Isolation and partial characterization of macrophages from bovine peripheral blood
by Goutam Mukherjee

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Science
Montana State University

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Goutam Mukherjee

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Macrophages were isolated from bovine peripheral blood and maintained in primary culture. They were cloned at 6th and 9th passage and maintained in culture. Various lymphokines were tested in vitro on the isolated and cloned cells for their ability to induce cytotoxic and antiparasiticidal activity against tumor cells (K562), Toxoplasma gondii and Eimeria bovis. The lines and clones stimulated with various lymphokines like Interferon-gamma, Tumor necrosis factor-alpha, Interleukin 2 and Concanavalin A supernatant, showed considerable cytotoxicity against tumor cells, T. gondii and E. bovis. Different clones varied widely in their activity against tumor cells and parasites. Nitroblue tetrazolium assay was performed to determine the level of oxidative burst in the lymphokine stimulated clones but no direct correlation could be established. Therefore it was supposed that mechanisms other than oxidative burst might be responsible in the killing process of tumors and parasites. Isolated macrophage line was observed to be heterogenous in their characteristics. Some of the clones like P9F4, and P9E4 were highly resistive to Toxoplasma growth without stimulation with any lymphokines. Other clones needed lymphokine stimulation to show any cytotoxicity. Some of the clones were refractory to any cytokine treatment, it may be so that they lacked the appropriate receptor. All the clones as a whole proved to be less favorable environment to Toxoplasma growth compared to the usual host cell line MRC5. The cells started to die out after around four months of continuous culture, probably due to the fact that they were a primary cell line.

INTRODUCTION

Monocytes and macrophages are important mediators of specific and non-specific immunity. They play important roles in antigen presentation during the immune cascade (1) as well as in host defense against a number of pathogens and tumor cells. Monocytes and macrophages are phagocytic, pinocytic and positively chemotactic, migrating toward dead and degenerating tissue (2). Monocytes can rapidly destroy intracellular pathogens such as bacteria, viruses and parasites (3,4).

Blood monocytes migrate to different tissues and transform into macrophages (5). Both monocytes and macrophages participate in host resistance against metastatic neoplastic cells, parasites and viruses (6).

Macrophages can be divided into three sub-populations based on their activation status. These are: resident (unstimulated), elicited, and activated. Resident macrophages are normal tissue macrophages which do not show the microbistatic ability of immune-activated macrophages (4,7). Elicited macrophages occupy an intermediate stage between resident and activated cells. They do not exhibit enhanced oxidative metabolism, but they will respond to certain receptor mediated stimuli such as interferon.

Many cytokines have been shown to have immunoregulatory effects on peripheral blood monocytes. These include tumor necrosis factor (TNF) alpha, the interferons (especially IFN-gamma), and many interleukins, of which interleukin-2 (IL-2) is best characterized. Following its identification and characterization, IL-2 has been given various names such as T cell growth factor (TCGF), thymocyte mitogenic factor (TMF) and killer cell helper factor (KHF). IL-2 was originally described as having an effect on T cell growth, but since then it has been found to be pleiotropic, affecting the generation and/or differentiation of stem cells, osteoblasts, B lymphoctes, natural killer (NK) cells, lymphokine activated killer cells and macrophages. Human IL-2 was first purified from culture supernatants of mitogen or alloantigen-activated T-cells and found to have molecular weights of 19-22 kd and 14-16 kd as determined by gel-permeation and SDS-PAGE respectively (8). The discrepancy in molecular weight was attributed to polymerization. In human the IL-2 molecule contains a single disulfide bond between amino acids 58 and 105, and chemical reduction of the bond or site-directed mutagenesis of these residues leads to loss of biological activity. Il-2 interacts with cells through receptors which might have high or low affinity. IL-2 appears to increase the oxidative metabolism of macrophages, but to a lesser magnitude than other cytokines.

IFN-gamma was first recognized for its antiviral activity

(9). IFN-gamma is pH 2 sensitive, whereas IFN-alpha and IFN-beta are not. The cDNA for human IFN-gamma was first isolated and characterized in 1981 (10). Prior to 1982 the molecular weight of IFN-gamma was thought to be 40,000-60,000 MW, but later Yip et. al. (11) showed that human natural IFN-gamma consists of two fractions: 20,000 and 25,000 Da which dimerize to give the biologically active form. Work with different animal systems has shown that the biological activity of IFN-gamma is strictly species specific. For example human IFN-gamma is inactive in the mouse or rat tumor model system. The murine IFN-gamma gene was isolated by screening a murine genomic-lambda library with the human cDNA sequence (12). The sequence homology between the murine and human IFN-gamma genes was 65%. The murine IFN-gamma gene was localized to chromosome 10 (13), which has several gene loci homologus to those found on human chromosome 12. The protein encoded by murine IFN-gamma gene shows only 40% homology with human IFN-gamma. Amino acid sequence for bovine and rat IFN-gamma have also been reported (14). The amino acid sequence of human, bovine, rat and murine IFN-gamma has very little homology which may account for the lack of cross reactivity. In contrast, closely related species such as the rat and mouse have 97% amino acid homology and rat IFN-gamma exhibits antiviral activity on murine cell lines.

In addition to its antiviral activity, IFN-gamma is an important immunomodulatory agent. It induces expression of

both class I and class II antigens of the major histocompatibility complex (15,14). IFN-gamma stimulates resting B cells to secrete antibody (16) and acts as a B cell differentiating agent (17). Resting macrophages sometimes require an initiation to exhibit cytotoxicity against tumor cell or intracellular parasites (18). IFN-gamma can induce elicited macrophages to respond to suboptimal concentrations of lipopolysaccharide (LPS,endotoxin), giving rise to enhanced tumor cell cytotoxicity (7). IFN-gamma can also act synergistically with lymphotoxin and TNF-alpha on macrophages to produce increased tumor cell lysis or parasite killing in vitro.

TNF-alpha was first described in 1975 by Carswell et al. (19) from serum of mice infected with Bacillus Calmette-Guerin (BCG). Most evidence indicates that TNF-alpha is produced by macrophages (20). When analyzed by reducing and non-reducing SDS-polyacrylamide gel electrophoresis, purified human TNF-alpha migrated as a single protein band at an apparent molecular weight of 17,000. Its isoelectric point is 5.3 and it is relatively insensitive to varying pH level and organic solvents but fairly sensitive to proteolytic enzymes (21). Both human and murine TNF-alpha share highly conserved amino acid sequences (over 79% homology) which may account in part for their cross reactivity. Both human and murine TNF-alpha have been cloned in yeast and the activities of the recombinant molecules have been shown to be similar to the

parent molecules. TNF-alpha induces expression of neutrophil antigens, enhances polymorphonuclear cell phagocytic activity and superoxide anion production, and acts synergistically with IFN-gamma in various cellular functions (21).

The differences in antimicrobial activity between resident and activated macrophages has been documented in many cases of intracellular parasitism. In murine Trypanosoma cruzi infection, trypomastigotes can escape from the phagosomes of resident macrophages and multiply free in the cytoplasm both in vivo and in vitro. Activated macrophages, however, can readily kill parasites in phagolysosomes before they escape to the cytoplasm (22).

Specific macrophage functions appear to vary in different experimental systems involving Toxoplasma gondii. Studies of monocyte-derived macrophages from different sources (alveolar, peritoneal and circulatory) indicate that macrophages can inhibit the growth of intracellular parasites by both oxidative and nonoxidative pathways (23,24). Non-oxidative macrophage activity involves the action of different peptides such as defensins and other small molecules (25). Such innate microbicidal ability of macrophages from different animals varies to a large extent (24). Studies with other coccidian parasites (e.g., Eimeria bovis) indicate further the importance of macrophages in protective immunity (26).

Macrophage activation can be brought about either with certain microorganisms or their products (endotoxins), or

through the action of macrophage-activating cytokines (27). Although IL-2, IL-3, TNF-alpha and granulocyte macrophage colony stimulating factor (GMCSF) can activate macrophages to kill tumors (28), parasites (29,37), viruses (14) and bacteria (14), IFN-gamma has been identified as the major macrophage activating cytokine to act against obligate intracellular parasites. Studies have recently identified the specific effects of these cytokines (22,30).

Normally, cytokines are secreted at the appropriate level for maintenance of normal immune functions. This, however, may not be the case in diseased or parasitized animals. In the acute phase of toxoplasmosis, there is augmented and then suppressed NK cell activity (31). The initial enhancement of NK cell activity is probably due to abnormal levels of IFN-gamma present systemically during the acute stages of infection (31).

Bovine coccidiosis, which is caused by Coccidian parasites of the genus Eimeria, causes major economic losses to farmers and ranchers in the United States (32,33). Of the 13 species of Eimeria that occur in the bovine, E. bovis and E. zuernii are by far the most pathogenic. According to the Veterinarians Annual Disease Report, coccidiosis appears to be the most important disease of Montana cattle caused by a single etiological agent (34). Bovine respiratory disease complex appears to be the most important disease of Montana cattle, but it has multiple etiologies (34). There is no

successful chemotherapeutic agent which can provide the host with long-lasting protection and no vaccine is yet available.

In case of obligate intracellular parasites, monocyte-macrophage mediated responses play a greater role (38). Both humoral and the cell-mediated immune responses are important in providing the host with protection against E. bovis (26,35,36). Recently lymphokines in supernatants from lectin-stimulated bovine T cells were found to be capable of inducing bovine macrophages to inhibit the multiplication of E. bovis and T. gondii in vitro (26,38). The active components of this crude T cell supernatant were neither GMCSF nor IFN-gamma, although 15% of its activity could be attributed to IFN-gamma. These studies indicate that this uncharacterized lymphokine may play an important role in immunity against E. bovis.

Hughes et al. (31) suggested that cytotoxic cells (T_C and NK) do not play a major role in host defense against Toxoplasma induced by oral parasitic inoculation. However, it is generally accepted that T cells play a fundamental role in protection against Toxoplasma which is mediated at least in part by lymphokines released by T helper cells. This could partially explain why Toxoplasma is such an important opportunistic pathogen of acquired immune deficiency (AIDS) patients in which infection of $CD4^+$ cells by HIV, effectively nullifies protection against Toxoplasma.

Macrophages appear to be the major effector cell population against Toxoplasma (38). Thus the study of normal and enhanced macrophage activity to kill or inhibit intracellular parasite growth is important to the overall understanding of host immunity against parasites. It has been suggested that host defense against Toxoplasma and Eimeria species may work in a similar fashion, i.e. by activating the macrophages to destroy the intracellular growth of the parasite (38). Conditions which are detrimental to Toxoplasma also appear to be lethal against Eimeria species.

In the present study, macrophages derived from peripheral blood monocytes were isolated and characterized according to their adherent properties, phagocytic ability, enzyme staining characteristics, major histocompatibility class (MHC) II antigen expression and physiological function. The isolated macrophages were then cloned and various clones were maintained in continuous culture. Different clones were then stimulated with various lymphokines eg., interferon-gamma, interleukin 2, Concanavalin A supernatant, macrophage activating factor and tumor necrosis factor. Tumoricidal and antiparasiticidal activity of these stimulated macrophage clones were studied against different tumor cell lines and Toxoplasma gondii and Eimeria bovis.

MATERIALS AND METHODS

Cell Line and Clones

Peripheral blood mononuclear cells (PBMC) were isolated from the fresh venous blood of a 1 year old bull calf. Blood was collected from the jugular vein into Vacutainers (Becton Dickinson, Rutherford, NJ) containing calcium heparin (50 U/15 ml) and diluted 1:2 in Hanks' balanced salt solution (HBSS; Flow laboratories, McLean, VA) supplemented with 10 mM ethylenediamine-tetraacetic acid (EDTA). Twenty five ml diluted blood was overlaid on 15 ml Ficoll/Hypaque (Sigma) in a 50 ml centrifuge tube (22°C), centrifuged (35 min. at 500 X g, 22°C), and the interface containing PBMC was collected and washed twice by centrifugation (300 X g for 10 min at 4°C) in RPMI-FBS (RPMI 1640; 10% defined fetal bovine serum [FBS]; 100U/ml penicillin G; 100 μg/ml streptomycin 1; 4 mM glutamine). All media and supplements were obtained from Flow Labs, except FBS which was obtained from Hyclone Inc.(Logan, UT). All washing steps were conducted at 4°C to reduce cell adherence to plastic.

The isolated PBMCs were suspended in 15 ml of RPMI-FBS and incubated in a gelatin coated (39) 75 cm^2 straight-neck tissue culture flask (Corning,Corning, NY) for 8 hr. at 37°C, 5% CO_2 ,95% air, after which excess medium and non-adherent cells were removed by aspiration. The remaining cells were

then maintained in RPMI-FBS in the same flask for an additional 6 days. At 12 hr. intervals, the cultures were rinsed with HBSS, fresh RPMI-FBS was added and the cultures incubated as above. In some cultures, supernatants from concanavalin A (Con A; Sigma, St. Louis, MO) stimulated PBMC were added to the cell monolayer at a 1:4 dilution in RPMI-FBS. When the cells reached 50-60% confluency, they were removed from the flask with a rubber policeman or trypsin-EDTA (Flow Laboratories) and passaged to new culture vessels. Thereafter the cultures were passaged at 3-4 day intervals or when they reached confluency. After six or nine passages, the cells were cloned by limiting dilution in flat bottom 96 well tissue culture trays (Costar, Cambridge, MA) at 100, 10, 1 and 0.1 cells/well in 200μl RPMI-FBS. After 3 weeks, the cells were removed with a rubber policeman from those wells showing single colony growth, suspended in 1ml RPMI-FBS and inoculated into a single well of a 24 well tissue culture plate (Falcon, Oxford. CA). When the cells reached confluency, they were removed from the 24 well plates with a rubber policeman, suspended in 5ml RPMI-FBS and inoculated into 25 cm^2 tissue culture flasks (Corning), and incubated as described above. After 10 days of incubation and at 5-day intervals thereafter, 100μl of medium was aspirated from each well and replaced with fresh culture medium. All lines and clones were suspended in 10% dimethyl sulfoxide in RPMI-FBS

and periodically preserved in liquid nitrogen.

Wright-Geimsa Staining

Air-dried blood smears were fixed in absolute methanol, stained for 1 min with Wright-Giemsa stain, rinsed in excess buffer for 8-10 min with gentle agitation, washed gently with tap water and air-dried (40).

Alveolar Macrophages

Alveolar macrophages were isolated by a procedure obtained from Dr. Denny Liggit (Immunex Corp., Seattle, WA). Cells were harvested from an 1 year old calf with a 4mm diameter beveline tube, the length of which was adjusted according to the size of the calf. The tube was lubricated with KY Jelly, passed gently through the nostril and trachea, into the lung. Sixty ml sterile pyrogen-free saline was passed gently but rapidly through the tube and was withdrawn immediately. The lavage was repeated until 200-250 ml fluid was collected in a sterile bottle. Cells recovered from the lung were then subjected to Histopaque (Sigma) separation as described above. The cells in the interface were collected and placed in 75 cm^2 flasks and cultured as described above for monocytes isolated from peripheral blood.

Mycoplasma Testing

All cell lines were tested for *Mycoplasma* infection with a Hoest *Mycoplasma* detection kit (Flow Laboratories). The protocol used was as described in Hoest Technical Bulletin (accompanying the kit) in which cells infected with *Mycoplasma* display spots of extranuclear green fluorescence when viewed with ultraviolet epifluorescent microscopy.

Parasites

Toxoplasma gondii (RH strain) tachyzoites were propagated in MRC5 cells (American Type Culture Collection, Rockville, MD) using established methods (41) with some minor alterations. Briefly, MRC5 cells were grown to near confluence in 25 cm^2 flasks (Costar), inoculated with 5ml RPMI-FBS containing 5 x 10^5 *T. gondii* trophozoites and incubated at 37^0C, 5% CO^2 , 95% air. After 96 hr, non adherent host cells and free *Toxoplasma* tachyzoites were removed by rocking the flask several times and decanting the solution which was then passed through a 27-gauge needle to liberate parasites from host cells. Parasites were counted and their viability was determined by trypan blue exclusion or by ethidium bromide-acridine orange staining in which the viable cells give green fluorescence, the dead cells give orange fluorescence (42).

The strain of Eimeria bovis used in this study was originally isolated by Dr. D. M. Hammond at Utah State University (Dr. C. A. Speer, personal communication). The parasite was maintained by serial passage in Holstein-Friesian bull calves.

Sporulated oocysts were collected in and supplied in previously described methods (43,54). Briefly, calves (usually 2 calves at a time) were inoculated orally with 3.5 to 5 X 10^4 sporulated oocysts of E. bovis. The calves were maintained in separate elevated metal feces collection stalls in which they were unable to turn around but could stand or lie down. Approximately 18 days following inoculation, feces (containing unsporulated oocysts of E. bovis) were collected in metal basins. Feces from infected calves were collected for 5 additional days. Oocysts were separated from the feces by sugar flotation, concentrated by centrifugation, and sporulated in aerated aqueous 2.5% (w/v) $K_2Cr_2O_7$. Sporulated oocysts were pooled and stored at 4^oC in 2.5% $K_2Cr_2O_7$. Microscopically oocyst preparations were estimated by hemocytometer count to consist of approximately 90% E. bovis and 10% other bovine eimerian species, especially E. ellipsoidalis, E. auburnensis, E. cylindrica and E. zuernii. For sporozoite isolation sporulated oocysts were treated with 5.25% aqueous sodium hypochlorite solution (Purex) for 1 hr at 22^oC, centrifuged at 200 X g for 10 min; resuspended in

sterile calcium-and magnesium-free HBSS, and washed two more times in HBSS.

Oocysts resuspended in HBSS and oocysts were broken by grinding (approximately 200 strokes) in a motor driven Teflon-coated tissue grinder to release sporocysts. The suspension which consists of sporocysts, oocysts walls and few intact oocysts was centrifuged at 200 X g for 10 min. The pellet was resuspended in 5ml excysting fluid [0.25% (w/v) trypsin 1/250, (Gibco, Long Island, NY) 0.75% sodium taurocholate, (Difco, Detroit, MI) in HBSS; pH 7.4] and incubated at 37^{o}C for 3 hr. Following incubation, the suspension was washed with HBSS and resuspended and passed over a nylon wool (Leuco-pak; Fenwal Laboratories, Deerfield, IL) column (44). The column elute contained mostly viable sporozoites with negligible contamination with sporocysts, oocysts and oocyst walls.

Cytokines

Concanavalin A supernatants (ConAS)

ConAS was generated by treatment of PBMC with Con A as described previously (35). Bovine PBMCs were isolated from three bull calves (6 month to 1 year old) as described above. Cells were adjusted to a concentration of 2 X 10^{6}/ml in RPMI-FBS and 1 ml was inoculated into each well of a 24 well plate. One ml RPMI-FBS containing 10, 5 or 2.5 microgram/ml was added to each well and the cultures were incubated at 37°C, 5% CO_2,

95% air. After 3 days, the medium containing ConAS was harvested, centrifuged (300 X g for 10 min at 4°C), aliquoted and stored at -80°C.

Macrophage activating factor (MAF)

The ConAS prepared as described above was used to prepare MAF by pH 2 dialysis (41). Supernatants were dialyzed first against glycine-HCl (pH 2; 0.1 M; 18h; 4^0C), then against PBS (pH 7.4; 24h;4^0C), and finally against HBSS (pH 6.9; 4h; 18^0C). ConAS was filter sterilized (0.22 μm) and stored in aliquots at -80°C.

Interleukin-2

Interleukin-2 (IL-2) was generated from the supernatant fluid of MLA144 (Gibbon thymoma) cells, which produce IL-2 spontaneously (42). Previous studies in our laboratory showed that MLA 144 growth and IL-2 secretion were optimal in RPMI 1640 supplemented with 2.5% NuSerum (Flow Laboratories), 1 ml/liter Mito+ (Collaborative Research Inc., Bedford, MA), 100 U/ml penicillin, 100 μgm/ml streptomycin and 4 mM glutamine, this medium (designated RPMI-NuS) was used to generate the IL-2 containing supernatants. Cells were grown in 100 ml RPMI-NuS in upright 75 cm^2 tissue culture flasks. After approximately 3 days of incubation the MLA 144 cells had usually reached stationary growth phase. At this time, the culture medium was decanted, centrifuged (300 X g for 10 min at 4°C) to remove cells, and concentrated 10-fold by dialysis

in Spectrapor dialysis tubing (Spectrum Medical Industries, Los Angeles. CA) against Aquacide Type III (Calbiochem, La Jolla, CA). Interleukin-2 was partially purified by 2-stage ammonium sulfate precipitation in which the proteins were first precipitated in 35% ammonium sulphate. The solution was then centrifuged (8000 X g for 20 min at 22^0C), the supernatant fluid adjusted to 65% ammonium sulphate and the proteins precipitated for 4 hr. After centrifugation, the pellet was resuspended in water and dialyzed against PBS (pH 7.4), 0.22 μm filter sterilized, and stored at -20^0C in 1 ml aliquots. The four fractions were 35P, 35S, 65P and 65S. The precipitate resulting from the 35% ammonium sulphate and centrifugation was 35P; the supernatant - 35S. The part of the 35S was then subjected to 65% ammonium sulphate and centrifuged resulting in 65P and 65S. All the parts were stored and later tested for IL-2 activity. The optimal dilution of IL-2 containing fraction was determined by following the growth of IL-2 dependent cells in different dilution. The optimal activity was found to be 3-4 units/ml (45).

Other cytokines

Human recombinant IFN-gamma (5 X 10^5 U/ml) was purchased from Collaborative Research Incorporated (Bedford, MA); human TNF-alpha (4000U/ml) from Endogen (Boston, MA); bovine recombinant IL-2 (3-4 U/ml) was a gift from Dr. Paul E. Baker

Monoclonal Antibodies

TH14B, specific for major histocompatibility class II antigens (MHC class II), is an IgG_{2a} that cross-reacts with cells from different species, including humans (46). CH137A is an IgM isotype which also has specificity for MHC class II antigens and cross-reacts with cells from other bovids such as eland and water buck (46).

Lymphocyte Function Assays

IL-2 activity was assessed by measuring the growth of IL-2 dependent T-cells (47). Cell growth was determined by the incorporation of ^{3}H-thymidine (^{3}H-TdR) into cells during a 4 hr pulse. The IL-2 dependent cell line was established in the laboratory using fresh PBMC as described above. Cells (2 X 10^6 /ml) were cultured in RPMI-FBS supplemented with 5 μg/ml Con A. After 3 days culture at 37^{0}C, 5% CO_2, cells were collected and subjected to discontinuous gradient centrifugation in Percoll (Pharmacia/LKB, Piscataway, NJ). Percoll was diluted in RPMI-FBS and layered into a 15 ml centrifuge tube as follows: 5 ml 40%, 1.5 ml 35%, 2.5 ml 31.5% and 2.5 ml 26% Percoll. Twenty million cells in 1.5 ml RPMI-FBS were overlayed on the gradient, and centrifuged for 35 min at 1500 X g at 22^{o}C. Cells from the 31.5%/35%, and 35%/40% interface were collected, washed twice in HBSS, left for 1hr at 37^{0}C, and washed again. These two fractions

contained mostly large blast cells and relatively few small lymphocytes (47). Blast cells were then passaged 3 to 4 times in culture medium containing IL-2 (3-4 U IL-2/ml). After thorough washing, the cells were plated at a density of 1 to 2 X 10^4 cells/well in 96 well culture plates and samples containing an unknown concentration of IL-2 were added at various dilutions. Positive controls utilized recombinant bovine IL-2; negative controls utilized RPMI-FBS.

Tumor Cell Cytotoxicity

Tumor cell cytotoxicity was assessed using an 18 hr ^{51}Cr-release assay (48).

Effector cells

Effector cells (different lines and/or clones of macrophages) were plated at 10^5 cells/200μl of RPMI-FBS in each well of flat bottomed 96 well culture plates (Costar) with various dilutions of different cytokines.

Target cells

Target cells (K562) were grown, in 10 ml RPMI-FBS suspension cultures in upright 25 cm^2 tissue culture flasks. Cells were harvested in log-phase growth and washed twice in serum-free RPMI by centrifugation at 300 X g for 10 min at 22^0C. After washing, 400 μCi of Chromium-51 (^{51}Cr) was added to the cells, which were incubated for 2 hr with agitation every 15 minutes. Following incubation, excess RPMI-FBS was

added and cells were washed twice with RPMI-FBS. Chromium-51 labelled cells were then suspended in fresh RPMI-FBS, incubated at 37°C for 1 hr to remove primary spontaneous isotope release, washed once again and resuspended in RPMI-FBS at a concentration of 10^5 cells/ml. For longer assays, such as 72 hr, the live ^{51}Cr- labelled cells were separated from the dead ones by centrifuging the cells over Histopaque (Sigma) and collecting the cells in the interface.

Assay

Following 72 hours of activation, 100μl supernatant from each well of effector cells were aspirated and replaced with 100μl of 10^4 ^{51}Cr-labelled target cells suspension. One hundred μl of labelled tumor cell suspension was added to plastic τ-counting tubes to assess total ^{51}Cr content. Spontaneous ^{51}Cr-release was assessed in target cell culture alone in RPMI-FBS. Following incubation of effector(E) cells with target(T) cells at an E:T ratio of 10:1 at 37°C for 18 hr, the plate was centrifuged at 500 X g for 10 min. One hundred μl of supernatant fluid were placed into supernatant harvest tubes (Flow laboratories) and prepared for τ spectroscopy in a Packard gamma-counter. The percentage specific lysis was assessed using an established formula for ^{51}Cr release (31).

$$\% \text{ release} = \frac{\text{ct/min in supernatant}}{\text{Maximum release from tumor cells}} \times 100$$

$$\% \text{ cytotoxicity} = \frac{\% \text{ effector cell release} - \% \text{ spontaneous release}}{\text{Maximum} - \% \text{ spontaneous release}}$$

Parasite Killing

Toxoplasma gondii Intracellular killing of *T. gondii* was measured by ^{3}H-uracil assay as described (49,26). Parasites (10^4/well in 100 μl) were added to previously stimulated macrophages (above) and incubated for 18 hours, after which the cultures were pulsed with 0.5 μCi of ^{3}H-uracil for 18 hours, harvested, and prepared for ß spectroscopy. Control cultures consisted of unstimulated macrophages infected with parasites, and cells and parasites cultured alone.

Eimeria bovis The antiparasitic response of the effector cells was measured by their ability to inhibit the intracellular development of *E. bovis* sporozoites to meronts and merozoites. Macrophage clones were grown to confluency in triplicate cultures in 24-well trays and treated with individual cytokines, combinations thereof or RPMI-FBS (as control) for 3 days before inoculation of sporozoites and

throughout the experiment. M617, a permissive cell line, was co-cultured as positive control. Cultures were monitored daily with phase-contrast microscopy for the development of sporozoites to meronts and merozoites. At 1 day after sporozoite inoculation, the culture medium was gently aspirated and replaced with the same type (cytokine etc.) of fresh medium. At 10 days after sporozoite inoculation, numbers of developing meronts were determined and photographs were taken of cells in all the wells.

MHC Class II Expression

Various cell lines and clones were suspended in RPMI-FBS and inoculated on coverslips in 24 well culture plates at the rate of 2 X 10^5 cells/well. Various concentration of different cytokines were added to the culture medium. The cells were allowed to grow to the point where individual cellular outline could be easily demarcated. At the end of the culture period (usually 2 days), the culture was gently washed twice with HBSS, treated with 1/2 ml blocking buffer (2% BSA, 0.2% NaN_3 in HBSS) for 30 min. at 22^0C, followed by treatment with 100μl of MAb solution (15mg/ml in blocking buffer) and then incubated in a humidified chamber at 22^0C for further 30 min. Following incubation, coverslips were washed 3 times in Phosphate buffered saline (PBS), pH 7.2 and 75 μl of fluorescein isothiocyanite (FITC) labelled goat anti-mouse μ specific antibody (Southern Biotech Assoc. Birmingham, AL)

was added, and incubated for 20 min at 4^0C. Cells were then washed once in PBS and once in distilled water. The coverslips were mounted on a glass slide with cell side down with 60% glycerol in PBS and viewed under an epifluorescence microscope (Nikon).

Latex Bead Incorporation

Cell preparations were adjusted to 2 X 10^6 cells/ml in HBSS and 1 ml of 1:50 dilution of latex particles (0.81μ diameter; Difco Laboratories, Detroit, MI) were added to 10 ml of cell solution. The mixture was incubated at 37^0C for 1 hr. with continuous agitation. Following incubation, the cells were washed 3 times with 50 ml HBSS. The degree of latex bead ingestion was determined visually with a phase contrast microscope (Nikon) (50).

Nitroblue Tetrazolium Assay

The assay was performed in 96 well trays. Five different clones (P6D10, P6F6, P6C6, P9E4 and GM4/9/4) were plated at the concentration of 10^3 cells/well in 200 microliter of RPMI-FBS or various cytokines. The cells were allowed to grow to the point where each cell was distinctly separated from others. One hundred microliter of NBT (0.1mg/ml of HBSS) was placed in the wells replacing 100 microliter of existing medium. The plate was then incubated for 15 min at 37^oC and then further 15 min at 22^0C. The percent positive cells were

then determined by viewing them under a phase contrast microscope by the presence of blue-black formazan granules (51).

RESULTS

Cell Isolation

Complete blood counts (CBC) were determined for three calves (Table 1). Following Histopaque isolation and Wright-Giemsa staining of PBMC, all white blood cells (WBC) were mononucleated; no polymorphonuclear cells were detected. Red blood cell (RBC) contamination was less than 1%. Only calf 289 had any detectable monocytes following PBMC separation (Table 1).

Table 1. Complete blood counts of peripheral blood from three calves.

Calf	WBC[a]	NEU[b,f]	MON[c,f]	EOS[d,f]	BAS[e,f]
287	8.4	74	1	5	-
289	9.0	76	3	4	1
29	8.7	62	1	11	1

[a]= total number of white blood cells (WBC) (X1000/ml of blood).
[b]= neutrophils
[c]= monocytes
[d]= eosinophils
[e]= basophils
f= percent of total WBC

Macrophage Cell Lines and Clones

After the PBMC culture had been incubated for 8 hr and then rinsed with HBSS, few adherent cells were present most

of which were epitheloid (Fig 1). After the cultures were incubated an additional week with regular rinsing, there were even fewer adherent cells (Fig 1) which had become more fibroblast in appearance (Fig 2). At 2 to 3 weeks after the addition of ConAS, the cells started to proliferate and became larger than in the cells in the initial isolation (Fig 2). Usually, if the ConAS was not added to the culture, viable cells were not recovered.

Monocytes isolated from PBMC were allowed to develop into macrophages which were then cloned by limiting dilution to a concentration of 0.1 cell/well (Table 2). Between 4 to 15 days, those wells showing cell growth were scored as positive.

Table 2. Cloning efficiency of macrophage cell line.

No. of cells/well	No. of wells/tray with cell growth	
100	96	96
10	96	96
1	96	96
0.1	11	9

Data obtained from the sixth passage of seven macrophage clones shown that P6F6 had the fastest rate of growth (Fig 3.), whereas after the 9th passage P9F5 had the fastest growth rate and P9F4 the slowest.

Cellular Characteristics

Greater than 99% of the cultured macrophage in each