ANALYSIS OF THE PHENOTYPE OF ELICITED MACROPHAGES CAPABLE OF IN VITRO PROLIFERATION

By

YAN CHEN

Bachelor of Medicine

Shanghai Second Medical University

Shanghai, China

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Thesis Advisor

Dean of the Graduate College

PREFACE

The goal of this study was to assess whether specific subsets of macrophages are involved in macrophage proliferation since it is known that macrophages are capable of proliferation. Subsets of murine thioglycollate-elicited peritoneal macrophages (TEM) based on differences in phagocytic function, cell surfaces markers (MAC-1, Ia and FcR) and cell size were analyzed for proliferating capacities in order to determine the phenotypes of macrophages capable of proliferation. In comparison, additional studies were performed using tumor associated macrophages (TAM) and resident peritoneal macrophages (RPM). Limiting dilution analysis (LDA) was applied to assess macrophage growth and determine proliferating frequencies. The results of these studies reveal that only specific subsets of macrophages are capable of optimal proliferation which is dependant upon M-CSF (macrophage-colony stimulating factors) or GM-CSF (granulocyte-macrophage-colony stimulating factors). The phenotypes of colony forming TEM, TAM and RPM are as follows: Phagocytic/FcR^{-}/MAC-1$^{+/-}$/Ia$^{+/-}$/small/large for TEM, MAC-1$^{+/-}$/Ia$^{+/-}$ for TAM and phagocytic for RPM.

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CHAPTER I

INTRODUCTION

Macrophages represent a heterogeneous population of cells and are found in virtually all tissues (5,22). They play a significant role in many physiological and pathological events within vertebrate animals (4,34). It is accepted that the maintenance of tissue macrophage level is probably due either to an influx of monocytes (immature macrophages) from the blood (5) or by proliferation of mature tissue macrophages in situ or by both mechanisms (8,14,15,26,41).

The proliferation of tissue macrophages has been recently recognized (1,2,8,13,14,15). However, the significance of this observation is still poorly understood due to unanswered questions concerning the complexity of macrophage subpopulations. For example, macrophages represent a heterogeneous population of cells in terms of level of differentiation and state of activation. It is unknown whether the proliferation of macrophages involves specific subpopulations or is a function of the whole population. To date, research concerning macrophage proliferation has been performed extensively on unfractionated populations of cells. Few studies have examined the proliferative capacity of subsets of macrophages.

To address this question, the growth of specific subsets of murine thioglycollate-elicited peritoneal macrophages (TEM) was

investigated. These macrophages exude into the peritoneal cavity from the circulation in response to thioglycollate injection. In this study, different subpopulations obtained following enrichment from TEM were cultured with macrophage-colony stimulating factor (M-CSF) and granulocyte-macrophage-stimulating factor (GM-CSF) in order to assess the proliferation of each subpopulation as measured by colony formation in vitro. As a comparison, additional studies on the growth of murine resident peritoneal macrophages (RPM) and tumor associated macrophages (TAM) were performed. Limiting dilution analysis (LDA) was applied to study the cell growth and determine the frequency of each subpopulation capable of proliferation.

Macrophages and the Mononuclear Phagocytic System

The mononuclear phagocyte system, consisting of monocytes and tissue macrophages, is the major cellular component of the reticuloendothelial system (22). It is a dynamic cellular system represented in virtually all tissues. This system has the potential to exert a modulatory role on tissue homeostasis and in local immunological and inflammatory responses (4,18,34).

In several general aspects, the mononuclear phagocytic system can be characterized. These include morphology, enzyme content, cell surface antigens, receptors, and functions.

Macrophages are spherical. They often have a single slightly indented nucleus containing prominent nucleoli (7). The centrosphere

region of these cells often contains abundant large phase dense lysosome granules (5,7).

Non-specific esterase, lysozyme, peroxidase and ecto-enzymes are all associated with macrophages (5). The activity of esterase with alpha-naphthyl butyrate or acetate as substrates has been used to identify mononuclear phagocytes. Lysozyme constitutes another useful marker of these cells. Peroxidase is also a useful marker to distinguish various developmental stages of mononuclear phagocytes. It is found in immature macrophages (monoblast, pro-monocytes, monocytes and exudate macrophages), but is absent in resident macrophages (5). The ecto-enzymes 5'-nucleotidase, leucine aminopeptidase, and alkaline phosphodiesterase I, which occur in the plasma membrane of these cells are also useful markers. 5'-nucleotidase is helpful in distinguishing normal (resident) macrophages from activated macrophages, whereas the other two ecto-enzymes increase upon activation (5).

The surface antigens found on macrophage membranes have been identified with monoclonal antibodies. MAC-1 is a mouse macrophage cell surface antigen which is recognized by the M1/70 rat anti-mouse monoclonal antibody (27). It is of general use in distinguishing macrophages from T and B lymphocytes (27). F4/80 is a membrane glycoprotein of mouse macrophages recognized by a rat hybridoma secreting antibody (3). F4/80 has the virtue of being remarkably stable. This marker is restricted to mature mouse macrophages and has been used to identify macrophages in situ (3). Ia is MHC class II antigen expressed on the macrophage cell

membrane. It is also present on other cells, such as B lymphocytes and activated T lymphocytes in the immune system.

FcR with multiple subtypes are the receptors for the Fc portion of IgG on macrophage cell surfaces. Along with a number of functional characteristics including phagocytosis, FcR is an intrinsic property of mononuclear phagocytes and certain other leukocytes (5).

Functionally, macrophages have the ability to engulf particulate materials, i.e., phagocytosis (30). Phogocytosis is a fundamental property of macrophages. These cells are also able to adhere actively to a solid substrate such as plastic, glass, or collagen. This property is often exploited as a way to separate macrophages from other leukocytes (10).

Mononuclear phagocytes originate from pluripotent stem cells in the bone marrow (reviewed in 5). The most immature type of cell within this lineage is the monoblast. In the mouse, the cell cycle of the monoblast is about 12h. After division one cell gives rise to two promonocytes. The promonocyte also divides only once (cell-cycle time about 16 h) and gives rise to two monocytes. Monocytes do not divide further in the bone marrow, and leave this tissue randomly within 24 h after they are formed. These cells remain in the circulation for 1-2 days, after which they leave this compartment and migrate to tissues where they differentiate into macrophages.

Macrophages have unique functional roles in tissues. First, they can interact with many extracellular molecules, proteins and polysaccharides, and internalize and submit them to intracellular metabolic changes (34). Second, macrophages are highly secretory

cells (4). The secretory products of macrophages include proteases, complement proteins, growth regulatory factors such as interleukin-1, and arachidonate derivatives. All of these molecules are important in inflammatory reactions. Third, macrophages interact with T lymphocytes and thereby promote immunological responses by antigen presenting and regulation (22). Fourth, macrophages have surface receptors for lymphokines, the regulatory proteins released by lymphocytes; which upon interaction with lymphokines, lead to novel properties included under the term "activation" (21). Activated macrophages are highly microbicidal and tumoricidal (22). Thus, the mononuclear phagocyte system is involved in infectious processes, in the modulation of immunological responses, and in inflammation.

Resident Peritoneal macrophages (RPM), Thioglycollate-elicited Macrophages (TEM), and Tumor Associated Macrophages (TAM)

Resident peritoneal macrophages (RPM) are the macrophages which reside in the peritoneal cavity in a steady state under normal hemostatic control. Peritoneal thioglycollate-elicited macrophages (TEM) are the macrophages which exude from the circulation in response to a peritoneal injection of thioglycollate. These cells are in an intermediate activation state (12). TEM have higher oxidative antimicrobial or cytocidial activity and Ia expression than RPM and a lower level of F4/80 expression (12).

Tumor associated macrophages (TAM) are found in tumor tissue and are presumably influenced by the tumor environment.

TAM have been isolated from tumors of widely diverse origins, and range from 2-60% of total isolated cells (22). Some studies propose that the entire TAM population of rapidly growing tumors can be accounted for by the influx of circulating monocytes, while others feel that proliferation of TAM at the tumor site must occur. Local proliferation and differentiation of TAM might be induced via growth factors produced locally by tumor cells. It has been reported that TAM have a higher level of mRNA transcripts of the c-fms protooncogene which encodes a tyrosine kinase which is probably identical to the M-CSF receptor (16). In this study, TAM did not express M-CSF transcripts whereas the tumor cells had high level of M-CSF mRNA (16). Other data show that some murine sarcoma cells transcribed the c-sfm gene coding for M-CSF. The supernatant from these tumor cell cultures induced the proliferation of bone marrow cells and macrophage IL-1 gene transcription. This suggests that M-CSF produced by tumor cells is related to the increase in the level of IL-1 activity in TAM and possibly their growth (35). The functions of TAM within tumors are poorly understood. Suppressor and cytotoxic activities as well as tumor growth-promoting functions have been demonstrated (22). The antitumor responses of TAM have also been observed. These were associated with an increase of class II-MHC antigen expression at the tumor site (18,19). On the other hand, growth stimulation by TAM has also been noticed. TAM from NFSA (a murine poorly immunogenic fibrosarcoma induced by 3-methylcholanthrene) which represent 80% of the cellular composition of this tumor, were shown to be growth stimulatory (6).

MAC-1, Ia and FcR

MAC-1, Ia and FcR are three of the markers found on macrophage membranes. The expression of these markers can vary among different macrophage populations.

MAC-1 is synthesized by macrophages (27). It is defined by the M1/70 rat anti-mouse monoclonal antibody. MAC-1 contains polypeptide chains of 170,000 and 95,000 mol wt. Blood monocytes, normal splenic and peritoneal macrophages, and peritoneal macrophages elicited by diverse agents all express MAC-1. MAC-1 is expressed on over 86% of resident peritoneal macrophages (RPM) as well as macrophages elicited by thioglycollate (TEM), Con A, lipopolysaccharide (LPS), or peptone (27). MAC-1 is present on exudate macrophages at early (1 day) and later (4 day) time points. It appears to be continuously expressed during maturation of monocytes to macrophages (27). Macrophage-like cell lines also express MAC-1 (27). The number of M1/70 binding sites per TEM is approximately 1.6×10^5 at saturation and 1.4×10^5 per RPM. This represents a high level of expression. Lymphocytes are MAC-1 negative (27). Thus MAC-1 can be used as a marker for differentiating macrophages from lymphocytes. Its ubiquitous and abundant expression on macrophages suggests that MAC-1 might play an important role in macrophage physiology and function (27).

FcR are the cell surface receptors expressed on mononuclear phagocytes which enable macrophages to recognize the Fc portion of IgG molecules of various subclasses. Fc receptors are involved in the selective binding and ingestion of foreign material. Murine Fc

receptors have been identified for IgG_{2a} (FcR1), IgG_1 and IgG_{2b} (FcR_2), and IgG_3 (FcR_3). IgG_{2b}, IgG_1 and IgG_{2a} are trypsin-insensitive receptors and IgG_{2a} is a trypsin-sensitive receptor (30). There are two obvious functions of macrophages that involve receptors for IgG: the removal of opsonized particulate material from the blood and tissues, and antibody-dependent cellular cytotoxicity (ADCC) (5). Inflammatory mediators, including prostaglandins (particularly PGE_2), are released after ingestion of antibody-antigen complexes or antibody-coated cells. Fc receptor interaction also stimulates the secretion of superoxide anion and various enzymes (30).

MHC Class II molecules are cell surface antigens expressed on subpopulations of monocytes and macrophages. The genes coding for these marker are located in the I region of the major histocompatibility gene complex (MHC) of the mouse. The MHC codes for two families of cell surface glycoproteins termed Class I and II (Ia) and certain complement proteins. Class II molecules in the mouse are heterodimers made of 35- and 29-kD chains (alpha and beta chains) found mainly on the surfaces of macrophages, B cells, and the Langerhans-dendritic cells of the skin and lymphoid organs (38). The expression of these glycoproteins is essential for macrophages to function as antigen-presenting cells during induction of immune responses since the interaction between accessory macrophages and the responding T cells is Class II-restricted. Class II expression appears transiently during macrophage maturation (24) and may be induced by cytokines, such as gamma interferon (25).

The Heterogeneity of Macrophages

The mononuclear phagocyte system comprises cells of considerable heterogeneity in terms of surface markers, functions and morphology. It is often uncertain whether heterogeneity reflects different stages of maturation or modulation by exogenous stimuli.

Macrophages express cell surface markers, such as MAC-1, Ia and FcR (19,27,30). However, expression varies among different macrophage subpopulations (6,18,25). The use of monoclonal antibodies against these markers makes it possible to distinguish subpopulations.

Heterogeneity among macrophages can also be shown by differences in function, such as phagocytosis (22). The main purpose of phagocytosis is digestion of foreign materials or damaged host tissues. Uptake of bacteria and particulate materials derived from host tissues, is important in host defense and in inflammation (30). Macrophages with or without phagocytic capability probably represent functionally different subpopulations due either to different stages of differentiation or to the existance of separate lineages.

In terms of morphology, heterogeneity can be demonstrated by cell size which in some cases has been correlated with differences in functions and expression of surface phenotypes (22). With regard to TAM, smaller macrophages have a considerably lower FcR avidity than the larger-sized cells and contain a greater percentage of peroxidase positive cells. The small-sized TAM contain the majority of Class II-positive TAM and have anti-tumor activity (6). Suppressor macrophages and macrophages with accessory cell

function also differ in size (29). Efficient antigen-presenting cells are found among smaller macrophages. In contrast, large macrophages often show potential for suppression of lymphocyte proliferation (29).

Macrophage Proliferation

How the level of tissue macrophages is maintained and whether tissue macrophages are capable of proliferation are not fully understood. Tissue macrophages are generally considered to be terminally differentiated cells with little, if any, ability to replicate. Therefore, monocyte influx is thought to be the main contributor to replacement of macrophage turnover with local division of tissue macrophages playing a minor role in renewal of the population (5). However, there are many recent studies which suggest the alternative concept that local division of alveolar macrophages, for example, contributes significantly to the renewal of tissue populations (2). The relative significance of local proliferation in tissue macrophage maintenance was also reported for Kupffer cells (liver macrophages, 26), TAM (8), and RPM (15). It was found that a large percentage of Kupffer cells present after induced macrophage hyperplasia were due to Kupffer cell replication and not macrophage immigration; A sizable proportion of TAM were also observed to proliferate (8). By morphologic appearance the replicating cells were mature macrophages, not immature progenitors (8). The maintenance of RPM in the steady state was dependent on proliferation of peritoneal macrophages in situ, not by influx (15). Thus, it is possible that more mature cells can participate in replication. A number of

cytokines have been shown to stimulate the proliferation of macrophages including M-CSF, GM-CSF and IL-3 (1,2,13,14,20,31).

To date, research on macrophage growth has been mostly limited to whole populations. There exists a need to determine whether there are specific subpopulations of macrophages involved in replication or wether all macrophages under the influence of specific environments can be induced to replicate.

This project focused on analyzing the proliferative potential of macrophage subpopulations. TEM, RPM and TAM population were analyzed in this study. Different separation methods were used to obtain subpopulations of macrophages with the following phenotypic pattens: MAC-1^{+}/MAC-$1^{+/-}$, Ia^{+}/Ia^{-}, FcR^{+}/FcR^{-}, Phagocytic-/Phagocytic$^{+/-}$, and small/large cells. These cells were then cultured in vitro.

Colony Stimulating Factors

Colony stimulating factors (CSFs) are specific regulatory glycoproteins that stimulate hematopoietic cell proliferation. In both mice and humans, four CSFs have been characterized that stimulate the production of different blood cell lineages specifically. Granulocyte-CSF (G-CSF) stimulates neutrophilic granulocytes and granulocytes. Granulocyte-macrophage-CSF (GM-CSF) stimulates both granulocytes and macrophages. Macrophage-CSF (M-CSF or CSF-1) stimulates macrophages. Multi-CSF (or Interleukin-3, IL-3) has not only the capacity to stimulate granulocyte and macrophage formation, but also an exceptional broad range of proliferative

effects, including actions on erythroid, megalokaryocytic, eosinophilic and mast stem cells (33).

Mouse GM-CSF was purified to homogeneity from mouse lung conditioned medium as a glycoprotein of 23,000 mol wt (36). Its cDNA was cloned and expressed by mammalian and bacterial cells. Recombinant GM-CSF has the typical in vitro properties of native GM-CSF (31).

Murine M-CSF(CSF-1) was purified to homogeneity from L cell conditioned medium as a glycoprotein of 70,000 mol wt. M-CSF is a dimer of two equal subunits (31). The structure of human and murine M-CSF are similar. Native and recombinant forms of human M-CSF, both manifest potent CSF activity on mouse bone marrow macrophage colony formation (32). In addition to its direct stimulation of macrophage progenitor cell proliferation, M-CSF also acts on more mature cells (32).

The receptors of M-CSF and GM-CSF exist on mononulear phagocytes. The receptor numbers for M-CSF are relatively high on some macrophage tumor cell lines, lower numbers are observed on mature monocyte-macrophages from various normal tissues (16). The M-CSF receptor is structurally related, and possibly identical, to the c-fms proto-oncogenes (21).

In addition to its role in hemopoiesis, M-CSF has been shown to stimulate a variety of functions in mature monocytes, bone marrow-derived macrophages, and tissue macrophages. Some of these functions include production of cytokines such as IL-1(35); enhancement of FcR expression (17); enhancement of tumor cell

killing (9); and induction of viral resistance and secretion of superoxide and H_2O_2 (11).

Limiting Dilution Analysis

Limiting Dilution Analysis (LDA) was chosen as a means of assessing the growth of cells. It is based on a statistical formula derived from Binomial and Poisson distributions. By LDA, the growth of rare cells is able to be determined since this assay is adapted to the analysis of rare events (28). This analysis is also capable of ruling out the effects of cellular interaction by examining the kinetics involved (28). Also the frequency of cell proliferation can be estimated with statistical confidence (23,28). In this study, LDA was used to determine wether subpopulations of TEM, RPM and TAM were able to proliferate in vitro. This analysis was also used to determine which phenotypes were responsible for the growth of macrophages and the frequencies of subpopulations which exhibited growth.

CHAPTER II

MATERIALS AND METHODS

Animals

Female C3H/HeN mice of about 6-8 weeks of age were obtained from Charles River of Wilmington, Massachusetts. All mice were 6-12 weeks old when used in experiments. Mice were maintained at a licensed facility at Oklahoma State University. Etherization was used to euthanize the mice, the advantage of this method is that it avoids the entry of blood into the peritoneal cavity as often occurs with cervical dislocation.

Culture Medium and Buffers

For all experiments, cells were cultured and diluted in complete RPMI medium (cRPMI). Powdered RPMI and other components to complete the medium were obtained from Sigma Chemical corporation, St. Louis, MO. To generate complete medium, 10.4 g powdered RPMI was reconstituted in one liter double distilled water with the following reagents: 2 g of sodium bicarbonate, 5% (vol/vol) heat inactivated fetal bovine serum (56^{0}C water bath for 30 minutes), sodium pyruvate (1.0 mM), non-essential amino acids (10 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). The pH was adjusted to 7.1 and the medium was

sterilized by filtration through 0.22 um exclusion sterile disposable filters (Fisher) and stored at 4°C until used.

Phosphate buffered saline (PBS) was used to dilute and wash cells and prepare reagent stock solution. It consisted of 2.7 g monobasic potassium phosphate ($KH_2PO_4 \cdot H_2O$, Fisher, Fair Lawn, NJ.), 4.1 g sodium chloride (NaCl, Baker Chemical Corporation, Phillipsburg, NJ.) and 5.6 g anhydrous sodium phosphate (Na_2HPO_4, Sigma) in one liter double distilled water. The pH of the solution was adjusted to 7.4. Sterilization was accomplished by autoclaving at 15 psi for 15 minutes.

Lysing Buffer (Tris-buffered ammonium chloride) was used to lyse sheep red blood cells (SRBCs). This solution consisted of 50 ml of a 2.06% (w/v) tris base solution (20.6 g/L, Sigma), that was adjusted to a pH of 7.6, combined with 450 ml of a 0.83% (w/v) solution of ammonium chloride (8.3 g NH_4 Cl/L, Sigma). The pH of the final solution was adjusted to 7.2 by HCL and then filtered through a 0.22 um sterile disposable filter.

M-CSF and GM-CSF

Recombinant human M-CSF (rhM-CSF) and recombinant murine GM-CSF (rmGM-CSF) were used in cell culture. rhM-CSF was obtained from Cetus Corporation, Emeryville, CA. at a concentration of 5 x 10^5 units/ml. It was diluted to 1 x10^4 units/ml in PBS and stored frozen at -20° C until use.

Recombinant murine GM-CSF was obtained from Immunex Corporation in Seattle, Washington at a concentration of 5 x 10^5/ml.

It was diluted to 1 x 10^4/ml in PBS and then stored at -20° C until used.

Sheep Erythrocytes

Sheep erythrocytes (SRBCs) were obtained from the College of Veterinary Medicine, Oklahoma State University and stored at 4° C. Prior to use in experiments, SRBC were washed with PBS. PBS was added to SRBC, mixed with SRBC gently, and centrifuged at 650x g for 8 minutes. The supernatant was removed. The above steps were repeated until the supernatant become transparent in color. SRBC were then diluted to the concentrations needed.

Monoclonal Antibodies.

Anti-sheep red blood cells, M1/70 and anti-Ia^k were the antibodies used in the study. Anti-sheep red blood cells (Accurate Chemical and Scientific Corporation, Westbury, N.Y.) consisted of an IgG fraction produced in rabbits. It was used in opsonizing SRBC. The Fc region on this monoclonal antibody is able to bind Fc receptors on macrophage surfaces. For opsonization, the antibodies were diluted to 1:400 with 5% SRBC and incubated in a 37° C water bath for 15 minutes. The SRBC were then washed once by centrifuging at 650x g for 8 minutes, after which the supernatant was discarded and fresh PBS was added.

Anti-Ia^k was obtained as a supernatant of the hydridoma cell line, 26-7-11S from The American Type Culture Collection (ATCC), Rockville, Maryland. This hybridoma was produced by fusion of $Sp^2/0$-Ag14 cells with spleen cells of an A.TH mouse immunized with

A.TL lymphocytes. This antibody is of the IgM class and reacts with Ia^k determinants on macrophages and other lymphoid cells. The medium used for hybrid cell culture was 90% Hybri-Care (Hyclone Laboratories, Logan, Utah) with 10% fetal bovine (heat inactivated) serum.

Anti-MAC-1 hybridoma supernatant was obtained from the M1/70 (anti-MAC-1) hybridoma which was formed by the fusion of mouse myeloma line NS-1 with spleen cells from DA rats immunized with C57BL/10 mouse spleen cells enriched for T lymphocytes. It is an IgG_{2b} antibody which reacts with the alpha chain of the murine macrophage specific antigen MAC-1. This hybridoma was also obtained from The American Type Culture Collection (ATCC), Rockville, Maryland. The medium used for culture of these cells was 90% Dulbecco's modified Eagle's medium with 10% fetal bovine serum (heat inactivated).

Isolation of RPM

RPM were obtained from normal C3H/HeN mice by peritoneal lavage with PBS. Mice were euthanized in an ether jar prior to removing the outer skin over the peritoneal cavity outer skin. Eight ml of cold PBS was injected to the peritoneal cavity and the cavity was gently massaged. Peritoneal cells within PBS were aspirated and transferred to a sterile tube kept on ice. This procedure was repeated once. The recovered fluid was pooled and centrifuged at 650x g for 10 minutes, after which the supernatant was removed and the pellet resuspended in 2 ml cRPMI and kept on ice. The cell concentration was determined by counting use of a hemocytometer. To get RPM ,

cells were then incubated at 37° C with 5% CO_2 in a culture dish (Baxter) at the concentration of 3 x 10^6/ml. The cells were cultured for 1 hour, which allowed RPM to adhere to the dish. Non-adherent cells were removed after incubation by gently rinsing with PBS three times. The adherent cells were obtained by adding 2.0 ml PBS to the dish and removing the adherent layer with a scraper (Baxter). Adherent cells were transfered to 10 ml medium in a sterile test tube, centrifuged at 650x g for 10 minutes, resuspended in 2.0 ml cRPMI medium and kept on ice. The cell viability was determined by preparing a mixture of 10 ul cell suspension, 40 ul trypan blue (0.1%) and 50 ul PBS and placing onto a hemocytometer. Cells stained by trypan blue were counted as dead since they were unable to exclude the stain. The adherent cells isolated by this method included approximately 11% of the total peritoneal cells obtained, of which the viability was over 95%. The harvest of peritoneal cells was about 5.3 x 10^6 cells/mouse.

Isolation of TEM

TEM were obtained from mice injected with thioglycollate broth. Thioglycollate broth was generated by adding powdered thioglycollate (Becton Dickinson and Company, Cockeysville, MD) to double distilled water (4.5% w/v) and autoclaving it at 15 psi for 15 minutes. Two ml of this broth were injected into the peritoneal cavity of each mouse three days before harvesting. To isolate TEM, the same procedures were performed as those for obtaining RPM, but without adherent enrichment since greater than 90% of the cells

recovered exhibited macrophage characteristics. The viability of TEM after isolation was above 95%.

Generation of Tumors and Isolation of TAM

1X-11-6 was the tumor used in this study. It was obtained from Dr. Jim Benson at the University of Oklahoma Tulsa Medical College. This tumor appears to be a fibrosarcoma by histological criteria (Jim Benson, unpublished results.). Tumor 1X-11-6 is a spontaneous tumor which arose from in vitro culture of murine placental tissue derived from a female C3H/HeN mouse. To passage tumors in C3H/HeN mice, 1X-11-6 tumor fragments were implanted by subcutaneous injection in the abdominal region. After 6-8 weeks, tumors grew to 1-2 cm in diameter and were ready for experiments. Two to three mice were euthanized for each experiment and the tumors excised. Following excision, the tumors were cut into fragments and were enzymatically dissociated into single cell suspensions. To accomplish this, tumor fragments were incubated in 0.1% (w/v) collagenase (Sigma) and dispase (Bohringer Mannheim, GmbH, W.-Germany) with mechanical stirring in a 37° C water bath for 1 hour. The dissociated tumor cell suspension was centrifuged at 650x g for 10 minutes. The pellet was resuspended in 2 ml complete RPMI. Cell concentrations were determined with the aid of a hemocytometer. For macrophage isolation, cells were incubated at a concentration of 3 x 10^6/ml in culture dishes (60 x15 mm) at 37° C and 5% CO_2 for 45 minutes, during which time TAM adhered to the dishes. Subsequently, the dishes were rinsed with PBS and the cell layer removed by using a scraper. The cell suspension

was then centrifuged at 650x g for 10 minutes and the cells resuspended in 2 ml medium. Cell viability was determined by trypan blue exclusion with the aid of a hemocytometer. Cells were kept on ice prior to use. With enzymatic separation and adherent enrichment, the harvest rate of viable TAM was about 1% - 9% of total tumor cells.

Separation of FcR^+ and FcR^- Subpopulations

After macrophages were obtained from different sources, including TAM, RPM and TEM, these cells were ready to be separated into subpopulations. For the separation of FcR^+ and FcR^- subpopulations, cells were diluted to concentrations of 5×10^6/ml to 1×10^7/ml. Two mls of cell suspension were then mixed with an equal volume of 5% opsonized SRBCs and incubated at 37^0 C for 30 minutes with vertical rotation. The mixed suspension was then gently layered above 3 ml of Ficoll-Hypaque (specific gravity 1.119, Sigma) and centrifuged at 1100x g for 20 minutes at room temperature. After centrifugation, the FcR^- cells which did not bind SRBCs were found at the medium-Ficoll interface. The supernatant above this layer was discarded and the FcR^- cells harvested. These cells were resuspended in 2.0 ml cRPMI medium following centrifugation. The FcR^+ macrophages were rosetted by antibody coated SRBCs and pelleted at the bottom of tube after centrifugation. These cells were harvested and resuspended in 2 ml medium. Both FcR^+ and FcR^- cell suspensions were washed at 650x g for 10 minutes one time and resuspended in 2.0 ml medium. The SRBCs bound to rosetted macrophages (FcR^+ subpopulations)

were lysed by adding 1 ml lysing buffer followed by the addition of 5 ml cRPMI to stop the reaction. The FcR^+ cell suspension was centrifuged again at 650x g for 10 minutes and the pellet resuspended in 2 ml cRPMI. The viability of both FcR^+ and FcR^- cells were determined and cell suspensions diluted in cRPMI to appropriate concentrations for limiting dilution analysis. By this process, the viable FcR^+ cells yielded about 8-18% out of total TEM and FcR^- cells, about 56-68%.

Preparation of Ia^- and $Ia^{+/-}$ Cells (negative selection)

Ia negative cells were obtained by lysis of Ia positive cells with monoclonal anti-Ia^k antibody and complement (fresh rabbit serum). Supernatant containing anti-Ia^k from hybrid cell culture was directly added to pelleted macrophages at cell concentrations of 1×10^6/ml. The cells were set on ice for 45 minutes to allow the monoclonal antibody to interact with Ia^k determinants on the surface of macrophages (Ia^+ population). The suspension was subsequently centrifuged and resuspended in complement solution (1:6 in medium, Cedarlane Laboratorie Limited, Hornby, Ontario, Canada). Cells were incubated in the presence of complement at 37^0 C with 5% CO_2 for 45 minutes in order for complement dependent lysis of Ia^k positive cells to occur. Following incubation, 10 ml of medium was added to the suspension to stop the reaction. The suspension was centrifuged at 650x g for 10 minutes and resuspended in 2 ml medium. $Ia^{+/-}$ cells (the mixed population) were treated by the same procedures as described above without adding anti-Ia^k antibody. Ia^- cells and $Ia^{+/-}$

cells (the mixed cell population) were diluted to concentrations needed for subsequent experiments. The viabilities of cells were determined prior to dilution.

Preparation of MAC-1^{-} and MAC-1$^{+/-}$ Cells (negative selection)

The MAC-1^{-} cells (the MAC-1^{+} deleted subpopulation) and Mac-1$^{+/-}$ cells (the mixed cell population) were prepared by the same procedures as above except that the monoclonal antibody used was anti-MAC-1 (M1/70) supernatant obtained from hybridoma cell culture. MAC-1^{-} and MAC-1$^{+/-}$ cells were diluted to specific cell concentrations and cultured. The viabilities of these cells were also determined prior to use in experiments. MAC-1+ cells from RPM by this isolating procedure consisted of about 84% of the total population.

Enrichment of Non-phagocytic Cell (negative selection)

Macrophage populations were depleted of phagocytic cells by using carbonyl iron (Sigma) and a cobalt magnet. Prior to the experiments, the iron powder was washed in ethanol, then distilled water, and autoclaved at 15 psi for 15 minutes. For phagocytic cell depletion, 4 mg of carbonyl iron was added to each ml of cell suspension at the concentration of 6-8 x 10^6 cells/ml and mixed thoroughly. The mixture was then incubated at 37°C for 30 minutes with mixing occasional mixing. Subsequently, the plastic tube containing the mixture was juxtaposed to one of the poles of a cobalt

magnet. The phagocytic cells which engulfed iron particles attached to the wall of tube due to magnetic attraction, while the non-phagocytic cells did not adhere. After incubation at 4^0 C for 10 minutes, the suspension containing non-phagocytic cells was transfered to a second plastic tube (with the first tube still standing on the magnet). The cell suspension on the magnet was resettled for a further 10 minutes at 4^0 C following removal of the magnet and transfered to another tube. Non-phagocytic cells (phagocytic$^-$ population) and the mixed cell population (phagocytic$^{+/-}$ population, without depletion) were then used for culture. Cell concentrations were adjusted according to the viability of cells.

Separation of Large and Small Cell Subpopulations

The large and small TEM were separated by a velocity sedimentation apparatus (Beckman) with fetal calf serum (FCS, Hazlenton Butchland Inc. Denver, PA) as the gradient material. The apparatus has three main parts: the sedimentation chamber, the gradient maker, and a small intermediate vessel. The sedimentation chamber is a cylindrical cavity with a conical base (cone angle at 30' to the horizontal). There are holes at the base of the chamber which regulate input and output of fluid. The chamber has a diameter such that each mm in the cylindrical region contains 11.4 ml of fluid. Prior to separation, the apparatus was sterilized by washing with ethanol and rinsing with sterile PBS. The separation was performed in a cold room at 4^0 C in order to inhibit cellular metabolism during the separation procedure. Twenty ml of TEM cells in PBS were

introduced from a small intermediate vessel into the chamber under a 30 ml layer of PBS which prevented disturbance of the cell bands by erratic movement of the rising fluid as the chamber was filling. A non-linear 15-30% FCS in PBS gradient, comprised of 30%, 25%, 20%, 17.5% and 15% FCS was introduced under the cell bands from the gradient maker. A small baffle was used to disperse the incoming jet of fluid which otherwise would produce mixing. The entire filling operation took about one hour. After three hours, during which cells settled by gravitational force, the chamber was drained. The fluid in the cone (120 ml) was discarded and the remainder of the gradient was collected in 15 ml fractions in disposable plastic tubes (Fisher). In this experiment, 20 fractions were collected. According to the plot of number of cells vs sedimentation velocity (fraction number), there were two peaks: the first one consisted of fractions 5,6,7 and 8 which were large sized macrophages (as determined by microscopic examination); the second one comprised of fractions 9,10 and 11 which apparently consisted of smaller macrophages and some lymphocytes. Each group of fractions was pooled respectively. The resulting cell suspensions were then centrifuged at 650x g for 10 minutes and the cell pellets resuspended in 2 ml medium each, after which the viable cell number of both large size and small size macrophage populations was determined.

Limiting Dilution Analysis

LDA was used to asses the proliferation of subpopulations of macrophages from TEM, TAM and RPM in culture. This type of analysis is based on a formula derived from the Binomial and Poisson

distributions. The zero term for this expression is $F_0 = e^{-u}$ (F_0: the fraction of nonresponding wells; u: the mean number of responding cells per well). Therefore the negative logarithm of the fraction of non-responding wells is linearly proportional to the mean number of responding cells added to each well (i.e. u=-In F_0). Thus if various cell concentrations are assessed for a given function, a plot of the cell input versus the negative In of F_0 will yield a straight line passing through the origin (i.e. Linearity or single-hit kinetics). In addition, when u=1 (there is an average of one responding cell per well), then $-F_0 = 0.37$ (1/e) and the frequency of responding cells can be estimated as the inverse of the cell input at which 37% of the cultures fail to respond (in this case, yield colonies). The Poisson distribution can only be applied to the type of analysis described above if the responding cell is truly limiting. If cellular interaction between two or more cell types of relatively similar frequency effects the outcome, then the frequency of nonresponse observed will not exhibit Poissonian behavior and a plot of the cell input versus the negative logarithm of the fraction of nonresponding cultures will not yield a straight line (i.e. multi-hit kinetics). This results from the fact that the probability of placing of two rare cells in a given well would be very small at lower cell input, but would increase dramatically at higher cell input.

In this study, macrophage subpopulations were separated by the differences of their phagocytic function, cell surface markers and cell size. Different subsets were then cultured with M-CSF or GM-CSF in microtiter plates (Fisher) as follows: phagocytic^{-}/phagocytic$^{+/-}$; FcR^{+}/FcR^{-}; Ia^{-}/Ia$^{+/-}$; MAC-1^{-}/MAC-1$^{+/-}$; and small/large. Sixty

wells were used for each concentration of each subpopulation of cells to insure the statistical reliability of the data obtained. The plates were cultured for 10 days with 100 ul cRPMI added to each well. At the end of the incubation period, the wells were scored for colony formation. Therefore non-responding wells consisted of those in which no macrophage colonies were observed. Scoring for macrophage colonies was facilitated by adding 50 ul of 0.2% opsonized SRBC to each well, gently rocking at a 37° C shaker for 60 min, and then examining the wells for colonies of rosetted cells with an inverted phase-contrast microscope. Only those wells with colonies containing six or more rosetted FcR^+ cells were considered positive (responding well). The data was then plotted on a semilog graph as macrophage concentration on the X axis and the In of the fraction of non-responding wells (wells without macrophage colonies) on the Y axis. The line drawn by LDA was then examined for straightness by linear regression analysis (Pearson's methods, (23)) and confidence intervals derived by least squares analysis obtained from a table. The coefficient of linearity derived from this analysis is at the 95% level of confidence (see Table 1).

By LDA, this study attempted to determine whether there are specific subpopulations capable of proliferation and forming colonies and the frequency of responding cells in each subpopulation if possible.

TABLE I

CONFIDENCE VALUES FOR THE PEARSON'S COEFFICIENT OBTAINED FROM LINEAR REGRESSION ANALYSIS OF THE DATA (95% CONFIDENCE)

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 from linear regression analysis of the data.

CHAPTER III

RESULTS

The primary goal for this study was to analyze the proliferation of murine macrophage subsets in culture. M-CSF and GM-CSF were used as growth factors. Subsets of macrophages from TEM, TAM and RPM were selected according to the presence or absence of phagocytic function, cell surface markers (including FcR, Ia and MAC-1), and cell size. LDA (limiting dilution analysis) and linear regression analysis were applied to assess macrophage growth statistically. LDA allows for the detection of proliferation of rare cell types as well as potential interactions with other cell types which enhance or inhibit proliferation. This type of analysis also indicates the frequency of macrophage proliferation which is confirmed by linear regression analysis if the response is determined solely by the presence or absence of a single responding cell. This study provided information concerning the proliferation of macrophage subsets and the frequencies of proliferating cells. This information is essential for an understanding of the regulation and significance of macrophage growth within tissues.

The Proliferation of Non-phagocytic and TEM Unseparated TEM in the Presence of M-CSF

Phagocytosis is one of the fundamental functions of macrophages. The first experiment was designed to compare the growth of phagocytic cells and non-phagocytic cells in the presence of M-CSF. Non-phagocytic cells were obtained by negative selection which involved depletion of phagocytic cells (as described in the Materials and Methods) from a population of TEM (thioglycollate elicited macrophages). Non-phagocytic cells and mixed cells were cultured in vitro respectively. Both groups of cells were diluted to concentrations of 1 x 10^4 cells/ml, 4 x 10^4 cells/ml and 8 x 10^4 cells/ml in completed RPMI medium. One hundred ul aliquots of each concentration were pipetted to each well to yield a total sixty wells. The culture medium contained M-CSF as a growth factor at a concentration of 1000 u/ml. Cells were cultured at 37^0 C with 5% CO_2 for 10 days. Control cultures contained medium without M-CSF. The plates were scored at the end of the 10 day incubation period by the detection of colonies containing FcR^+ cells by addition of opsonized SRBCs (as described in the Materials and Methods). The data was collected and plotted on Figure 1 and 2 by LDA. The results showed that cultures containing unseparated cells cultured with M-CSF yielded positive responses in terms of FcR^+ colony formation. The fraction of positive wells observed increased with increasing cell concentrations. Linear regression analysis using the Pearson's method yielded a coefficient of 0.998 for the unseparated population. At the 95% level of confidence with 2 degrees of freedom, this indicated that the data fit a straight line (see table 1). If an LDA plot

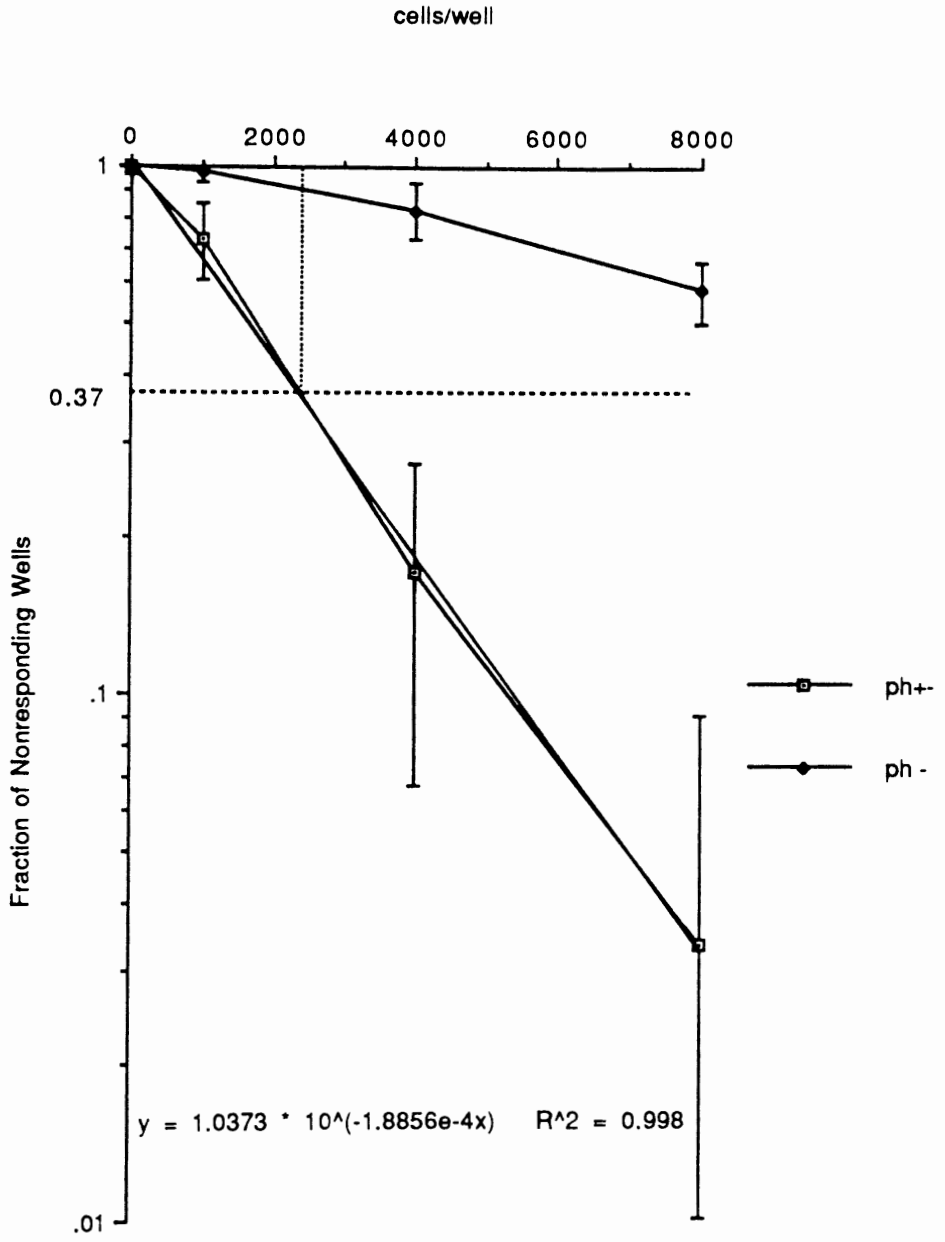

Figure 1. Limiting dilution analysis of the proliferation of phagocytic+- (ph+-) and Phagocytic- (ph-) TEM in the presence of M-CSF

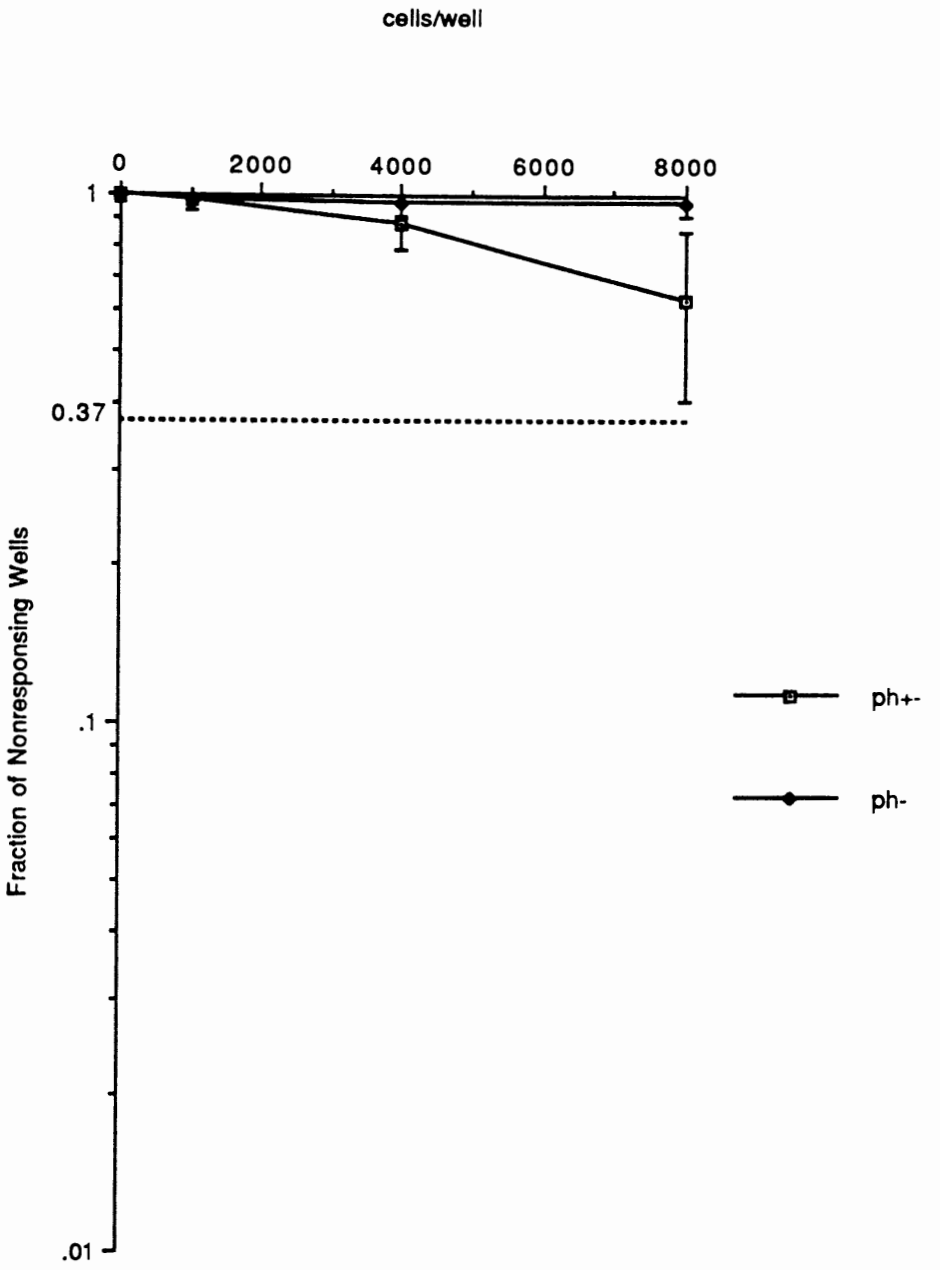

Figure 2. Limiting dilution analysis of the proliferation of phagocytic+- (ph+-) and phagocytic- (ph-) TEM in the absence of M-CSF

passes through the origin and yields a straight line, it satisfies the condition of single hit kinetics and indicates that proliferating cells were not influenced by other cell types. Therefore the frequency of responding cells could be determined in this case. The frequency is equal to the reciprocal of the point on the X axis (cell input) where the line crosses the point on the Y axis such that 37% of the wells yield no response (contain no FcR^+ colonies). In this experiment a straight line passing through the origin was observed which fit single hit kinetics. This line indicated a frequency of 1/2300 (1 cell responded in 2300 cells). The cultures containing non-phagocytic cells in the presence of M-CSF contained a lower frequency of positive wells. Upon culture without M-CSF, these cells did not yield colonies Figure 2). However, in control cultures without M-CSF, unseparated TEM did generate FcR^+ colonies but at a low frequency (Figure 2). The results obtained suggest that phagocytic cells account for the majority of proliferation observed among TEM. In addition M-CSF was required for optimal proliferation.

The Proliferation of Non-phagocytic RPM and Unseparated RPM in the Presence of M-CSF

RPM (resident peritoneal macrophages) represent a normal macrophage population at steady state without inflammatory stimulation, while TEM contain recruited macrophages, most of which exist in an intermediate activation state (12). RPM were analyzed for the proliferation of non-phagocytic cells and unseparated cells in order to compare the characteristics of colony forming RPM with those of TEM. RPM were enriched by adherence to plastic dishes.

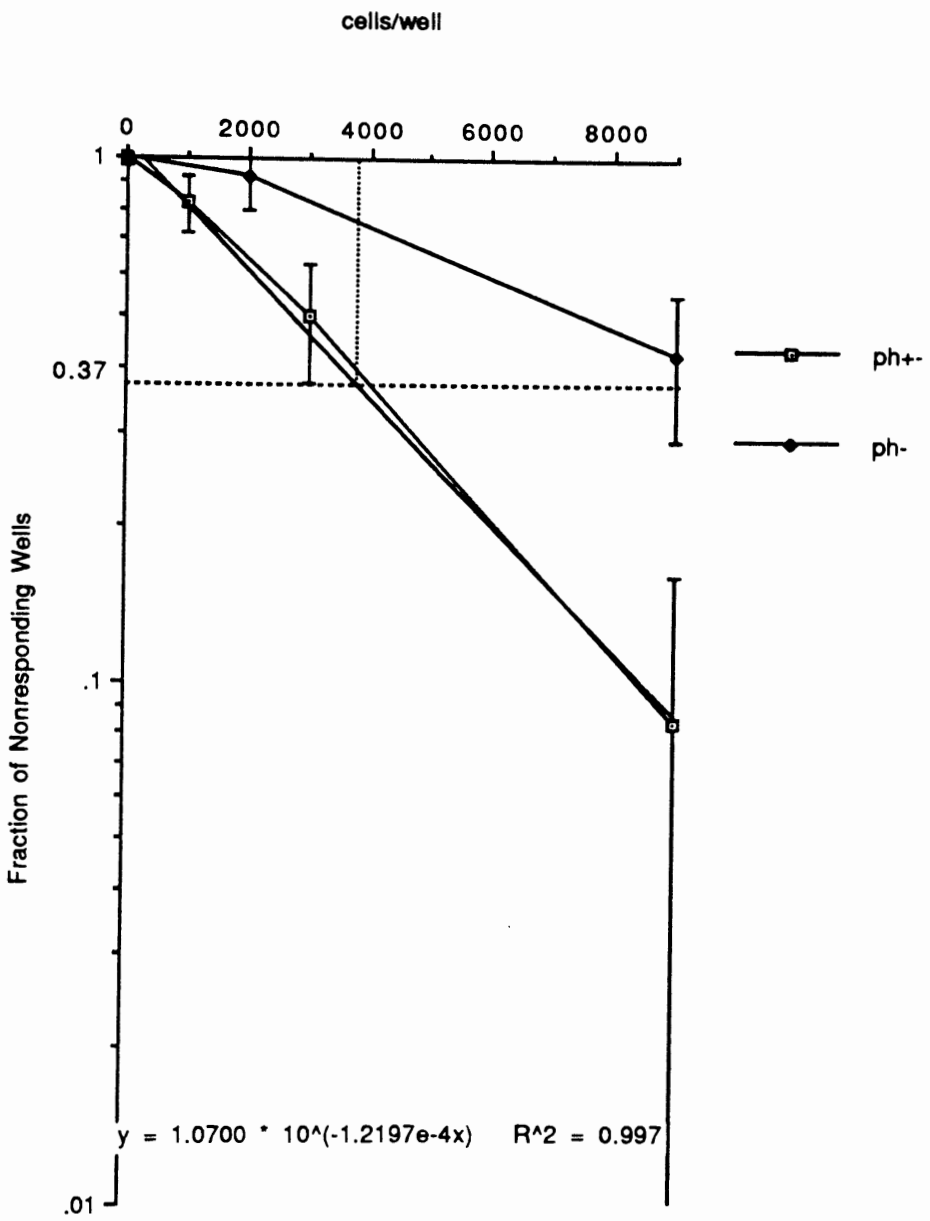

Figure 3. Limiting dilution analysis of the proliferation of phagocytic+- (ph+-) and phagocytic- (ph-) RPM in the presence of M-CSF

Following isolation, these cells were enriched for non-phagocytic cells as described as above. Subsequently non-phagocytic cells were cultured at concentrations of 2000 cells/well and 9000 cells/well. Unseparated RPM containing both phagocytic and non-phagocytic cells were cultured at concentrations of 1000 cells/well, 3000 cells/well and 9000 cells/well. M-CSF was added to all cultures at a concentration of 1000 u/ml. Other culture conditions were the same as described previously. The data from this experiment were plotted in Figure 3. As shown, the unseparated population containing both phagocytic and non-phagocytic cells yielded colonies. The coefficient from the data of mixed cells by linear regression analysis was 0.997. At the 95% level of confidence with 2 degrees of freedom, it fits a straight line, crossing the line at which 37% of wells were not responding with an X axis value of 3800. Thus, the frequency was about 1/3800, meaning 1 cell responded in 3800 cells since the line passed through the origin. A lower frequency of colonies was observed within cultures of non-phagocytic cells. This result was similar to that obtained for TEM cultures with the exception that the TEM yielded an overall higher frequency of proliferation than RPM.

The Proliferation of FcR^+ and FcR^- Populations of TEM in the Presence of M-CSF

FcR found on macrophage surfaces are receptors for the Fc portion of IgG molecules. The difference in the level of expression of this receptor can be used to divide macrophages into FcR^+ and FcR^- subpopulations. Both populations were studied for their capacity for proliferation. The procedures for separation of FcR^+ and FcR^- cells

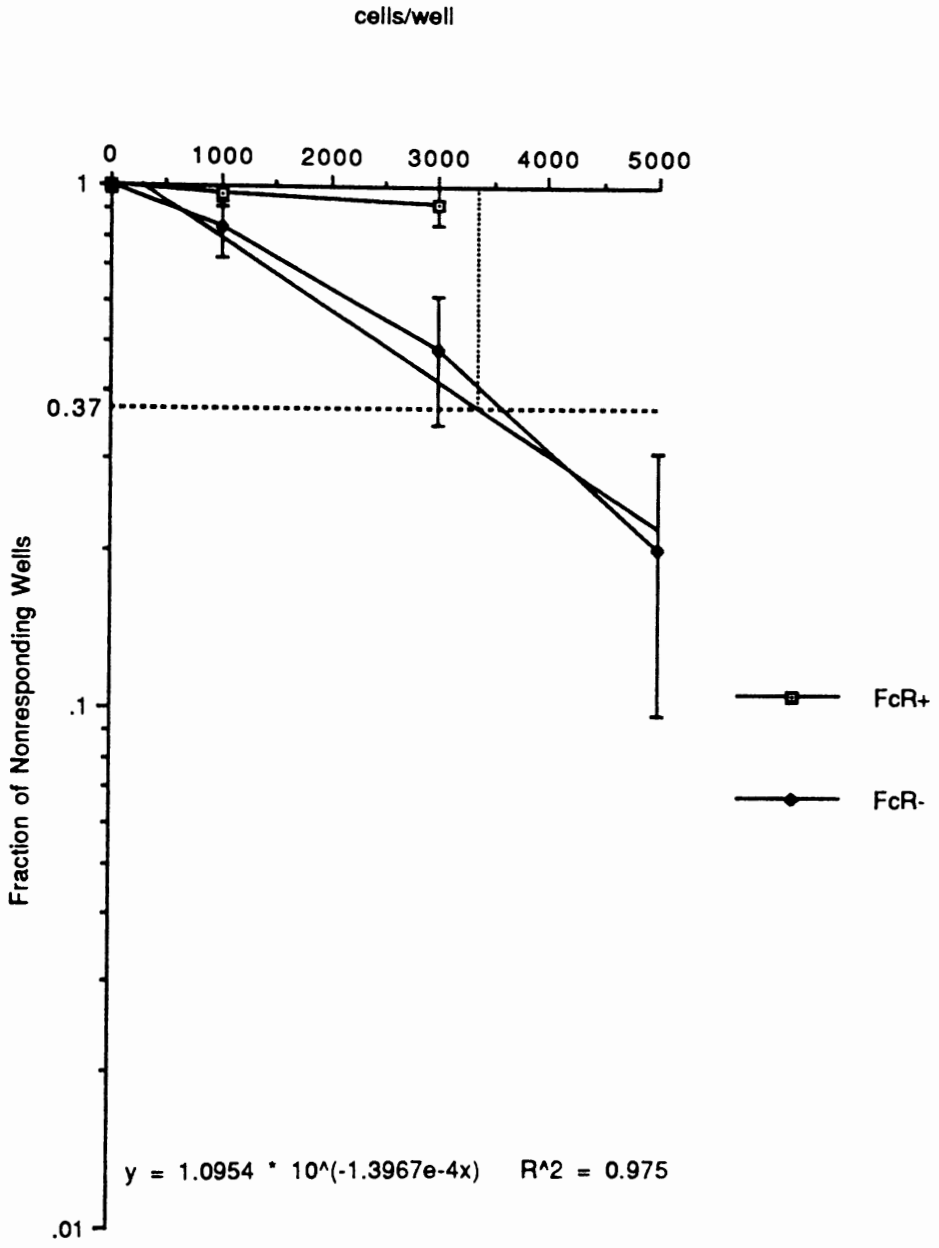

Figure 4. Limiting dilution analysis of the proliferation of FcR+ and FcR- TEM in the presence of M-CSF

were described in Chapter II. Briefly, TEM were incubated with opsonized SRBCs to allow FcR^+ cells to bind to SRBCs. Following centrifugation on a layer of Ficoll-Hypaque, SRBCs binding cells (FcR^+) were physically separated from non-binding cells (FcR^-). Both cell types were then harvested and washed. Both FcR^+ and FcR^- cells were cultured with M-CSF at a concentration of 1000 u/ml. The cell concentrations of FcR^+ cells employed were 1000 cells/well and 3000 cells/well. For FcR^- cells the concentrations were 1000 cells/well, 3000 cells/well and 5000 cells/well. All cultures were incubated at 37^0 C with 5% CO_2 for 10 days. At the end of the culture period, opsonized SRBCs were added to each well to facilitate scoring for colony formation. The data was collected and plotted in Figure 4. As shown, the FcR^- population yielded positive responses. The coefficient obtained by linear regression analysis of the data points from the cultures of FcR^- cells was 0.975. This value was at the 95% level of confidence with 2 degrees of freedom for linearity. Single hit kinetics were not observed, however, since the line did not cross the origin. The approximate frequency observed was 1/3300. The FcR^+ population showed little response. The data from this experiment indicated that FcR^- cells played the major role in TEM proliferation in the presence of M-CSF.

The Proliferation of FcR^+ and FcR^- Populations of TEM in the Presence of GM-CSF

In order to compare the proliferation of FcR^+ and FcR^- TEM in the presence of M-CSF with that of GM-CSF, an experiment similar to that described above (was performed with the exception that GM-

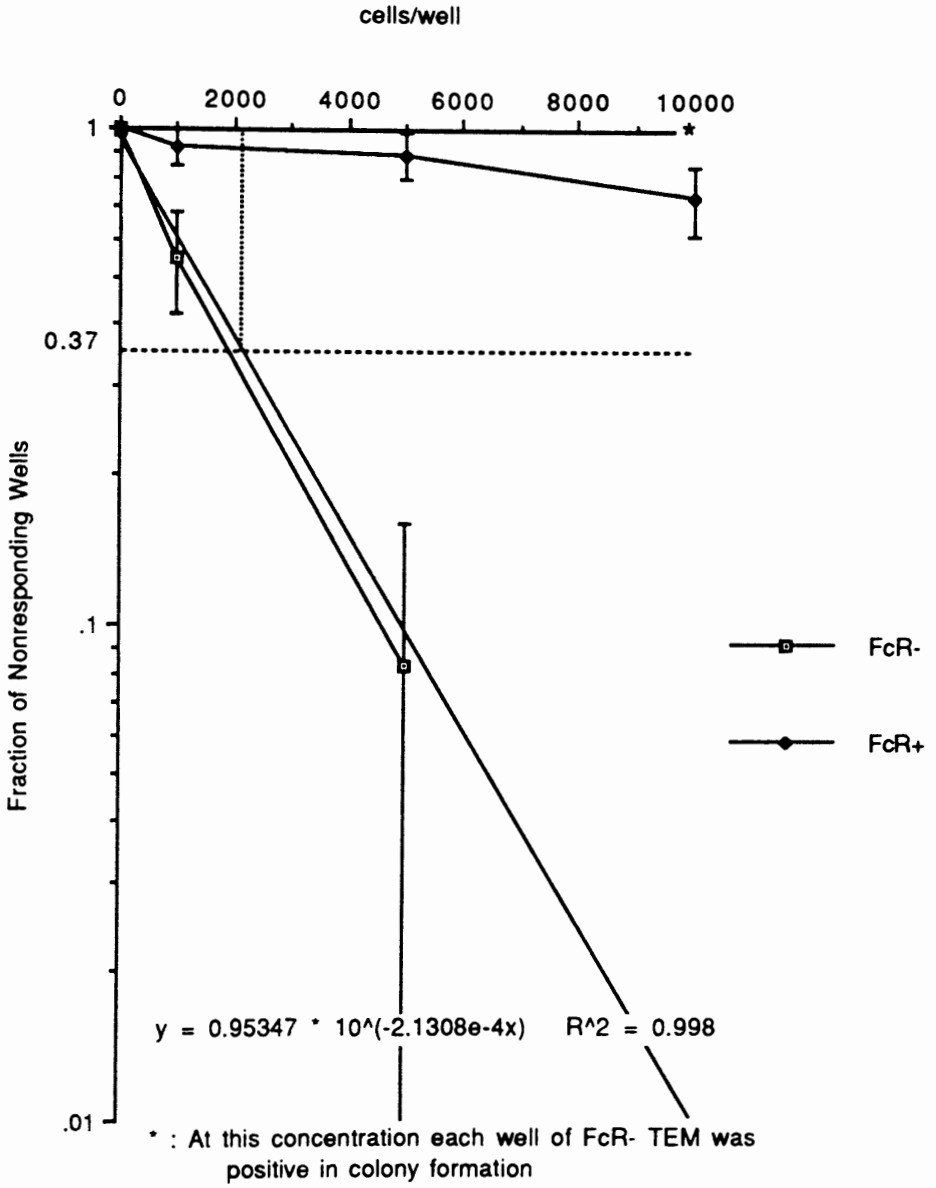

Figure 5. Limiting Dilution analysis of the proliferation of FcR + and FcR - TEM in the presence of GM-CSF

CSF was added instead of M-CSF. FcR^+ and FcR^- cells were obtained from TEM as previously described. Cells of each subset were cultured at three concentrations: 1000 cells/well, 5000 cells/well and 10,000 cells/well. GM-CSF was added at a concentration of 100 u/ml. Following culture, cells were incubated with opsonised SRBCs and scored as performed above. The data generated are shown in Figure 5. The FcR^- population yield a significantly higher response which was statistically different from that of FcR^+ cells. All wells containing FcR^- cells responded at the concentration of 10,000 cells/well (this was not plotted on Figure 5). Linear regression analysis of the points plotted yielded a coefficient of 0.996, indicating a straight line at the level of 95% confidence with 1 degree of freedom. The line passing through the origin crossed the line at which 37% of the wells were negative in response with the X axis value of 2200, indicating a frequency of 1/2200. In contrast, FcR^+ cells did not show a positive response. These data were similar to those obtained from the culture of FcR^+ and FcR^- populations using M-CSF as a growth factor, except that the frequency of cell

proliferation among FcR- cells was higher in the presence of GM-CSF as compared to M-CSF. The above data suggested that FcR^- cells of TEM were contained in the subpopulation capable of proliferation in the presence of M-CSF or GM-CSF.

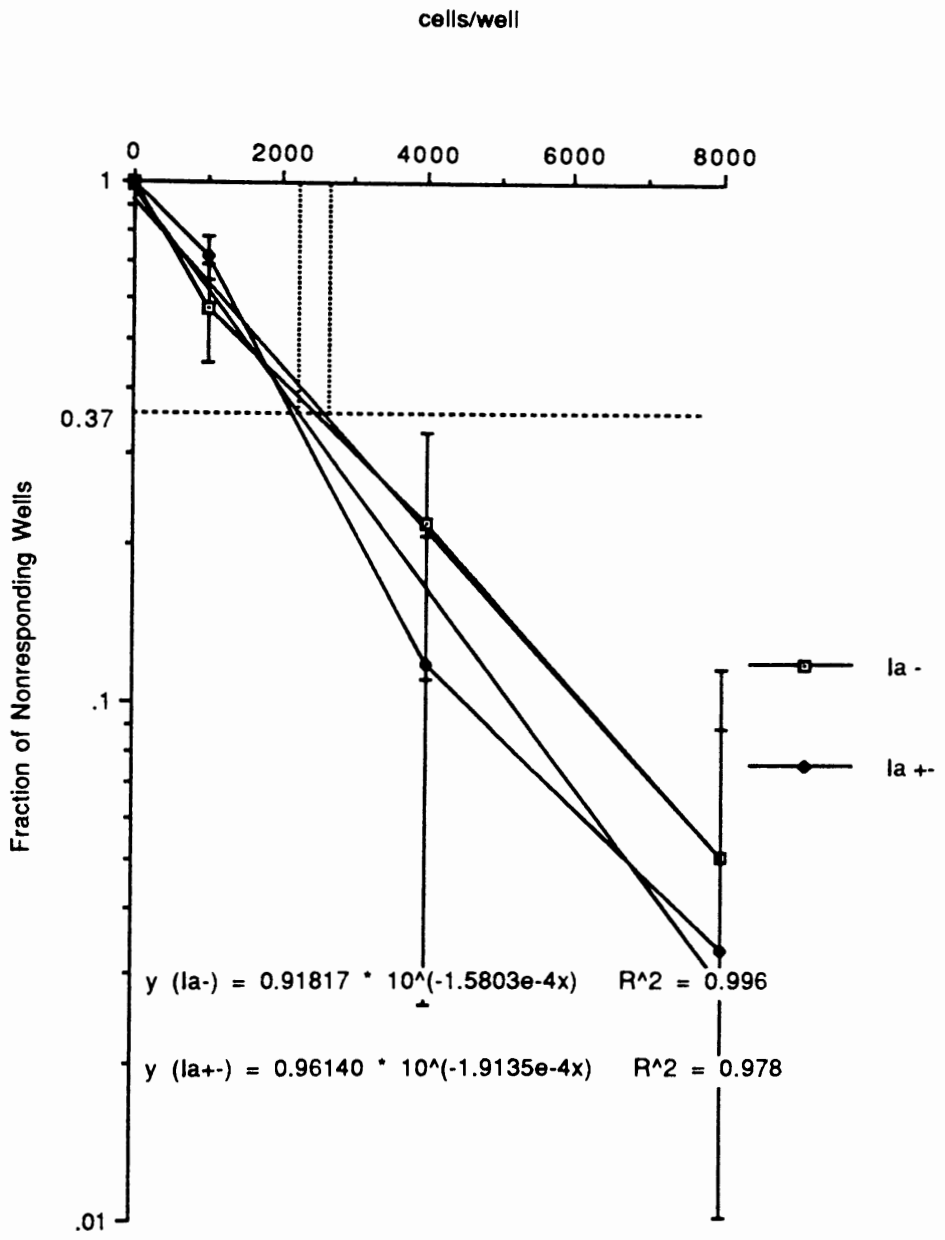

Figure 6. Limiting dilution analysis of the proliferation of Ia+- and Ia- TEM in the presence of M-CSF.

The Proliferation of Ia$^-$ and Unseparated TEM in the Presence of M-CSF

MHC class II molecules (Ia) are expressed on the surfaces of some but not all macrophages (5). Ia plays a role in antigen presentation and other macrophage functions. The expression of Ia by macrophages may vary at different stages and under different environmental stimulations. The following study was designed to assess the Ia expression of colony forming macrophages. Ia$^-$ and unseparated TEM were used. Ia$^-$ cells were obtained by specifically lysing Ia$^+$ cells with anti-Ia^k antibody and complement. Ia$^-$ and unseparated TEM were cultured with M-CSF in the medium at a concentration of 1000 u/ml. Both groups were cultured at concentrations of 1000 cells/well, 4000 cells/well and 8000 cells/well. After 10 days of culture, all wells were scored for colony formation. Both groups of cells yielded positive response. The data from this experiment was plotted on figure 6. The coefficients from the results of Ia^{+-} and Ia$^-$ cell culture were 0.978 and 0.996 respectively which fit in both cases a straight line at the level of 95% confidence with 2 degrees of freedom. The line from the data of unseparated TEM cell cultures passed through the origin yielding a proliferation frequency of 1/2200. The approximate frequency of Ia$^-$ cell proliferation was measured to be 1/2600. The results showed that Ia$^-$ and unseparated cells were similar in proliferation, suggesting that the difference in expression of Ia among TEM did not affect TEM growth in the presence of M-CSF, or that colony precursors expressed low levels of Ia.

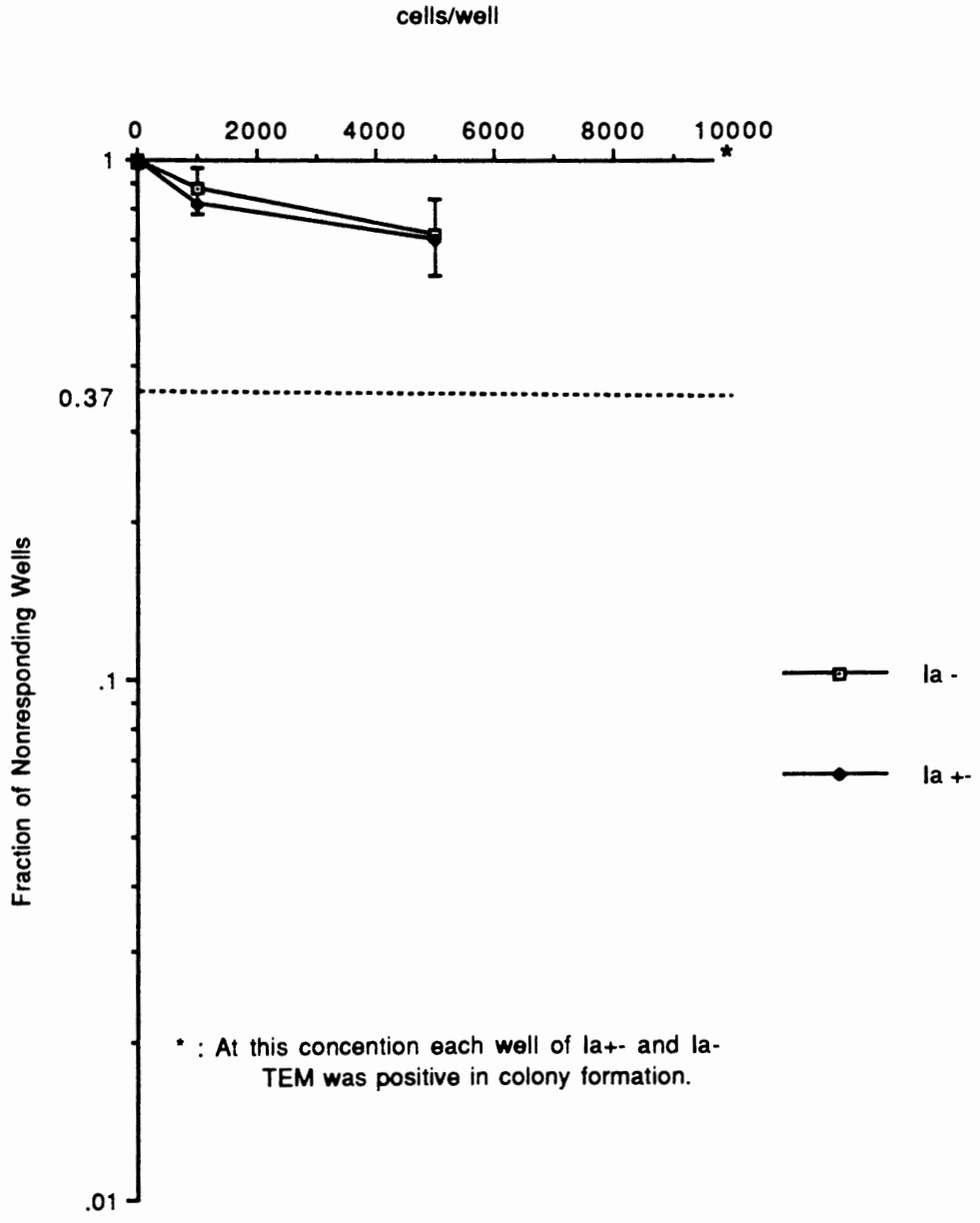

Figure 7. Limiting dilution analysis of the proliferation of Ia+- and Ia- TEM in the presence of GM-CSF

The Proliferation of Ia$^-$ and Unseparated TEM in the Presence of GM-CSF

GM-CSF was applied as the growth factor in place of M-CSF in the following experiment for the sake of comparison. Ia$^-$ and unseparated TEM were diluted to concentrations of 1000 cells/well, 5000 cells/well and 9000 cells/well. GM-CSF was added to the cultures at a concentration of 100 u/ml. Similar culture conditions and scoring procedures were performed as employed above. The results of this experiment (Figure 7) showed that for Ia$^-$ and unseparated cell culture below 5000 cells/well, the colony frequency was low. However, at the concentration of 10,000 cells/well, all wells contained FcR$^+$ colonies (These data points were not plotted on figure 7). The results demonstrated that Ia$^-$ and unseparated cells had similar capacities for proliferation as was observed for culture containing M-CSF. In contrast to the data of Ia$^-$ and Ia^{+-} TEM cultured with M-CSF, no straight line or single hit kinetics were obtained from this experiment.

The Proliferation of Ia$^-$ and Ia^{+-} Populations of TAM in the Presence of M-CSF

The growth of TAM (tumor associated macrophages) was analyzed for the sake of comparison with that of TEM. TAM represent a unique population of macrophages because they are influenced by an environment containing transformed cells. In addition, the total number of TAM within growing tumors often increases rapidly to numbers which often exceed that of the number

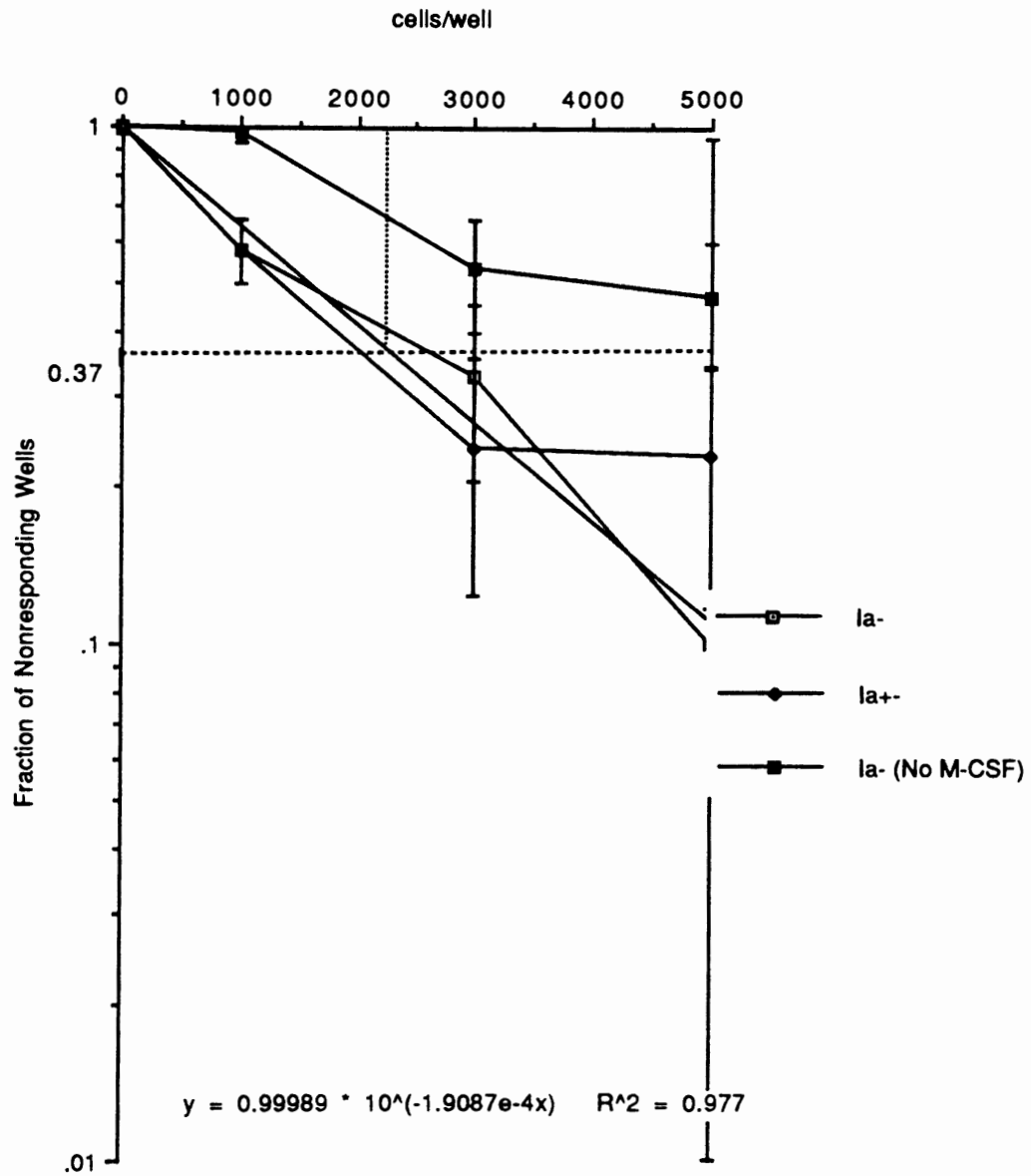

Figure 8. Limiting dilution analysis of the proliferation of Ia+- and Ia- TAM in the presence of M-CSF and Ia- TAM in the absence of M-CSF

of macrophages within an entire normal mouse. Therefore TAM may possess unique growth properties. In order to examine the phenotype of colony forming TAM, these cells were isolated from 1X-11-6 tumor bearing mice and separated from tumor cells by adherence. Ia^- TAM were prepared by depletion of Ia^+ cells by anti-Ia^k antibody and complement. Ia^- and control cells were then cultured at concentrations of 1000 cells/well, 3000 cells/well and 5000 cells/well. M-CSF were added to the cultures at a concentration of 1000 u/ml. Another group, consisting of Ia^- TAM was cultured in the absence of M-CSF, at the same three concentrations as described for M-CSF containing cultures. After 10 days incubation, Data were obtained and plotted after 10 days incubation (Figure 8). Ia^- and control cell cultures both exhibited positive colony formation. Ia^- TAM cultured without M-CSF exhibited a lower number of colonies. By linear regression analysis the data derived from Ia^- cell cultures showed a straight line with a coefficient of 0.977 at the level of 95% confidence with 2 degrees of freedom. This line also fitted single hit kinetics and yielded a frequency of 1/2200 for the proliferation of Ia^- TAM. No straight line was observed from the data of control cells. The results showed that Ia^- and whole TAM cell populations were both capable of proliferation in the presence of M-CSF similar to what was observed for TEM.

The Proliferation of MAC-1^- and Unseparated TEM in the Presence of M-CSF

MAC-1 is a specific surface marker found on macrophages and granulocytes. The proliferation of MAC-1^- and unseparated TEM

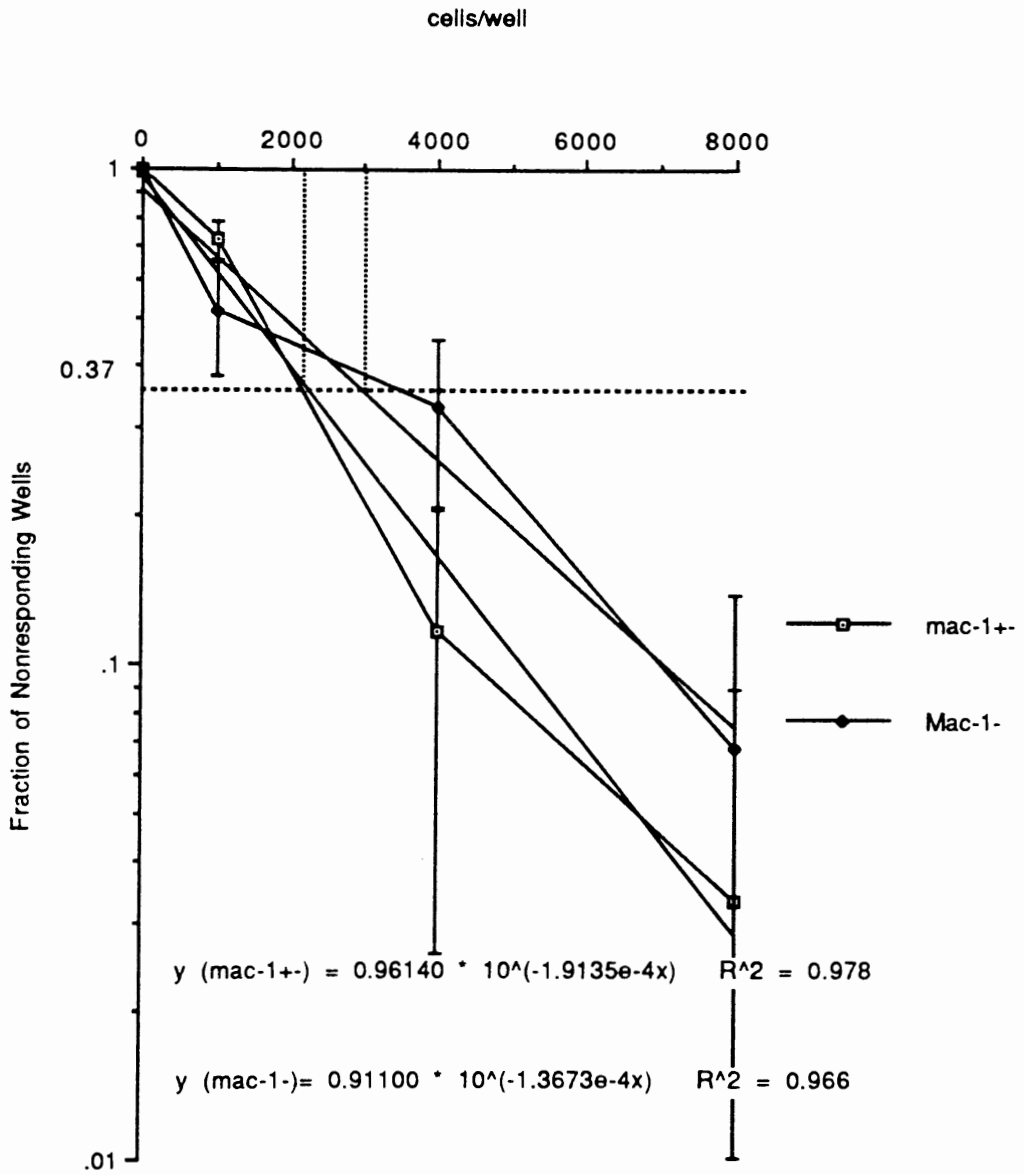

Figure 9. Limiting dilution analysis of the proliferation of MAC-1+- and MAC-1- TEM in the presence of M-CSF

were studied to assess whether MAC-1 expression might correlate positively or negatively with colony forming capacity among TEM. MAC-1$^-$ cells were obtained by depletion of MAC-1$^+$ cells from the whole population with anti-MAC-1 antibody M1/70 and complement (incubated with complement). MAC-1$^-$ and control cells were cultured at concentrations of 1000 cells/well, 4000 cells/well and 8000 cells/well in the presence of 1000 u/ml M-CSF. The results of this study showed that both MAC-1$^-$ and control cells yielded positive responses, which are plotted on Figure 9. Data obtained from MAC-1$^-$ and control cultures both fitted the linearity requirement for straight lines with a coefficient of 0.978 for the former and 0.966 for the later. In both cases, these coefficients fit linearity. The line plotted from the data of control cultures passed through the origin, yielding a proliferating frequency of 1/2100. The line generated from the data obtained from MAC-1$^-$ cultures also fitted single hit kinetics and yielded a frequency of 1/3000. The results indicated both MAC-1$^-$ and control TEM are capable of proliferation with MAC-1$^-$ cells having lower proliferating frequency.

The Proliferation of MAC-1$^-$ and Control TEM in the Presence of GM-CSF.

In order to assess the MAC-1 phenotype of colony forming cells which respond to GM-CSF, MAC-1$^-$ and control TEM were cultured at concentrations of 1000 cells/well, 5000 cells/well and 9000 cells/well. Culture conditions and scoring procedures were performed as above. Data were collected and plotted in Figure 10. As shown, both MAC-1$^-$ and control cell cultures yielded positive responses. At

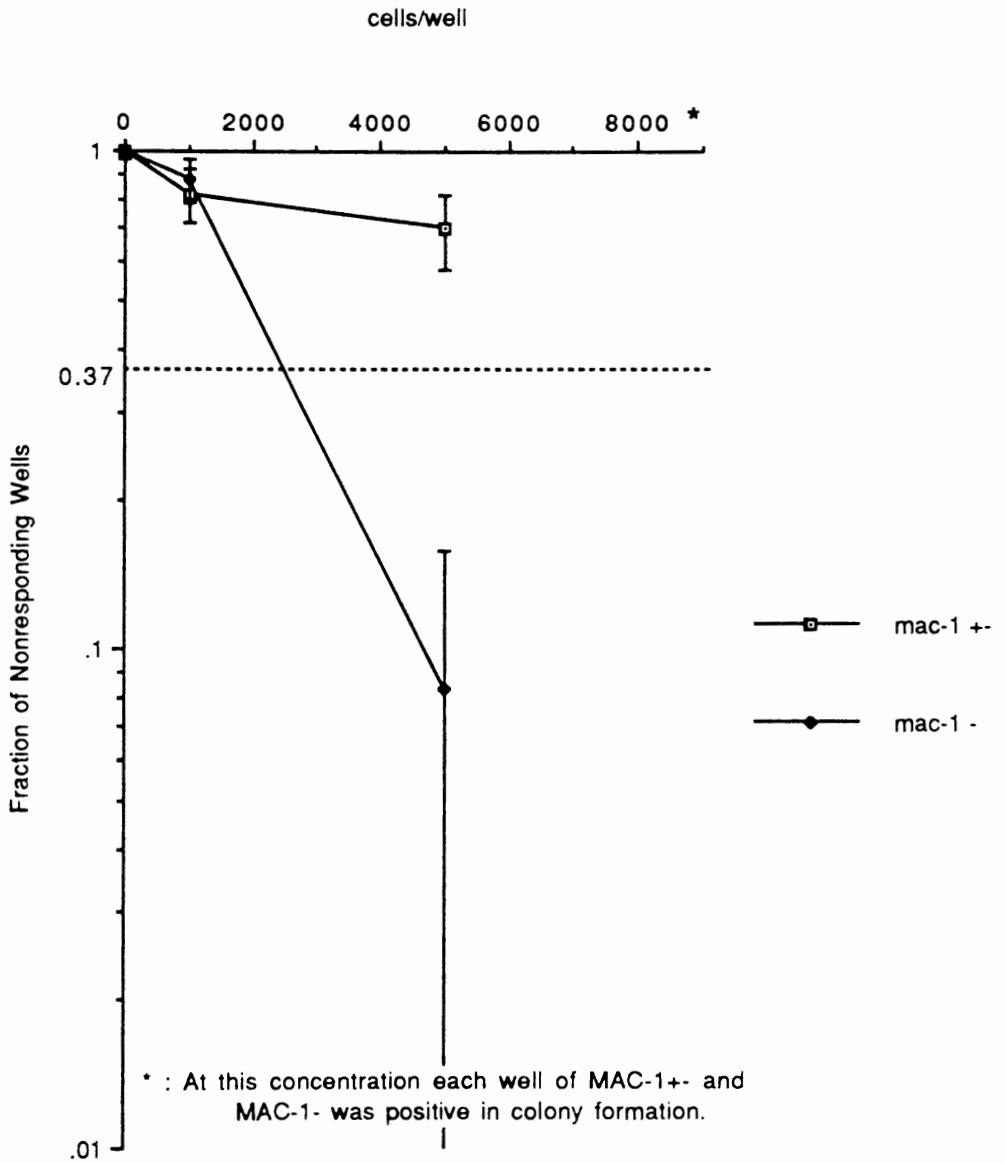

Figure 10. Limiting dilution analysis of the proliferation of MAC-1+- and MAC-1- TEM in the presence of GM-CSF

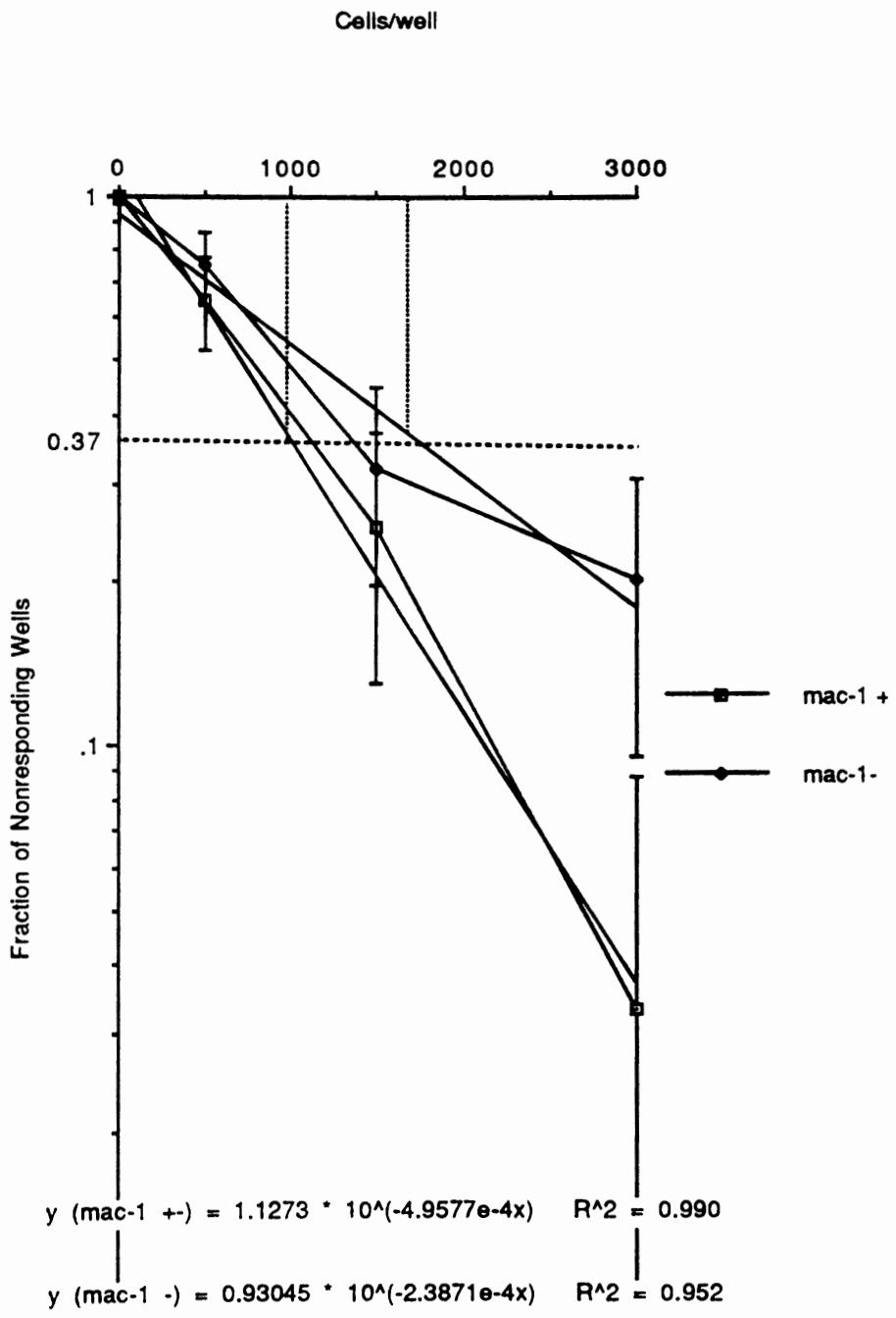

Figure 11. Limiting dilution of the proliferation of MAC-1+- ans MAC-1- TAM presence of M-CSF

the concentration of 9000 cells/well, all wells of both groups contained FcR+ colonies (these data points were not plotted on figure 10). In contrast to the data obtained from MAC-1- and control TEM cultures with M-CSF, no straight line was observed for either set of data in this experiment.

The Proliferation of MAC-1- and Control TAM in the Presence of M-CSF

MAC-1- TAM and control cells were studied for proliferation in the presence of M-CSF. TAM were isolated from 1X-11-6 tumor bearing mice by enzymatic separation and adherence. MAC-1- cells were obtained by depleting MAC-1+ cells from the whole population with M1/70 supernatant (containing anti-MAC-1 antibody) and complement. MAC-1- and control cells were cultured at concentrations of 500 cells/well, 1500 cells/well, and 3000 cells/well with 1000 u/ml M-CSF. The results of this experiment were plotted in figure 11. As shown, both groups yielded high frequencies of colony containing wells. Two straight lines were obtained due to the fact that the coefficient was 0.990 from the data from control cell cultures and 0.952 from the data generated from MAC-1- cell cultures. The approximate frequency was 1/1700 for MAC-1- cells and 1/1000 for control cells. The results suggested that MAC-1- and MAC+/- cell may both be capable of proliferation unless responder cells express low levels of MAC-1.

The Proliferation of Small, Large and Mixed TEM in the Presence of M-CSF

Small and large macrophages may be functionally different. Whether the size of macrophages is related to growth is uncertain. Small, large and mixed populations (with both small and large macrophages) were studied for their proliferation. Separation of small and large macrophages was accomplished by velocity sedimentation with fetal calf serum as the gradient material. The detailed procedure was described in Chapter II. Fractions containing small and large macrophages were pooled after velocity sedimentation separation. A mixed population was obtained by combining equal numbers of small and large macrophages. Small, large and mixed cells were diluted and cultured at concentrations of 1000 cells/well, 3000 cells/well and 8000 cells/well. After the end of the 10 days culture period, opsonized SRBCs were added to enable scoring of FcR^+ colonies. The results were collected and plotted in Figure 12. As shown, all three groups of cells yielded large frequencies of colony containing wells. By linear regression analysis, all data fitted straight lines with a coefficient of 0.988 for data of large cells, 0.978 for small cells and 0.984 for mixed cells. The frequencies of cell proliferation were 1/5100 for large cells, 1/7100 for small cells and 1/3800 for mixed cells. The mixed cells had the highest response and small cells had the lowest response. However, no statistically significant differences were observed between large and small cells. The results indicated that both large and small TEM (or intermediate TEM) were capable of proliferation.

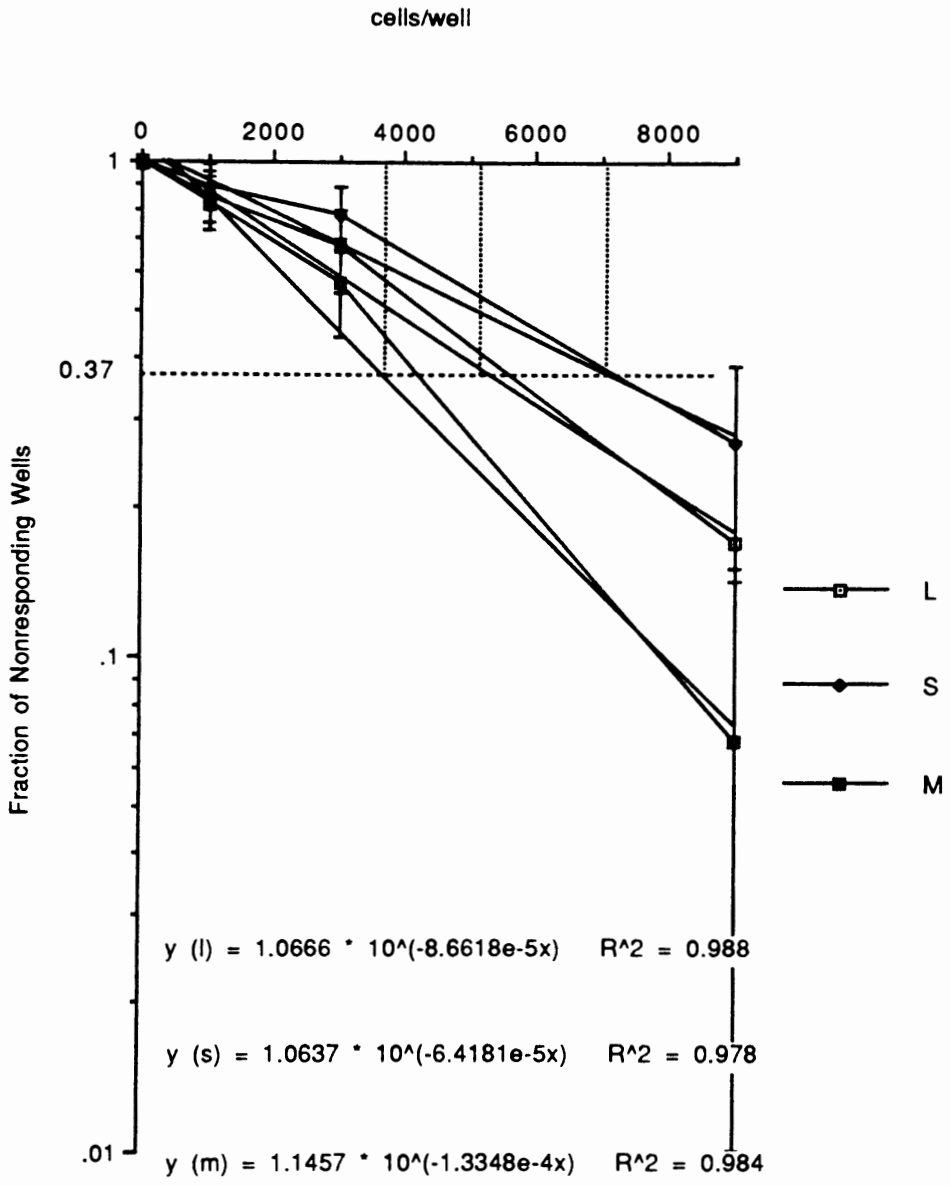

Figure 12. Limiting dilution analysis of the proliferation of large, small and medium sized TEM in the presence of M-CSF

and complement. Monoclonal antibodies and complement were added to wells which contained large, small and mixed cells at the beginning of the culture period at a concentration of 1000 cells/well. One hundred microliters of anti-Ia^k antibody (hybridoma supernatant) was added to 24 wells of each population and 100 ul of anti-MAC-1 antibody (M1/70, hybridoma supernatant) was added to another 24 wells of each. After incubation at room temperature for 30 minutes, 100 ul of 1:10 diluted rabbit complement was added to each well and incubated for another 30 minutes at 37^0 C. Subsequently, 100 ul of trypan blue was added to each well for 5 minutes on ice. Dead and viable colonies were examined under a phase contrast microscope. The results showed that the average proportion of dead colonies and viable colonies were 7.3:1 in the wells treated with anti-MAC-1 antibody M1/70, and 1.6:1 in the wells treated with anti-Ia^k antibody. This indicated that the majority of cells with FcR^+ colonies expressed MAC-1 and Ia. The proportion was higher for MAC-1 than for Ia. This was expected since MAC-1 is believed to be present on the vast majority of macrophages while Ia expression varies among macrophage populations.

Summary

Together, the results of these studies indicate that TEM, TAM and RPM are capable of proliferation. Some subsets appear to be highly capable of proliferation in vitro while others do not (These results are summarized in Table 2). The following populations appeared to be capable of proliferation: phagocytic TEM and RPM, FcR^- TEM, Ia^+/Ia^- and $MAC\text{-}1^+/MAC\text{-}1^-$ TEM and TAM, and large & small

be capable of proliferation: phagocytic TEM and RPM, FcR^- TEM, Ia^+/Ia^- and MAC-1^+/MAC-1^- TEM and TAM, and large & small subsets of TEM. Non-phagocytic TEM, RPM and TAM, and FcR^+ TEM do not proliferate to a significant extant. From the data above, the phenotype of TEM capable of proliferation is: Phagocytic/FcR^-/MAC-$1^{+/-}/Ia^{+/-}$. The optimal proliferation of macrophages was observed only in the presence of M-CSF or GM-CSF, which is in an agreement with published data that suggest that growth factors such as M-CSF and GM-CSF are required for macrophage growth in culture (1,2,13,14,20,32,41).

TABLE II

SUMMARY TABLE OF THE PROLIFERATION
OF SUBSETS OF TEM, TAM AND RPM

	Subpopulations	Response	Frequency	CSFs
TEM	Phagocytic+/-	+	1/2300	M-CSF
	Phagocytic-	-		M-CSF
	FcR+	-		M-CSF
	FcR-	+	(1/3300)	M-CSF
	Ia+/-	+	1/2200	M-CSF
	Ia-	+	(1/2600)	M-CSF
	MAC-1+/-	+	1/2100	M-CSF
	MAC-1-	+	1/3000	M-CSF
	Large	+	1/5100	M-CSF
	Small	+	1/7100	M-CSF
	Large & small	+	1/3800	M-CSF
	FcR+	-		GM-CSF
	FcR-	+	1/2200	GM-CSF
	Ia+/-	+		GM-CSF
	Ia-	+		GM-CSF
	MAC-1+/-	+		GM-CSF
	MAC-1 -	+		GM-CSF
TAM	Ia+/-	+		M-CSF
	Ia-	+	1/2200	M-CSF
	MAC-1+/-	+	(1/1000)	M-CSF
	MAC-1 -	+	(1/1700)	M-CSF
RPM	Phagocytic+/-	+	1/3800	M-CSF
	Phagocytic-	-		M-CSF

(): The frequencies in these cases were estimated since the data did not fit a single hit kinetics.

CHAPTER IV

DISCUSSION

The goal of this study was to assess whether specific subsets of macrophages proliferate, since it is known that macrophages are capable of proliferation (8,15,37,39). Subsets of macrophages based on differences in phagocytic function, cell surfaces markers (MAC-1, Ia and FcR) and cell size were analyzed for proliferating capacities in order to determine the phenotypes of macrophages capable of proliferation.

Phagocytic cells and non-phagocytic cells differed with regard to proliferation. Cultures of mixed cells showed much higher levels of proliferation than did non-phagocytic cells among TEM and RPM cell populations. Non-phagocytic cells of TAM showed very low levels of proliferation also (data were not shown). These results suggest that non-phagocytic cells have less capacity for proliferation. Another possibility might be that phagocytic cells act as helper cells, which, for example, produce cytokines necessary for non-phagocytic cell proliferation. Such a possibility could explain the lower level of proliferation observed among TEM and RPM depleted of phagocytic cells. This possibility is unlikely, however, since these cultures contained M-CSF or GM-CSF at concentrations previously demonstrated as optimal for growth of whole population (14). It is possible that other cytokines may be involved in growth of certain

subpopulations. Comparing the frequencies, 1/2300 TEM produced colonies and 1/3800 for RPM. These results indicate that TEM exhibited a higher potential for proliferation than RPM in our hands.

FcR^+ and FcR^- TEM exhibited different proliferative capacities with FcR^- cells yielding a much higher response. These results indicate FcR^- cells play a major role in TEM proliferation. However, other possibilities might exist. First, FcR^- cells might be contaminated with FcR^+ cells with low expression of FcR which were not removed from FcR^- cells. The proliferation might be due to FcR^+ cells in the presence of help from FcR^- cells. Since growth factor was present in these cultures can give cells optimal growth (14), the above explanation is considered unlikely. Second, FcR^+ cells might undergo a change in their growth capacity following isolation procedures in which FcR^+ cells sustained hyperosmotic pressure and IgG (anti-SRBC) bound by cell surface FcR. However, FcR^+ TAM isolated by the same procedures were observed to proliferate at similar frequencies as compared to TAM isolated by adherence (14). In a separate study, no functional changes were detected after this type of separation (6). Related results (Dr. Burnham, unpublished data) have shown that FcR^- RPM were capable of proliferation and that FcR^+ RPM had less growth capacity, which is consistent with this study. In contrast to TEM and RPM, FcR^+ TAM exhibit a higher level of proliferation. FcR are acquired during differentiation of cells of the monocyte/macrophage lineage (5). Therefore, TEM capable of growth probably represent less mature cells than those among TAM capable of proliferation. This is consistent with the fact that TEM represent cells which are recently derived from the circulation (39). The

proliferating frequency observed among FcR$^-$ cultures is 1/2200 in the presence of GM-CSF and 1/3300 in the presence of M-CSF. In this case, GM-CSF may have induced more responding cells than M-CSF. An alternative possibility is that in the presence of M-CSF, some cells are growth inhibited.

Ia positive and negative TEM and TAM appear to play similar roles in proliferation as evidenced by the results which show that mixed cells and Ia$^-$ cells from TEM or TAM exhibited similar levels of proliferation. However, alternative explanations exist. Ia$^-$ cells might be contaminated with low expression Ia$^+$ cells which can not be completely lysed by antibodies and complement such that these contaminating Ia$^+$ cells proliferate. In this case, if the percentage of Ia$^+$ cells in the whole population is not extremely low, the proliferating frequency of mixed cell cultures should have been significantly higher than Ia$^-$ cultures above, which is not consistent with the results of this study.

Both MAC-1$^+$ and MAC-1$^-$ cells proliferated according to the data derived from TEM and TAM cultures. The results of the studies showed significant colony formation among both mixed and MAC-1$^-$ cell cultures but with slightly higher frequencies in the former. Among TEM, MAC-1 expression is over 80% (27) so that most of cells in the mixed population are MAC-1$^+$. It is possible that MAC-1$^+$ cells have higher capacity for proliferation than MAC-1$^-$ cells. This would explain why mixed cell cultures showed slightly higher proliferating frequencies than did MAC-1$^-$ cultures. However, both MAC-1$^+$ cells and MAC-1$^-$ are capable of proliferation.

There was no statistical difference between large and small TEM in terms of proliferating frequencies, but a slightly higher frequency was observed among cultures of large TEM. The relatively lower frequency of proliferating TEM observed in this experiment may be due to the separation procedures, which might have affected macrophage metabolism and growth. Combining large and small cells led to an increase in proliferating frequency as compared to cultures of large or small macrophages. It is possible that interaction between small and large cells leads to optimal proliferation.

From all the results of this study and other data, TEM capable of proliferation are phagocytic, FcR^-, MAC-$1^{+/-}$, $Ia^{+/-}$, and small and large; for TAM these cells are FcR^+, MAC-$1^{+/-}$ and $Ia^{+/-}$; and for RPM they are phagocytic, FcR^-, and $Ia^{+/-}$. In conclusion, the phenotype of TEM capable of proliferation is: Phagocytic/FcR^-/MAC-$1^{+/-}$/$Ia^{+/-}$ small/large. These results, in addition to those of previous studies, suggest that the phenotype of TAM capable of growth is: FcR^+/MAC-$1^{+/-}$/$Ia^{+/-}$ and the phenotype of RPM capable of growth is: Phagocytic/FcR^-/Ia^+.

M-CSF and GM-CSF are growth factors capable of enhancing the proliferation of macrophages. In the absence of M-CSF or GM-CSF in cultures, cell responses were definitely low. These results are consistent with other reports (1,2,14,37). Both M-CSF and GM-CSF act on macrophage proliferation with GM-CSF acting on granulocyte proliferation as well. During fetal development, M-CSF appears earlier than GM-CSF (33) and also acts on more mature macrophages (32). Receptors for M-CSF and GM-CSF are present on macrophage surfaces (31,37). However, combining M-CSF and GM-CSF, the

enhancement of macrophage proliferation is not additive (14,40). M-CSF and GM-CSF appeared to be different according to the data observed in this study. In the presence of M-CSF, single straight lines or single hit kinetics were yielded from data, indicating that proliferation is by rare cells types without interaction with other cell types. In contrast, in the presence of GM-CSF, no straight line or single hit kinetics were observed from the data on $Ia^{+/-}$ /Ia^{-} and MAC-$1^{+/-}$/MAC-1^{-} proliferation. These results indicate that cellular interaction occurred between different proliferating cell types. It is possible that GM-CSF induced more than one type of macrophages which proliferated in response to GM-CSF so that multi-hit kinetics was observed but not single-hit kinetics. At higher cell input, cultures might contain more cell types than that at lower cell input which led to higher responses at the former. GM-CSF might also stimulate multiple types of cells to proliferate by indirectly acting upon helper cells which produced substances necessary for colony forming cells. Another possibility would be that in the presence of GM-CSF, some inhibitors suppress macrophage proliferation especially at low cell input cultures. This might explain the data derived from the cultures of $Ia^{+/-}$ and Ia^{-} TEM (in Figure 7) which shows that at low cell concentrations macrophage proliferation is not high. However, when cell concentrations increase the responses increase dramatically.

In addition to M-CSF and GM-CSF, other cytokines may also affect macrophage proliferation, such as multi-CSF (IL-3) which enhances proliferation of macrophages and other blood cells (31).

Macrophage growth may be subject to regulation by other factors besides M-CSF and GM-CSF which may induce macrophage subsets that M-CSF and GM-CSF do not. Therefore, higher frequencies and other phenotypes may proliferate in vivo. Also, growth of same macrophages could be inhibited under the experimental conditions employed.

Proliferation of macrophages was determined by colony formation. It is necessary to test whether these colony forming cells are macrophages since other cells such as lymphocytes and granulocytes might be present. The evidences are that first, these colonies all express FcR, by which opsonized SRBCs bound to cell surfaces in the colony scoring procedure. Second, most of these cells express MAC-1 and some express Ia on their cell surfaces. These results were as expected since most macrophages express MAC-1 and a portion of them express Ia. Expression of these markers was examined indirectly by adding anti-MAC-1 or anti-Ia antibodies with complement to cultures after scoring colonies, to lyse MAC-1 and Ia positive cells (see Chapter II). Third, morphological observations also suggest that these cells are macrophages.

The other problem which should be noted in scoring colonies is that some clusters of FcR^+ cells observed in macrophage cultures might be due to clumping rather than proliferation. However, many clusters of macrophages are only observed during the first few days of incubation. Most of these, if not all, disappear as non-responding cells die. Therefore, such false colonies are almost certainly rare.

The frequencies of macrophage proliferation might be underestimated since colony forming macrophages were indicated by

FcR expression. FcR are expressed upon macrophage maturation or differentiation. At the end of culture period, some macrophages expressed FcR, and some may not, which might not have been detected. Thus, the proliferating frequencies might be higher than observed.

Variation in proliferating frequencies of the same subsets of macrophages but from different experiments was observed. It is possible that isolation or separation methods applied in experiments to obtain specific subsets may affect macrophage growth in different ways. For examples, MAC-1$^-$ and Ia$^-$ cells are enriched by complement lysis, non-phagocytic cells are enriched by phagocytic depletion, large and small cells are obtained by velocity sedimentation. These procedures themselves vary so that it is not unexpected that various frequencies resulted from different experiments. However, data from the same experiments and related macrophage subsets in each experiment revealed the proliferating potential of specific macrophage subsets. From all the results, the phenotypes of macrophages capable of proliferation were determined as described previously.

The key problem in this study is to identify subsets of macrophages analyzed. Subsets of macrophages are determined or isolated depending on specific terms needed for analyzing so that subsets such as MAC-1$^{+/-}$/MAC-1$^-$, Ia$^{+/-}$/Ia$^-$, phagocytic/mixed population and FcR$^+$/FcR$^-$ were prepared and analyzed respectively in this study. It should be noted that a single macrophage may express multiple cell surface markers and possess many functions. The ideal way to study macrophage subsets would be to identify all

the surface markers and functions on specific macrophage subsets before analyzing their proliferation. Due to technical limitations so far, this has not yet been accomplished. However, the potential proliferation of related subsets was determined by individual experiments. Furthermore, by analyzing all the results, the phenotypes of macrophages capable of proliferation were determined.

Numerous recent studies indicate that macrophages are able to proliferate (1,2,8,13,14,15,20,26). How macrophage proliferation is regulated, and which phenotypes are responsible for proliferation is still poorly understood. This study focused on the proliferation of macrophage subsets. According to the results, macrophage proliferation is mainly due to specific subsets and not by cells of the whole population. M-CSF or GM-CSF are required to yield optimal proliferation. This study provides further information which is important to reveal insights concerning the nature of macrophage proliferation.

Macrophages play significant roles in tissues especially in immune responses and anti-tumor reactions (21,22). Functionally, macrophages are heterogeneous with some subsets being responsible for host defense (6,21,29). Data derived from the culture of TEM subsets suggest that immature cells (FcR^-) are responsible for colony formation. Other studies (Dr. Burnham, unpublished data) suggest that colony formation by RPM is also due to immature cells. It would be interesting to study whether or how these less mature subsets are induced, whether they represent cells which have immigrated from the blood or other tissues, or whether they are produced by

proliferation in situ. It would also be interesting to determine whether proliferation of certain subsets is related to their functional requirement based on the conclusions obtained from this study.

In summary, this study analyzed proliferation of TEM subsets in the presence of M-CSF or GM-CSF. In comparison, additional studies were performed using TAM and RPM. LDA was applied to assess macrophage growth and determine proliferating frequencies. The results of these studies reveal that only specific subsets of macrophages are capable of optimal proliferation which is dependant upon M-CSF or GM-CSF. The phenotypes of colony forming TEM, TAM and RPM are as follows: Phagocytic/FcR^-/$MAC\text{-}1^{+/-}$/$Ia^{+/-}$/small/large for TEM, FcR^+/$MAC\text{-}1^{+/-}$/$Ia^{+/-}$ for TAM and Phagocytic/FcR^-/Ia^+ for RPM. This is the first report on TEM subsets and phenotypes capable of proliferation and is important in understanding the nature of macrophage proliferation and functions.

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VITA

Yan Chen

Candidate for the Degree of

Master of Science

Thesis: ANALYSIS OF THE PHENOTYPE OF ELICITED MACROPHAGES CAPABLE OF IN VITRO PROLIFERATION

Major Field: Microbiology

Biographical:

Personal Data: Born in Shanghai, China, March 22, 1963.

Education: Graduated from Nanyang Model High School, Shanghai, China, in July, 1981; received Bachelor of Medicine Degree from Shanghai Second Medical University at shanghai in July, 1987; completed requirements for the Master of Science degree at Oklahoma State University in May, 1993.

Professional Experience: Intern, Renji Hospital, Shanghai Second Medical University, August, 1985, to July, 1987; Teaching Assistant, Department of Microbiology and Molecular Genetics, Oklahoma State University, August, 1991, to December, 1992.