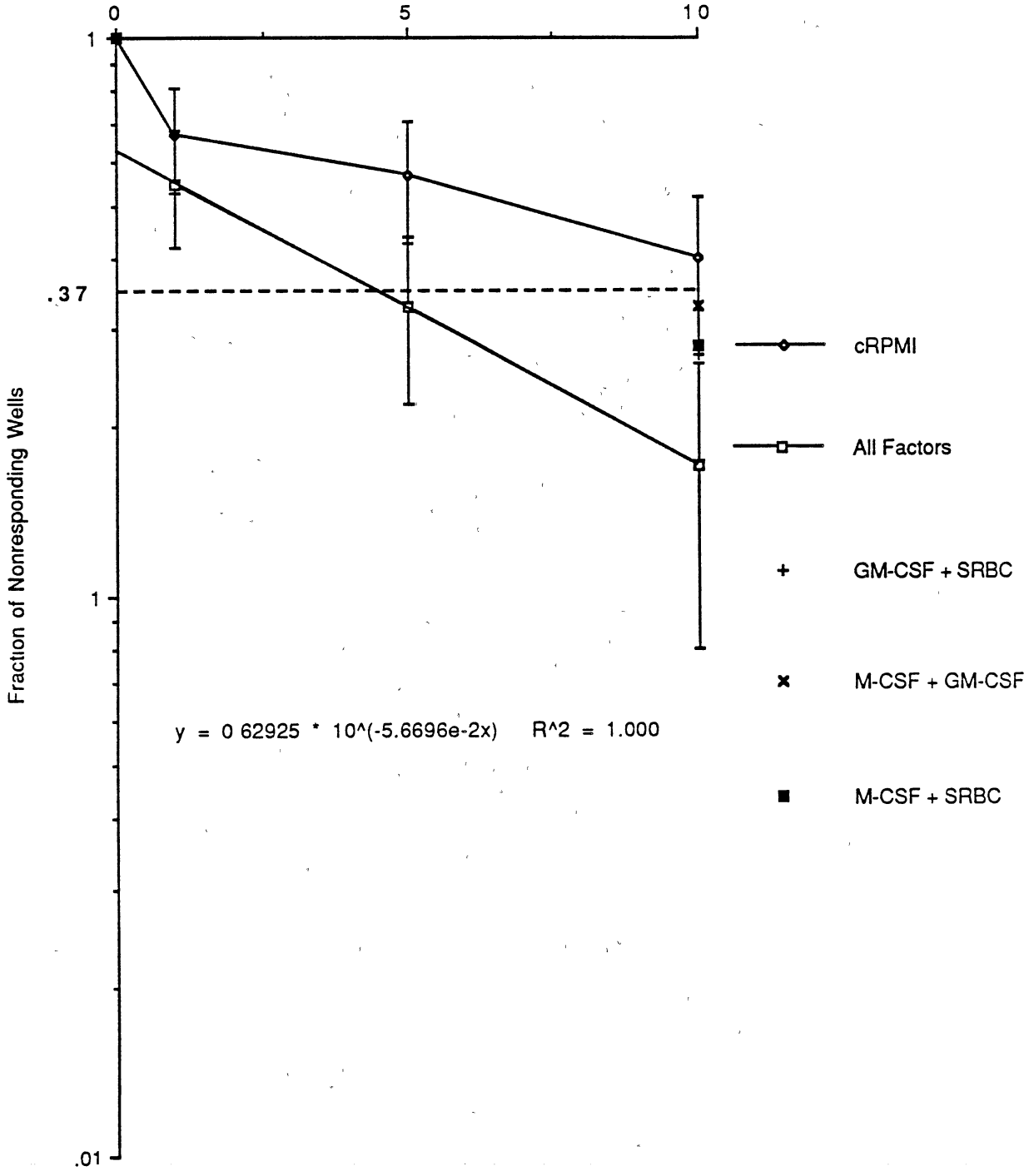

Figure 13 Limiting dilution analysis of the effect of crude 5% M-CSF, 10% GM-CSF, and 1%SRBC on colony formation of MC-4 TAM.

first, at concentrations of 100, 500, and 1000 U/ml with a single cell concentration of $1x10^4$RPM/well. A negative control of RPM in cRPMI alone was also used. Results showed a sharp increase in colony formation with the maximum (100%) achieved at concentrations of 500 U/ml and 1000 U/ml (see Figure 14.). For subsequent LDA experiments, a concentration of 1000 U/ml of rM-CSF was used.

Recombinant GM-CSF was also tested, but at different concentrations. Examination of recent literature showed that most researchers utilizing rGM-CSF in growth experiments employed a concentration of 100 to 200 U/ml (13,31,32). Therefore, concentrations of 25, 50, 100, and 200 U/ml were tested on a single cell concentration of $1x10^4$ RPM/well. A negative control of RPM prepared in cRPMI alone was also included. After 11 days of culture, the results showed that optimal colony formation (100%) was achieved at concentrations of 50 U/ml and greater (see Figure 15.). For subsequent LDA, 100 U/ml of rGM-CSF was utilized.

Response of RPM to Recombinant Cytokines and SRBC

After the optimal concentrations of the two recombinant cytokines had been determined, LDA was employed to analyze the frequency of RPM and TAM that would respond to both cytokines individually, in combination with one another, and in combination with SRBC. As with the crude cytokines, RPM were examined first to provide a reference for the results obtained upon analysis of TAM. Analysis of rM-CSF was accomplished initially, as had been done previously in

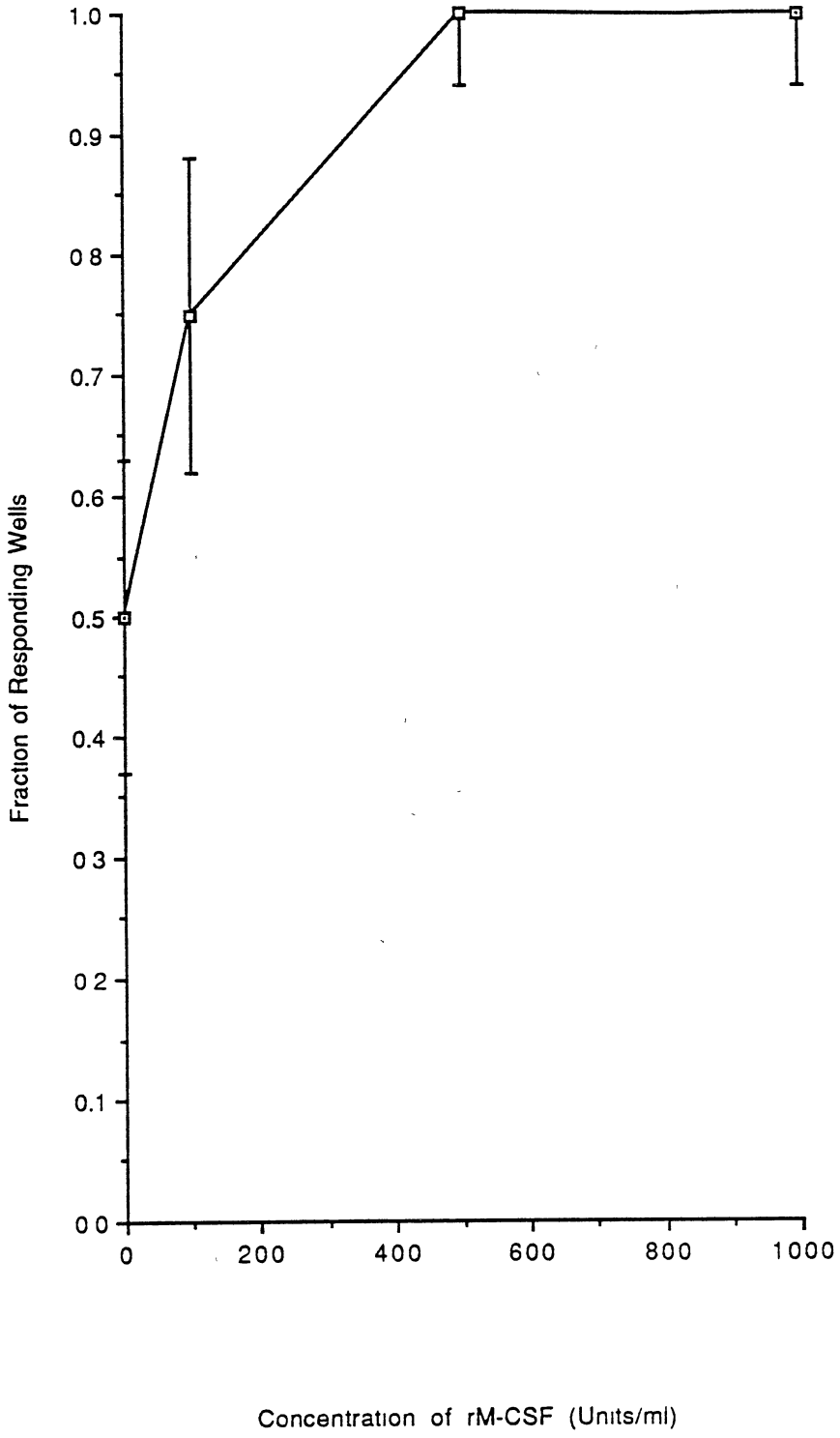

Figure 14 Effect of different concentrations of recombinant M-CSF on colony formation of RPM (10,000 cells/well).

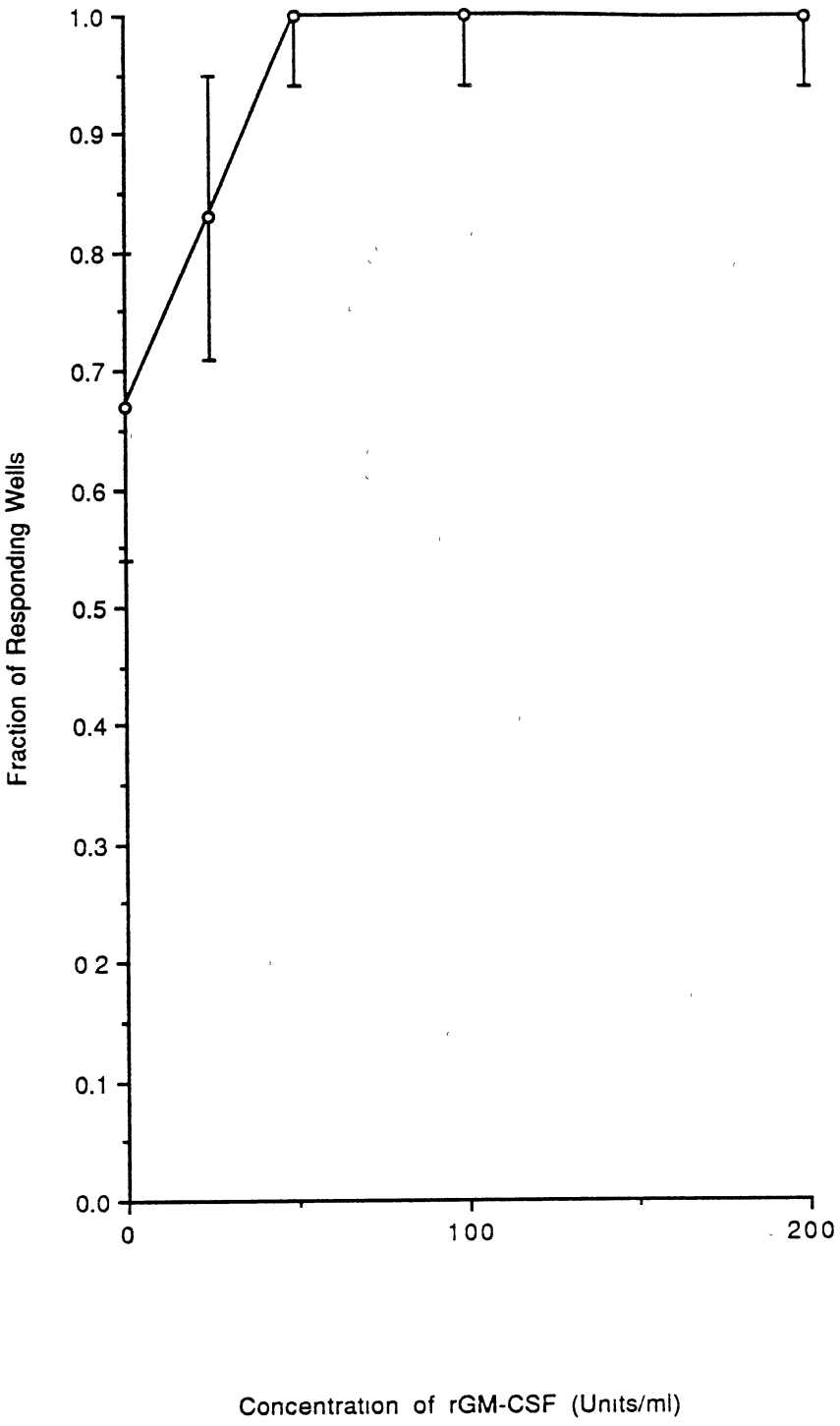

Figure 15. Effect of different concentrations of recombinant GM-CSF on colony formation of RPM (10,000 cells/well).

analysis of the crude cytokines. Cell concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ RPM/well were cultured in cRPMI alone and in cRPMI containing 1000 U/ml of rM-CSF. Results obtained after 11 days of culture showed that rM-CSF did increase proliferation of RPM, with the line drawn through the data points revealing a Pearson coefficient of linearity of 0.996 (see Figure 16.). Single hit kinetics were not indicated since the line did not pass through the origin. A frequency of approximately 1 in 1500 TAM were proliferating in response to rM-CSF.

The remaining cytokine, rGM-CSF, was then examined. Cell concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ RPM/well were prepared in cRPMI alone and in cRPMI containing 100 U/ml of rGM-CSF. Results obtained after 11 days of culture revealed that rGM-CSF did enhance proliferation of RPM, with a frequency of 1 in 2250 cells responding (see Figure 17.). Linearity was demonstrated with linear regression analysis of the data, and indicated a single hit event (the line passed through the origin)

The combination of rM-CSF and rGM-CSF was examined next. Cell concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ RPM/well were cultured in cRPMI alone and in cRPMI containing 1000 U/ml rM-CSF and 100 U/ml rGM-CSF. In addition, rGM-CSF and rM-CSF were tested individually on cultures containing a single cell concentration of $5x10^3$ RPM/well. Examination of the data obtained from this experiment showed that the combination of rM-CSF and rGM-CSF did not significantly

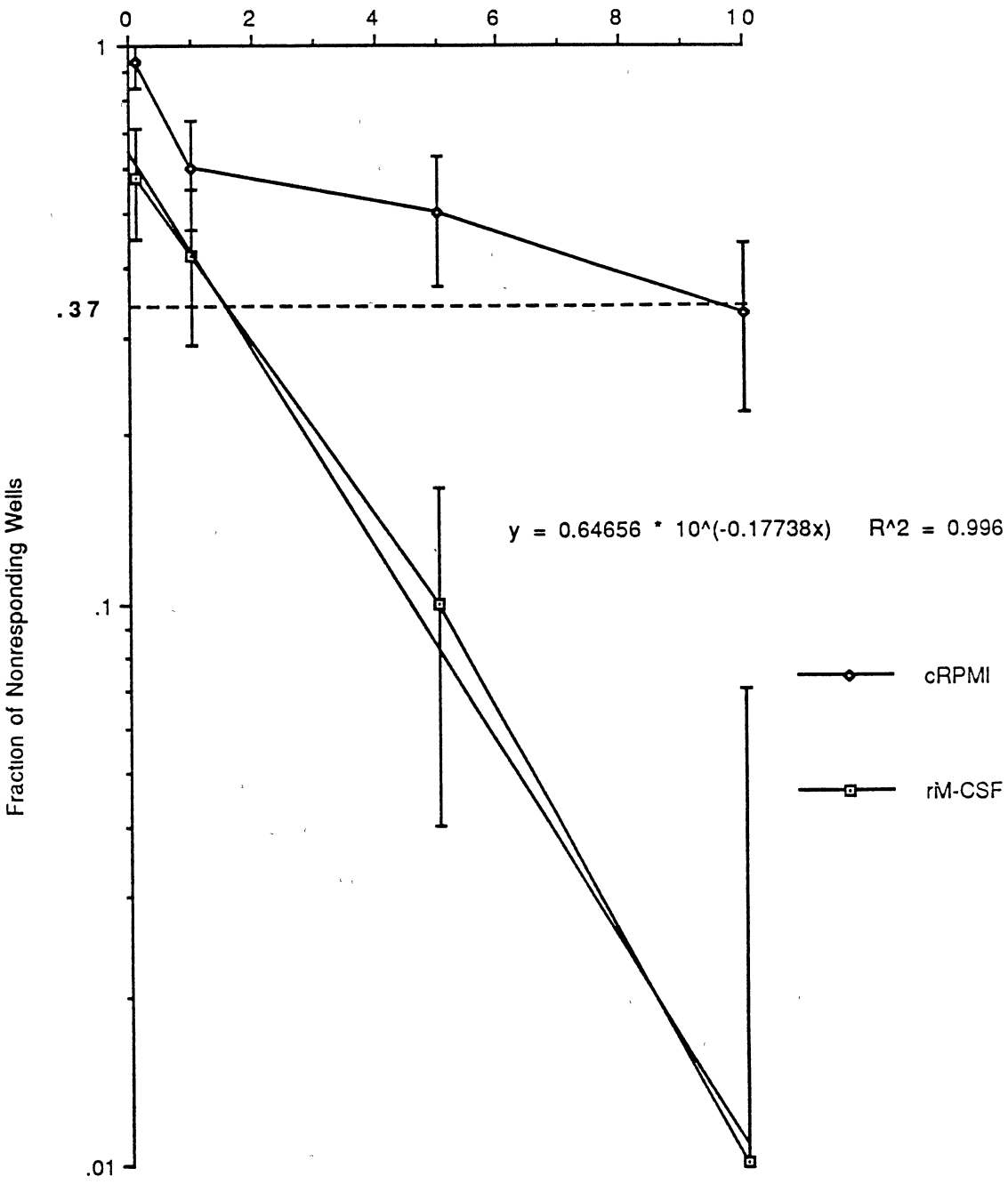

Figure 16. Limiting dilution analysis of the effect of rM-CSF (1000 U/ml) on colony formation of RPM.

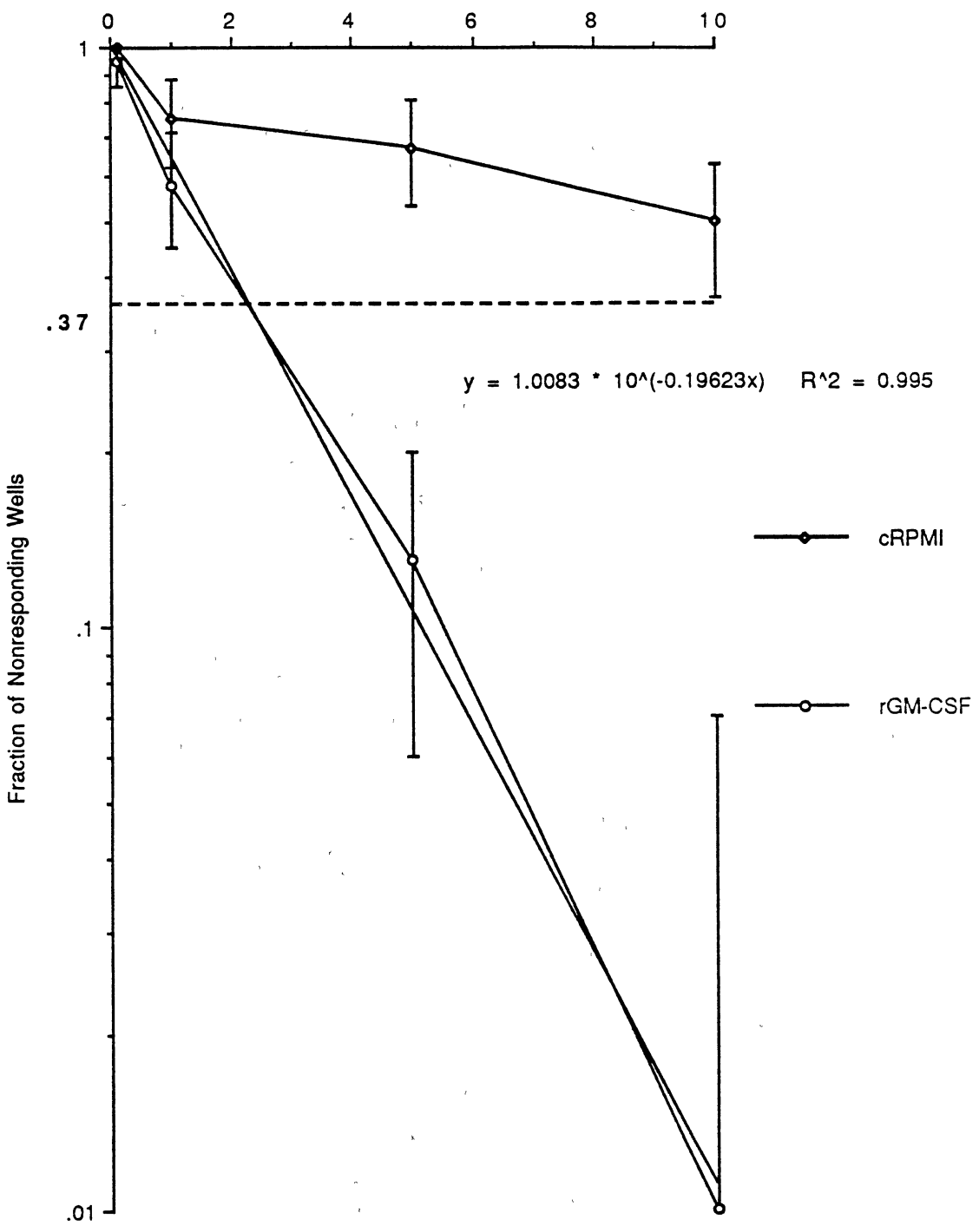

Figure 17. Limiting dilution analysis of the effect of rGM-CSF (100 U/ml) on colony formation of RPM.

enhance the proliferation of RPM. Although a valid frequency could not be determined, an approximate frequency of 1 in 2250 RPM responded (see Figure 18.). Linear regression analysis of the data indicated a coefficient of 0.974. Single hit kinetics were not followed since the line did not pass through the origin.

The effect of both recombinant factors and SRBC on proliferation of RPM was then examined. Cell concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ were prepared in cRPMI alone and in cRPMI containing 1000 U/ml rM-CSF, 100U/ml rGM-CSF, and 1% vol/vol SRBC. The three different combinations of two factors (rM-CSF + rGM-CSF, rM-CSF + SRBC, and rGM-CSF + SRBC) were tested on a single cell concentration of $5x10^3$ RPM/well. The data was collected after 11 days of culture and plotted (Figure 19). Linear regression analysis of the data for the cultures containing all three factors yielded a coefficient of 0.996, which indicated linearity. The results in this experiment showed that the combination of all three produce a better response than any combination of just two factors. The frequency of responding cells determined from the data, however, was 1 in 2500 RPM, which is comparable to the frequency determined for the combination of rM-CSF and rGM-CSF from Figure 18.

The results of this set of experiments demonstrates that RPM respond to pure recombinant factors, M-CSF and GM-CSF, with much higher frequencies of proliferating cells than was observed with the crude natural cytokines. The combination of the recombinant factors, however, did not show enhancement

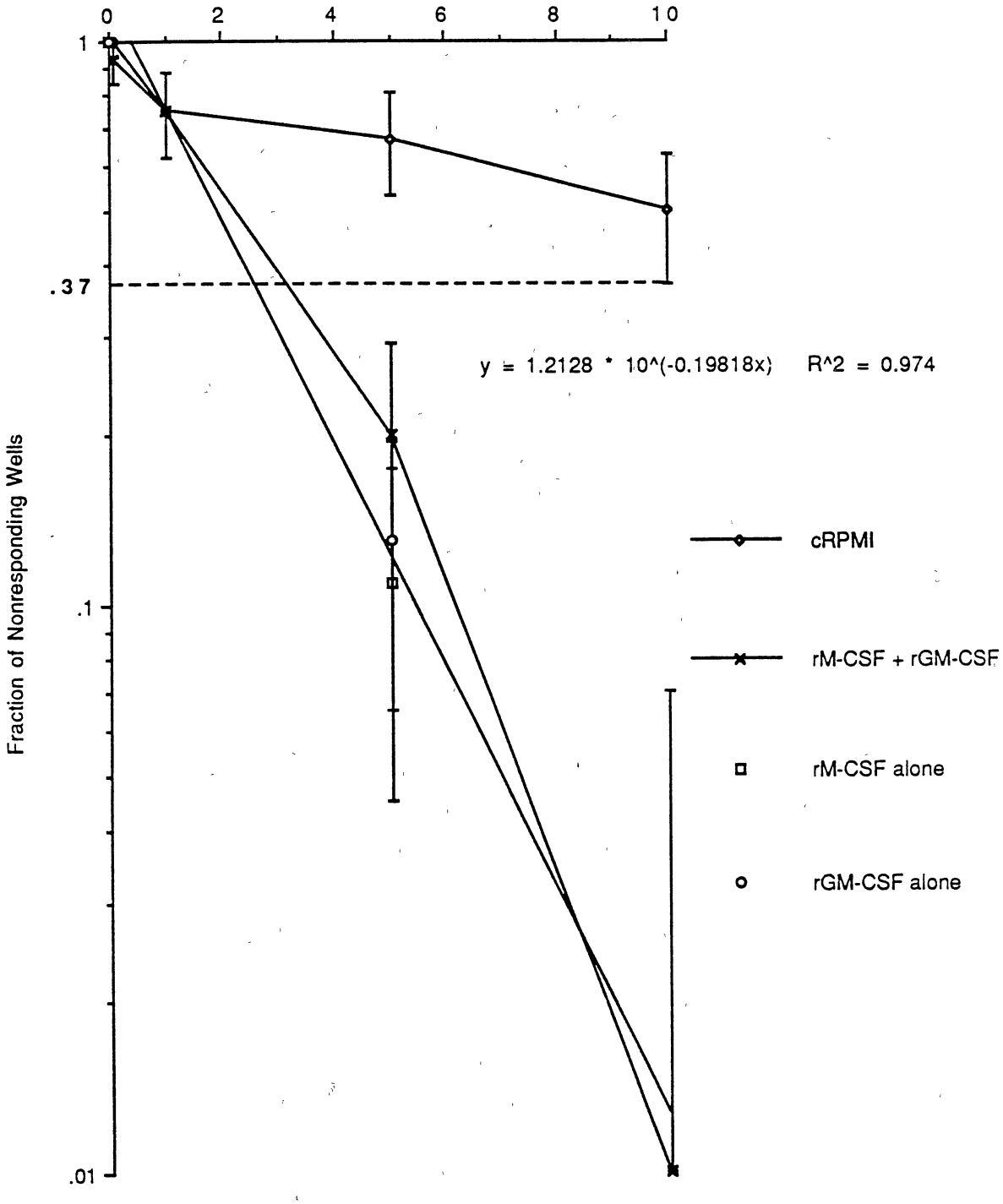

Figure 18. Limiting dilution analysis of the effect of rM-CSF (1000 U/ml) and rGM-CSF (100 U/ml) on colony formation of RPM.

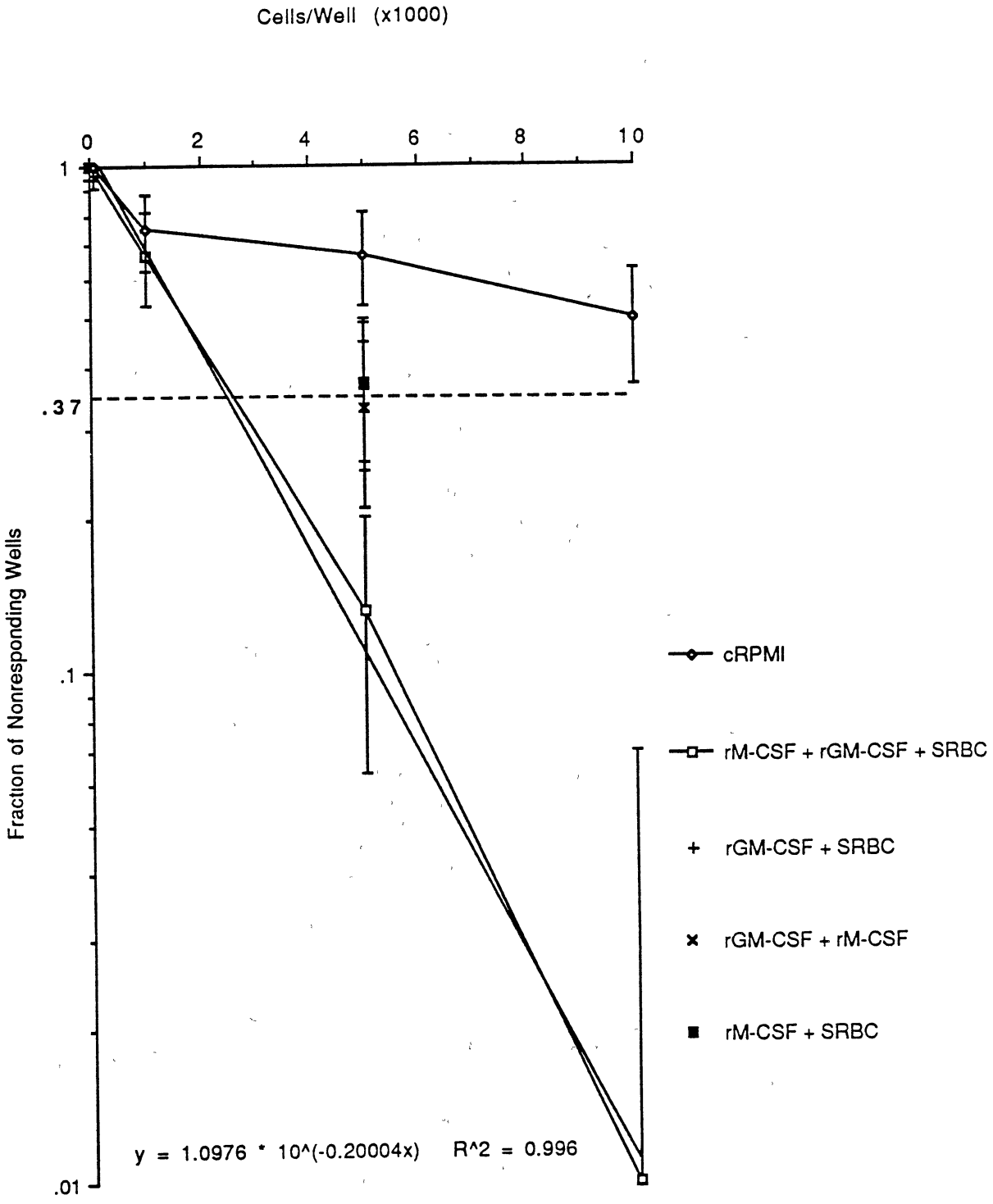

Figure 19. Limiting dilution analysis on the effect of rM-CSF (1000U/ml), rGM-CSF (100U/ml), and 1% SRBC on colony formation of RPM.

of proliferation over either factor alone, nor did the combination of both rM-CSF and rGM-CSF with SRBC. Since the concentrations of the cytokines that were utilized generated 100% response at $1x10^4$ cells/well, it may be that every possible cell capable of proliferating was responding in the presence of either factor alone and no further enhancement was possible.

Response of TAM to Recombinant Cytokines and SRBC

As with the RPM, the effect of rM-CSF on TAM proliferation was determined first. Macrophages were isolated from an MC-4 tumor and cultured at concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ in cRPMI alone and cRPMI containing 1000U/ml of rM-CSF. The wells were scored for colony formation after 11 days and the data plotted in Figure 20. The line drawn through the points on the graph indicates that rM-CSF does enhance proliferation of TAM, although a valid frequency could not be determined. Linear regression analysis yielded a coefficient of 0.996 for the line drawn through the points for the cells in cRPMI containing rM-CSF. This line did not pass through the origin, indicating that the addition of rM-CSF did not generate a single hit event.

Recombinant GM-CSF alone was then tested for its effect on proliferation of TAM. Macrophages isolated from an MC-4 tumor were cultured in cRPMI alone and cRPMI containing 100 U/ml rGM-CSF at concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and

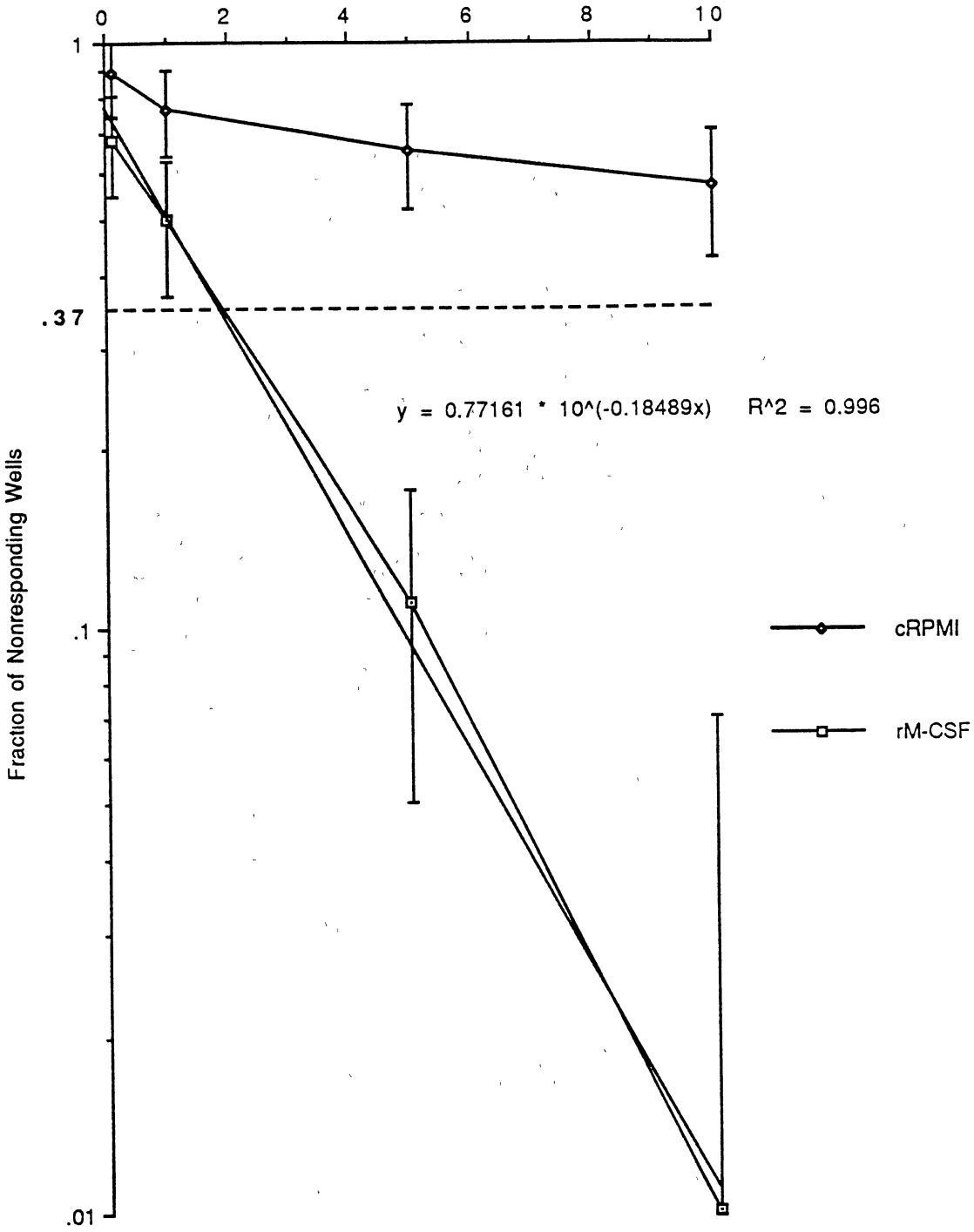

Figure 20. Limiting dilution analysis of the effect of rM-CSF (1000U/ml) on colony formation of MC-4 TAM.

$1x10^4$ cells/well. Results obtained 11 days later indicated (see Figure 21.) that rGM-CSF, like rM-CSF, did enhance colony formation of TAM, with 1 in 2250 TAM responding when rGM-CSF was added to the medium. The line was examined with linear regression analysis and represented a single hit event.

The combination of rM-CSF and rGM-CSF was then tested on TAM isolated from an MC-4 tumor. TAM were used at concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ cells/well cultured in cRPMI alone or in cRPMI containing rM-CSF (1000 U/ml) and rGM-CSF (100 U/ml). The two factors were also tested individually at a single cell concentration of $5x10^3$ cells/well. The wells were scored for colony formation after 11 days and the results plotted in Figure 22. The lines drawn through the data points showed that the combination of the two factors produced a better proliferative response than either factor alone, with 1 in 1750 TAM responding. The following experiment involved examining the effect of combining all three factors. TAM isolated from a 1X-11-6 tumor were prepared in cRPMI alone and in cRPMI containing rM-CSF (1000 U/ml), rGM-CSF (100 U/ml), and SRBC (1% vol/vol) at cell concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ cells/well. The three different combinations of two factors (rM-CSF + rGM-CSF, rM-CSF + SRBC, rGM-CSF + SRBC) were each tested at a single cell concentration of $5x10^3$ cells/well. After 11 days the data was collected and the results plotted (Figure 23). The lines

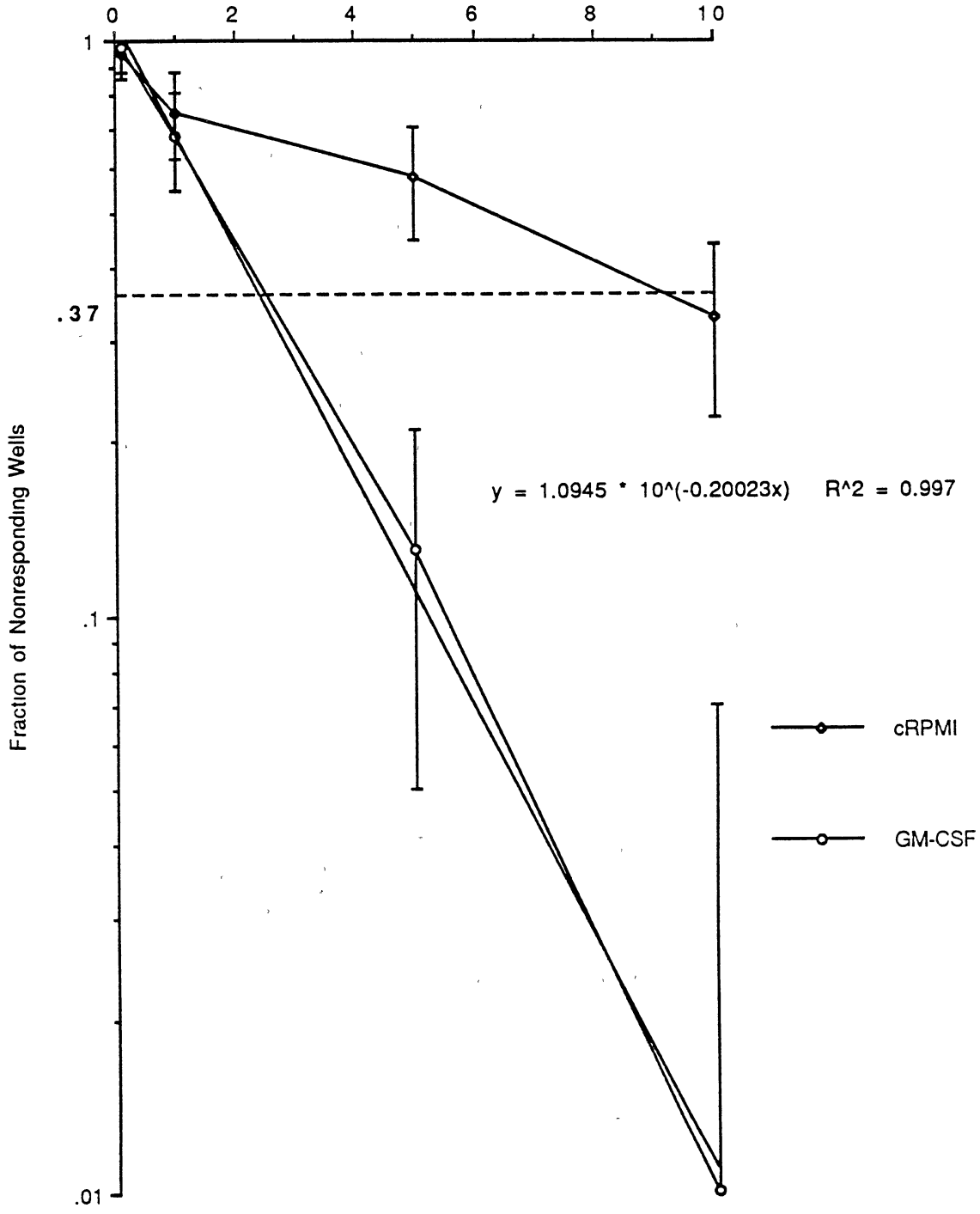

Figure 21. Limiting dilution analysis of the effect of rGM-CSF (100U/ml) on colony formation of MC-4 TAM.

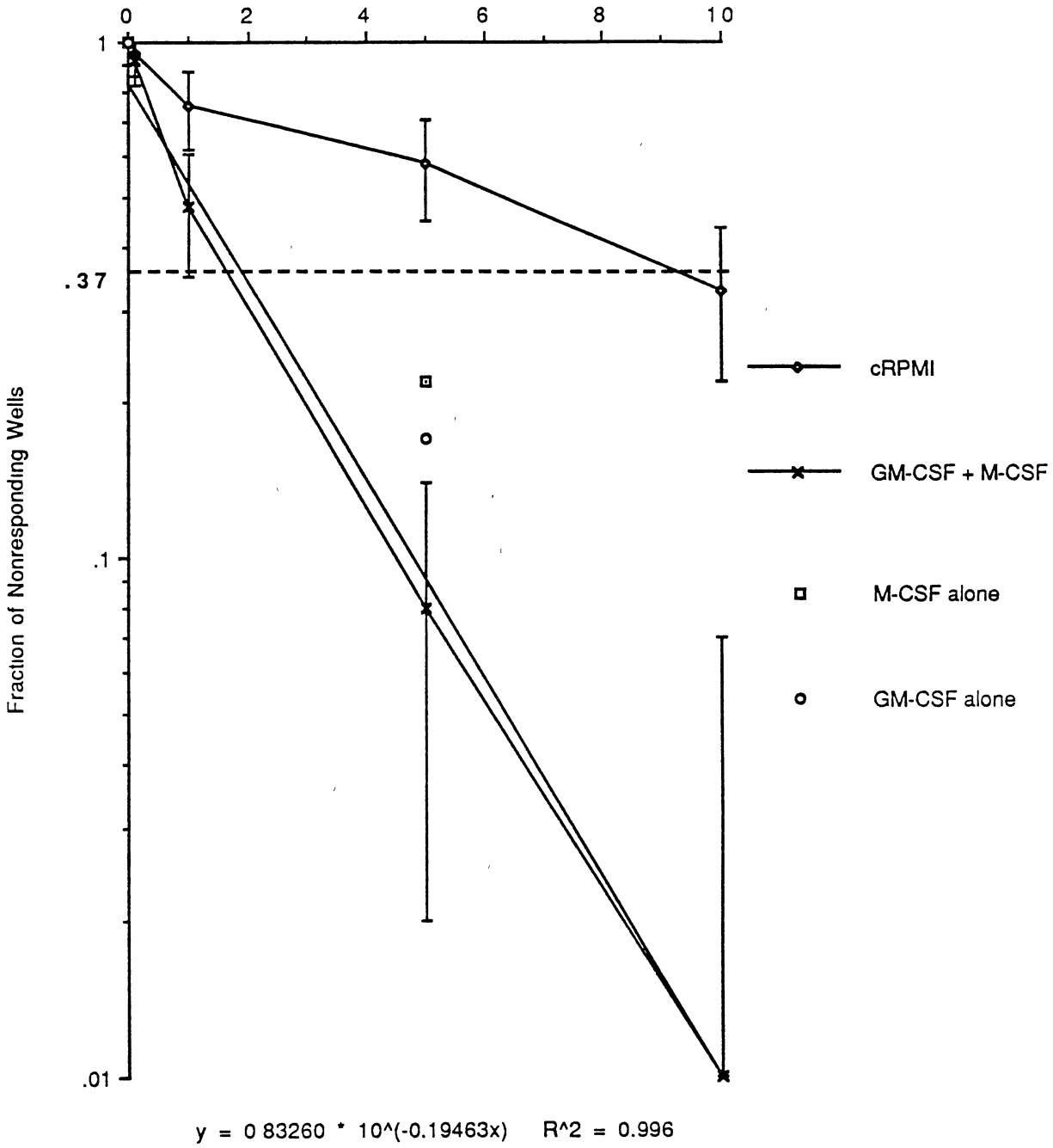

Figure 22. Limiting dilution analysis of the effect of rM-CSF (1000 U/ml) and rGM-CSF (100 U/ml) combined on colony formation of MC-4 TAM.

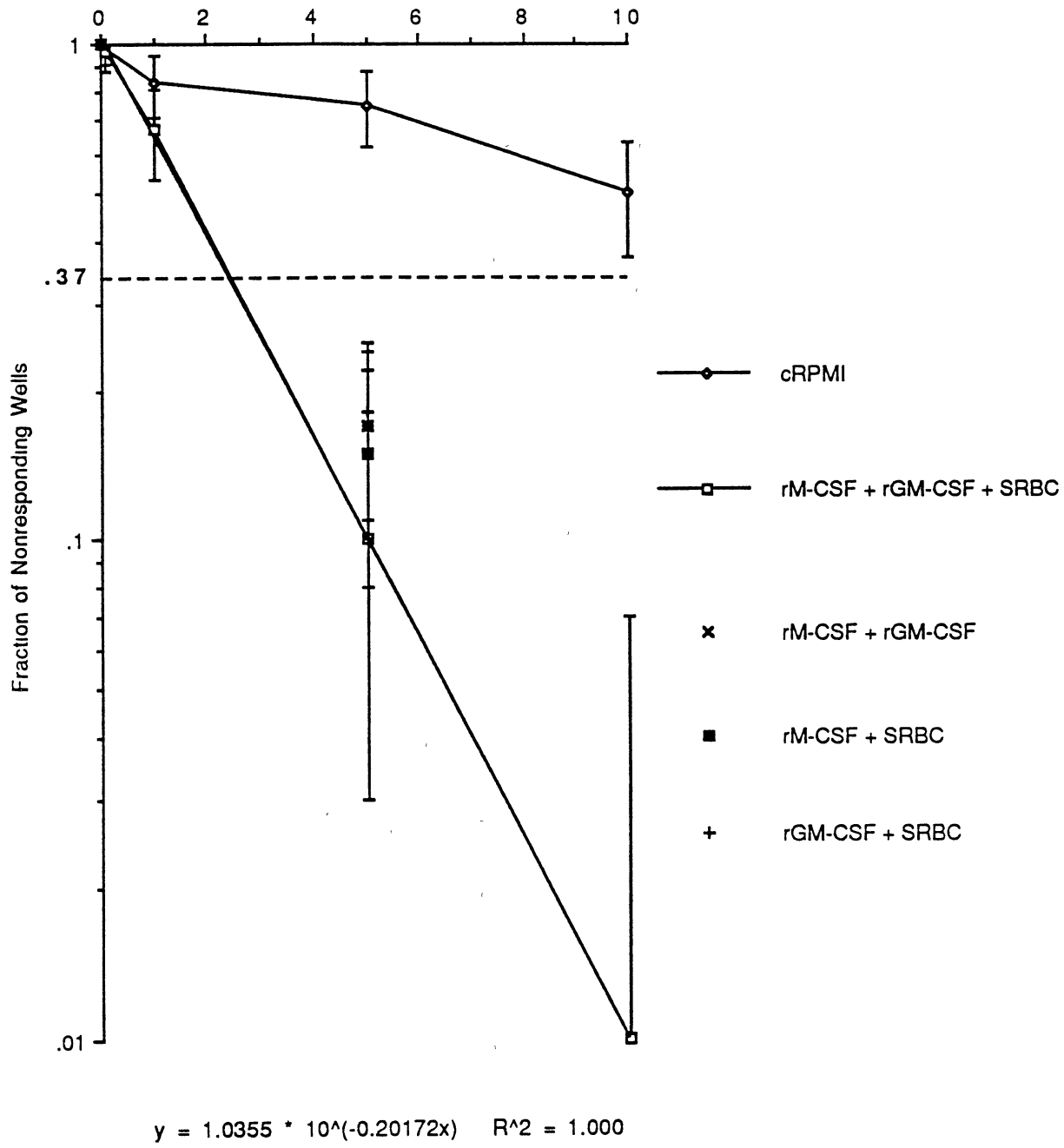

Figure 23 Limiting dilution analysis of the effect of rM-CSF (1000 U/ml), rGM-CSF (100 U/ml), and 1% SRBC on 1X-11-6 TAM.

obtained indicate that the combination of all three factors did exhibit enhancement of proliferation above that of the various combinations of two factors. However, the frequency of 1 in 2250 responding cells determined from the graph in Figure 23. does not appear to be significantly different from the result obtained for rM-CSF and rGM-CSF in Figure 22. Linear regression analysis of the data in Figure 23 yielded a coefficient of 1.000. In addition, the line obtained from the data of the cultures combining all three factors passed through the origin, indicating that a single hit event had occurred.

The results obtained from this group of experiments confirm that TAM do respond to M-CSF and GM-CSF. As with the experiments examining the effect of the recombinants on RPM, the combination of the two recombinants and the recombinants with SRBC did not enhance proliferation over the effect of the factors alone. This would indicate that, as with the RPM, the total number of cells capable of proliferating were responding with either factor alone and further enhancement was not possible.

Dose Response Assays of Tumor Supernates

Once it had been determined that TAM would proliferate <u>in vitro</u> in response to colony-stimulating factors, the question turned to whether or not the supernates of tumors could enhance proliferation of TAM and RPM. It was also of interest to examine the tumor supernates for the existence of

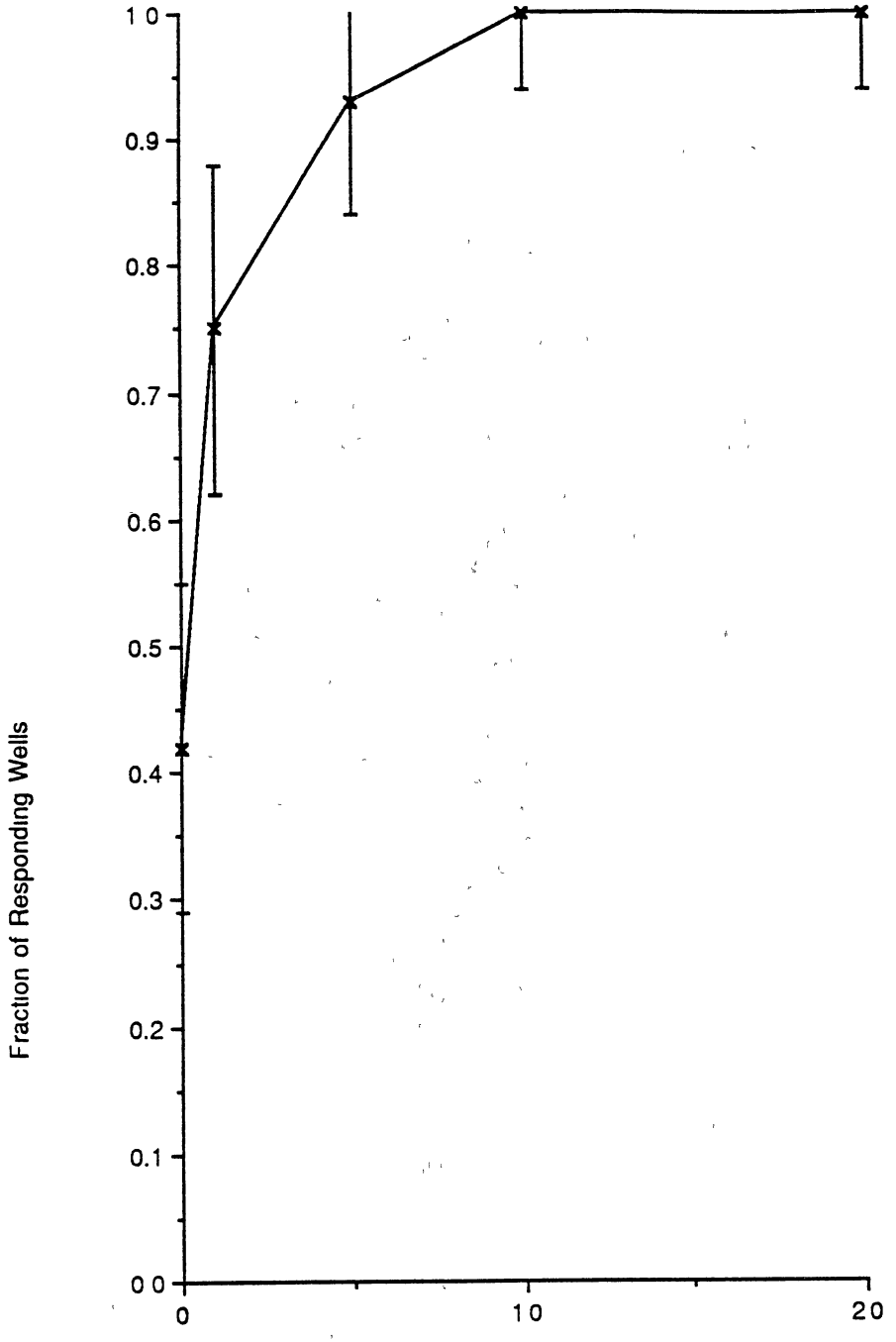

Figure 24. Determination of the minimum concentration of 1X-11-6 supernate required for optimal colony formation of 1X-11-6 TAM (10,000 cells/well).

M-CSF and GM-CSF activity. This would indicate whether or not tumors might effect the quantity of TAM in the tumor. In order to analyze this possibility, the optimal concentrations of the tumor supernates for colony formation of RPM were initially determined.

The supernate from an in vitro culture of an 1X-11-6 tumor was tested first, at concentrations of 1, 5, 10, and 20% (vol/vol) in cRPMI. TAM were isolated from a 1X-11-6 tumor and used at a single concentration of $1x10^4$ cells/well for all of the concentrations of the supernate tested, along with a negative control of TAM in cRPMI alone. The results were plotted (Figure 24) and showed a sharp increase in proliferation as the concentration of 1X-11-6 tumor supernate increased, with the level of proliferation reaching the maximum (100%) at a concentration of 10% 1X-11-6 supernate in cRPMI.

The supernate from in vitro cultures of an MC-4 tumor were tested at concentrations of 1, 5, 10, and 20% (vol/vol) in cRPMI. Macrophages were isolated from an MC-4 tumor and used at a single concentration of $1x10^4$ cells/well for all of the concentrations of MC-4 supernate tested. A negative control of cells cultured in cRPMI alone was also employed in this experiment. The wells were scored after 11 days and the results plotted (Figure 25). These results indicated an increase in proliferation as the concentration of MC-4 supernate increased, although not as dramatically as that seen for the supernate from the 1X-11-6 tumor. Optimal

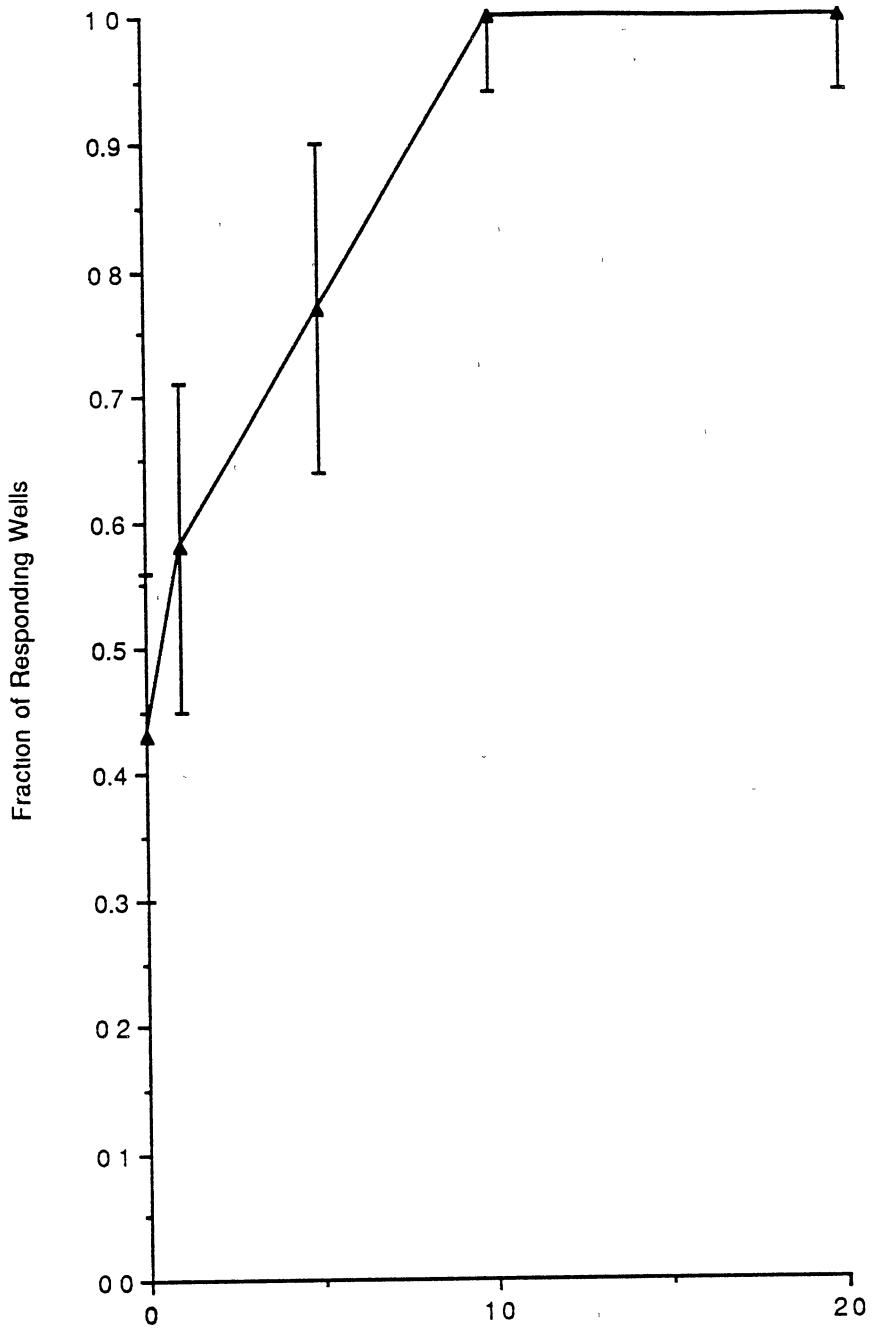

Figure 25. Determination of the minimum concentration of MC-4 supernate required to produce optimal colony formation in MC-4 TAM (10,000 cells/well)

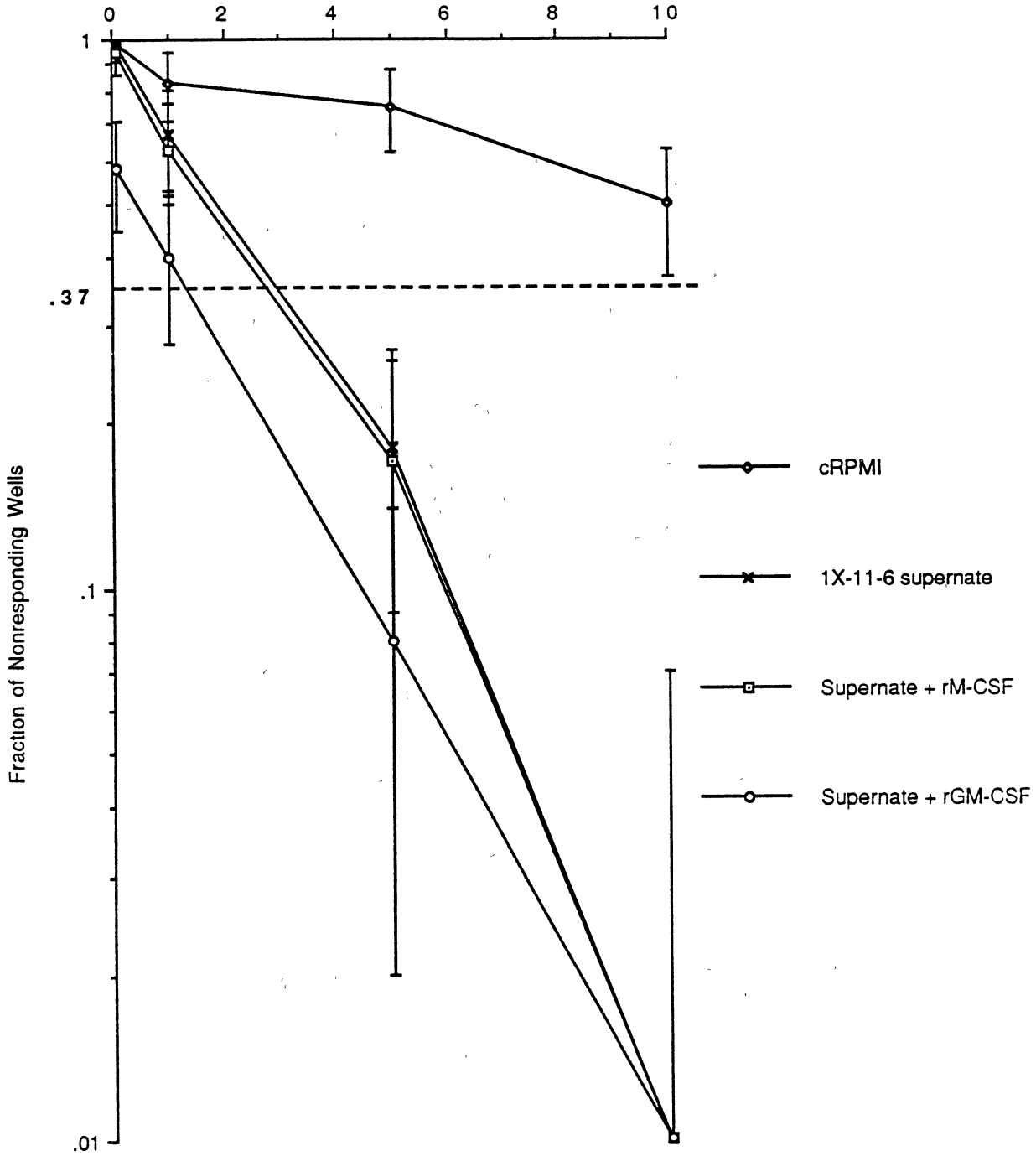

Figure 26. Limiting dilution analysis of the effect of adding rM-CSF and rGM-CSF to 1X-11-6 tumor supernate on colony formation of 1X-11-6 TAM.

response was achieved at 10% (vol/vol) MC-4 supernatant fluid in cRPMI, the same concentration of 1X-11-6 tumor supernate which exhibited maximum enhancement.

Response of TAM to Tumor Supernates and Cytokines

Limiting dilution analysis was utilized to examine the effect of 1X-11-6 supernate alone or supernate plus rM-CSF or rGM-CSF on TAM isolated from a 1X-11-6 tumor. TAM isolated from a 1X-11-6 tumor were cultured at concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ cells/well in cRPMI, cRPMI containing 10% (vol/vol) 1X-11-6 supernate, cRPMI containing 10% 1X-11-6 supernate and 1000 U/ml rM-CSF, or cRPMI containing 10% 1X-11-6 supernate and 100 U/ml rGM-CSF. Results obtained after 11 days of culture (see Figure 26) indicated that 10% 1X-11-6 supernate did increase proliferation, resulting in a frequency of 1 in 2900 TAM responding. The combination of 1X-11-6 supernate and rM-CSF showed the nearly identical result of 1 macrophage out of 2750 responding. The combination of 1X-11-6 supernate and rGM-CSF, on the other hand, showed a substantial increase over both the 1X-11-6 supernate alone or combined with rM-CSF, with 1 in 1400 TAM proliferating in response.

The effect of MC-4 supernate alone and in combination with the two cytokines was then tested on TAM from an MC-4 tumor. Concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ cells/well were prepared from TAM isolated from an MC-4 tumor, in cRPMI alone, 10% (vol/vol) MC-4 supernate in cRPMI, 10% MC-4 supernate and 1000 U/ml rM-CSF in cRPMI, and 10%

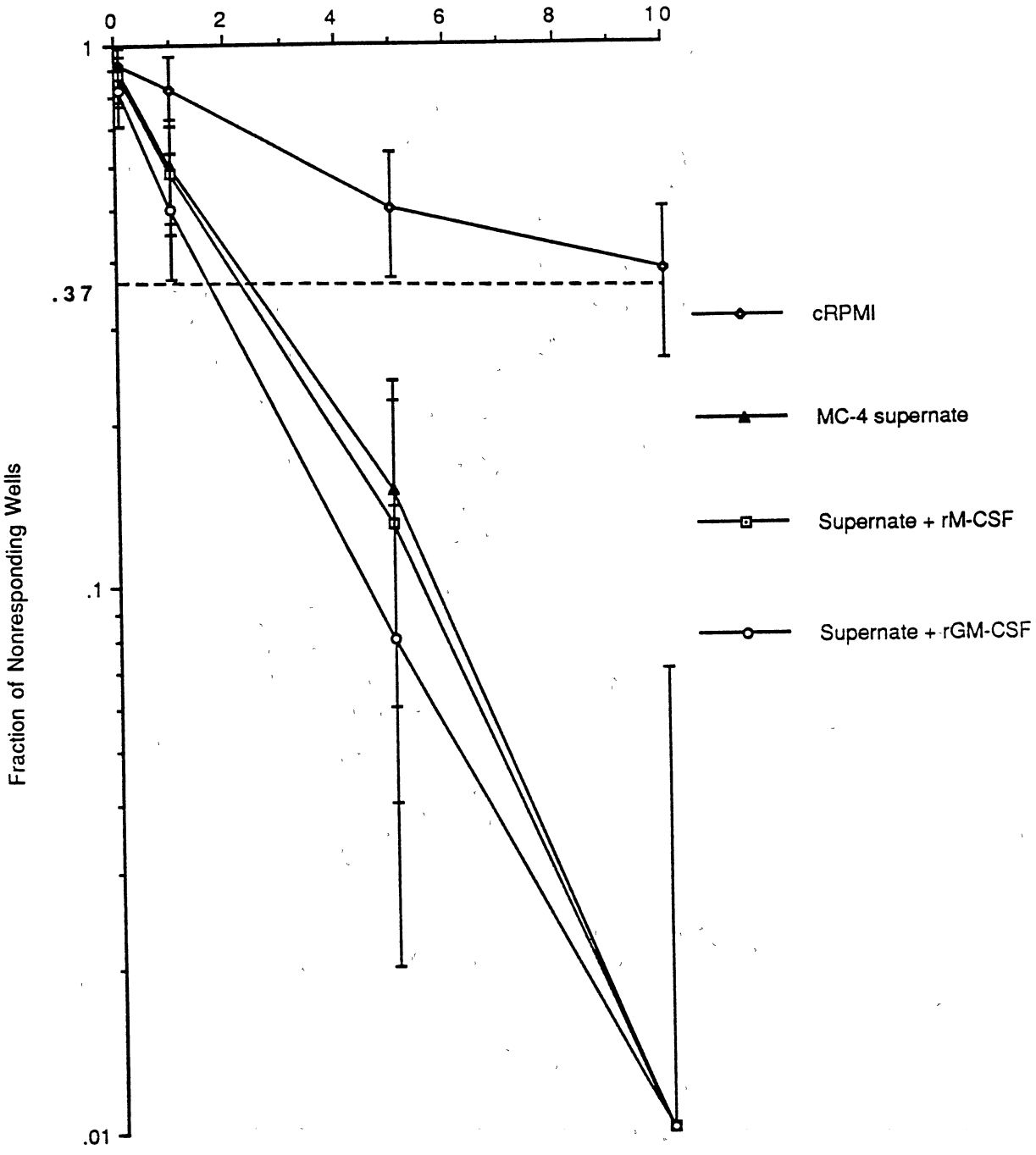

Figure 27. Limiting dilution analysis of the effect of adding rM-CSF and rGM-CSF to supernate from an MC-4 tumor on colony formation of MC-4 TAM.

MC-4 supernate and 100 U/ml rGM-CSF in cRPMI. The results (Figure 27) were nearly identical to those from the previous experiment (Figure 26). A frequency of 1 in 2500 TAM responded to the supernate alone, and 1 in 2250 responded when rM-CSF was added to the supernate. The additon of rGM-CSF to the MC-4 supernate resulted in 1 in 1600 TAM proliferating, an increase almost to the same degree as that seen in Figure 26 for the 1X-11-6 supernate combined with rGM-CSF.

The effect of 1X-11-6 supernate alone and in combination with the two cytokines was tested on RPM to discern if a difference in response would occur. RPM were prepared at concentrations of $1x10^2$, $1x10^3$, $5x10^3$, and $1x10^4$ cells/well in cRPMI, 10% 1X-11-6 supernate in cRPMI, 10% 1X-11-6 supernate and 1000 U/ml rM-CSF in cRPMI, and 10% 1X-11-6 supernate and 100 U/ml rGM-CSF in cRPMI. Results (see Figure 28) showed the same pattern as that seen with the 1X-11-6 TAM. Supernate from the 1X-11-6 tumor enhanced proliferation to the same degree as when rM-CSF was added with the supernate; a frequency of 1 in 2500 responded. As in the results seen with the TAM, the addition of rGM-CSF with the supernate resulted in an increase over the supernate alone, with 1 in 1200 RPM responding to the combination. This set of experiments indicates that the supernates from both tumors contain M-CSF activity but not GM-CSF activity.

Summary

The results from all of these experiments indicate that

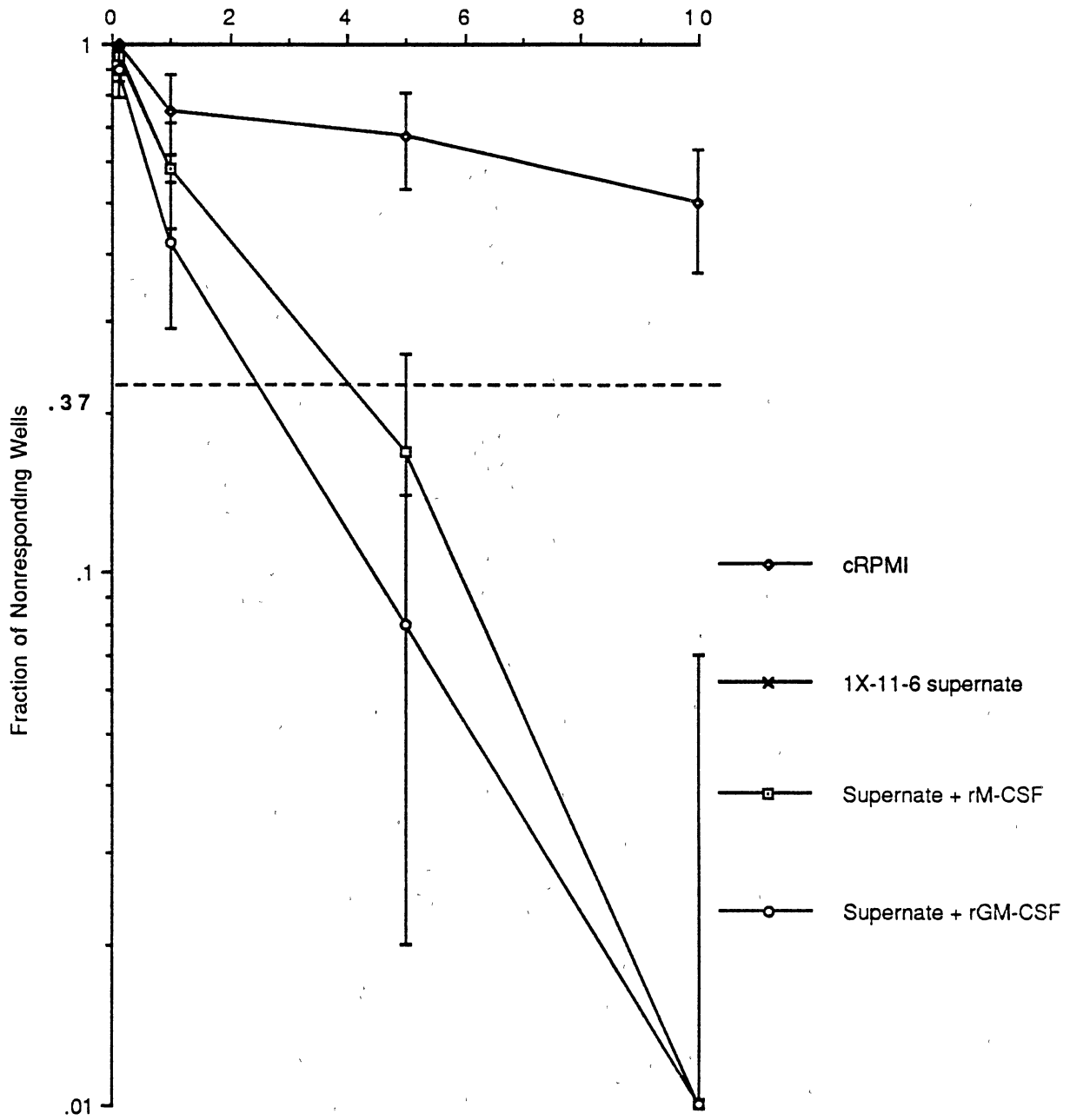

Figure 28. Limiting dilution analysis on the effect of adding rM-CSF and rGM-CSF to 1X-11-6 tumor supernate on colony formation of RPM (10,000 cells/well). The data for the supernate + rM-CSF is superimposed over the data for the supernate alone.

a subset of RPM and TAM do proliferate, and that the addition of M-CSF, GM-CSF, and SRBC to the medium enhances the frequency of cells that proliferate. In addition, combinations of these three factors do produce greater proliferation than any of the three factors alone, although not always significantly. These results have been summarized in Table 2, and indicate several points of interest. The frequencies of responding cells observed among both RPM and TAM are very similar, indicating the possibility that TAM populations are derived from populations of resident macrophages and not those of inflammatory macrophages. Alternatively, both RPM and TAM could be derived from an immediately common progenitor, and would be similarily differentiated and responsive to colony-stimulating factors. Also, these results suggest that the effect of combining M-CSF and GM-CSF may be additive and not synergistic on proliferative responses of the cells tested. Finally, the supernates of two tumors have been shown to contain M-CSF-like activity and not GM-CSF activity, which indicates that these two tumors may be capable of stimulating macrophage proliferation <u>in vivo</u>.

	Source of Cells		
		TAM	
	RPM	MC-4 tumor	1X-11-6 tumor
Treatment			
cRPMI	<1.0	<1.0	<1.0
SRBC	2.2	5.0[a]	2.8[a]
Crude cytokines			
M-CSF	2.2[a]	2.1[a]	2.2[a]
GM-CSF	1.6	1.4[a]	1.6[a,b]
Combinations			
M-CSF + GM-CSF	3.6[a]	2.0[a,b]	2.8[a]
M-CSF + GM-CSF + SRBC	2.0	2.2[a]	2.5[a,b]
Recombinant cytokines			
M-CSF	6.6[a]	5.0[a]	5.5[b]
GM-CSF	4.4	4.4	3.7[b]
Combinations			
M-CSF + GM-CSF	4.4[a]	5.7[a]	7.1[b]
M-CSF + GM-CSF + SRBC	4.0	5.0[a,b]	4.4

a. This value is estimated since single hit kinetics were not observed in this case.
b. This result was not shown previously in the Results section.

Table II. Summary table of the frequencies of FcR^+ colony forming cells among RPM and TAM populations (converted to responding cells/10,000).

CHAPTER IV

DISCUSSION

The results of this study show that macrophages, both from normal mice and isolated from tumors, are capable of proliferation *in vitro* consistent with previous reports (3,17,22). This proliferation occurred whether or not M-CSF, GM-CSF, or SRBC were part of the culture medium, although the presence of any of these three factors substantially enhanced the ability of RPM and TAM to form colonies. The response to M-CSF was approximately the same as to GM-CSF in enhancing proliferation of macrophages in either the crude natural or recombinant preparations of the two factors. SRBC were more potent than the crude natural preparations of either cytokine, but less effective than the recombinant factors.

M-CSF has been demonstrated to be capable of enhancing proliferation of macrophages when added to the culture medium (5,24,31) and the results of the experiments in this study add further evidence to these observations. RPM and TAM both showed increased amounts of colony formation when incubated in medium containing either the crude natural or recombinant forms of M-CSF. Since populations of TAM have recently been shown to express receptors for M-CSF on their surface (3), it was not an unexpected observation that TAM did respond to

this agent However, this is the first report of enhancement of TAM growth by M-CSF.

Macrophages from both populations also proved capable of responding to the crude and recombinant forms of GM-CSF. The frequency of RPM and TAM that responded to GM-CSF was slightly less than the frequency of these two populations that responded to M-CSF The recombinant GM-CSF was of murine origin whereas the recombinant M-CSF was of human origin, which could explain the difference in frequencies that was seen. In the crude preparations the difference could be due the difference in sources; M-CSF was obtained from a *in vitro* culture of a tumor cell line which constitutively produces M-CSF, and GM-CSF was obtained from an *in vitro* culture of normal murine lungs which were unstimulated as regards to GM-CSF production. In addition, the difference could be due to the presence of inhibitors or unknown colony-stimulating factors that might comprise part of the normal *in vitro* environment of the cells.

SRBC were also shown to enhance proliferation of both RPM and TAM when added to the culture medium. Although the reason for this effect is unknown, the appearance of macrophage colonies in 'clearing zones' in the lawn of SRBC coating the well could indicate that phagocytosis of foreign particles (the SRBC) can drive proliferation of macrophages. It is also possible that cell contact is important in proliferation of macrophages, and that the higher the concentration of SRBC, the better the response of the macrophages due to increased cell density. Finally, it could

also be possible that SRBC may prevent iron depletion of the medium by other macrophages present in the cultures and thereby aid macrophage proliferation (27).

The combinations of the three factors tested produced some very interesting results. It has been reported recently in several articles that M-CSF and GM-CSF are able to synergize and generate a greater effect than either one alone (4,15) The results from the combination of the crude preparations of the two cytokines do not support or agree with these findings. The combinations tested showed a substantial increase in colony formation in RPM and TAM over either cytokine alone which appeared to be additive (see Figures 7, 12, and Table 2). The combination of the recombinant cytokines, however, showed very little increase in proliferation of RPM and TAM over that of either recombinant factor alone (Figures 18 and 22). If only one subset of the macrophage population that comprise RPM and TAM is capable of responding and only this subset carries the receptors for both M-CSF and GM-CSF, then these results can be explained. The crude natural preparations, although at their optimal concentration for the proliferative response, may contain suboptimal concentrations of M-CSF and GM-CSF for stimulating every possible cell to divide. When the two are added together, the suboptimal doses of both cytokines are able to work together to produce a much greater response. With both recombinant M-CSF and/or GM-CSF the use of the optimal concentrations to stimulate every cell in the subset

to proliferate may have masked a synnergistic effect. There may have been enough of both cytokines that either one alone can stimulate every cell in the subset, so that when the two were mixed, only a very small increase was seen because the additional cytokine was unable to stimulate any additional cells to undergo division. Although LDA cannot accurately determine whether the effects of combinations are synergistic or additive, the results of these experiments do seem not to agree with the literature and suggest that the effect was additive in these experiments.

The combination of the two colony stimulating factors with SRBC resulted in no real increase in proliferation of RPM or TAM over that observed in the presence of rM-CSF and rGM-CSF. The reason for these results is probably the same as what was suggested to have occurred with the combination of the two recombinant cytokines; maximum proliferation of the macrophages in the two populations had already been achieved and further enhancement was not possible. It is also possible that different subsets of the macrophage population are stimulated to proliferate by the cytokines and SRBC, and that an increase in one population inhibits an increase in the other.

The presence of colony-stimulating factor activity in tumors was also determined in this study. Supernates of the two tumors were examined for their ability to enhance proliferation of TAM and for the presence of M-CSF and GM-CSF activity in the supernates. In the examination of supernates from both the 1X-11-6 tumor and the MC-4 tumor, the presence

of M-CSF activity but not GM-CSF activity was detected. In the development of macrophages from the bone marrow, both M-CSF and GM-CSF act on macrophage precursors (31). It is widely accepted that GM-CSF acts on an earlier precursor than M-CSF, a progenitor that can become either a granulocyte or a macrophage (31). Since M-CSF does stimulate proliferation of mature macrophages (1,24,25,31) and if the populations of RPM and TAM contain mature macrophages, than the presence of M-CSF activity and not GM-CSF activity in the tumor is consistent with the functional roles of these factors. However, it must be kept in mind that GM-CSF may still be present within the tumor in vivo since this factor has been shown to be released by endothelial cells and epithelial cells (24).

Another interesting observation derived upon examination of the supernatant fluids of the tumors was the concentration of the M-CSF in the tumor. A comparison of the crude preparation of M-CSF produced by the L cell line used in the first experiments with the two tumor supernates shows that the concentration of M-CSF activity in the tumor supernates is considerably higher. This would indicate that both tumors may be stimulating the highest possible level of macrophage proliferation in vivo. Since the tumors are actively growing, it is unlikely that the tumors would be benefited by generation of tumoricidal macrophages. Some function(s) of macrophages that could promote the tumor may be stimulated along with proliferation.

Another notable observation of this study is that the two tumors, which were different in etiology, behaved so similarly in regards to TAM proliferation. Both appeared capable of stimulating the maximum possible proliferation of the subset of TAM capable of growth. It is assumed that in both tumors this would promote some function of macrophages beneficial to tumor growth and survival. This observation confirmed reports in the literature that the accumulation of macrophages is considered to be tumor-dependent and not host-dependent (7,13,14). The observation also indicated a potentially identical mechanism used by the two tumors to enhance growth and integrity. Further investigation into this path could lead to a novel immunotherapeutic strategy for cancer applicable to a wide variety of tumors. For instance, colony-stimulating factors might possibly be added to a tumor, stimulating tumoricidal functions of TAM (19).

The frequencies of responding RPM and TAM were virtually the same throughout the entire study, indicating that the two different populations may be related in some way. Comparison of TAM to thioglycollate-elicted macrophages (TEM) showed very different proliferative capacities between the two (Kim Burnham, unpublished results), in which TEM exhibited much less capacity for growth than RPM or TAM. This indicates that TAM may be derived from either the normal resident populations of macrophages or from the same pool of circulating monocytes as RPM and not the populations elicted in an immune response. This is consistent with other reports in the literature that TAM obtained from progressively

growing tumors are neither activated nor tumoricidal (7,13,14).

Investigation of the ability of TAM to proliferate and the potential for this proliferation to account for the large numbers of macrophages found in tumors was one of the original goals of this study. The frequencies of TAM determined to proliferate *in vitro* indicated that only a rare subset of TAM was responsive. However, this does not rule out proliferation of existing or recruited macrophages as the primary source of TAM. In this study, only two factors (M-CSF and GM-CSF) were examined for stimulation of growth of TAM *in vivo* were examined. Many more factors known to stimulate proliferation of macrophages could potentially affect the situation *in vivo*, including IL-2, IL-3, and phospholipids.

Examination of these other factors may reveal other subsets of TAM capable of growth and that proliferation is the primary source of TAM. Other synergistic and/or additive effects of various signals might be revealed. It is equally possible that both the influx of macrophages from the blood and proliferation of macrophages already in the tumor may account for the numbers of TAM.

Another noteworthy observation from the results was the occurance of single hit events in the data. With the crude natural cytokines, single hit events were only seen when the two were combined with SRBC From the type of line resulting in these experiments when single hit kinetics were not seen,

this would indicate that some other population of cells present in both the populations of TAM and RPM or factors present in the crude preparations inhibited the response. Since RPM did not contain any tumor cells, this inhibition could potentially be caused by another subpopulation of macrophages. When the crude cytokines were combined with SRBC, this inhibition was completely overridden, which could be due to the prevention of iron depletion of the medium by the presence of SRBC as previously mentioned. In later experiments with the recombinant cytokines and tumor supernates, this inhibition was either reduced or not evident.

There were two key problems with this project, namely the potential effect of the protocol utilized for isolating TAM and insuring that the colonies examined under the microscope were indeed colonies of macrophages. The protocol used for isolating TAM had several features that could have led to problems. First, it was assumed that TAM capable of proliferation were FcR+ and would rosette with opsonized SRBC Secondly, lysis of the SRBC by hyperosmotic pressure at the conclusion of the isolation procedure could likewise lyse or metabolically shock macrophages in the suspension; on the average 50 to 60 percent of the total number of cells in the suspension before the application of the isolation procedure were lost. Lastly, it was also possible that the use of opsonized SRBC could result in activation of TAM which could have resulted in frequencies of proliferation completely unlike what may occur <u>in vivo</u> However,

repetition of an experiment utilizing adherence to plastic to isolate TAM showed approximately the same results as TAM isolated by opsonized SRBC (results not shown) In addition, RPM were isolated without the use of opsonized SRBC and showed roughly the same frequency of responding cells as TAM, indicating that the TAM were not activated by the opsonized SRBC.

The primary method of determining that the colonies of RPM and TAM were indeed macrophages involved rosetting the cells with opsonized SRBC and scoring as positive for growth colonies consisting of rosetted cells. However, it should be noted that other murine cells, particularly T lymphocytes, are capable of rosetting SRBC (whether opsonized or not). Demonstration that the colonies examined were indeed macrophages was achieved by identifying two additional macrophage markers, MAC-1 and I-A, in some of the experiments that were performed (results not shown) Addition of antibody specific for I-A followed by lysis with complement eliminated nearly all of the FcR+ colonies (80%) Addition of magnetic beads coated with anti-MAC-1 antibody and scoring for rosetting of the beads revealed roughly the same frequency of macrophages responding as did scoring with opsonized SRBC.

There are several directions that can be taken in future research based on these results. First, neutralizing antibodies which are specific against M-CSF and GM-CSF can be added to the tumor supernates to verify the presence of M-CSF

and not GM-CSF and to examine the supernates for the presence of other colony-stimulating factors. Others have reported tumoricidal activity of macrophages exposed to rM-CSF (19), so another possible avenue to examine would be to add rM-CSF and rGM-CSF to cultures of RPM and TAM and look for the production of tumor necrosis factor. Other populations of macrophages, such as those found in the spleen, lymph nodes, and bone marrow, could be examined for their ability to proliferate when grown in culture containing M-CSF, GM-CSF, and SRBC. A mechanism for the effect of SRBC on proliferation of RPM and TAM needs to be defined; cell density, phagocytosis of foreign particles, and prevention of iron depletion should be examined. In addition, the effect of combining M-CSF and GM-CSF might be better identified as synergistic or additive by combining suboptimal doses of the recombinant factors. Other cytokines need to be examined for their ability to induce or inhibit proliferation of macrophages. IL-3, also known as multi-CSF, should be investigated, as well as IL-2. Phospholipids have been reported as capable of stimulating proliferation *in vitro* (18) and should also be examined by limiting dilution analysis for their effect on RPM and TAM. It would also be interesting to examine the effects of potential inhibitors of macrophage-mediated cytostasis in an effort to block potential inhibition of macrophage growth. Furthermore, all of these factors should be examined for their ability *in vivo* to stimulate proliferation of macrophages both in tumors and in normal mice.

In summary, this study constitutes the first report in which colony-stimulating factors have been shown to enhance the frequency of TAM capable of *in vitro* proliferation. The populations of TAM examined have been shown to be very similiar to normal resident macrophages (RPM) in the frequency of cells that respond. This study also indicates that TAM capable of proliferation constitute a relatively rare subset of the TAM population, however, this subpopulation of cells may be very important due to their potential capacities for growth and interaction with other TAM.

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