DETERMINATION OF THE FREQUENCY AND CYTOTOXIC ACTIVITY OF TNF-SECRETING SUBPOPULATIONS OF TUMOR ASSOCIATED MACROPHAGES

By

RICHARD POTTS

Bachelor of Science

in Arts and Sciences

Oklahoma State University

Stillwater, Oklahoma

1988

Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1991

DETERMINATION OF THE FREQUENCY AND CYTOTOXIC ACTIVITY OF TNF-SECRETING SUBPOPULATIONS OF TUMOR ASSOCIATED MACROPHAGES

Thesis Approved:

Thesis advisor

Dean of the Graduate College

PREFACE

Limiting dilution analysis was utilized as a method to estimate the frequency of murine tumor associated macrophages (TAM) and normal splenic macrophages that secrete tumor necrosis factor (TNF) before and after prestimulation with bacterial lipopolysaccharide (LPS). In addition, an MTT cytotoxicity assay utilizing anti-TNF polyclonal antibody (α-TNF) to block the TNF activity of TAM was used to determine if TNF secretion is solely responsible for the tumoricidal activity of TAM. Finally, combinations of LPS, macrophage-colony stimulating factor (M-CSF), and gamma- interferon (IFN-γ) were assessed for their effects on the enhancement of the tumoricidal activtity of TNF-secreting tumor associated macrophages (TAM). Analysis of the data generated in this study show that only rare subpopulations of TAM and normal splenic macrophages can be induced to become cytotoxic. This cytotoxic activity is due to the secretion of tumor necrosis factor (TNF) and, in both cases, the frequency of these macrophages exhibiting cytotoxic activity is strikingly similar. Further analysis of data from MTT cytotoxicity assays suggest that the LPS, M-CSF and IFN-γ alone or in specific combinations can significantly enhance the cytotoxic activity of TNF-secreting subpopulations of tumor associated macrophages.

I am most grateful to my wife Becky and my daughter Jamaica for their love, praise and support. The successful completion of this thesis was, above all, a direct result of the inspiration provided by their kindness, patience and unselfish sacrifices. I would also like to express my gratitude and sincere

appreciation to my mother-in-law and father-in-law for their love, moral support and financial assistance. Also, I would like to gratefully acknowledge my graduate advisor, Dr. Kim Burnham. I will always owe my professional and academic success to him. Even if I tried for a thousand years, I could never properly express my gratitude for his friendship, patience, support as well as his expert advice and guidance throughout my graduate career. Last, but certainly not least, I want to express my gratitude to Dr. Mary Grula and Dr. Jim Blankmeyer for their friendship, advice and guidance while serving on my graduate committee.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Macrophages found within solid tumors are usually referred to as tumor associated macrophages (TAM). These unique macrophages can comprise as few as two or as many as eighty percent of some solid tumors. In addition, Mantovani and his coworkers have shown that the relative percentage of TAM remains constant throughout most of the tumor life cycle, even during rapid growth stages (9). The most reported and perhaps most important TAM-mediated activities are enhancement of tumor growth and tumoricidal activity (15). However, the precise relationship between TAM and their specific tumor hosts remains a matter of conjecture.

To date, no report can be found in which the frequency of TNF-secreting subsets of TAM has been determined. It would therefore be interesting to determine the frequency of activated and non-activated TAM that secrete TNF and kill tumor cells. Regulation or manipulation of this tumoricidal activity, using biologic factors and recombinant cytokines, may eventually lead to a new approach to treatment of solid tumors as well as a better understanding of the activity and cytotoxic capabilities of normal tissue macrophages.

The statistics-based limiting dilution analysis developed by Leftkovits is one experimental approach that can be employed to determine the frequency of TNF-secreting subpopulations of tumor associated macrophages (12).

Macrophages

Macrophages are part of the mononuclear phagocytic component of the immune system in higher animals. When certain functional genes have been activated by receptor-ligand interactions, macrophages are effectors of cell-mediated immunity and are capable of many specific functions (29). These functions include production of various factors that influence the growth of other cell types including macrophages. In addition, other factors, shown to be important in the processes of intracellular killing and phagocytosis, are also produced by macrophages. These factors are usually associated with cytotoxic functions that require activation by cell to cell contact (8, 10, 27). In this case, macrophages attach themselves to and destroy foreign particles or bacteria through production of toxic oxygen metabolites, acid hydrolases and lysosomal degradation of the phagocytized organism or particle. At the same time, macrophages are very effective antigen presenting cells (APC's) (29). During the phagocytosis process, they can process and present foreign antigen to thymus-derived helper T lymphocytes (T_H cells). This usually results in the induction of a cascade of immune reactions, regulatory activities and the activation of a complete immune response (29). These and other functions are highly dependent upon effector to target ratios and time. Macrophages have a finite state of activation which decays rapidly over a period of hours. Cytotoxic activity is therefore a totally reversible and highly regulated function (29).

Some agents, such as growth factors, are constitutively produced and secreted by macrophages and represent biologically active factors or cytokines that are important in extracellular functions. Other factors in this category are secreted only when macrophages have been activated. Macrophage activating agents include IFNγ, pyrogens, acid hydrolases, prostaglandins and comple-

ment components (10). In addition, macrophages are capable of repairing tissue damage through the production of factors which stimulate growth and angiogenesis (29). The production of interleukin-1 (IL-1) and other factors by macrophages can lead to induction of fever and inflammatory responses (29). Macrophages also have the ability to secrete factors which play important regulatory roles. For instance, they can produce factors such as lymphocyte activating factor (LAF) which can either suppress or stimulate lymphocyte-mediated immune responses (10). They can also produce macrophage-colony stimulating factor (M-CSF), interleukin-2 (IL-2) and platelet-derived growth factor (PDGF). All these factors can stimulate the growth and proliferation of a variety of cells such as fibroblasts and other macrophages (10, 25). However, some macrophage functions can have negative effects on the host. For instance, macrophages can cause tissue damage at the site of a wound or bacterial invasion. This is primarily attributable to their production of certain enzymes and products of oxidative metabolism (29). Of course, the functional activities of macrophages depend upon time, their state of activation, their functional roles, effector to target cell ratios and, most importantly, their production and release of biologically important factors and cytokines (29).

Macrophage Morphology and Ontogeny

Macrophages are ubiquitous throughout the tissues and organs of higher organisms (29). Some of the more interesting morphological characteristics that are common to most macrophages include their nuclear and cytoplasmic characteristics. Macrophages have a kidney-shaped nucleus and a large amount of cytoplasm that contains many lysosomes which are primarily responsible for their phagocytic activity. In addition, macrophages may express a variety of cell surface markers. This marker expression can be

constitutive or in response to cellular or microenvironmental factors. Some of these markers include adherence (MAC-1), antibody (Fc) and interleukin-2 (IL-2) receptors , class I and class II major histocompatibility complex molecules (MHC), and lymphocyte function associated antigen-3 (LFA-3) (29).

Two interesting functional characteristics which macrophages share are their ability to rapidly adhere to various types of glass or plastic surfaces and their ability to form rosettes with opsonized sheep erythrocytes through Fc recptor binding (31). These and other functional characteristics (such as phagocytosis) are widely used as a means of isolation of macrophages from a mixed population and they can also be used as a means of macrophage identification (31).

Normal tissue macrophages are fully differentiated effector cells that are derived from short-lived circulating monocyte precursors. The ultimate source of macrophages is the bone marrow. Pluripotent stem cells are born in the marrow and follow a maturation pathway that leads to the development of monoblasts. These, in turn, undergo cell division to produce progeny that become promonocytes. Promonocytes quickly produce monocyte progeny that enter the blood stream. After circulating in the blood stream for several days the undifferentiated monocytes can enter specific tissues and, after differentiation and maturation, become resident tissue macrophages that are capable of a somewhat longer life span (about five weeks) as well as tissue-specific biological functions (32).

It is not clear whether macrophage subpopulations are the product of one specific precursor or the product of different precursors. It is clear that, among their most important functions, macrophages are capable of promoting the growth and proliferation of many cell types through the production of colony stimulating factors. Macrophages are also capable of direct bacterial

phagocytosis as well as the induction of cytostasis and cytolysis of tumor cells through production and secretion of tumor necrosis factor (15).

Macrophage-Mediated Cytostasis and Cytotoxicity

Activated macrophages are capable killing highly antigenic tumor cells when direct cell to cell contact between effector and the target cell occurs (8, 27). This is due to the cytostatic and cytotoxic activities of macrophages. Some of the mechanisms involved include the transfer of lysosomes to the tumor target, production of neutral proteases which appears to induce cytostasis of tumor targets and toxic oxygen metabolites such as superoxide anions and hydrogen peroxide which can be directly cytotoxic or mutagenic to tumor target cells (27). One of the most important cytokines produced by activated macrophages is tumor necrosis factor (TNF) (27). In fact, some macrophages, that have been infected with intracellular microorganisms, are capable of tumoricidal activity (27). This activity is primarily due to activation of the macrophage by lipopolysac-charide (LPS) that may be found on the cell surface of the infecting organism or introduced in reagent form into experimental systems (27). Macrophage activation seems to induce effector to target binding by up-regulating macrophage binding receptor synthesis (27). This, in turn, can induce the production and secretion of TNF by the macrophage. Consequently, some cytotoxic macrophages can then mediate the lysis of sensitive tumor cells by direct cell to cell contact with macrophage membrane-associated TNF (16). In contrast, secreted or extracellular TNF can also promote lysis of tumor cells within twenty-four hours in a manner which is independent of cellular contact (27).

Tumor Necrosis Factor

Lymphocytes, macrophages and other cells of the immune system are capable of producing a variety of biologically-active cytokines. Cytokines, of course, are cellular messengers that can have a variety of effects including the activation or suppression of target cells. These include colony stimulating factors, lymphotoxin (LT), gamma interferon (IFN-γ), and tumor necrosis factor (TNF) (5). The terms cachectin and tumor necrosis factor are often used interchangeably to designate a homologous molecule of about seventeen kilodaltons (kd). TNF-α (cachectin) is a polypeptide hormone produced by macrophages (5). More recently it has been noted by Aggarwal that TNF-α and TNF-β (a term often used to denote lymphotoxin) compete for the same cellular receptor (3). This shared characteristic suggests that lymphotoxin and TNF-α may share a common heritage within cellular effector pathways (3).

TNF-β (LT) and TNF-α (cachectin) have many closely related biological properties in common (5). They can play a substantial role in the induction of fever, diarrhea and hypotension which are the same symptoms observed during the course of endotoxin-induced shock (5). TNF-α can alter host metabolism by suppressing the lipoprotein lipase activtity of adipocytes. TNF-α is also capable of inducing cachexia which is the general wasting of a host that has been afflicted with a chronic disease. The term cachectin is perhaps more appropriately applied to the overall activity of these biologically important hormones. Finally, cachectin (TNF-α) can lyse TNF-sensitive tumor cells and the term tumor necrosis factor (TNF) is perhaps more correctly applied in this case (5). Tumor necrosis factor (TNF) may therefore provide the key to understanding the complex nature of solid tumor systems and may also provide a powerful weapon in cancer therapy (3, 5).

Regulation of TNF Production

Prostaglandin E2 (PGE2) has been shown to be an important autocrine regulator of TNF production. Kristein et al. have shown that activated macrophages produced high levels of TNF and low levels of PGE2. However, when this activation state declined, so did TNF production and, at the same time, the levels of PGE2 correspondingly increased to normal ranges (11).

Mechanisms of TNF Activity

Although the exact mechanisms of TNF-induced cytolysis remains a matter of conjecture, some interesting theories have recently emerged. The first step in killing is most likely the binding of TNF to cell receptors (3). However, it should be noted, that many cells within heterogeneous populations express TNF receptors but, are not sensitive to the cytolytic effects of TNF (30). One major result of TNF-induced cytotoxicity is the indirect degradation of DNA that was observed in many types of tumor target cells (2).

TNF as a Theraputic Agent

Regardless of the mechanism of TNF-induced cytoxicity the use of tumor necrosis factor (TNF) as a chemotheraputic agent in cancer therapy may be promising. Balkwill has shown an 85% survival rate of nude (immuno-compromised) mice that had been given an intraperitoneal tumor injection then treated with human recombinant-TNF and gamma interferon (IFNγ) (4). IFNγ has been shown to induce the production of TNF receptors by target cells (3). This may explain the synergistic effect of combined IFN-γ and TNF treatment (3). However, Balkwill also noted that either treatment alone was ineffective (4). Besides the most obvious effects of tumor necrosis factor

(TNF) on tumor growth, TNF may actually select for metastatic behavior by depleting only TNF-sensitive tumor cells. Such a selection may result in survival of tumor cell subsets that are resistent to destruction by TNF (9). Since TNF can be produced by normal tissue and tumor associated macrophages much more needs to be learned about this facinating cytokine.

Tumor Associated Macrophages

Unlike normal tissue macrophages, tumor associated macrophages (TAM) reside in transformed (tumor) tissue and can represent a substantial portion (up to 80%) of a tumor mass. The ratio of TAM to tumor cells remain constant during tumor growth and, like their normal macrophage counterparts, appear to share many of the same biological functions (15). Some of the functions they share include the production of biologically important factors, expression of cell surface molecules (such as MHC II and Fc receptors), antigen presenting capabilities, secretion of TNF and cytotoxic activity (9, 14, 15).

TAM Heterogeneity

Tumor associated macrophages (TAM) are characteristically quite heterogenous within their tumor environment. However, two phenotypically distinct subpopulations have been described (16). These TAM differ in size and are separated on the basis of velocity sedimentation. One subpopulation, consists of relatively large, peroxidase-negative TAM that express high levels of MHC II molecules and high avidity Fc receptors (FcR). The other subpopulation consists of smaller TAM that are peroxidase-positive, have lower avidity FcR and may represent less mature TAM (15). In fact, Moore and McBride have suggested that the smaller TAM may mature into the larger ones and, in the process, lose their peroxidase activity (16).

Proliferation of TAM

Even during rapid tumor growth, TAM can constitute a substantial portion of the tumor mass (9). It is unclear whether TAM are the progeny of circulating monocytes (which may have entered the tumor environment from the bloodstream) or a product of intratumoral proliferation or both (14). However, TAM have recently been shown to express receptors for M-CSF (19). More recently, TAM, from solid murine tumors, demonstrated an increased capacity to proliferate in vitro when treated with recombinant M-CSF (20). It is interesting to note several investigators have demonstrated the induction of various differentiation activities of mature macrophages after activation with M-CSF (19, 22).

TAM Functional Activities

TAM have many interesting functional characteristics that may help provide insight into the functions and activities of all macrophages. TAM have been shown to be capable of secreting cellular growth enhancement molecules and cytokines (14, 15). These may include procoagulants (PCA's) which can lead to the increased formation of fibrin and may subsequently aid tumor cell motility, the formation of intratumoral vascular networks (angiogenesis) and the inducement or suppression of effector cell entry into the tumor mass (14). These and other TAM functions such as production of platelet-derived growth factor (PDGF) and interleukin-1 (IL-1) parallel the growth enhancement activities of normal tissue macrophages (7, 14). However, TAM reside in tumor tissue and, in some cases, may therefore help promote the growth and proliferation of potentially lethal tumor masses (14).

Much like their normal tissue counterparts, TAM can produce agents which may aid in tumor invasion and metastasis. These include protease

enzymes like elastase and collagenase as well as toxic oxygen metabolites (14). Some of these metabolites like superoxide and hydrogen peroxide (H_2O_2) can be mutagenic (1). These metabolites can aid in the tumoricidal effects of TAM in vivo and, at the same time, they can have the effect of minimizing host defenses by increasing tumor heterogeneity through mutagenesis (14).

Perhaps the most interesting, yet intriguing, function of some tumor associated macrophage subsets is their ability to become tumoricidal when activated (15). TAM, found at secondary sites of tumor invasion, can produce and secrete TNF and are thus quite often tumoricidal. In addition, they are usually present in higher ratios at these sites and seem to have the ability to partially block tumor metastasis (14).

Limiting Dilution Analysis

Until recently, the elucidation of cellular activity was limited in its scope by the need for an assay with the sensitivity to accurately detect very small amounts of activity and, at the same time, distinguish between functionally distinct subpopulations. Limiting dilution analysis (LDA) can be employed to elucidate kinetic events. Consequently, when single-hit kinetics are observed, LDA can be used to determine the frequency of TNF-secreting TAM, within a heterogeneous TAM population, which exhibit cytotoxic or tumoricidal activity (12). Limiting dilution analysis (LDA) can also be used to determine whether tumoricidal TAM require cell to cell interactions with other phenotypes before expressing their activity. This requirement would indicate multi-hit relationship and would be reflected, in the LDA, as a non-linear event (13). The LDA is a statistical analysis based on binomial (dependent) and Poisson (discrete) distributions. This unique analysis permits the investigator to extrapolate the specific activity of a small experimental

population of tumor associated macrophages to an entire population of tumor associated macrophages when there is a linear or single-hit relationship (13). Because sufficient data, concerning the frequency of TAM that secrete TNF is currently unavailable, a limiting dilution analysis (LDA) was employed in this study because it allows for the estimation of the frequency of TNF-secreting TAM within an experimental population (12).

The major goal of this study is to use a limiting dilution analysis to determine the frequency of experimental murine splenic macrophages and MC-4 and 1X-11-6 TAM that secrete TNF in response to LPS. In addition, anti-TNF antisera (anti-TNF-α) will be used to block the cytotoxic activity of these TAM in an effort to show direct evidence that TNF is secreted by TAM and is responsible for their cytotoxic activity. Finally, LPS, M-CSF and IFN-γ will be assessed by an MTT assay as well as dot plot and mean absorbance comparison analysis for their effects on the cytotoxic activity of 1X-11-6 TAM. The results of this study should provide additional data concerning the cytotoxic activity of TAM and further insight into the functions of normal tissue macrophages.

CHAPTER II

METHODS AND MATERIALS

Experimental Animals

Female, C3H/HeN mice were obtained from Charles River (Wilmington, Massachusettes) and used as experimental animals throughout this study. All mice were about seven weeks old and kept at an animal care facility at Oklahoma State University. Prior to organ and tumor removal, etherization (as opposed to other methods which would allow blood to enter the peritoneal cavity) was used in all experiments as a humane method of euthanasia .

Media, Buffers and Reagents

All cells, used in experiments and in the generation of cytokines, were diluted and cultured in complete RPMI (cRPMI) medium. Powdered RPMI as well as the components to complete the medium and insure its microbial quality were obtained from Sigma Chemical Corporation (St.Louis, MO). Complete RPMI was prepared in the following manner. Powdered RPMI was reconstituted in reverse osmosis/double distilled water with 2 grams of sodium bicarbonate per liter of prepared medium. Sodium pyruvate (1.0 mM), L-glutamine (2 mM), 5% (v/v) fetal bovine serum (heat inactivated for 30 minutes in a water bath at 56^0 C), nonessential amino acids (those that were not used to supply nitrogen) (1.0 mM) were used to complete the RPMI.

In addition, amphotericin B (2.5 mg/ml), penicillin (100 mg/ml), streptomycin (0.1 mg/ml) and gentamycin sulfate (50 mg/ml) were added to inhibit microbial contamination during incubation and storage. After the pH of the cRPMI was adjusted to 7.2, the completed medium was then filter sterilized through 0.45 μm sterile disposable filters (Fisher, Sci. Co., Norcross, Ga) into sterile (autoclaved, 15 psi/15 min) 500 ml glass bottles and stored at 4^0 C prior to use.

Freezing medium was used throughout this study to preserve cell lines. Concentrated cells were aseptically dispensed into 1 ml aliquots of freezing medium and stored in a liquid nitrogen-cooled chamber at -273^0 C. Freezing medium was prepared by adding 9.5 milliliters of calf serum (Sigma, 95%) to 0.5 milliliters of anhydrous dimethyl sulfoxide (DMSO) (Sigma, 5%), filter sterilizing the mixture through 0.22 μm sterile disposable filters (Fisher, Inc.) into sterile (autoclaved) screw-capped test tubes and storing the medium at 4^0 C until needed.

Buffers were primarily used in this study to prepare reagents and wash, dilute or lyse cells. Phosphate buffered saline (PBS, 0.05 M PO_4) was used as a diluting and washing buffer and to prepare reagents. PBS was prepared in one liter quantities by adding monobasic potassium phosphate (Fisher, $KH_2PO_4 \cdot H_2O$, 2.7 g/L), sodium chloride (Baker Chem. Co., Phillipsburg, NJ., NaCL, 4.1 g/L) and anhydrous sodium phosphate (Sigma, Inc. Na_2HPO_4 10.57 g/L) to one liter of double distilled water, adjusting the pH to 7.4 and sterilizing the PBS by autoclaving at 15 psi for 15 minutes.

Lysing buffer (Tris-buffered ammonium chloride) was used to lyse sheep red blood cells (SRBCs) during the process of isolating macrophages (see isolation of tumor associated macrophages, page 19). Lysing buffer was prepared by adding 50 ml of a 2.06% (w/v) sterile H_2O solution of tris base

(Sigma, 20.6 g/L), that had been adjusted to a pH of 7.6, to 450 ml of an 0.83% (w/v) solution of ammonium chloride (Sigma. 8.3 g NH_4 CL/L). Following thorough mixing, the pH of the lysing buffer was adjusted to 7.2 with HCL and, after adjustment of the pH, the lysing buffer was filter sterilized through 0.45 μm sterile disposable filters (Fisher) into sterile (autoclaved, 15 psi./15min) 500 ml bottles.

The enzyme reagents used in this study were used and prepared in the following manner. Collagenase-dispase is a mixture of enzymes and was used as an agent to enzymatically dissolve the connective tissue of tumor fragments and produce single tumor cell suspensions. Collagenase-dispase (0.10% w/v) was prepared by adding 0.10 grams of collagenase (Sigma, St. Louis MO, 0.001 g/ml) and 0.10 ml of dispase (Boehringer Mannheim, GmbH, W. Germany, 0.001 g/ml) to 100 ml of sterile PBS, adjusting the pH to 7.4 and immediately filter sterilizing the resulting mixture through 0.22 μm sterile disposable filters into sterile (autoclaved) 150 ml bottles. The collagenase-dispase was then aseptically aliquoted, in 25 ml quantities, into sterile screw-capped test tubes and preserved, at -20^0 C, prior to use.

Trypsin-EDTA was used in this study to enzymatically remove confluent cell layers during cell culture maintenance. Trypsin-EDTA was prepared by adding 0.25 grams of trypsin (Sigma, 0.25% w/v) and 0.0292 grams of anhydrous ethylene diamino tetraacetic acid (EDTA) (Sigma, 1 mM) to 100 ml of sterile PBS, adjusting the pH to 7.4 and filter sterilizing through sterile 0.22 μm filters into sterile 150 ml reagent bottles and stored at 4^0 C prior to use.

MTT was used as a colorimetric reagent in dose response and cytotoxicity assays in this study. MTT [Sigma, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] is a disubstituted tetrazolium salt that can be biochemically converted to formazan crystals by viable (living) cells. Following

formation, the crystals can be solubilized with isopropanol-HCL (0.04 N). The product (solubilized formazan) can then be quantitatively detected with the aid of an ELISA plate reader at a wavelength of 570 nanometers. MTT was prepared in the following manner. Fifty milligrams of MTT (Sigma, 5 mg/ml) was added to 10 ml of sterile PBS, the mixture filter sterilized through 0.22 μm sterile disposable filters into sterile 20 ml screw-capped test tubes and stored at 4^0 C until needed.

Isopropanol-HCL (0.04 N) was used, as previously discussed, to solubilize formazan crystals and was prepared by aseptically mixing 100 ml of anhydrous isopropanol (2-propanol) (EM Sci., Inc., Cherry Hill, NJ., 100%) with 0.333 ml of concentrated, aquaeous hydrochloric acid (MCB, Cincinnati, OH., 12 N). Following preparation, isopropanol-HCL was stored in a flameproof cabinet until needed.

To determine the frequency of activated macrophages that secrete tumor necrosis factor (TNF) and to evaluate the cytotoxic activity of TAM, several agents were used to stimulate TAM, and other effector cells, to secrete TNF. These agents were prepared in the following manner. Bacterial lipo-polysaccharide (LPS) (Sigma, St. Louis, MO) was aseptically diluted in sterile PBS. LPS was then added to TAM cells at a concentration of 5μg LPS/ml to stimulate TNF secretion. Recombinant mouse gamma-interferon (IFN-γ) (kindly provided by Genentech, Inc., San Francisco, CA., 200,000 units/ml) and recombinant Macrophage-colony stimulating factor (rM-CSF) (kindly provided by Cetus, Emeryville, CA., 5×10^5 units/ml) were also used to stimulate TNF secretion. Both were prepared by diluting a portion of the stock solutions of IFN-γ and M-CSF to appropriate concentrations in sterile PBS substituted with 1% fetal bovine serum. Stock solutions were preserved

by freezer storage at -70^0 C and aliquotes of diluted IFN-γ and M-CSF were immediately used in LDA and other assays.

Whole rabbit anti-mouse TNF-α antisera (anti-TNF-α) (Genzyme, Boston MA., 1 x 10^6 neutralizing units/ml) was used to show that TNF secretion is responsible for the the cytotoxicity activity of TAM. This was accomplished by using anti-TNF-α antisera to neutralize the TNF activity of TAM. Anti-TNF-α antisera was prepared by aseptically diluting a portion of stock solution to the appropriate concentration in sterile RPMI medium and used immediately. Again, the stock solution of anti-TNF-α was preserved by freezer storage.

Cell Culture Maintenance

WEHI-164 tumor cells (American Type Culture Collection, Rockville, MD) are methylcholanthrene-induced mouse fibrosarcomas. WEHI-164 cells were originally established, after subcutaneous injection of BALB/C mice with 3-methylcholanthrene, by M. Rollinghoff and N.L. Warner. WEHI-164 tumor cells and cells from a very TNF-sensitive WEHI-164 tumor cell clone (2/F2), generated in Dr. Kim Burnham's lab by limiting dilution cloning, were used in this study as target cells. During periods of use, cultures of target cells were maintained in 50 ml tissue culture-treated cell culture flasks (Fisher), periodically replenished with cRPMI and incubated at 37^0 C in an atmosphere of 95% air and 5% CO_2. When culture growth approached monolayer confluency (as determined through phase contrast microscopy), the cells were subsequently subcultured within 24 hours in the following manner. The depleted medium was discarded and the culture gently rinsed with 5-10 ml of phosphate buffered saline (PBS) (0.05 M PO_4, pH 7.4). To remove the confluent cell layer, 1 ml of trypsin-EDTA (0.25% w/v trypsin and 1 mM

EDTA in PBS) was pipetted into the flask then the flask was incubated at 37^0 C for 10 minutes. After incubation, 5 ml of cRPMI was added to the flask to stop the enzyme reaction, the flaskwas subsequently tapped to remove additional cell masses and rinsed with 5-10 ml of PBS. Prior to incubation, 30 ml of cRPMI was added to allow new culture growth.

Isolation of Splenic Macrophages

Splenic macrophages were obtained sugically from Normal C3H/HeN mice. Mice were euthanized in an ether jar prior to removing the peritoneal cavity skin. The peritoneal cavity was then opened and the spleens surgically removed. The spleens were transferred to a small (60 x 15 mm) sterile culture dish (Baxter, Inc., Grand Prarie TX.) containing cold (4^0 C) PBS and kept on ice prior to removing splenic macrophages. In the hood, splenic macrophages were squeezed out of the spleen sac with gentle pressure using a sterile scalpel and forceps. Once the cells were obtained the depleted spleen sac and large tissue fragments were discarded. The crude macrophage suspensions were then transferred to a large (10 ml) sterile screw-capped test tube and washed by centrifugation at 650 g for 10 minutes. After centrifugation, the supernatants were discarded and, depending on the experiment, the concentrated splenic macrophages were diluted to the appropriate concentration in cRPMI.

Generation of Crude Tumor Necrosis Factor

Crude natural tumor necrosis factor (TNF) preparations were generated in 20 ml of cRPMI by culturing LPS-treated spleen cells from normal mice in the following manner. To induce the production of TNF by normal splenic macrophages, LPS (5µg/ml) was added to the diluted macrophages. The LPS-

treated suspensions were then dispensed, in 20 ml aliquots, into sterile 15 mm culture plates and incubated 24 hours at 37^0 C. After incubation, the supernatants were harvested and centrifuged at 650 g for 10 minutes to pellet residual cells. The crude TNF suspensions were then dispensed, in 20 ml aliquots, into sterile test tubes and stored at -20^0 C until needed.

Sheep Erythrocytes

Sheep erythrocytes (SRBCs) were obtained from Organon Teknika (Durham, North Carolina) and from the college of Veterinary Medicine at Oklahoma State University. SRBCs were stored at 4^0 C prior to use in experiments. Ten percent solutions of SRBCs were prepared for use in experiments by first washing the SRBCs in the following manner. PBS was added to the SRBCs with gentle mixing. To concentrate the SRBCs, the mixture was centrifuged for 10 minutes at 650 g (1500 RPM). Following centrifugation, the cloudy supernatant was gently removed with a 5 ml pipette and replaced with an equal volume of PBS. The procedure was then repeated three or more times until the supernatant became clear. After washing, the concentrated SRBCs were then diluted to 10% v/v in PBS and used in appropriate experiments.

Generation of Tumor Associated Macrophages

The tumor associated macrophages (TAM) used in this study were derived from two different sources. The first source for TAM was a tumor line designated as MC-4. MC-4 tumors were generated by injecting normal C3H/HeN female mice with methylcholanthrene (MCA) (Sigma). Briefly, 1 mg of MCA was dissolved in 50 µl of olive oil and subcutaneously injected at the ventral surface of each mouse. Subsequent passage of the MC-4 tumor

line into secondary hosts was accomplished by subcutaneous injection of 2-3 small MC-4 fragments into normal C3H/HeN mice. The second source for TAM was a tumor designated as 1X-11-6 and was obtained from Dr. James Beeson at the University of Oklahoma at Tulsa Medical College. Tumor line 1X-11-6 arose spontaneously from an in vitro culture of C3H/HeN placental tissue. Subsequent passage of the 1X-11-6 tumor line was accomplished in the same manner as the the MC-4 line. Tumors were excised from euthanized mice two to four weeks after the tumors were implanted into secondary hosts. Following excision, the tumors were enzymatically dissociated into single cell suspensions. This was accomplished by cutting the tumors into fragments and incubating them in a mechanically stirred mixture of 0.1% w/v collagenase plus 0.1% w/v dispase at 37^0 C for approximately one hour. The dissociated tumor cell suspension was then centrifuged at 650 g for 10 minutes and, following centrifugation, the pellet was then resuspended in 2 mls of cRPMI. The cell concentration was then determined with a hemacytometer and diluted (depending on the method of isolation and experiment) to the appropriate concentration.

Isolation of Tumor Associated Macrophages

To determine the most efficient and practical method for isolating TAM, three methods were used. At first, TAM were isolated by density gradient exclusion over ficoll hypaque gradient reagent. Single cell suspensions from dissociated tumor fragments were diluted to 2 $x10^7$ cells/ml then mixed with an equal volume of 5% Rabbit anti-SRBC-Ig (Sigma) coated SRBCs (opsonized SRBCs) and incubated on a vertical rotator at 37^0 C for 30 minutes. The percentage of rosetted cells (TAM) was then determined with a hemacytometer. Rosetted cells were separated by gently layering the rosetted cell

suspension over 3 mls of ficoll hypaque (Sigma Inc., specific gravity 1.119) and centrifuging for 20 minutes at 1100 g. Following centrifugation, the supernatant was discarded, the harvested pellet resuspended in 2 mls of cRPMI medium and the ficoll hypaque separation, centrifugation and harvesting process was repeated. Once again the percent of rosettes were determined with the aid of a hemacytometer. The suspension was then centrifuged at 650 g for 10 minutes to concentrate the rosetted cells. To release TAM, SRBCs were lysed by adding one milliliter of sterile double distilled water followed immediately by the addition of 5 mls of cRPMI to prevent the lysis of TAM. The TAM were pelleted by centrifuging the cell suspension at 650 g for 10 minutes. After centrifugation TAM pellets were resuspended in 2 mls of cRPMI and counted with the aid of a hemacytometer. After counting, TAM were diluted to appropriate concentrations and used as effector cells in limiting dilution analysis or other assays.

The second method used to isolate TAM was Magnetic bead hybrid rosetting (MBHR). In this procedure, sheep anti-rat IgG coated M-450 magnetic beads (dynabeads) (DYNAL A.S. Corp., Oslo, Norway) were allowed to bind specifcially to opsonized SRBCs to produce dynabead rosettes (DBRs). Dynabead rosettes were immediately added to a TAM containing tumor cell suspension and allowed to bind to Fc receptor positive (FcR^+) TAM. This process which subsequently produced magnetic bead hybrid rosettes (MBHR). MBHR were immunomagnetically isolated from tumor cells suspensions in the strong magnetic field of a DYNAL MPC-1 magnetic partical concentrator (DYNAL, coehesive force: 8250-8750 oersted). After washing the MBHR five times in the magnetic field with cold (4^0 C) PBS, the SRBCs were lysed with lysing buffer. Used Dynabeads were recovered in the magnetic field and the isolated TAM recovered in the MBHR effluent. After recovery, TAM were

washed in PBS, concentrated by centrifugation at 650 g for 10 minutes and resuspended in 5 ml of cRPMI. Following resuspension, the freshly isolated TAM were counted with the aid of a hemacytometer, diluted to appropriate concentrations in cRPMI and used as effector cells in limiting dilution analysis.

The third and final method used to isolate TAM was adherence (6). The adherence method was used to positively select for (desirable) cells such as splenic and tumor associated macrophages and to simultaneously exclude (undesirable) tumor cells or non-adherent cells . Briefly, heterogeneous tumor cell suspensions were aseptically added to 60 x 15 mm tissue culture-treated culture dishes (Baxter) at a concentration of 3×10^6 cells per milliliter of cRPMI medium then incubated for 30 minutes at 37^0 C in an atmosphere of 95% air and 5% CO_2. Incubation for 30 minutes allowed time for macrophages to adhere to the bottom of the culture dish. However, the incubation period was not long enough for most tumor and other undesirable cells to adhere. Consequently, undesirable cells were removed after incubation by gently rinsing the culture dishes 5-6 times with sterile PBS. After washing, adherent cells were recovered by adding 2 ml of fresh, sterile PBS to the washed plates and gently removing the adherent layer with a cell scraper (Baxter). Adherent cell suspensions were then added to sterile 10 ml test tubes and concentrated by centrifugation at 650 g for 15 minutes. Following concentration, adherent cells were resuspended in 1-2 ml of sterile cRPMI and counted on a hemacytometer. Portions of the adherent cells were then tested for purity by methods which included microscopic morphological analysis and rosetting a portion of the adherent cells with opsonized SRBCs to determine the percent rosettes. Adherent cells were then diluted to appropriate concentrations in cRPMI and used immediately in limiting

dilution analysis, dose response and MTT cytotoxicity assays. Adherence proved to be the most practical method of isolating TAM in terms of speed, convenience, costs and accuracy and was therefore used exclusively as a method of choice, for isolating TAM and other effector cells, in the latter stages of this study.

Generation of Highly TNF-Sensitive WEHI-164 Tumor Cell Clones

To determine the frequency of TAM that secrete TNF as well as the cytotoxic activity of TAM, it was necessary to generate extremely TNF-sensitive target clones. Cloning was accomplished by diluting TNF-sensitive WEHI-164 tumor cells to a concentration of 3 cells/ml in cRPMI, distributing the cells at a frequency of one cell for every two wells by aliquoting 100 µl of cell suspension into each well of a sterile 96 well microtiter plate. Following distribution, the plates were incubated at 37^0 C and monitored daily for the appearance of single cell distributions, then allowed to grow to confluency. Confluent cell layers that were derived from single cells were subsequently removed by trypsinization. The resulting clone suspensions were then washed in sterile PBS, resuspended in 2 ml of cRPMI and replated into sterile 24 well microculture plates. The clones were then incubated at 37^0 C and allowed to grow to confluency. Following four to seven days of incubation, the confluent clones were once again removed by trypsinization, washed in PBS, resuspended in cRPMI and reincubated in 30 ml of cRPMI in 50 ml tissue culture flasks. The clones were subsequently incubated at 37^0 C and monitored daily prior to testing for their sensitivity to TNF. Following testing, clones were concentrated, resuspended in 1 ml of freezing medium and cryogenically preserved in a liquid nitrogen chamber.

MTT Assays

In this study, colorimetric (MTT) assays were used as a basis for cytotoxicity and dose response assays as well as limiting dilution analysis. More specifically, MTT assays were used throughout this study to photometrically quantitate absorbance values generated by viable target cells. This data was used to determine the cytotoxic activity of TAM and to measure the extent of killing of 2/F2 target cells (TNF-sensitive WEHI-164 clone) by TAM and other macrophage supernatants. After target cells had been incubated with supernatants, MTT (Sigma, 5 mg/ml) was added to target wells and the target plates reincubated for 6 hours at 37^0 C. Incubation allows time for conversion of the MTT reagent to formazan crystals by viable cells. Following incubation, the formazan product was then solubilized with isopropanol-HCL (0.04 N). Solubilization produces a colored product that can be quantitated, at a wavelength of 570 nm, on an ELISA plate reader. The intensity of the color (absorbance or optical density) of each well is directly proportional to the amount of formazan produced by viable target cells and is therefore inversely proportional to the number of sensitve target cells that had been killed by macrophage super-natants (17). Once the absorbance data, generated in MTT assays, was collected it was collated and analyzed on a dot plot graph to distinguish responding wells from nonresponding wells. The data from dot plot analysis was then used as a basis for limiting dilution analysis as well as cytotoxicity and dose response assays.

Limiting Dilution Analysis

Limiting Dilution Analysis (LDA) is a statistical analysis developed by Ivan Leftkovits (13). Using the data generated from an experimental population,

LDA is often used to make accurate assessments of the cellular functions and activities of an entire population. In this study, LDA was used to estimate the frequency of macrophages that secrete tumor necrosis factor. LDA is based on a formula derived from Binomial and Poisson distributions. Briefly, the formula for the zero term of this distribution is defined by the equation $F_o = e^{-\mu}$ where; F_o is the fraction of nonresponding wells and μ is the mean number of responding cells per well (13). However, it is only possible to apply Poisson distributions if responding cells are truly limiting. If they are not truly limiting, a straight line is not observed and multi-hit kinetics are implied. This type of cooperativity violates the assumptions which govern Poisson distributions. A multi-hit event indicates that the response to be measured is modulated by other factors or other cell types. This is because the probability that two or more rare cell types will be placed in the same well increases exponentially with the cell density (13).

Linearity or single-hit kinetics is attained when the mean number of responding cells is directly proportional to the negative logarithm of the fraction of nonresponding wells. Single-hit events are therefore expressed mathematically as $\mu = -\ln F_o$ and, in this case, a straight line that dissects the origin is observed when cell concentrations are plotted against the log of F_o. It is important to note that if single-hit kinetics (linearity) are observed, μ is equal to 1 when F_o is equal to 0.37. This means that there is an average of one responding cell per well when 37% of the wells are non-responding (13). Consequently, when linearity is observed, the inverse of the cell concentration where 37% of the wells do not respond can be used as an accurate estimation of the frequency of responding cells (13). In this study, responding cells secrete TNF. LDA was used to measure the frequency of effector cells (untreated or LPS-prestimulated TAM or splenic macrophages) that are

capable of secreting TNF. Briefly, wells that had been diluted in cRPMI, were set up in 96 well tissue culture-treated microtiter plates (Fisher). To insure that the data would be statistically significant, sixty wells of both effector and corresponding target plates were used at each effector cell concentration. In every experiment wells containing treated or untreated cells were used as comparison controls. In addition, twelve wells, containing no cells, were used to establish absorbance baselines. In this case, the supernatants from the effector plates were transferred to corresponding cell-free target control wells. Effector plates were subsequently incubated. After incubation, supernatants were transferred from the effector wells to corresponding target wells. After 48 hours of incubation an MTT assay was performed on each target plate. The absorbance data was collected from these assays and plotted on a dot plot as the concentration of effector cells versus target well absorbance values. An absorbance response line was then established on the dot plot by determining the mean absorbance of the controls (C) and subtracting one standard deviation (S.D.). This line has no statistical significance and is used to simply delineate responding wells (those having at least one + cell) from non-responding wells. Dot plot data were subsequently plotted as the macrophage concentration versus F_O on an LDA graph. The resulting line that is generated on the LDA was then examined for statistical significance by Pearson's method of linear regression analysis (26). Following this analysis, the coefficient of linearity (at the 95% level of confidence) was determined by the least squares method (see Table I.). Experimental results were, depending on the degrees of freedom, accepted or rejected on the basis of linearity and statistical significance.

TABLE I

VALUES FOR PEARSON'S COEFFICIENTS DETERMINED BY LINEAR REGRESSION ANALYSIS OF THE DATA AT THE 5% LEVEL OF SIGNIFICANCE

[1]Degrees of Freedom	[2]Value of R^2
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

[1]Degrees of freedom are equal to the number of data points in the line (excluding the origin) minus 1.

[2]Value of R^2 represents the Pearson's coefficient (26) determined by linear regression analysis of the data.

CHAPTER III

RESULTS

Determining the frequency of TAM that secrete TNF was the primary goal of this study. Limiting dilution analysis was exclusively utilized to accomplish this goal because it allows for the detection of both common and rare cell types with specific activities. In this case, limiting dilution analysis was used to estimate the frequency of TAM with TNF-secreting activity. In addition, limiting dilution analysis can also be used to determine whether other cell types are positively or negatively influencing this activity. The second major goal of this study was to measure the cytotoxic activity of TAM in response to the biological agent LPS as well as M-CSF and IFN-γ cytokines. To accomplish this goal, it was necessary to determine the amount of killing of sensitive 2/F2 target cells by supernatants from TAM that were prestimulated with LPS, M-CSF and IFN-γ. Here, an MTT cytotoxicity assay was used to determine the mean absorbances of treated and untreated target wells. Comparison of mean absorbances were then used to determine the percent killing of target cells and thus estimate the change in the cytotoxic activity of TAM.

Response of WEHI-164 Tumor Targets to Supernatants from C3H/HeN Murine Splenic Macrophages

It was assumed that tumor target cells had to be very sensitive in order to use a limiting dilution analysis to detect a single TAM, within an

experimental population, with TNF secreting capabilities. Consequently, it was first necessary to determine the sensitivity of heterogenous WEHI-164 tumor cells to TNF. Sensitivity testing was accomplished by limiting dilution analysis (LDA). Briefly, 50 µl of supernatants from 24 hour bulk splenic macrophage cultures were added to corresponding wells containing WEHI-164 target cells. Spleen cell suspensions that contained splenic macrophages (effector cells) were prestimulated with LPS (5 µg/ml) and, prior to incubation at 37^0 C in a 5 % CO_2 incubator, aliqouted into effector plates at concentrations of 100, 300 and 500 cells per well. Target cells were added to 60 test wells of each corresponding target plate and used at a single concentration of 2.5×10^3 cells/well. Twelve control wells (C) were also added to target plates to establish a mean absorbance response line. Also, twelve additional control wells of this type were added to the 300 cell/well target plate and used to determine whether target cells interfere with test absorbance readings. In addition 12 control wells were added to target plates to establish absorbance baselines. In this case, controls contained target cells that were not pulsed with macrophage (effector) supernatants. Effector cell supernatants were added to the target test wells and all target plates were then incubated for 48 hours at 37^0 C. After incubation, an MTT cytotoxicity assay was performed. on the target plates as described in Chapter II, Methods and Materials.

Following the collection of the absorbance data dot plot analysis showed that the mean absorbance of the 12 control wells minus one standard deviation (C - S.D.) provided an absorbance response line of 0.087 and was used to distinguish responding from non-responding wells (figure 1.). The mean absorbance value of the additional controls was 0.007. This value indicated that tumor target cells were significantly lysed during solubilization and do not alter the absorbance values generated in the MTT assay.

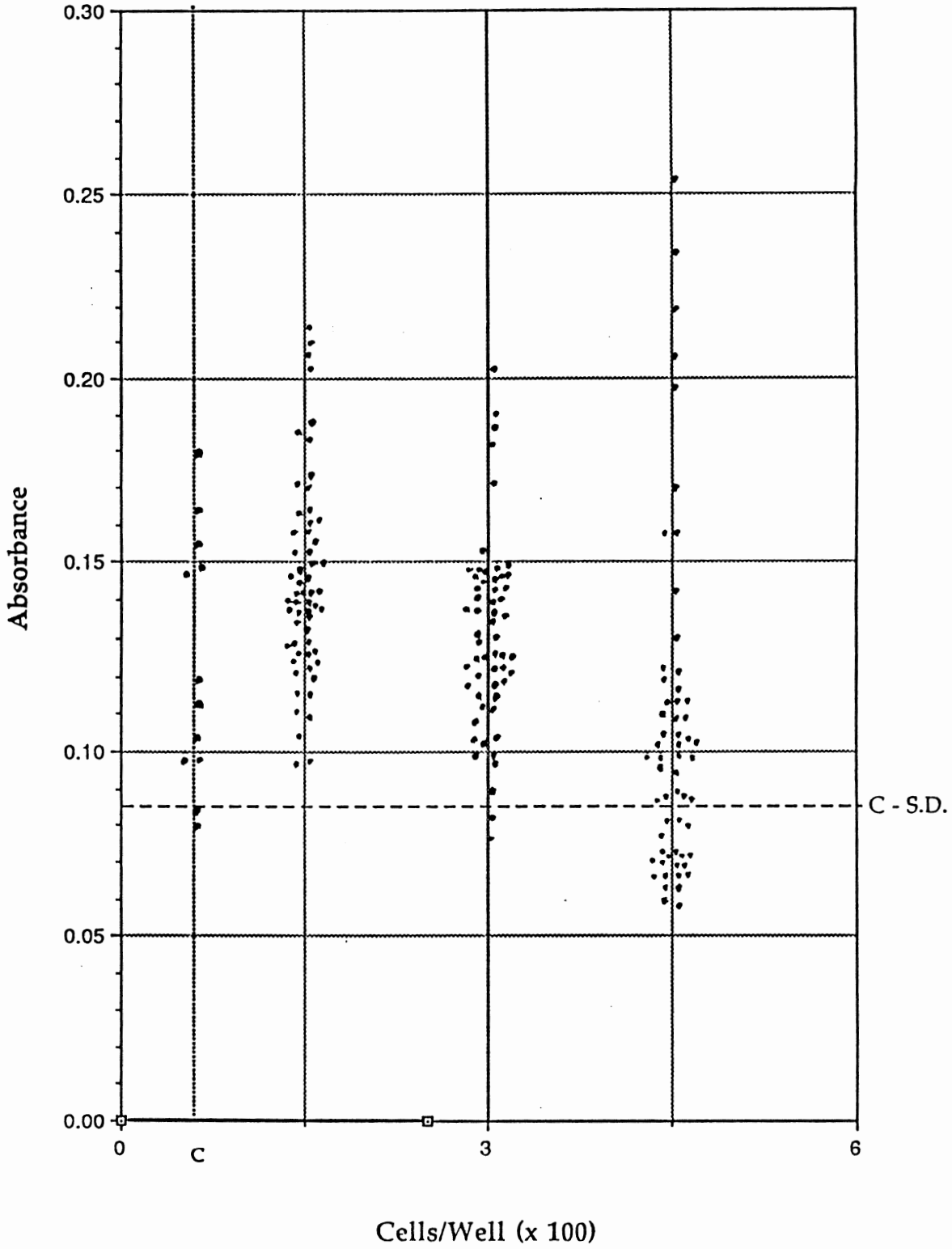

Figure 1. Dot Plot Analysis of the Effects of Supernatants from LPS-Prestimulated Splenic Macrophages on WEHI-164 Tumor Targets.

Absorbance values of the test wells decreased as effector cell concentration increased. This decrease indicated that WEHI-164 tumor target cells are increasingly killed at higher concentrations of effector cells.

Limiting dilution analysis of the dot plot data, generated by spleen cell (macrophage) supernatants, showed that the LDA plot did not yield a straight line, single-hit kinetics were not observed and the frequency of TNF-secreting splenic macrophages could not be determined (figure 2.). The results, plotted on the LDA graph, also included 95% confidence intervals and, in this case, the results indicated that heterogenous WEHI-164 tumor targets lacked the necessary TNF sensitivity to be used in this study.

Response of TNF-Sensitive WEHI-164 Clones to Crude Natural TNF

The results of the previous experiment indicated that our assay lacked sensitivity and therefore indicated the need to generate a highly TNF-sensitive tumor cell clone. This clone would be used as a target throughout this study to estimate the frequency and cytotoxic activity of TNF-secreting TAM. Generation of sensitive clones was accomplished by testing WEHI-164 tumor cell clones for their sensitivity to killing by crude natural TNF supernatants. Briefly, 50 µl of 1.25×10^4 cell/ml cRPMI suspension of each clone, generated in the protocol described in chapter II, Methods and Materials, were added to 12 wells of a sterile 96 well microtiter plate. Subsequently, 50 µl of a 1:20 solution of crude TNF was added to 6 wells of each 12 well test series. In contrast, 50 µl of cRPMI was added to the remaining 6 wells as comparison controls. Also, 50 µl each of cRPMI and crude 1:20 TNF supernatant was added to 12 additional, cell-free wells of each test plate and used to establish absorbance baselines. Target plates were

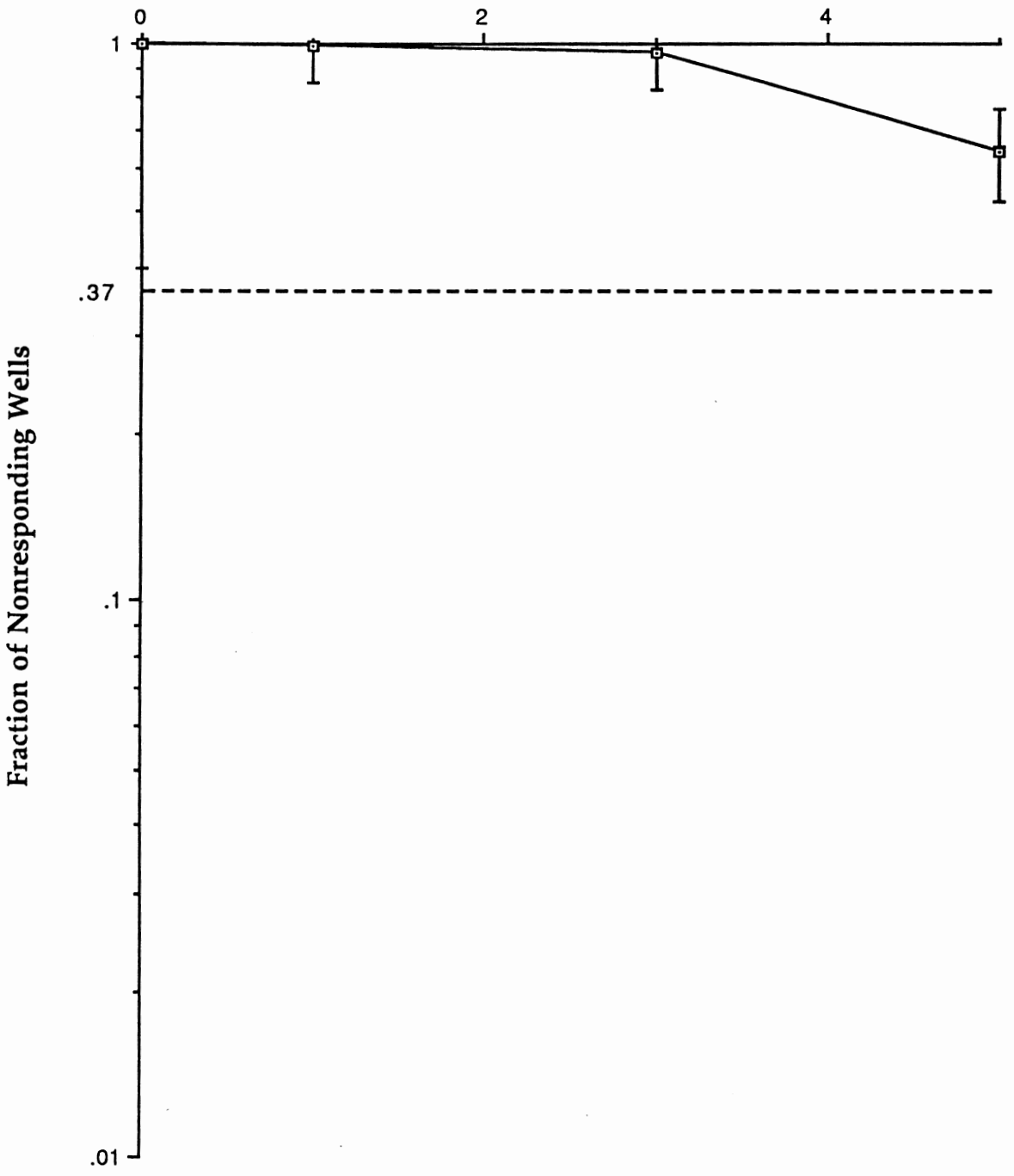

Figure 2. Limiting Dilution Analysis of the Effects of Supernatants From LPS-Prestimulated Splenic Macrophages on WEHI-164 Tumor Targets.

then incubated for 48 hours at 37^0 C. After incubation, an MTT cytotoxicity assay was performed (as previously described) on each target plate. The absorbance data were then analyzed to determine the sensitivity of each test clone to TNF. Sensitivity, in this case, was measured as the percent killed (%killed). The results showed that the most sensitive clone was those designated as 2/F2 (see Table II). Here, 92% of the 2/F2 cells were killed in the presence of macrophage supernatants. Highly sensitive 2/F2 clone cells were subsequently used as tumor targets in this study because they seemed to be sensitive enough to detect one effector cell, within a heterogeneous experimental population, with TNF secreting capabilities,

Response of Sensitive 2/F2 Tumor Targets to Supernatants from C3H/HeN Murine Splenic Macrophages

Because they were most sensitive to macrophage supernatants, cells of WEHI-164 clone 2/F2 was used in the following experiment as tumor target cells to determine the frequency of splenic macrophages with TNF-secreting activity. This experiment was performed exactly as the first experiment was except LPS-pretreated macrophages were aliqouted into effector plates at concentrations of 200, 400 and 600 splenic macrophages per well. After the 24 hr incubation period, 50 µl of effector supernatants were added to each well of the corresponding 2/F2 target plates. Following incubation of target plates and the performance of an MTT assay, absorbance data was recorded and analysed by dot plot and limiting dilution analysis.

Dot plot analysis indicated that maximum killing of sensitive 2/F2 tumor targets occurs at the lowest concentration of splenic macrophages (Figure 3.). This implies that 2/F2 clones are indeed very sensitive to TNF. At higher concentrations of effector cells ($\geq$ 200 cells/well) the absorbance values also

TABLE II

SUMMARY OF THE EFFECTS OF CRUDE NATURAL TNF ON WEHI-164 CLONES

Clone	Mean Absorbance of [1]Untreated Clones	Mean Absorbance of [2]Treated Clones	[a]%Killing
2/F2	0.0948	0.0073	92.3
2/G8	0.0585	0.0070	88.0
2/F8	0.1023	0.0160	84.3
2/D1	0.0923	0.0147	83.6
2/D7	0.0575	0.0110	80.9
2/F3	0.0697	0.0140	79.9
2/G3	0.0380	0.0117	69.2

[1]Untreated: WEHI-164 clones were incubated in cRPMI only.

[2]Treated: WEHI-164 clones were incubated with 1:20 crude TNF.

[a]%Killing = percent change in absorbance (% ΔAbs) where;

$$\% \Delta Abs = \frac{\text{Mean Abs [control] - Mean Abs [treated]}}{\text{mean absorbance of contol}} \times 100\%$$

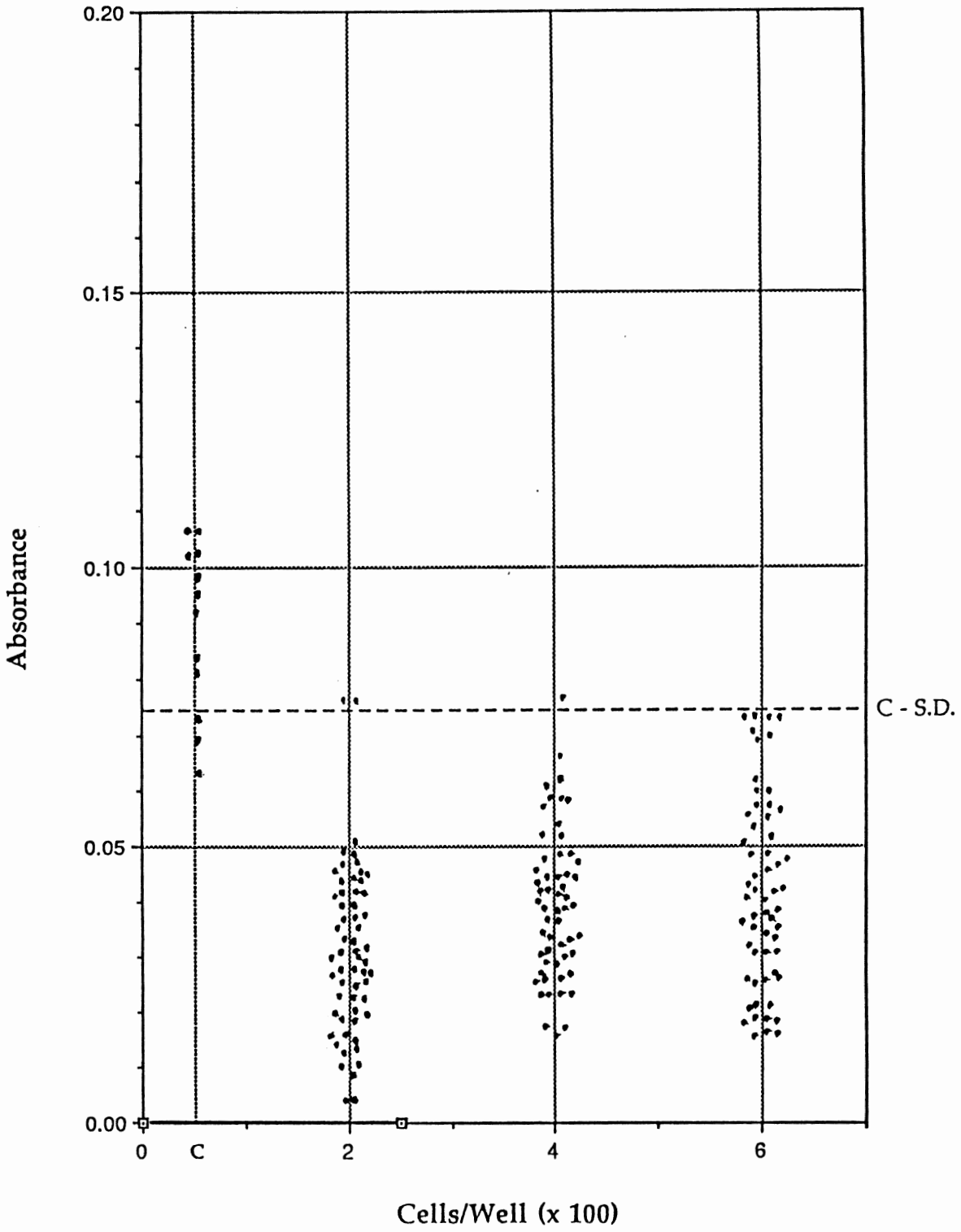

Figure 3. Dot Plot Analysis of the Effects of Supernatants from LPS-Prestimulated Splenic Macrophages on 2/F2 Tumor Targets.

indicate total killing. However the absorbance values at these effector cell concentrations begin to increase indicating that effector wells may have contained contaminating or other interfering cell types. These cell types may have enhanced the growth of targets or blocked signal transduction of TNF-secreting macrophages. Since higher concentrations of macrophage suspensions would be expected to contain correspondingly more contaminating cell types it is possible that a lower maximum concentration of effector cells should be used in future experiments.

Limiting dilution analysis (LDA) showed that single-hit kinetics are not observed and the frequency of macrophages that secrete TNF cannot be determined (Figure 4.). The LDA results also indicated that most of the sensitive 2/F2 targets are killed at the lowest concentration of splenic macrophages (200 cells/well). At the highest effector concentrations (≥ 200 cells per well), no additional 2/F2 killing occurred. In fact, LDA values actually increased slightly. This also indicated that effector cell concentrations may be contaminated with cells that enhance the growth or block the killing of 2/F2 targets in some way. The LDA showed that 2/F2 targets are very sensitive to TNF. In fact, the data showed that supernatants, generated at the lowest concentration of effector cells, were responsible for the greatest amount of killing of 2/F2 targets. Here, the fraction of nonresponding target wells was 0.033 or about three percent. This suggested that target cells were killed in approximately 97% of the target wells. However, because single-hit kinetics were not attained, the LDA indicated the need to isolate macrophages in higher purity.

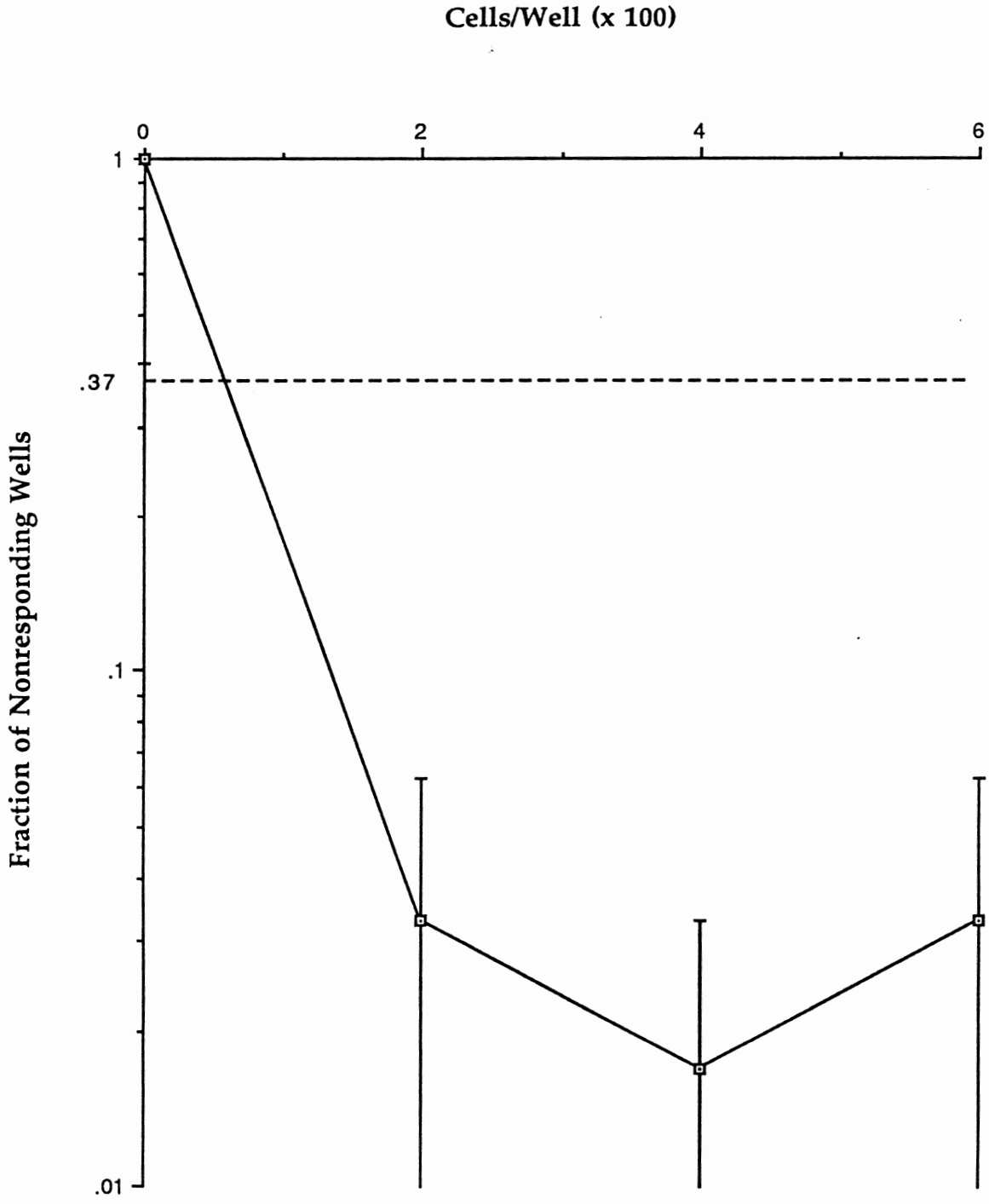

Figure 4. Limiting Dilution Analysis of the Effects of Supernatants from LPS-Prestimulated Splenic Macrophages on 2/F2 Tumor Targets.

Response of Sensitive 2/F2 Tumor Targets to Supernatants from 1X-11-6 TAM and 1X-11-6 Tumor Cells

To determine if the lack of sensitivity encountered in the previous experiment was a function of effector cell purity, 1X-11-6 TAM and 1X-11-6 tumor cells were used as effector cells in the following experiment. The results, using 1X-11-6 TAM and tumor effector cells, were also compared with the results generated in the previous experiment by normal splenic macrophages supernatants. With few exceptions, the 1X-11-6 experiment was set up as in the previous experiment. Briefly, TAM were isolated, as outlined in Chapter II, Methods and Materials, from tumor cell suspensions by rosetting TAM with opsonized SRBC followed by density gradient centrifugation over Ficoll-hypaque. The percent of rosetted cells (FcR^+ TAM) was calculated to be 60%. Since some macrophages may not be capable of rosetting opsonized SRBCs, it was not surprising that the percent of TAM, determined by microscopic morphological analysis was significantly greater. In this case, 75-80% of the isolates were morphologically identical to macrophages. These morphological characteristics included a kidney-shaped nucleus as well as large amounts of cytoplasm. Following isolation of 1X-11-6 TAM, tumor cells (non-rosetted cells) were isolated from the same density gradient. This was accomplished by removing the non-rosetted cell layer with a pipette. 1X-11-6 tumor cells were then washed twice with sterile PBS and concentrated by centrifugation at 650 g for 15 minutes. After centrifugation, the tumor cell pellet was resuspended in 2 ml of cRPMI. Following isolation, both cell types were diluted to concentrations of 200, 600 and 1,000 cells per well in cRPMI, treated with LPS and plated as before. When the absorbance of 2/F2 target wells from both parts of the experiment

had been recorded, the results were again analyzed by dot plot and limiting dilution analysis.

Dot plot analysis of the effects of 1X-11-6 tumor cell supernatants on sensitive targets showed that these supernatants enhance the growth of 2/F2 targets (Figure 5.). Because the absorbance values indicated that enhancement of target cell growth was occuring, one standard deviation was added to the mean absorbance of the 12 control wells to establish the absorbance response line (C + S.D.). The results showed that the absorbance values of target wells increased as the concentration of effector cells increased. Here, absorbance values indicated that tumor cell supernatants had enhanced the growth of 2/F2 targets.

Limiting dilution analysis of the first portion of this experiment showed that single-hit kinetics were attained when 1X-11-6 tumor cells were used as effector cells (see Figure 6.). Linear regression analysis (LDA) of the line, plotted through the LDA data, revealed a coefficient of 0.993. The frequency of 1X-11-6 tumor cells that enhanced the growth of TNF-sensitive 2/F2 targets was subsequently determined to be approximately 1/825. Since 1X-11-6 tumors are derived from transformed fibroblasts, enhancement of target cell growth seems reasonable. Colony stimulating factors, that are known to be potent stimulators of cell proliferation, have been shown to be produced by fibro-blasts (25). Of course, it is possible, although not as likely, that 2/F2 tumor targets might account for some, if not all, of the enhancement of growth of target cells. If this is true the growth, in this case, may simply reflect the growth pattern of 2/F2 target cells in the presence of addtional tumor cell supernatants.

In the second portion of this experiment, limiting dilution analysis of the absorbance data, generated by TAM supernatants, showed that single-hit

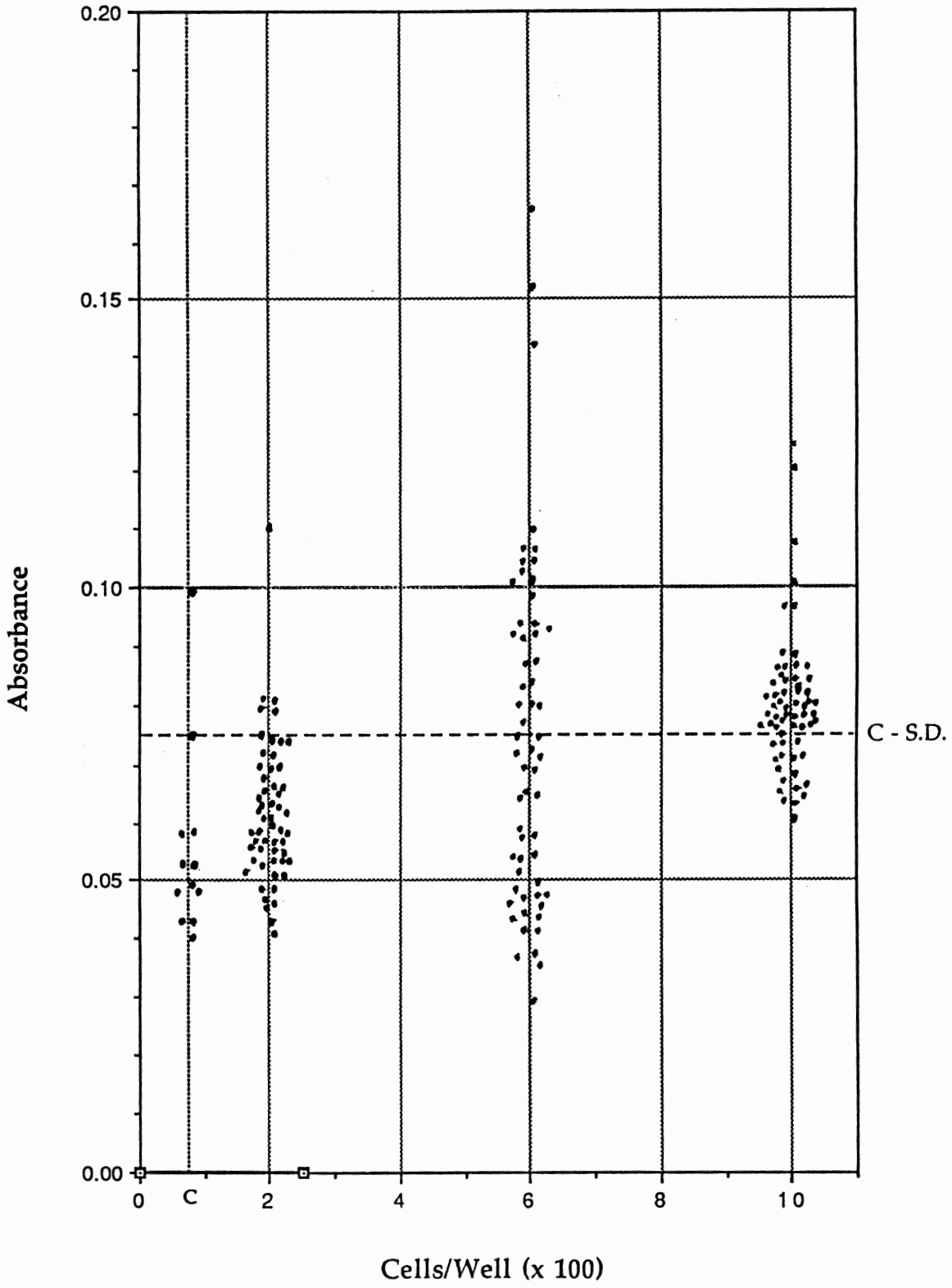

Figure 5. Dot Plot Analysis of the Effect of Supernatants from LPS-Prestimulated 1X-11-6 Tumor Cells on 2/F2 Tumor Targets.

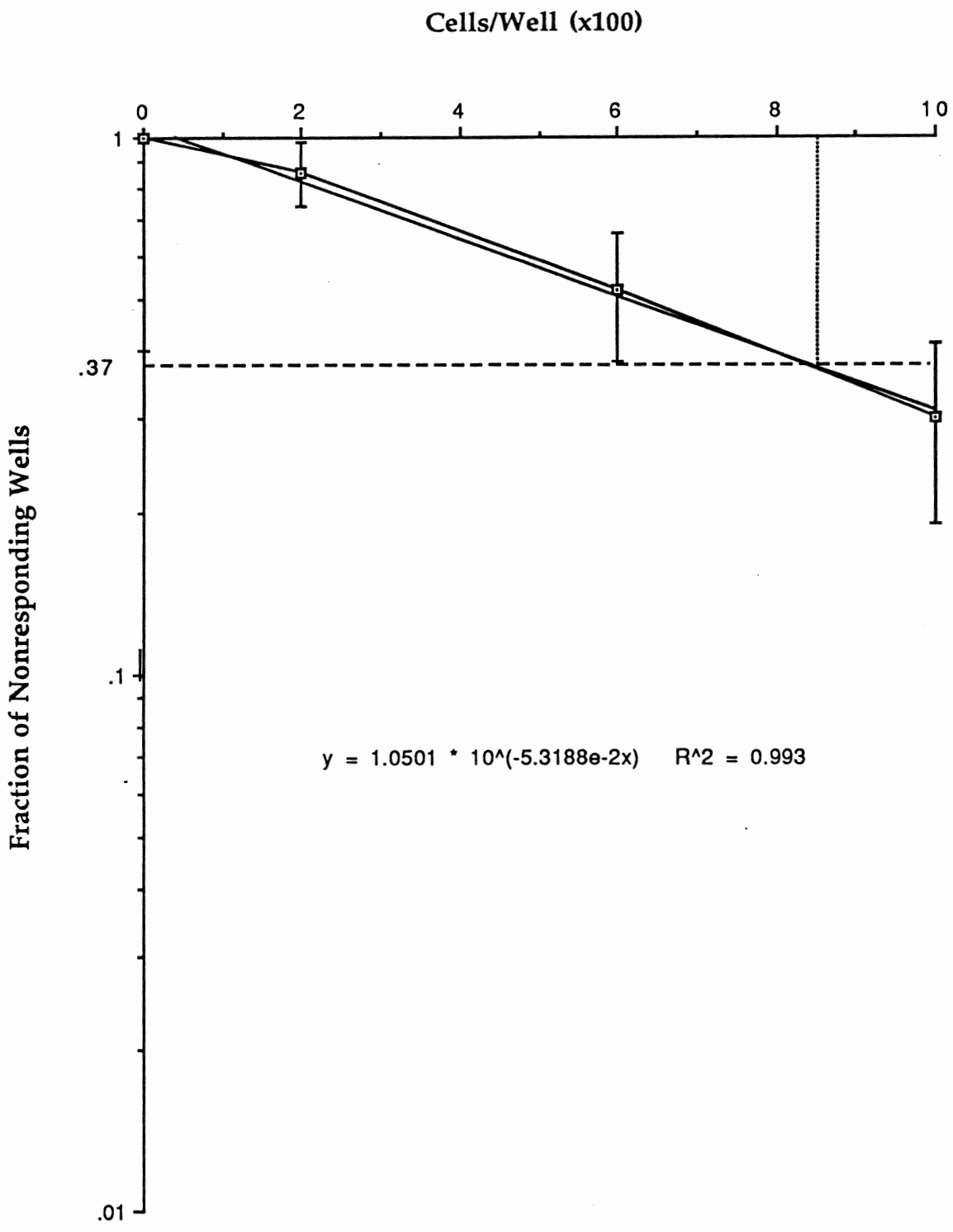

Figure 6. Limiting Dilution Analysis of the Effects of Supernatants from LPS-Prestimulated 1X-11-6 Tumor Cells on 2/F2 Tumor Targets.

kinetics were not observed and the frequency of TNF-secreting 1X-11-6 TAM could not be determined (Figure 7.). The LDA results, generated by these supernatants, revealed that no killing could be detected even at the lowest concentration of TAM (200 cells/well). The results indicated that the purity of the effector cell population was inadequate and the Ficoll Hypaque isolation technique did not produce sufficiently pure TAM.

Response of Sensitive 2/F2 Tumor Targets to Supernatants from Untreated MBHR-Isolated 1X-11-6 TAM

To test the effects of LPS on the TNF secreting activities of tumor associated macrophages (TAM) as well as the degree of purity of macrophage isolates, TAM were isolated from 1X-11-6 tumor cell suspensions by magnetic bead hybrid rosetting (MBHR) and not prestimulated with LPS. The results of the previous experiment indicated that isolation of TAM by Ficoll Hypaque exclusion resulted in the TAM populations that are somewhat impure (75-80% at best) and lacked the sensitivity that was necessary to determine the frequency of TNF-secreting TAM. MBHR isolation of 1X-11-6 TAM was performed by the method outlined in Chapter II, Methods and Materials. Briefly, sheep α-rat-Ig coated magnetic beads (dynabeads) were allowed to bind to opsonized SRBCs to produce dynabead rosettes (DBRs). A TAM containing 1X-11-6 tumor cell suspension was then mixed, at a ratio of 1: 4, with DBRs to produce a MBHR suspension. MBHRs were then immunomagnetically isolated from the suspension in an MPC-1 particle concentrator. MBHRs were susequently washed with PBS and, following lysis of SRBCs the used dynabeads were recovered in the MPC-1. Following the recovery of used dynabeads the 1X-11-6 TAM were recovered in the lysing effluent and washed in sterile PBS. TAM were then resuspended in 5 ml of cRPMI, counted and

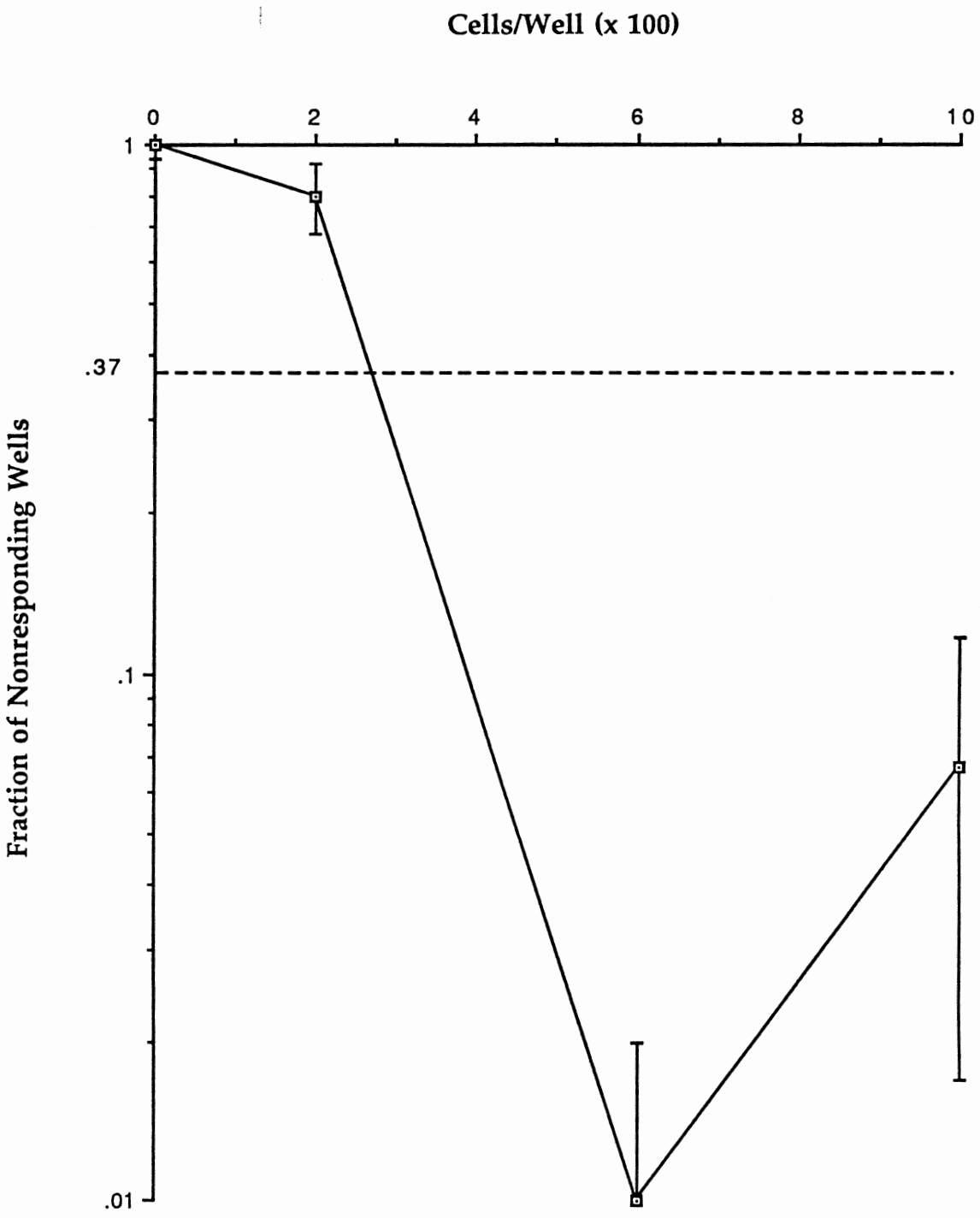

Figure 7. Limiting Dilution Analysis of the Effects of Supernatants from LPS-Prestimulated 1X-11-6 TAM on 2/F2 Tumor Targets.

diluted to concentrations of 200, 400 and 600 cells/well. To determine the relative purity of MBHR-isolated TAM, small portions of TAM suspension were examined morphologically and rosetted with opsonized SRBCs. Morphological analysis revealed that 85-90% of the isolates were TAM. However, the percent of rosetted TAM (FcR^+) was calculated at 63 percent. 1X-11-6 TAM were subsequently aliquoted into 96 well microtiter plates and incubated for 24 hours. Following incubation, TAM supernatants were added to 2/F2 target wells and the MTT assay portion of the experiment completed as previously described.

Limiting dilution analysis of the results showed that the LDA values, generated by untreated 1X-11-6 TAM, remained virtually constant. This suggested that prestimulation of TAM with LPS was necessary to stimulate significant TNF secretion (Figure 8.). However, the results did not indicate whether isolation of cells by the MBHR technique provided pure TAM populations because single-hit kinetics were not observed and the frequency of TNF-secreting 1X-11-6 TAM could not be determined.

Response of Sensitive 2/F2 Tumor Targets to Supernatants from Untreated and LPS-Prestimulated MBHR-Isolated 1X-11-6 TAM

Since the results of the previous experiment indicated that prestimulation of TAM with LPS was necessary to induce TNF secretion, the following experiment was performed to confirm this finding and to determine if the isolation of TAM by the MBHR technique would actually provide TAM of higher purity. This experiment was performed exactly as the previous experiment except that MBHR-isolated 1X-11-6 TAM were either prestimulated with LPS (5 μl/ml) or left untreated prior to use. In addition, TAM were aliquoted into effector plates at concentrations of 100, 200 and 400 cells per

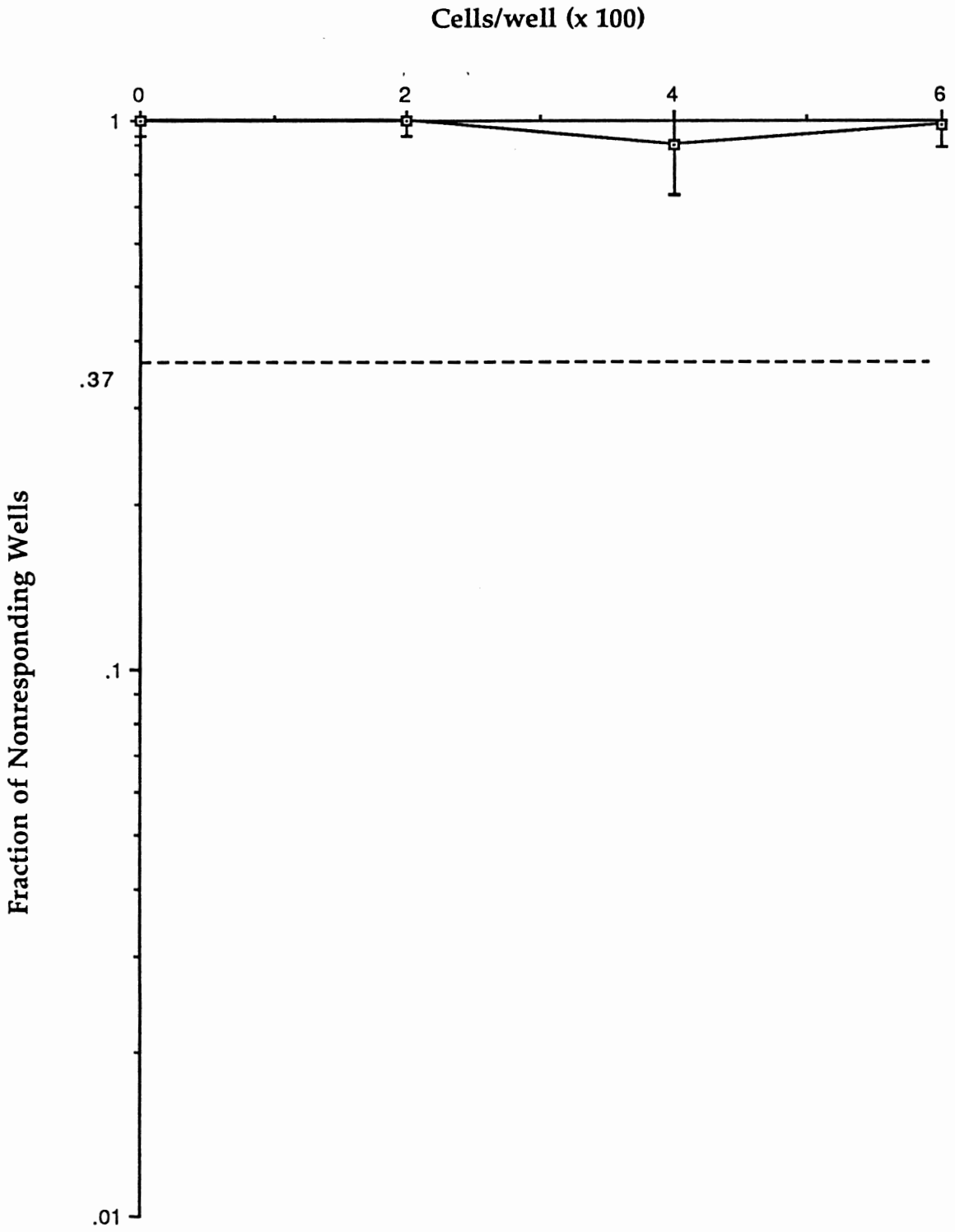

Figure 8. Limiting Dilution Analysis of the Effects of Supernatants from Untreated 1X-11-6 MBHR-Isolated TAM on 2/F2 Tumor Targets.

well to determine the effects of lowering the concentrations of TAM. Also, 24 control wells were added to untreated effector plates to determine whether additional control wells will significantly alter position of the response line. Analysis of the purity of the isolates showed that approximately 61% of the 1X-11-6 TAM rosetted with opsonized SRBCs. However, morphological analysis showed that 90% of the isolates were macrophages.

Dot plot analysis of the absorbance data showed that the absorbance values changed in proportion with TAM concentrations (Figure 9.). At the highest TAM concentration (400 cells/well) most target cell killing ceased and, in this case, the absorbance values increased significantly.

Limiting dilution analysis of the data indicated that TAM purity was very adequate (Figure 10.). In addition, LDA revealed that single-hit kinetics are attained at lower TAM concentrations (≤ 200 cell/well) (Figure 11.). Here, the highest concentration of TAM (400 cells/well) was ignored. Linear regression analysis of the results revealed a coefficient of linearity (R^2) of 0.993. If MBHR-isolated TAM were pure enough, it seemed clear that, at higher concentrations (≥ 200 cells/well), the MBHR-isolated TAM populations may have contained cells that interfered with the assay. Of course, these experimental populations were not 100% pure and, at higher concentrations, could have contained interfering cell types.

Dot plot analysis indicated that untreated (-LPS) 1X-11-6 TAM subpopulations did not secrete significant amounts of TNF (Figure 12.). In fact, absorbance values remained virtually constant as the concentration of untreated 1X-11-6 TAM increased. The data therefore indicated that no significant amount of growth enhancement or killing of 2/F2 target cells could be detected. Here, no significant increase in absorbance values could be detected, even at the highest concentration of TAM. In fact, there was a

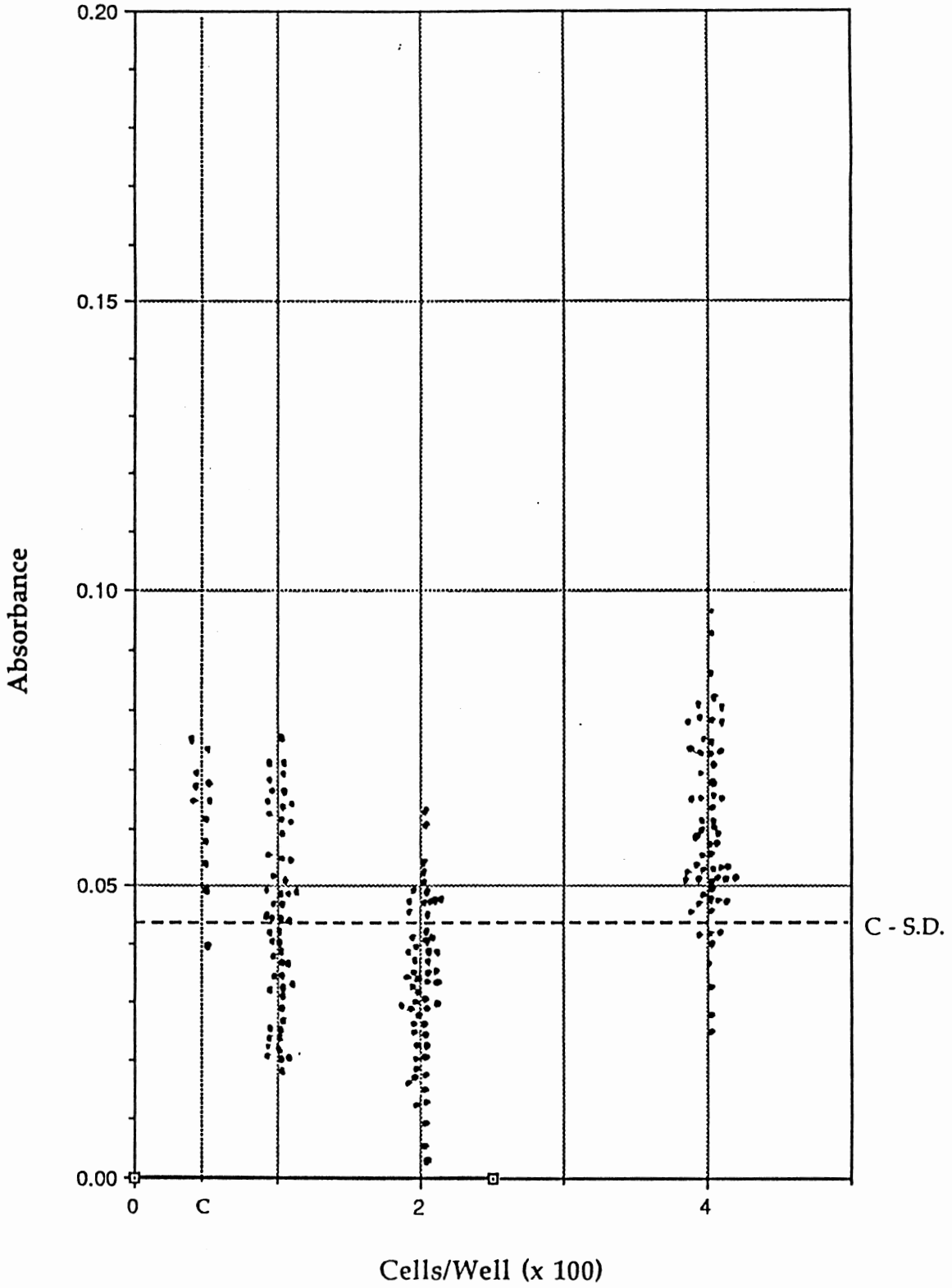

Figure 9. Dot Plot Analysis of the Effects of Supernatants from MBHR-Isolated LPS-Prestimulated 1X-11-6 TAM on 2/F2 Tumor Targets.

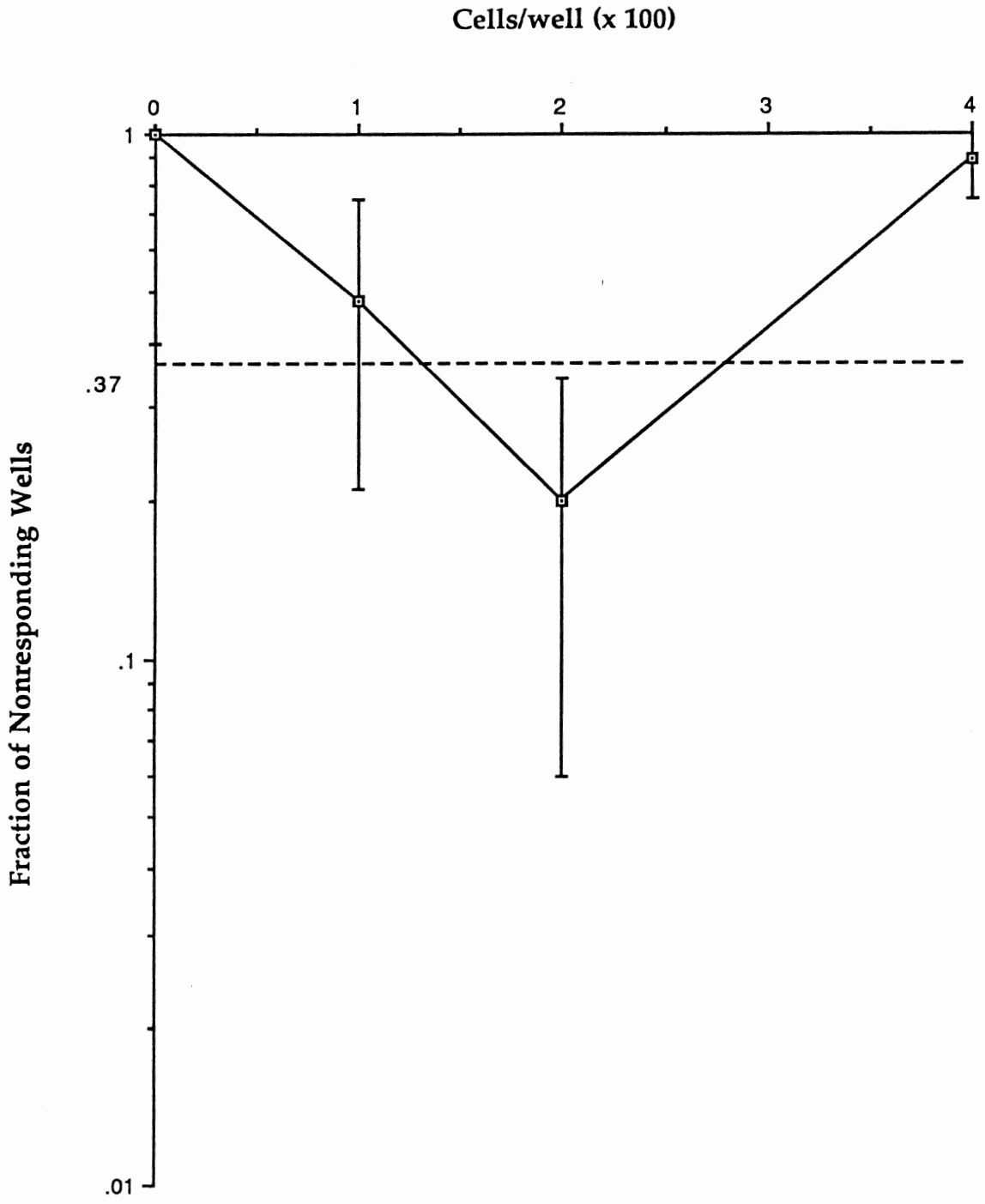

Figure 10. Limiting Dilution Analysis of the Effects of Supernatants from MBHR-Isolated LPS-Prestimulated 1X-11-6 TAM on 2/F2 Tumor Targets.

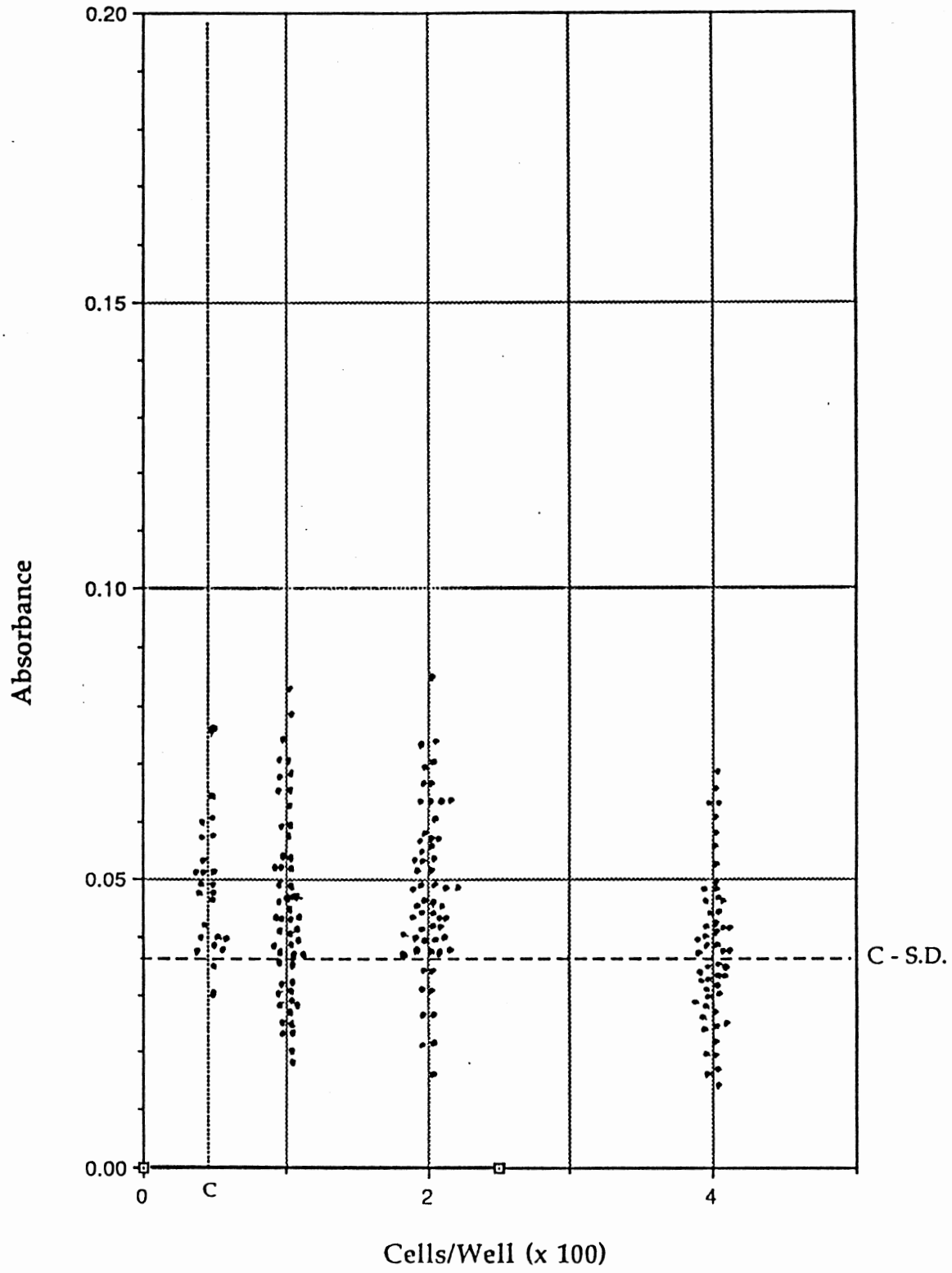

Figure 12. Dot Plot Analysis of the Effects of Supernatants from MBHR-Isolated 1X-11-6 TAM on 2/F2 Tumor Targets.

somewhat significant decrease in absorbance values at the highest concentration. In addition, the use of 12 additional control wells (24 total) did not significantly change the position of the absorbance response line. In this case, the absorbance response line for untreated TAM (- LPS) decreased by only 0.008 and, when compared to the line for prestimulated TAM, indicated that 12 control wells are sufficient to establish the response line (see Figure 9 and Figure 12).

Limiting dilution analysis of the absorbance data generated by untreated 1X-11-6 TAM showed that these cells secrete very little, if any, TNF (Figure 13.). In this case, LDA values remained fairly constant at all concentrations of 1X-11-6 TAM, single-hit kinetics were not observed and the frequency of TAM that secrete TNF constitutively or in response to ubiquitous LPS, could not be determined. Comparison, by LDA, of the results generated by prestimulated (+ LPS) 1X-11-6 TAM supernatants with those generated by untreated (- LPS) 1X-11-6 TAM supernatants indicated that LPS is needed to stimulate TNF secretion (Figure 13.). The fraction of nonresponding wells for 2/F2 targets that were pulsed with untreated TAM supernatants remained consistently high at all concentrations. These values ranged from 80-95% (0.80 to 0.95) and indicated that, at best, only 20% of the 2/F2 target wells exhibited killing. This occurred after corresponding target wells had been pulsed with supernatants from untreated TAM that had been aliquoted at 200 TAM per well. In contrast, 80% of the target wells exhibited killing at this concentration when corresponding target wells were pulsed with LPS-prestimulated TAM supernatants. In addition, the results showed that higher concentrations of TAM greater (≥ 200 cells/well) may contain macrophage subtypes or other contaminating cells because target cell killing was almost totally blocked beyond this point. Here, 89% (0.89) of the target wells were nonresponding.

Response of Sensitive 2/F2 Tumor Targets to Supernatants from Untreated and LPS Prestimulated MBHR-Isolated MC-4 TAM

Since the results of the previous experiment indicated that isolation of TAM (effector cells) by the MBHR technique provided experimental populations of significant purity, the following experiment was performed to determine if this technique would also provide pure experimental populations of other TAM. In this case, MC-4 TAM, obtained from a methylcholanthrene-induced murine skin tumor were used as effector cells. This experiment was performed (using both untreated and LPS-prestimulated MC-4 TAM) to compare the results generated by MC-4 TAM, with those generated by 1CX-11-6 TAM. Following isolation by the MBHR technique, MC-4 TAM were tested for purity. The results showed that 61% rosetted and approximately 90% were morphologically identified as macrophages. MC-4 TAM were subsequently aliquoted into effector plates at concentrations of 200 and 400 cells/well and incubated. Following the MTT assay, the absorbance data was then collected and collated prior to analysis.

Limiting dilution analysis of the results, generated by prestimulated and untreated MC-4 TAM supernatants, also showed that LPS is needed to stimulate the secretion of TNF by MC-4 TAM (Figure 14.). The fraction of nonresponding target wells, that were pulsed with untreated TAM supernatants, remained consistently high at all concentrations. These values remained above 90% (0.90) and indicates that, at best, only 5-7% of the 2/F2 target wells exhibited killing. In contrast, 65% of the wells contained killed clones at this concentration when corresponding target wells were pulsed with prestimulated 200 cell/well MC-4 TAM supernatants. These results compare very closely with the results generated, in the previous experiment, by 1X-11-6 TAM. Furthermore, the results showed that higher

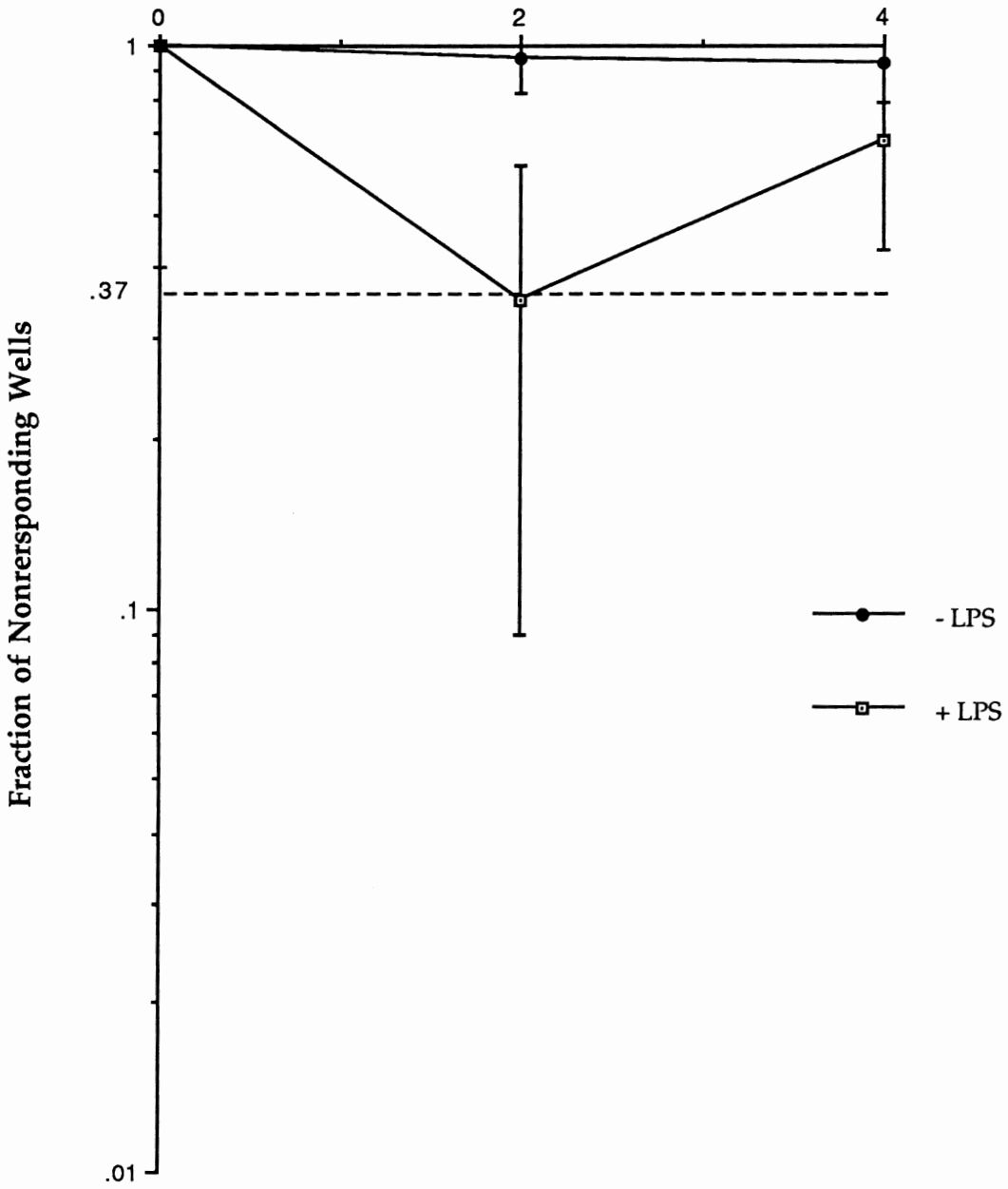

Figure 14. Limiting Dilution Analysis of the Effects of Supernatants from Untreated (- LPS) and LPS-Prestimulated (+ LPS) MBHR-Isolated MC-4 TAM on 2/F2 Tumor Targets.

concentrations of MC-4 TAM (≥ 200 cells/well), may have contained contaminating macrophages or other cell types because target cell killing decreased significantly beyond this point. In this case, 68% (0.68) of the target wells were nonresponding.

Response of Sensitive 2/F2 Tumor Targets to Supernatants from LPS-Prestimulated Adherence-Isolated 1X-11-6 TAM

Although the MBHR isolation technique (used in the two previous experiments) provided TAM of significant purity, it also proved to be tedious, time-consuming and very difficult to perform. The following experiment was therefore performed using TAM that were isolated by the adherence method outlined in Chapter II, Methods and Materials. The adherence method was used in this case to positively select for 1X-11-6 TAM and simultaneously exclude (undesirable) tumor cells or non-adherent cells. Briefly, heterogeneous 1X-11-6 tumor cell suspensions were aseptically added to tissue culture-treated culture dishes at a concentration of 3×10^6 cells/ml in cRPMI medium. The cells were then incubated for 30 minutes at 37^0 C. Incubation for 30 minutes allowed enough time for TAM, but not tumor cells, to adhere to the bottom of the culture dishes. Non-adherent cells were subsequently removed by washing plates with PBS buffer. After washing, adherent cell (TAM) layers were recovered with the aid of a cell scraper. Adherent cell suspensions were then concentrated by centrifugation, resuspended in cRPMI and counted on a hemacytometer. Portions of the adherent cells were then tested for purity by rosetting and microscopic morphological analysis. The results showed that 71% of the adherence-isolated TAM rosetted opsonized SRBCs and approximately 95% had marophage morphologies. Adherent cells (TAM) were then diluted to

appropriate concentrations in cRPMI, prestimulated with LPS and used as effector cells. This experiment was performed as before except that TAM were used in concentrations of 50, 100, 250 and 400 cells/well. These concentrations were used to help determine optimum concentrations, the frequency of TNF-secreting 1X-11-6 TAM, and to compare the results, at the highest concentration with the results obtained with MBHR-isolated 1X-11-6 TAM.

Limiting dilution analysis showed that when corresponding target wells were pulsed with prestimulated TAM supernatants, that had been aliquoted at 200 cells/well, 80 % of the target wells exhibited killing (Figure 15.). Here, only 20 % (0.20) of the target wells were nonresponding. LDA results also showed that higher concentrations of TAM (≥ 200 cells/well) may have contained macrophage subtypes or other contaminating cells because target cell killing decreased significantly beyond this point. The data suggest that 95% of the target wells were nonresponding when corresponding target wells were pulsed with 400 cell/well TAM supernatants.

Linear regression analysis of the LDA data showed that when the highest TAM concentration (400 cells/well) was ignored single-hit kinetics were observed (Figure 16.). Since there were two degrees of freedom, the coefficient of linearity (R^2) was determined to be 0.981. The frequency of TNF-secreting 1X-11-6 TAM was then calculated to be approximately 1/170.

This experiment was subsequently repeated. However, in this case, to minimize interference by contaminating cells, adherence-isolated, LPS-prestimulated 1X-11-6 TAM were aliquoted into effector plates at a maximum concentration of 200 cells/well. As in the previous experiment, an MTT cytotoxicity assay was performed. The absorbance data were subsequently collected and analysed by LDA.

Linear regression analysis of the LDA results showed that single-hit

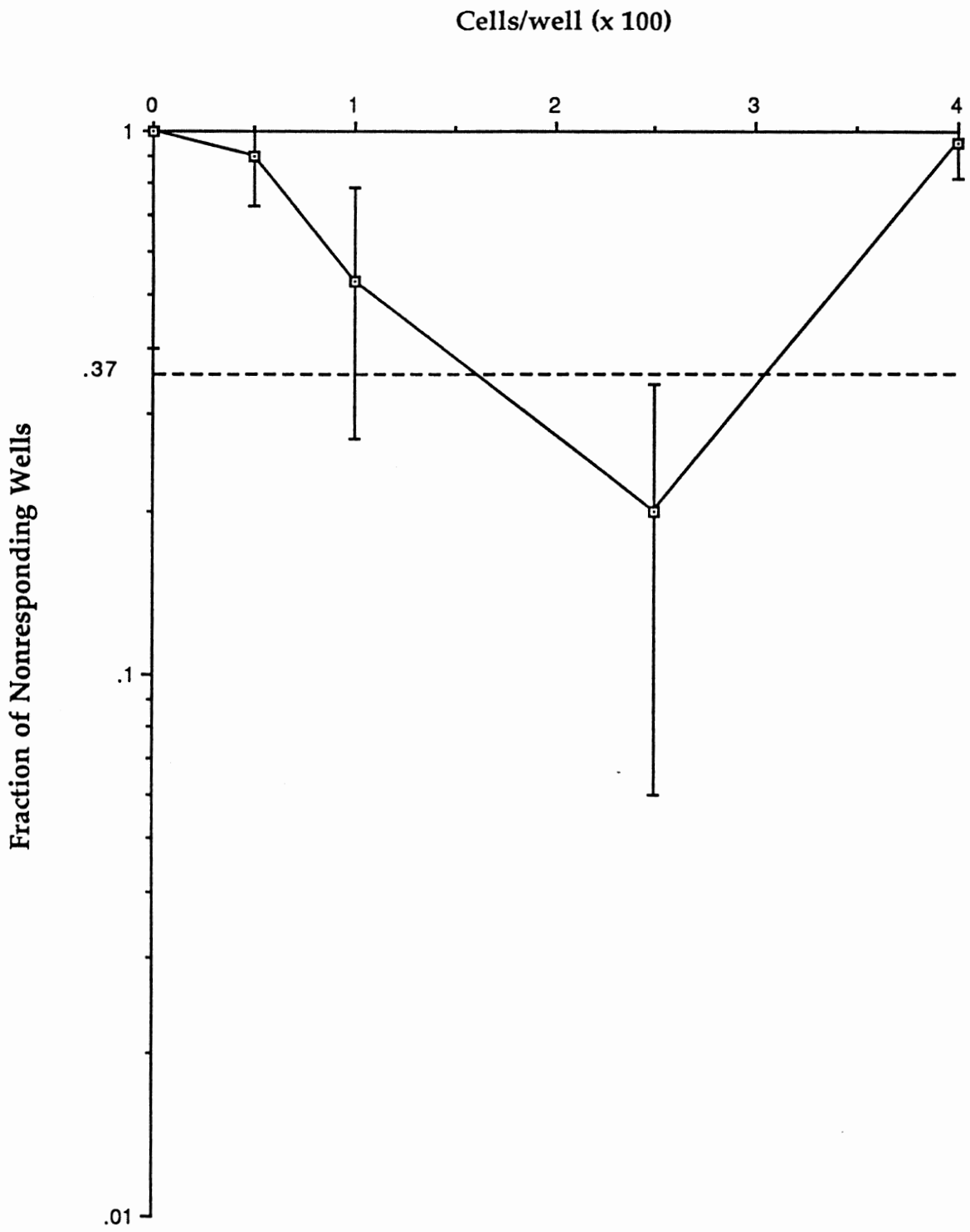

Figure 15. Limiting Dilution Analysis of the Effects of Supernatants from LPS-Prestimulated Adherence-Isolated 1X-11-6 TAM on 2/F2 Tumor Targets.

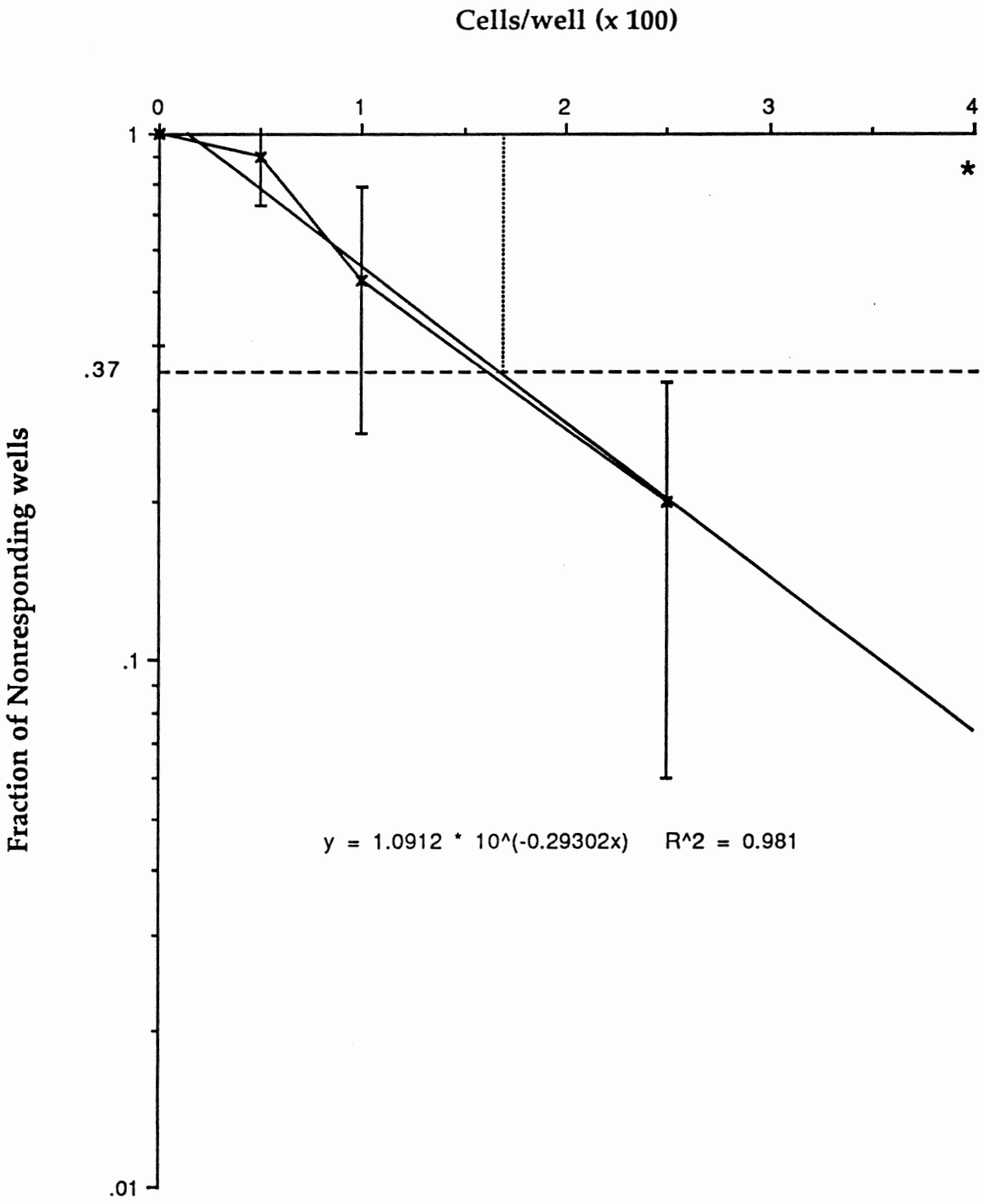

Figure 16. Linear Regression Analysis of the LDA Results Generated by Supernatants from LPS-Prestimulated Adherence-Isolated 1X-11-6 TAM

kinetics were attained and the frequency of TNF-secreting 1X-11-6 TAM was estimated to be 1/170 (Figure 17.). In addition, after being pulsed with 200 cell/well TAM supernatants, 33% (0.33) of the corresponding target wells were nonresponding This indicated that 67% of the target wells contained killed 2/F2 tumor target cells.

Response of Sensitive 2/F2 Tumor Targets to Supernatants from LPS-Prestimulated Adherence-Isolated MC-4 TAM

In a parallel experiment, performed exactly as the previous experiment, LPS-prestimulated MC-4 TAM were used as effector cells. MC-4 TAM were also isolated by the adherence method and aliquoted into effector plates at concentrations of 75, 125 and 200 cells/well.

Limiting dilution analysis of data, generated by the MC-4 TAM supernatants showed that single-hit kinetics were observed (Figure 18.). Linear regression analysis of the LDA results showed that the coefficient of linearity (R^2) was 0.986 and the frequency of TNF-secreting MC-4 TAM was determined to be 1/200. In addition, 65% of the corresponding 2/F2 target wells contained killed cells after being pulsed with 200 cell/well MC-4 TAM supernatants. This compares closely with the previous results for 1X-11-6 TAM supernatants where 67% of the 2/F2 target wells exhibited killing.

Response of Sensitive 2/F2 Tumor Targets to Supernatants from LPS-Prestimulated Adherence-Isolated C3H/HeN Murine Splenic Macrophages

As a comparsion, a similar, parallel experiment was performed exactly as the previous experiment except that LPS-prestimulated splenic macrophages were used as effector cells. This experiment was used to determine

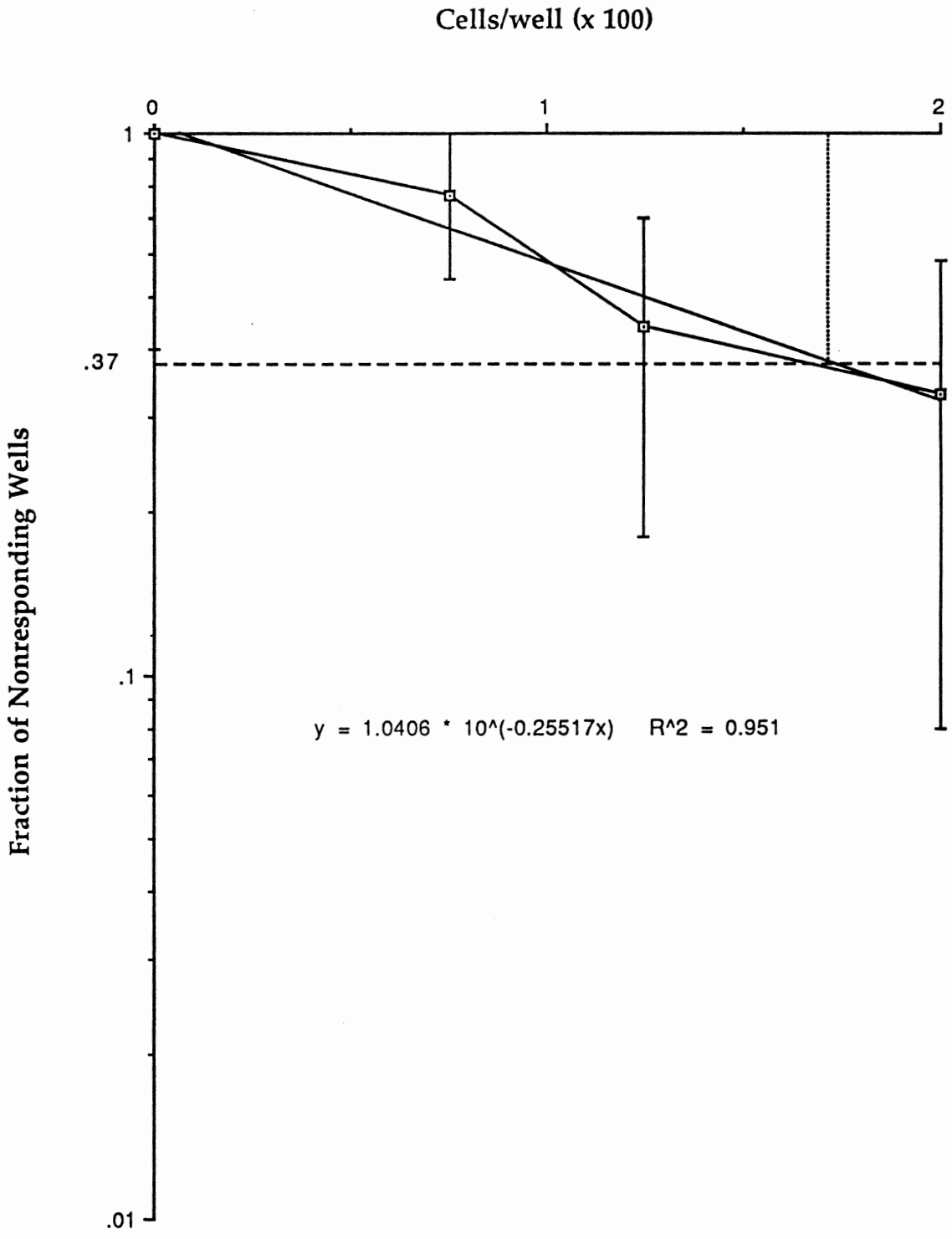

Figure 17. Limiting Dilution Analysis of the Effects of Supernatants from LPS-Prestimulated Adherence-Isolated 1X-11-6 TAM on 2/F2 Tumor Targets.

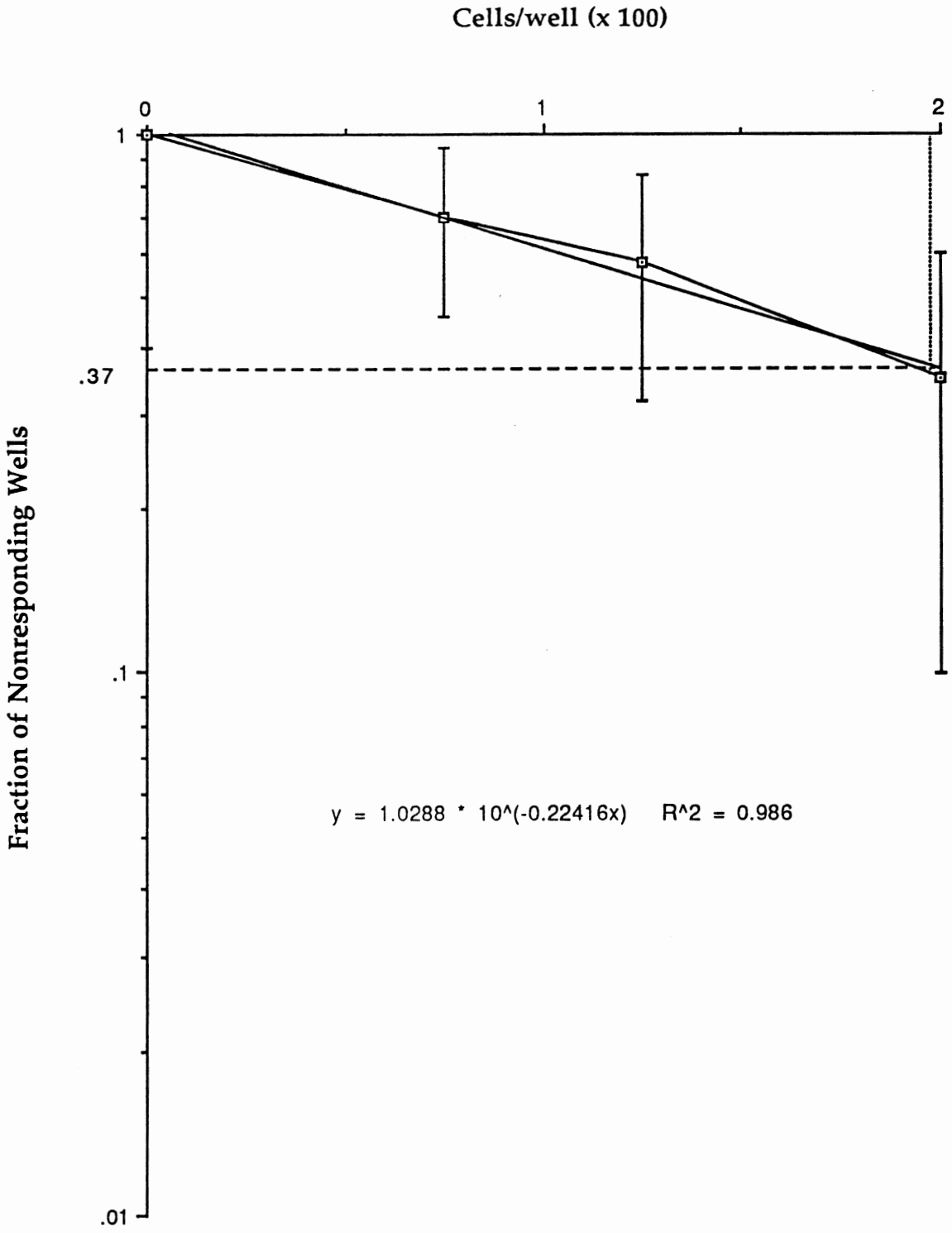

Figure 18. Limiting Dilution Analysis of the Effect of Supernatants from LPS-Prestimulated Adherence-Isolated MC-4 TAM on 2/F2 Tumor Targets.

what functional similarities, if any, exist between normal splenic macrophage and tumor associated macrophage (TAM) populations.

Limiting dilution analysis of the data, generated by the splenic macrophage supernatants showed that single-hit kinetics were observed (Figure 19.).

Linear regression analysis of the LDA results (also see Figure 19.) showed that the coefficient of linearity (R^2) was calculated to be 0.980 and the frequency of TNF-secreting splenic macrophages was estimated to be 1/150. In addition, 77% of the corresponding target wells contained killed 2/F2 target cells after being pulsed with 200 cell/well macrophage supernatants. Comparison of the data, generated by adherence-isolated TAM and normal spenic macrophage supernatants, suggests that they have nearly equivalent TNF-secreting activities. The frequencies of macrophages that secrete TNF ranged from 1/200 (0.5%) for MC-4 TAM to 1/150 (0.7%) for normal murine splenic macrophages. The results from the three previous experiments also showed that the adherence method of isolating macrophages from solid tissues proved to be the most practical method in terms of speed, convenience, costs and accuracy of results. The adherence technique was used as a method of choice to isolate TAM in the remainder of this study.

Response of anti-TNF-α-pretreated 2/F2 Tumor Targets to Supernatants from MC-4 and 1X-11-6 TAM

The results, generated in the preceding three experiments, suggested that rare subsets of prestimulated TAM were capable of killing 2/F2 tumor target cells. However, the results of these experiments did not indicate how much, if any, of the observed target cell killing was actually due to secretion of TNF by TAM. The following assay was designed to address this question. Briefly, MC-4 and 1X-11-6 TAM were isolated by the adherence technique. TAM were

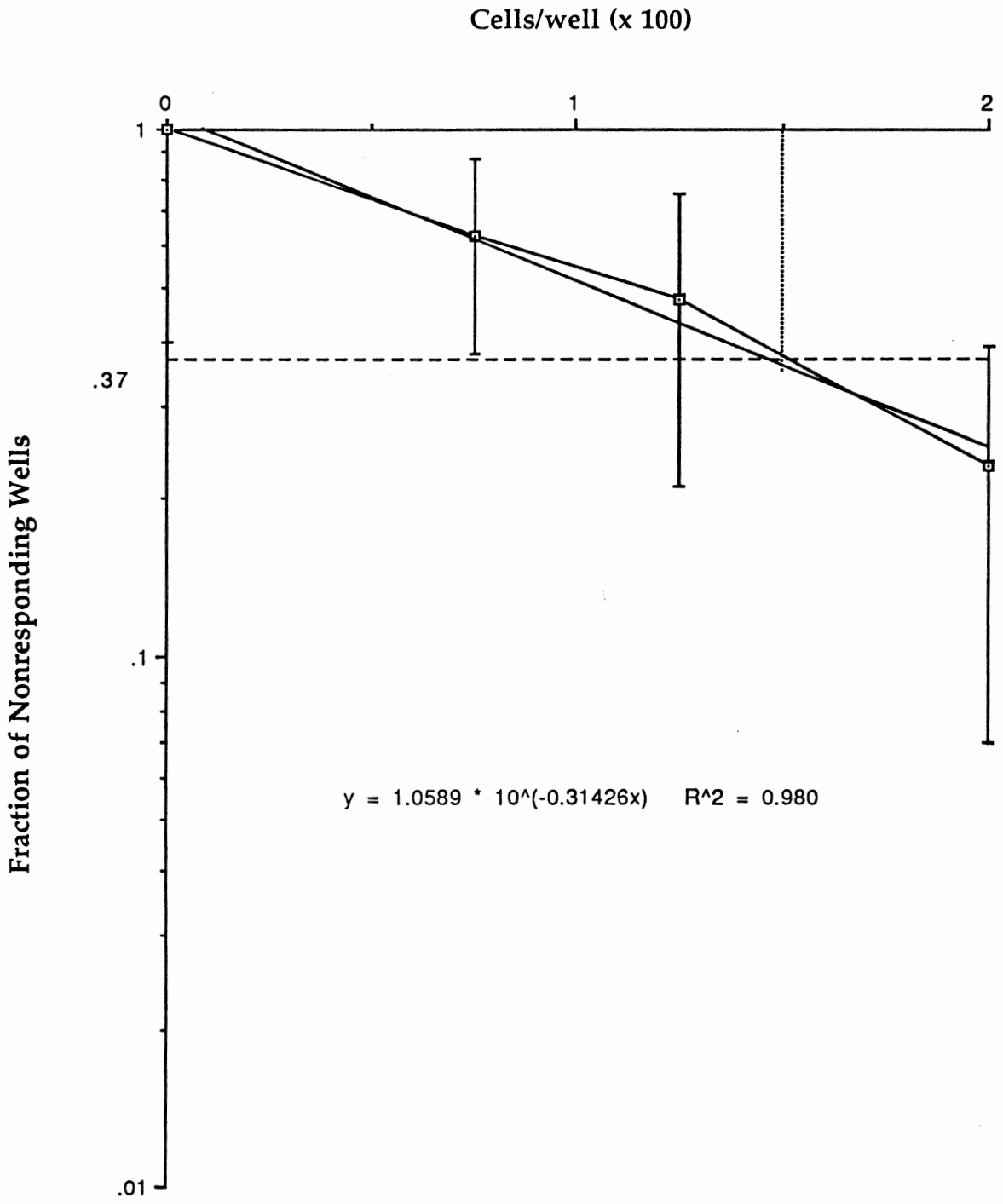

Figure 19. Limiting Dilution Analysis of the Effects of Supernatants from LPS-Prestimulated Adherence-Isolated Splenic Macrophages on 2/F2 Tumor Targets.

then prestimulated with LPS (5 μg/ml) and aliquoted into effector plates. At the same time, 2/F2 tumor target cells were aliquoted, at 1.25 x 10^4 cells/ml, into 60 wells of each corresponding target plate. As in previous experiments, controls were added to target plates to establish baseline absorbances and an absorbance response line. Effector and target plates were then incubated at 37^0 C. After 22 hours of incubation, target wells were either left untreated, treated with whole rabbit anti-TNF-α serum (anti-TNF-α, 2000 neutralizing units/ml) to block TNF activity or treated with whole rabbit anti-bovine serum (Rα-BS). R-α-BS antisera was used to test whether normal components in the cRPMI medium are capable of blocking TNF activity. Following the addition of antisera to corresponding wells, target plates were reincubated for 2 hours to allow time for antibody binding. Following reincubation, target wells were pulsed with corresponding TAM supernatants and reincubated for an additional 6 hours. After this final incubation period, an MTT assay was performed and the absorbance data collected and analysed by dot plot and comparative analysis.

The results of the experiment, summarized in Table III, showed that anti-TNF-α antisera blocks all of the killing of 2/F2 tumor targets by TAM supernatants (Table III.). In this case, none (0%) of the antibody-treated targets were killed by 1X-11-6 TAM supernatants. In contrast, 65% of the corresponding untreated target cell cultures exhibited killing. These targets were pulsed with prestimulated 1X-11-6 TAM supernatants but had not been pretreated with anti-TNF-α antisera. Similar results were obtained with MC-4 TAM supernatants. Here, only 2% of antibody treated target wells contained killed targets. In contrast, 68% of the untreated target wells exhibited killing. The fact that an insignificant amount (2%) of the antibody treated target wells showed killing probably reflects normal cell death or loss of viability.

TABLE III

SUMMARY OF THE EFFECTS OF WHOLE ANTI-TNF-α ANTISERA ON THE TNF ACTIVITY OF MC-4 AND 1X-11-6 TAM

TAM	Treatment[a]	% Killed[b]
MC-4	anti-TNF-α	2
MC-4	Rα-BP	83
MC-4	Control	68
1X-11-6	anti-TNF-α	0
1X-11-6	Control	65

[a]Targets were: 1.) incubated with LPS-prestimulated TAM supernatants only (control). or 2.) pretreated with 10 µl of 10^{-1} dilute whole Rabbit anti-Bovine Serum [to test whether cRPMI medium components such as fetal calf serum will block killing] then subsequently incubated with LPS prestimul-lated TAM supernatants (Rα-Bs). or 3.) pretreated with anti-TNF-α antisera (2000 neutralizing units/ml) [to show that TNF was secreted by TAM and to determine if TNF was solely responsible for MC-4 and 1x-11-6 TAM tumoricidal activity] then subsequently incubated with LPS-prestimulated TAM supernatants

[b]% Killing = Fraction of responding wells x 100 % = 1-F_0 x 100 %; where F_0 is the fraction of nonresponding wells.

However, it is more likely that the antibody failed to bind to some of the targets. The results gave direct evidence that killing of targets by TAM supernatants was due solely to the secretion of TNF by prestimulated TAM. These results also correlate favorably with results of prior experiments in which 65-67% of the target wells responded after treatment with LPS-prestimulated TAM supernatants. Also, the results suggested that 83% of the target wells, that were pretreated with Rα-BS antisera then pulsed with MC-4 TAM supernatants, exhibited killing. This comparison suggested that an additional 15% of the R-α-BS pretreated target wells (83%-68%) showed killing. Since Rα-BS could have bound to components of fetal calf serum (FCS) in the cRPMI medium and, since FCS components should account for at least some of the the growth enhancement activity of the medium, it is possible that Rα-BS may have blocked some of this growth enhancement activity. Rα-BS antisera may have therefore acted to synergistically enhance the effects of TNF.

Response of 2/F2 Tumor Targets to Supernatants from 1X-11-6 TAM that had been Prestimulated with Various Doses of IFN-γ

The second major goal of this study was to estimate the cytotoxic activity of tumor associated macrophages in response to LPS, macrophage-colony stimulating factor (M-CSF) and interferon-gamma (IFN-γ). Optimal concentrations of LPS and M-CSF that were required to activate TAM had been previously established (14, 20). However, the required optimal dose of IFN-γ needed to be established. This was accomplished by utilizing an MTT cytotoxicity assay. Briefly, 1X-11-6 TAM were arbitrarily chosen and used as effector cells. These were isolated by the adherence technique and prestimulated with either LPS (5 μg/ml) or concentrations of IFN-γ which ranged

from 20 to 20,000 units/ml. Prestimulated TAM were then aliquoted, at 200 cells/well, into effector plates. In addition, a duplicate effector plate was used to eliminate the possibility that product (IFN-γ) carryover would kill 2/F2 tumor targets. The duplicate plate contained TAM that had been prestimulated with 200 units/ml of IFN-γ. However, the wells of this duplicate plate were thoroughly washed after 5 hours of incubation with sterile PBS to remove IFN-γ. At the same time, 2/F2 tumor target cells were aliquoted, at 1.25×10^4 cells/ml, into corresponding target plates. As in previous experiments, additional wells of the target plates were aliquoted with target cells or cRPMI medium and used as controls to establish a mean absorbance response line and absorbance baselines. Effector and target plates were then incubated at 37^0 C. After 24 hours of incubation, target wells were pulsed with supernatants from corresponding effector plates. Also, an additional target plate was pulsed with supernatants from the washed effector plate. Target plates were then incubated for 48 hours, an MTT assay was performed and the absorbance data collected.

Dose response analysis of the absorbance data showed that the mean absorbance of target wells decreased significantly when they were treated with supernatants from TAM that were prestimulated with increasingly higher doses (200-20,000 units/ml) of IFN-γ (Figure 20.). Comparison of the results showed that supernatants, produced by TAM that had been prestimulated with a low dose (20 units/ml) of IFN-γ, had little or no effect on 2/F2 targets. In fact, this observation compares closely with the results for the baseline controls. The mean absorbance of these control wells, was 0.587. However, at higher doses (≥ 200 units/ml), mean absorbance of target wells decreased dramatically as the concentration of IFN-γ increased. Also, IFN-γ itself does not kill tumor target cells because the mean absorbance of target wells, that

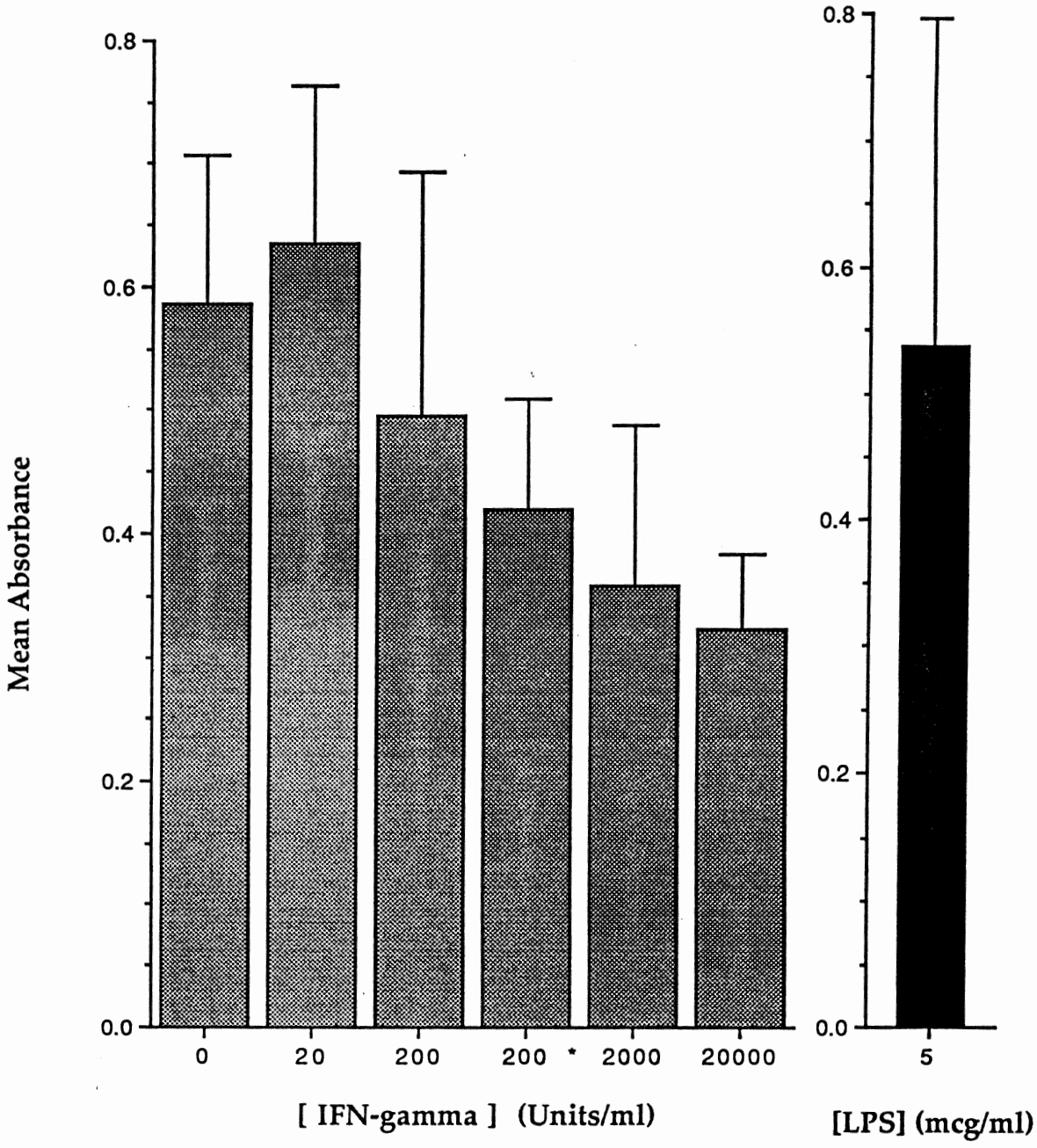

Figure 20. Dose Response Assay of the Effects of Supernatants from IFN-gamma-Prestimulated 1X-11-6 TAM on 2/F2 Tumor Targets.

had been pulsed with supernatants from the washed effector plate (200*), was lower than the mean absorbance of target wells that were pulsed with supernatants from the corresponding unwashed effector plate. Here, a comparison of the results indicated that supernatants from the washed plate are more efficient at killing target cells. However, it is more likely that membrane-associated TNF was not removed when the IFN-γ was washed from the wells of the duplicate effector plate. In this case, the additional TNF, that could have accumulated during the extended incubation period (5 hours), may account for the observed additional decrease in target well mean absorbance. The optimum concentration of IFN-γ was determined to be 2,000 units/ml. At this concentration, mean absorbance was reduced by almost 40% (0.587 - 0.358/0.587 X 100%). At the highest concentration of IFN-γ (20,000 units/ml), there was only a slight reduction in target well mean absorbance. In this case, mean absorbance only decreased an additional 3.5%. However, this slight decrease required a 10-fold increase in the concentration of IFN-γ and did not merit using the highest concentration as an optimal dose.

Response of 2/F2 Tumor Targets to Supernatants from LPS, M-CSF and IFN-γ-prestimulated 1X-11-6 TAM

The final experiment in this study was designed to estimate the cytotoxic activity of prestimulated TAM. Again, 1X-11-6 TAM were arbitrarily chosen and used as effector cells. To determine their cytotoxic activity, 1X-11-6 TAM were prestimulated with LPS, M-CSF and IFN-γ. These were used, alone or in specific combinations, to stimulate the secretion of TNF. This experiment was performed in much the same manner as the previous experiment. However, a washed duplicate efffector plate, to test for product carryover, was not needed and, was not used. TAM were prestimulated with single or

combination doses of M-CSF (10,000 Units/ml), IFN-γ (2,000 Units/ml) and LPS (5 μg/ml) prior to incubation. Prestimulated TAM were then aliquoted into effector plates and incubated for 24 hours. Wells of target plates, containing 1.25×10^4 2/F2 cells/ml, were then pulsed with corresponding effector (TAM) supernatants, incubated 48 hours, pulsed with MTT and, following a 6 hour reincubation period, solubilized with isopropanol-HCL. Target well absorbance data were subsequently read and analysed.

The results of this experiment, summarized in Table IV, showed that prestimulation of 1X-11-6 TAM with LPS, M-CSF and IFN-γ induces the production of supernatants with the greatest cytotoxic activity (see Table IV). The cytotoxic activity of prestimulated TAM was estimated by calculating the percent change (decrease) in mean absorbance. Comparative analysis of the cytotoxic activities of TAM supernatants indicated that prestimulation of 1X-11-6 TAM with LPS, M-CSF and IFN-γ produces a 35% increase in cytotoxic activity. In contrast, cytotoxic activity increased by only 3.3% when TAM were prestimulated with LPS alone. M-CSF alone was only slightly more effective than LPS in increasing cytotoxic activity of TAM. Here, M-CSF only increased the cytotoxic activity of TAM by 4%. This slight increase seems reasonable since M-CSF may stimulate the proliferation of TAM as well as TNF-secreting subsets of TAM. In fact, M-CSF, alone or combined with other factors, seemed to have only a slight additive effect on the increase in the cytotoxic activity of TAM supernatants. In comparison, when 1X-11-6 TAM were prestimulated with IFN-γ there was a corresponding 27% increase in cytotoxic activity. Comparison of the results, generated by IFN-γ-prestimulated TAM with those generated by LPS-prestimulated TAM, indicated that IFN-γ was 8 times more efficient at increasing the cytotoxic activtiy of 1X-11-6 TAM. It seemed clear that IFN-γ was a powerful stimulant of the cytotoxic activity of TAM.

TABLE IV

SUMMARY OF THE EFFECTS OF LPS, M-CSF AND IFN-γ ON THE CYTOTOXIC ACTIVITY OF 1X-11-6 TAM SUPERNATANTS

Treatment[a]	Mean Absorbance[b]	Cytotoxic Activity[c] (% Δ Mean Absorbance)
Control[1]	.296	0.0
Untreated[2]	.277	0.0
LPS	.268	3.3
M-CSF	.266	4.0
LPS +M-CSF	.249	10.1
M-CSF+IFN-γ	.208	25.0
IFN-γ	.202	27.1
IFN-γ +LPS	.186	33.0
LPS+M-CSF+IFN-γ	.180	35.0

[a] Targets were: 1.) Incubated with cRPMI or 2.) Pulsed with non-prestimulated 1X-11-6 TAM supernatants or 3.) Pulsed with prestimulated 1X-11-6 TAM supernatants.

[b] Mean Absorbance = total absorbance/60

[c] Cytotoxic activity (% Δ Mean Absorbance) was estimated by calculating the percent change (decrease) in mean absorbance

where; Cytotoxic activity = % Δ Mean Absorbance

$$\% \Delta \text{ Mean Absorbance} = \frac{\text{Untreated Mean Abs - Treated Mean Abs}}{\text{Untreated Mean Abs}} \times 100\ \%$$

[1] Control: targets were incubated with cRPMI medium only.

[2] Untreated: targets were pulsed with non-prestimulated 1X-11-6 TAM supernatants.

However, it is not clear whether IFN-γ simply stimulated an increase in TNF secretion by existing TNF-secreting TAM subsets or stimulated additional 1X-11-6 TAM to secrete TNF or both. It is also possible that IFN-γ induced TAM to produce other, unreported, factors that may have rendered 2/F2 targets more susceptible to the normal cytotoxic activity of TAM.

Summary of Results

The results of this study show that, in the absence of cell to cell contact, both splenic and tumor associated macrophages must be prestimulated to exhibit significant cytotoxic activity. The results also indicate that rare subsets of 1X-11-6 tumor cells enhance the growth of TNF-sensitive 2/F2 tumor target cells (Figure 6.). This evidence suggests that tumor cells may have the capacity to enhance the proliferation of other cell types, including TAM, in vivo. Also, the results show that rare subsets of splenic macrophages and TAM are capable of TNF secretion after prestimulation with LPS. The frequencies of both normal and tumor associated types of TNF-secreting macrophages are strikingly similar. This similarity suggests that TAM are probably not derivatives of elicited macrophage populations. Furthermore, the complete blocking of TNF activity with anti-TNF-α antibody provides direct evidence that TNF is secreted by prestimulated MC-4 and 1X-11-6 TAM and is solely responsible for their cytotoxic activity. In addition, the prestimulation of TAM with single and combination doses of LPS and M-CSF slightly enhances the cytotoxic activity of TAM. Prestimulation of TAM with IFN-γ, alone or in combination with LPS and M-CSF can greatly enhance the secretion of TNF and, therefore, the cytotoxic activity of TAM.

CHAPTER IV

DISCUSSION

The results of this study show that an average of approximately 1/180 1X-11-6 TAM, MC-4 TAM and murine splenic macrophages secrete tumor necrosis factor and are threrefore cytotoxic for tumor target cells. These results provide the first reported estimates of the frequency of TAM that secrete TNF. The determination of these frequencies is important because the existence of low frequencies of cytotoxic TAM suggests the existence of low effector to target ratios in vivo. It is therefore reasonable to conclude that, in the presence of functionally diverse cell populations within rapidly growing tumors and, in the absence of stimulation, the frequency of cytotoxic TAM would be too low to facilitate tumor rejection in vivo.

In other respects, the results of this study are consistent with previous observations and show that, in the absence of cell to cell contact, both murine splenic macrophages and tumor associated macrophages must be induced to secrete TNF (2, 27, 28). Bacterial LPS has been previously shown to stimulate the tumoricidal or cytotoxic activity of normal and tumor associated macrophages (27). Also, McIntosh et al have shown that stimulation of macrophage cytotoxic activity is a multistage process in vivo (21). Macrophages exhibit little or no cytotoxic activity in the absence of stepwise exposure of macrophages to activating factors, such as IFN-γ and LPS (21). In this study, the cytotoxic activity of experimental macrophages was enhanced

significantly in the presence of these factors and adds supporting evidence to McIntosh's data.

Our results also show that rare subsets of 1X-11-6 tumor cells enhance the growth of TNF-sensitive 2/F2 tumor target cells (Figure 6.). Approximately 1/825 of these tumor cells were capable of enhancing the growth of targets. This evidence suggests that tumor cells have the capacity to enhance the proliferation of other cell types, including TAM. It is possible that the observed enhancement of 2/F2 target cell growth could have been produced by contaminating cell types. However, since the purity of the 1X-11-6 tumor cells was not determined, the results of this experiment are reasonable because single-hit kinetics were observed. Also, since the 1X-11-6 tumor is a fibrosarcoma, the results presented here may further support a previous report which suggests that fibroblasts produce potent colony stimulating factors (25). In fact, a more recent report shows that M-CSF activity was detected in 1X-11-6 and MC-4 tumor cell supernatants and, in addition, these supernatants dramatically enhanced the proliferative response of 1X-11-6 and MC-4 TAM targets (20).

In order to determine the frequency of cytotoxic macrophage subsets within experimental populations, it is evident, from the results generated in this study, that macrophages must be isolated in high purity. In this regard, the method of isolation seems to be the most critical parameter. Isolation of TAM by density gradient centrifugation over ficoll-hypaque produced experimental TAM populations that were, approximately 80% pure. In this case, single-hit kinetics were not observed and the frequency of cytotoxic TAM could not be determined. In contrast, TAM that were isolated by MBHR and adherence methods were at least 90% pure. Here, isolates were pure enough

to allow for the determination of the frequency of TNF-secreting macrophage subsets by limiting dilution analysis.

Two noteworthy observations were encountered in this study. First, as previously mentioned, the percent purity of macrophage isolates depended on the method of isolation and was determined (in part) by SRBC rosetting. The percent of macrophages which rosetted opsonized SRBCs was, on average, approximately 60-65%. In sharp contrast, the percent of these cells, that were morphologically identified as macrophages, varied considerably. In this case, the purity ranged from 75-80% for density gradient isolates to approximately 95% for the adherence isolates. This observation suggests that, although as many as 95% of the isolates were morphologically identified as macrophages, approximately 40% of the isolates were incapable of rosetting SRBCs. This indicates that SRBC rosetting is not a good method of determining the percent purity of experimental macrophage populations. Since macrophages are known to fuse with other macrophages and form multinucleated cells it is possible that TAM, which do not express Fc receptors (FcR^-), are actually the fusion products of TAM and transformed cells. Also, since TAM reside in an abnormal environment, it may be possible that FcR^- TAM are transformed macrophages. Of course, it is more likely that FcR^- TAM actually represent TAM with reduced FcR avidity. In fact, evidence reported by Moore and McBride suggests that less mature TAM exhibit lower FcR avidity and this avidity varies from tumor to tumor (15, 16). It is reasonable to speculate that the frequency of TAM with low FcR avidity will therefore dictate the percent of TAM that can be identified by the SRBC rosetting technique. TAM might be isolated in higher purity by binding antibody to a cell surface marker (i.e. F4/80 or MAC-1) that is common to all macrophages and using fluorescence activated cell sorting techniques (FACS).

Once pure (≥ 99%) macrophage populations are isolated, they could be tested for purity by the SRBC rosetting technique. If an average of 40% of these FACS isolates do not rosette opsonized SRBCs, the results would suggest that a significant proportion do not possess Fc receptors or, at least, have low FcR avidity. This might also suggest that the TAM populations used in this study would be expected to contain FcR^-, FcR^+ and low avidity FcR^+ TAM subsets. Of course these may or may not have affected the results of this study.

The second noteworthy observation here was that, even though 1X-11-6 and MC-4 TAM are diverse in etiology, both had virtually the same capacity to secrete TNF. This observation suggests that the results of this study may apply to a wide range of tumors. Also, less than 1% of experimental MC-4 and 1X-11-6 TAM populations secreted TNF and, in addition, significant quantities of TNF were only secreted after TAM had been prestimulated with LPS or other factors. This observation implies an absence of recognition of the tumor by the host immune system. However, it should be noted that the TAM used in this study were from rapidly growing (progressive) tumors. Therefore, the evidence presented here may not apply to tumors in early stages of development. In fact, Burnham has shown that in vivo tumor establishment is antagonized by the presence of macrophages and, in contrast, macrophages augmented the growth of tumors at later stages of progression (Dr. Kim Burnham, unpublished results). Additional reports show that TAM secrete growth factors (7, 15), and, enhance the growth of some established tumors in vivo (15). Burnham and Reed have shown that super-natants from MC-4 and 1X-11-6 tumors stimulate the in vitro proliferation of their respective TAM (20). Since the results of our study suggest that the frequencies of TNF-secreting MC-4 and 1X-11-6 TAM are too low to inhibit tumor growth, it seems reasonable to conclude that, during progression, TAM

and their tumor hosts may achieve a stable yet complex functional relationship that is mutually beneficial.

The results of this study also shows a striking similarity in the frequencies of 1X-11-6 and MC-4 TAM as well as normal splenic macrophage subsets which secrete TNF. This similarity suggests that an ontogenic relationship may exist between these etiologically distinct macrophages. These experimental macrophage subsets may therefore have arisen from the same circulating monocyte pool. Also, in contrast to inflammatory macrophages, which are elicited during an immune response and fully activated for cytotoxic activity, the macrophages used in this study secrete significant quantities of TNF only after prestimulation. This evidence suggests that 1X-11-6 and MC-4 TAM are not derivatives of elicited macrophage populations. In fact, Burnham and Reed have shown that MC-4 and 1X-11-6 TAM exhibited the proliferative characteristics of resident macrophages and, therefore, do not behave like elicited macrophages (20).

It is clear that higher concentrations ($\geq$ 200 cells/well) of macrophage isolates may contain cells that produce factors which block or override the cytotoxic activity of macrophages. In most cases, where the frequency of TNF-secreting TAM was determined, the LDA results of this study showed that nearly all cytotoxic activity was lost at the highest concentrations of macrophages. Contaminating cells may be macrophage subsets which produce factors that enhance the growth of 2/F2 targets or block the effects or production of TNF by prestimulated macrophage subsets. This seems reasonable since Moore and McBride have shown that TAM exhibit functional heterogeneity (16). This heterogeneity may help account for the loss in cytotoxic activity because TAM are known to produce a variety of growth enhancing factors (7, 14). In contrast, macrophages are also capable of

producing prostaglandin E2 (PGE2) which is suspected of playing a role in down-regulating TNF production (11). Indeed, it is possible to test for the involvement of PGE2 by adding indomethacin to TAM cultures to inhibit PGE2 production. The restoration of cytotoxic activity in such a way would suggest that PGE2, produced by TAM subsets, is capable of regulating TNF production. Inhibition of TNF production by contaminating tumor cells could also explain the observed loss of TAM cytotoxic activity. In fact, Rybski and Scuderi et al have shown that that tumor supernatants do inhibit TNF production (23). However, the results presented in this study show that a frequency of approximately 1/825 1X-11-6 tumor cells can enhance the growth of sensitive 2/F2 tumor targets. Assuming that the loss of cytotoxic activity was due to enhancement of target cell growth and, since macrophage cytotoxic activity was almost totally lost at concentrations of 400 cells per well, it seems that this concentration would not contain enough contaminating tumor cells to cause the the observed loss in cytotoxic activity. One way to determine what cell types are causing this loss would be to use 100% pure experimental macrophage populations in future studies. As discussed earlier, this would probably require the investigator to use a cell surface marker that is common to all macrophages. If the cytotoxic activity of supernatants, produced by pure macrophage populations, is still lost at high cell concentrations ($\geq$ 200 cells/well), the results would indicate that diverse macrophage subsets are responsible for the loss in cytotoxic activity. Of course, if cytotoxic activity is restored, the results would indicate that sufficient numbers of other contaminating cells are present.

This study also showed that the cytotoxic effects of TAM supernatants were completely blocked when 2/F2 tumor targets were initially pretreated with anti-TNF-α antisera. This provided direct evidence that TNF is secreted

by prestimulated MC-4 and 1X-11-6 TAM and is solely responsible for their observed cytotoxic activity. It also lends support to evidence presented in a separate study by Lord and Wilson et al. They howed that the cytotoxic activity of EMT6 TAM supernatants was also blocked by anti-TNF antibody (33). In our study visual observations showed that many of the target wells, that had been pulsed with 200 cell/well TAM supernatants, exhibited marked decreases in cell density. Since TNF has been shown to alter target cell metabolism by suppressing lipoprotein lipase activity, and since the enzymes associated with energy metabolism (i.e. NADH) are known to convert MTT (used in our assays) to formazan product, these observations indicate that target cells are killed by TNF and not simply metabolically suppressed. It is interesting to note that whole rabbit anti-bovine serum (Rα-BS) was used in this portion of our study to test whether components of the cRPMI medium are capable of blocking TNF activity. The results showed that 83% of the target wells, that were pretreated with anti-bovine serum, exhibited killing. In contrast, only 68% of the those target wells, that were not pretreated with R-α-BS antisera prior to the addition of TAM supernatants, exhibited killing. This comparison suggests that an additional 15% of the R-α-BS pretreated target wells showed killing. It is possible that targets were simply more sensitive to the effects of TNF in the presence of R-α-BS. However, since Rα-BS may have bound to specific fetal calf serum (FCS) components in the cRPMI medium, it is more likely that Rα-BS may have blocked the growth enhancement or anti-TNF activity of these components. The results therefore support the finding that human serum, in the presence of super-natants from fibroblast and tumor cell cultures, suppresses the secretion of TNF by blood mononuclear cells (23). R-α-BS may therefore have acted to seemingly enhance or intensify the secretion of TNF. In this regard, it is

possible to test whether R-α-BS antiserum can bind to FCS components and block their growth enhancement properties. This can be resolved by performing an experiment in which 2/F2 target plates are incubated in the presence of FCS^+ and FCS free medium. In this case, half of the target plates would be treated with R-α-BS antiserum. MTT assays could then be performed on the target plates. If analysis of the data shows that growth enhancement of target cells does occur but is substantially reduced in the presence of R-α-BS antiserum the results would show that the antiserum does block the activity of FCS components and may account for the 15% increase the target cell killing generated by TAM supernatants.

Additional data generated in this study, show that the cytotoxic activity of TAM was substantially increased in the presence of recombinant forms of IFN-γ and M-CSF. IFN-γ and M-CSF are known to stimulate TNF secretion by macrophages, (21, 22). However, in our study M-CSF was only slightly more effective than LPS at enhancing the cytotoxic activity of TAM. In sharp contrast, recombinant IFN-γ, alone or in combination with LPS and M-CSF, stimulates a significant increase in TAM cytotoxic activity. Although TAM have recently been shown to express M-CSF receptors on their surface (22), the results of this study represent the first comprehensive report of the enhancement of TAM cytotoxic activity in the presence of M-CSF and IFN-γ. Since both prestimulated normal and tumor associated macrophages have been shown to secrete TNF (14, 33) and, since the results presented in this study provided direct evidence that TAM can be induced to secrete TNF, it is reasonable that the cytotoxic activity of TAM, as described above, is accurate. The mean absorbance values, generated in the MTT assay, are proportional to target cell viability. Cytotoxic activity was measured, in this case, by calculating the percent change in the mean absorbances of untreated versus

treated target wells. It is interesting to note that only an additional 8% increase in the cytotoxic activity of 1X-11-6 TAM was observed when TAM were prestimulated with LPS, M-CSF and IFN-γ. This 8% increase in cytotoxic activity represents a rather small increase over the cytotoxic activity observed for IFN-γ alone. This leveling off suggests that TAM may reach a cytotoxicity threshold. Above this threshold, it is possible that no additional TAM can be induced to secrete TNF. Of course, it is also possible that no additional TNF can be secreted by existing TAM. Additional assays may be used in future correlative studies to test whether IFN-γ, M-CSF or other factors induce increases in the frequency of TNF-secreting TAM subspecies or simply induce existing subsets to increase their secretion of TNF. In fact, to determine the cytotoxic activity of TAM, a cytokine specific immunospot (ELISPOT) assay (24, 28) may offer a practical alternative to the LDA and MTT assays that were used in this study. The ELISPOT assay is derived from the enzyme-linked immunosorbant assay or ELISA. However, unlike the ELISA, the ELISPOT assay is run in the solid phase. Briefly, macrophages can be allowed to adhere to the bottom of tissue culture-treated plastic dishes. Contaminating (tumor) cells would then washed from the dishes. After washing, TAM can be harvested, washed, resuspended in medium and counted using a hemacytometer. Macrophages can then be allowed to adhere to the bottom of culture dishes or the wells of 96 well microtiter plates, incubated in the presence of prestimulating factors (i.e. LPS and IFN-γ) and allowed time to produce TNF. An appropriate anti-TNF-α antibody could then be added to the culture dishes or plate wells and allowed to bind to secreted TNF. This antibody would serve as the primary (1^{o}) antibody. After completion of the initial stages of the assay using ELISA techniques, soluble substrate can then be suspended in molten but cooled (37^{0} C) agarose gel and added to the plate. Sufficient time

would then be allowed for the gel to harden and for the enzyme-substrate reaction to occur. The enzyme-substrate reaction results in the production of a visible colored product wherever macrophages have produced TNF. Since the total number of adherent macrophages would have been previously determined, the frequency of TNF-secreting TAM could then be calculated by determining the number of colored spots. In addition, the change in both the frequency of TNF-secreting TAM as well as changes in the cytotoxic activity of TAM, in response to LPS, IFN-γ and other factors, could be determined. Here, changes in frequencies can be determined by visual inspection. Also, since the diameter of the colored spots is proportional to the amount of TNF secreted, changes in cytotoxic activity could be determined by calculating the area of each spot. Changes in the amount of TNF produced or the number of TAM that secrete TNF would naturally be expected to be a function of the type of factors used to prestimulate macrophages. In future studies, it may also be possible to use the ELISPOT to test the accuracy of the data generated in this study and, at the same time, to determine whether their are any inherent, yet unidentified, problems with this study.

There were several potential problems of importance encountered in this study. The first potential problem is the possibility that removal of adherent macrophages from culture dishes may have somehow affected the overall functional performance of these effector cells. Macrophages may have been affected in such a way that an accurate assessment of the frequency of TNF-secreting macrophages and their cytotoxic capabilities, in vivo, may not be precisely portrayed. However, the results, generated with both adherence and MBHR-isolated TAM, proved to be virtually indistinguishable. This seems to indicate that neither macrophage isolation technique has any great effect on macrophage functions. In fact, it wasn't until macrophages were isolated in

relatively pure form, using these techniques, that it became possible to determine the frequencies of TNF-secreting macrophage subsets.

Since bacterial LPS stimulates the production of TNF by macrophages and, since it is ubiquitous in nature, the second potential problem with this study is the possibility that the cRPMI medium may have inherently contained small amounts of LPS. Contamination of the media with LPS could induce macrophages to secrete additional TNF. This may result in an increase in cytotoxic activity and may not accurately represent the true cytotoxic capability of TAM. It is possible to use an ELISPOT or MTT assay to test for the presence of ubiquitous TNF in the growth medium. The growth medium, used to culture IFN-γ-prestimulated macrophages, could be pretreated with anti-LPS antiserum. In this case, a decrease in cytotoxic activity would indicate the presence of LPS in the medium. Steps could then be taken to help insure the exclusion of ubiquitous LPS from the media. This might best be accomplished by taking extra precautions in the preparation of growth medium prior to use. Of course, it might be possible to use these techniques in conjunction with anti-LPS antiserum to establish the amount of additional cytotoxic activity that is attributed to ubiquitous LPS. This additional activity (if any) could then be accounted for when the cytotoxic activity of macrophages is calculated.

The Mean absorbance values of controls varied from experiment to experiment and may be an additional source of error in this study. Variations in mean absorbance values may be due to the fact that outer wells tended to dry out during incubation. These wells generally tended to have slightly higher absorbance values than wells near the center of the target plates. However, since it was not always possible to use fresh medium and since medium components have a finite half-life and lose activity over time it is

conceivable that, the variations in mean absorbance of controls may be the indirect result of the loss of growth enhancement activity of stored medium.

To summarize, this study represents the first reported evidence in which the frequency of TNF-secreting TAM subpopulations has been established. The determination of these frequencies shows that only rare subsets of both TAM and splenic macrophages are capable of secreting TNF. Direct evidence has also been presented which shows that prestimulated TAM do indeed secrete TNF and this secreted TNF is solely responsible for the killing of sensitive 2/F2 tumor targets. Also, the data suggest that the cytotoxic activity of TAM , in response to LPS, M-CSF and IFN-γ, can be greatly enhanced. Since IFN-γ and M-CSF are naturally occurring cytokines, the increased enhancement of the cytotoxic activity of TAM in response to individual or combined doses of these cytokines may prove to be an effective approach to cancer immunotherapy in vivo.

LITERATURE CITED

1. Adams, D.O., Johnson, W.J., Florito, E., and Nathan, C.F. 1981. Hydrogen peroxide and cytolytic factor can interact synergistically in effecting cytolysis of neoplastic targets. J. Immunol., 127, 1973.

2. Adams, D.O., and Nathan, C.F. 1983. Molecular mechanisms operative in cytolysis of tumor cells by activated macrophages, Immunol. Today, 4: 166.

3. Aggarwal, B.B., Eessalu, T.E., and Hass, P.E. 1985. Characterization of receptors for human tumor necrosis factor and their regulation by γ-Interferon, Nature, London, 318: 665.

4. Balkwill, Francis R., B.G. Ward, E. Moody and W. Friers. 1987. Theraputic potential of tumor necrosis-a and g-interferon in experimental human ovarian cancer. Cancer Research, 47: 4755-4758.

5. Beutler, B. and A. Cerami. 1986. Cachectin and tumor necrosis factor as two sides of the same biological coin, Nature, 17: 584-588.

6. Dougherty, G.D. and McBride, W.H.1986. Accessory cell activity of murine tumor associated macrophages, J. Natl. Cancer Inst. 76: 541-548.

7. Duel, T.F. and Huang, J.S. 1984. Platelet derived growth factor: structure function and roles in normal and transformed cells, J. Clin. Invest., 74: 669-676.

8. Espevik, T., and Nissen-Meyer, J. 1987. Tumor necrosis factor-like activity on paraformaldehyde fixed monocyte monolayers, Immunology, 61: 443.

9. Fulton, A.M. 1988. Tumor associated macrophages. Macrophages and Cancer, (ed., Heppner, G.H. and A.M. Fulton), pp. 97-111.

10. Keller, R. 1976. Susceptibility of normal and transformed cell lines to cytostatic and cytocidal effects exerted by macrophages, J. Nat. Cancer Inst. 56: 369-374.

11. Kunkel, S.L., Wiggins, R.C., Chensue, S.W. and Larrick, J. 1986. Regulation of macrophage tumor necrosis factor production by prostaglandin E2. Biochem. Biophys. Res. Commun. 137: 404.

12. Lattime, E.C., A. Stoppacciaro, and O. Stutman. 1988. Limiting dilution analysis of TNF producing cells in C3H/HeJ Mice, J. Immunol., 141: 3422-3428.

13. Lefkovits, I., and H. Waldmann. 1979. Limiting Dilution Analysis of cells in the immune system. Cambridge University Press.

14. Mantovani, A., et al. 1986. Origin and regulation of tumor associated macrophages: the role of tumor-derived chemotactic factor, Bioch. et Biophys. Acta, 865: 59-67.

15. McBride, W.H. 1986. Phenotype and functions of intratumoral macrophages. Bioch. et Biophys. Acta 865 : 27-41.

16. Moore, K. and McBride, W.H. 1980. The activation state of macrophage subpopulations from a murine fibrosarcoma, Int. J. Cancer 26: 609-615.

17. Mosmann, T. 1983. Rapid Colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods, 65: 55-63.

18. Nissen-Meyer, J., R. Austgulen and T. Espevik. 1988. Synergistic growth-inhibitory activity of tumor necrosis factor and alpha interferon: contribution to the monocyte-derived cytostatic activity towards human leukemia K562 cells, Scand. J. Immunol., 28: 627-637.

19. Ralph, P., and I. Nakoinz. 1987. Stimulation of macrophage tumoricidal activity by the growth and differentiation factor CSF-1. Immunol. 105: 270-279.

20. Reed, Doug and Kim Burnham. 1991. Tumor associatd macrophages share in vitro growth characteristics with resident but not elicited macrophages, J. Leuk. Biol., In press.

21. Russell, S.W., Doe, W.F., and McIntosh, A.J. 1977. functional characterization of a stable noncytolytic stage of mcrophage activation in tumors. J. Exp. Med., 146 : 1511.

22. Sampson-Johannes, A., and J.A. Carlino. 1988. Enhancement of human monocyte tumoricidal activity by recombinant M-CSF. J. Immunol. 141:3680-3686.

23. Scuderi, P., R.A. Rippe, A.B. Raitano, and J. Rybski. 1989. Human sera and culture supernatants from human tumors and diploid fetal fibroblasts suppress tumor necrosis factor in vitro. J. Leuk. Biol., 46: 34-40.

24. Sedgwick, J.D. and P.G. Holt. 1983. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. J. Immunol. Methods, 57: 301-309.

25. Stanley, E.R. 1981. Colony stimulating factors. In The Lymphokines, (ed., Hadden, J.W., and W.E. Stewart), pp. 101-132.

26. Steel, R.G.D., Torrie, J. H. Linear Regression. In Principals and Procedures of Statistics: A Biometrical Approach, Mcgraw Hill, New York, NY. 1980, pp. 239-269, 597.

27. Stewart, C.C., A.P. Stevenson and J. Hibbs. 1988. Effector mechanisms for macrophage-induced cytostasis and cytolysis of tumor cells. In Macrophages and Cancer, (ed., Heppner, G.H., and A.M. Fulton), pp. 39-59.

28. Takashi, T and K. Hiroshi et al. 1990. Detection of individual mouse splenic T cells producing IFN-γ and IL-5 using the enzyme-linked immunospot (ELISPOT) assay. J. Immunol. Methods, 128: 65-73.

29. Takemura, R., and z. Werb. 1984. Secretory products of macrophages and their physiological functions, Am. J. Physiol. 246: C1-C9.

30. Urban, J.L., H.M. Shepard, J.L. Rothstein, B.J. Sugarman, and H. Schreiber. 1986. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages, Proc. Natl. Acad. Sci. USA, 3: 5233.

31. van Furth, R., Z.A. Cohn, J.G. Hirsch, J.H. Humphrey, W.G. Specter, and H.L. Langevoort. 1972. The mononuclear phagocytic system: a new classification of macrophages, monocytes and their percursor cells, WHO Bulletin 46: 845-852.

32. van Furth, R. 1981. Identification of mononuclear phagocytes: overview and definitions. In Methods for Studying Mononuclear Phagocytes, (ed., Adams, D.O., P.J. Edellson, and H.Koren), pp. 243-252.

33. Wilson, K.M., Siegal, G., and Lord, E.M. 1989. Tumor necrosis factor-mediated cytotoxicity by tumor associated macrophages, Cellular Immunol., 123: 158-165.

VITA

Richard D. Potts

Candidate for the Degree of

Master of Science

Thesis: DETERMINATION OF THE FREQUENCY AND CYTOTOXIC ACTIVITY OF TNF-SECRETING SUBPOPULATIONS OF TUMOR ASSOCIATED MACROPHAGES

Major Field: Microbiology

Biographical:

Personal Data: Born in Nashville, Tennessee on December 31, 1948 to Alton and Helen Potts.

Educational Data: Attended Central High School in Nashville, Tennessee. Received a GED Diploma in June, 1968; received a Bachelor of Science Degree in Microbiology from Oklahoma State University inMay, 1988. Completed requirements for a Master of Science Degree in Microbiology at Oklahoma State University in July, 1991.

Professional Experience: Teaching Assistant for undergraduate Microbiology and Immunology (seven semesters). Department of Botany and Microbiology, Oklahoma State University, August, 1988 to December, 1990.