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Dissection of Microphage Activation Using T Cell Hybridoma Derived Lymphokines

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DISSECTION OF MACROPHAGE ACTIVATION USING T CELL HYBRIDOMA DERIVED LYMPHOKINES

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ADCC	- antibody-dependent cellular cytotoxicity
AMP	- adenosine monophosphate
APD-I	- alkaline phosphodiesterase I
ATP	- adenosine triphosphate
BCG	- Bacillus Calmette-Guerin
BM	- bone marrow
B. pertussis	- Bordetella pertussis
BSA	- bovine serum albumin
Ca^{2+}	- calcium ion
Con A	- concanavilin A
CO_2	- carbon dioxide
CP	- cytolytic protease
C. parvum	- Corynebacterium parvun
CR1	- complement receptor 1
CR2	- complement receptor 2
CR3	- complement receptor 3
CSF	- colony stimulating factor
C3	- third component of complement
DMEM	- Dulbecco's modified minimum essential medium
EtOH	- ethanol
FCS	- fetal calf serum
HAT	- hypoxanthine, aminopterin, thymidine (hybridoma selective medium)
HB	- hybridoma supernatants containing HB101 serum-free medium
HBSS	- Hank's balanced salt solution
HEL	- hen egg lysozyme
HGPRT	- hypoxanthine guanine phosphoribosyl transferase

H_2O_2	- hydrogen peroxidase
IgE	- class E immunoglobulin
IgG	- class G immunoglobulin
IL-1	- interleukin - 1
IL-2	- interleukin - 2
i.p.	- intraperitoneal
LAP	- leucine aminopeptidase
LK	- lymphokine
LPS	- lipopolysaccharide
MAF	- macrophage activating factor
Mg^{2+}	- magnesium ion
MHC	- major histocompatibility complex
M. tuberculosis	- Mycobacterium tuberculosis
N. fowleri	- Naegleria fowleri
NH_4Cl	- ammonium chloride
O_2^-	- superoxide anion
P. acnes	- Propioibacterium acnes
PBS	- phosphate buffered saline
PEC	- peritoneal exudate cells
PG	- prostaglandin
PGE	- prostaglandin of the E series
PPD	- purified protein derivative
RIA	- radioimmunoassay
RT	- room temperature
S	- hybridoma supernatants containing 10% fetal calf serum
SRBC	- sheep red blood cells

TCA	- trichloroacetic acid
TCGF	- T cell growth factor
TG	- thioglycollate
T. gondii	- Toxoplasma gondii
T-3	- T cell hybridoma 3
T-9	- T cell hybridoma 9
U	- units
α-IFN	- alpha interferon
β-IFN	- beta interferon
-IFN	- gamma interferon
5'N	- 5'nucleotidase

ABSTRACT

DISSECTION OF MACROPHAGE ACTIVATION USING T CELL HYBRIDOMA DERIVED LYMPHOKINES

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Macrophage phenotype/function can be modulated by various T-cell lymphokines (LK). The alteration of macrophage phenotype is a result of LK concentration, duration of exposure, and the level of macrophage activation when obtained from in vivo sources through elicitation by either sterile irritants or immune cellular mechanisms. In order to dissect macrophage activation into discrete signals T cell hybridomas were constructed by fusing HAT sensitive BW5147 cells with nylon-wool purified, con A stimulated T cells. The resulting T cell hybrids were screened for their ability to: (a) protect macrophages from the cytopathic effect of Naegleria; (b) induce class II MHC gene product (Ia antigen) expression; (c) increase cytostasis and tumoricidal activity; and (d) alter ectoenzyme profiles on either resident or thioglycollate (TG) elicited macrophages. Two hybridomas (T-3 and T-9) were selected for further evaluation because of their activity patterns. Supernatants from T-3 and T-9 were compared with cloned γ-interferon (γ-IFN) for alteration of biological activities. Both T-3 and T-9 were able to protect resident macrophage cells from Naegleria but had no protective effect on TG-macrophages. T-9 supernatant had patterns of activity similar to γ-IFN while T-3 patterns were different. The addition of anti-γ-IFN removed T-9 cytostatic activity while not affecting T-3 induced activity. The LK inducing protection from the cytopathic effect of Naegleria

lysate is not γ-IFN but another molecular moiety. It was also shown that γ-IFN does not protect TG-macrophages from the destructive effects of adenylate cyclase produced by Bordetella pertussis. We conclude that activation of macrophages for the destruction of tumor cells and activation for protection against amoeba and bacteria occur via different biological pathways. Furthermore, we have proposed an association between the cell cycle and the responsiveness of resident and TG-elicited macrophages to specific LK.

INTRODUCTION

When Metchnikoff first introduced the term "macrophage" in 1883 the major function of these cells was considered to be the rapid phagocytosis of foreign particles. Now over a century later, the role of the mononuclear phagocyte in host defense has been found to be far more complex, playing an important role in resistance to attack by parasites, tumors, and microorganisms (31,47,52,75).

The major emphasis of this work is directed toward macrophage effector functions against microbial infection and neoplastic cells. While the activation of macrophage for these two mechanisms occurs through similar stages, the inducing signals appear to be different (31,21,113,114). Furthermore, macrophage populations appear to exhibit variable degrees of efficiency in performing these functions (113).

The macrophage has also been implicated in a variety of other functions both immunological and nonimmunological. Their secretory function has been well documented (91). The macrophage is also of considerable importance because of its role in the regulation of the immune response (97,98,126) and may also play a role in vascularization and wound healing (97,113).

The diversity of macrophage function has lead to attempts to describe discrete stages of activation, generally defined by the degree of microbicidal or tumoricidal activity. These stages have been characterized as resident, inflammatory (responsive, primed), or activated. Normal, unstimulated peritoneal macrophages, are considered resident macrophages. Macrophages elicited by i.p. injection of a sterile agent such as thioglycollate, casein, or protease-peptone are inflammatory macrophages. These inflammatory macrophages are functionally "responsive" to LK(s). After LK treatment these cells are prepared to become tumoricidal or microbicidal and are considered

primed. Exposure of these primed macrophages to a second signal such as endotoxin or LK produces a fully activated macrophage. Injection of Calmette-Guerin bacillus (BCG) or C. parvum (P. acnes) also results in the elicitation of peritoneal macrophage which have tumoricidal activity.

Mononuclear phagocytes are an extremely heterogeneous population of cells. They differ according to a number of biochemical and histochemical parameters and also differ markedly according to their tissue distribution (lungs, liver, bone, spleen, connective tissue, serous cavities) (29,97). Even within one location, macrophages vary in their morphology, state of differentiation, and functional parameters (97,113).

There have been many attempts to explain the functional heterogeneity of the macrophage. Daems (29) has suggested that while inflammatory macrophages originate from bone-marrow-derived blood monocytes, the resident macrophage is a self-renewing population. This hypothesis is based upon observations that resident macrophages regularly enter mitosis and give rise to progeny of their own kind. Van Furth (29) believes that all macrophages originate from bone-marrow and that inflammatory stimuli and lymphocyte derived biological modifiers, lymphokines (LK), have a direct effect on the macrophage and induce functional heterogeneity in a homogenous population of bone marrow precursor cells. Bursuker and Goldmann (13) have proposed that a heterogeneous population of bone marrow-derived macrophage-precursor cells exists and that subsets of these precursors are differentially expanded under normal and inflammatory conditions. A heterogeneous population of monocytes then enters the circulation and under the influence of LK they migrate into local tissues. It is at this level where the tissue macrophage interacts with local factors which determine the final functional state. A similar proposal suggests that functional heterogeneity arises at the level of the monocyte and that

monocytes give rise to separate lineages of macrophages which eventually result in true macrophage populations that reside in specific tissues or organs (112). Both of these models result in terminally differentiated subpopulations in which transition from one subpopulation to another would be severely restricted and require retrodifferentiation to the level of a common precursor (112).

Sorg and Newmann (112) have proposed a model in which macrophages pass sequentially through a series of intermediate, stable phenotypes. Under this mechanism terminally differentiated macrophages can rearrange their genetic program and switch to phenotypes that can reenter into the cell cycle. While it is not clear which of these models is correct, the use of biochemical markers to define distinct stages of macrophage differentiation and purified LK as tools for regulating macrophage homeostasis, should allow for the manipulation of macrophage effector functions and study of its regulation.

Origins of Mononuclear Phagocytes

The mononuclear phagocytes are of bone marrow (BM) origin arising from a pluripotential stem cell common to all hematopoietic cells (29). As this stem cell becomes committed, it gives rise to the monoblast. The murine monoblast has a cell cycle time of 12 hours and divides to give rise to two promonocytes (29). The resulting promonocytes have a cell cycle of 16 hours. These cells give rise to the monocytes which leave the BM into the peripheral blood after 24 hours and do not normally undergo any further cell division (29). After approximately 48 hr mature monocytes will randomly emigrate from the blood into various tissues and serous cavities and become macrophages (29,97,134).

Monoblasts are difficult to identify by current techniques; however, Nichols et al. (82) reported that they contain no granules and others report that they lack any functional activity usually associated with monocytes (123). The promonocyte is a large cell, (10-15 μm in length) with a large

nuclear/cytoplasmic ratio, and prominent nucleoli. The azurophil granules are immature and are peroxidase positive as are the cisternae of the rough endoplasmic reticulum (RER) and the Golgi complex (83). It also contains the lysozomal enzymes arylsulfatase and acid phosphatase (82). In the bone marrow monocyte, the RER and the Golgi complex are peroxidase negative and there is the appearance of a second peroxidase negative granule. No arylsulfatase reactivity is present and acid phosphatase activity is greatly reduced. The nucleus is large with a horseshoe shape and the nuclear/cytoplasmic ratio is decreased in comparison to the promonocyte (83). The mature blood monocyte continues to form peroxidase negative granules and exhibit cellular membrane ruffling (83).

Distinct morphological and biochemical changes accompany the development of monoblasts into mature monocytes (6). These changes are associated with the acquisition of functional character. As promonocytes develop into blood monocytes and ultimately into tissue macrophages there is a progressive increase in the number of receptors for the Fc portion of immunoglobulins and the C3b components of complement (6,134). There is also an increased capacity for pinocytosis and phagocytosis (6) and an increasing number of the cells become esterase positive. The development of these five markers occurs in the majority of bone marrow derived mononuclear phagocytes and are the basis by which they are identified (6). In addition to these general features of the mononuclear phagocytes, there are many biochemical properties and functions acquired in response to inflammation that distinguish the resident tissue macrophage from the inflammatory macrophage.

Normally, as mononuclear phagocytes leave the BM they are fairly well developed, express high levels of Ia antigen, and functionally responsive to lymphokine. As they migrate into the tissues they appear to become down-

regulated or suppressed (105). It is possible that in the absence of activating stimuli, the macrophage autoregulates its own suppression. Resident macrophages, which are functionally at rest, produce substantial amounts of prostaglandin E (predominantly PGE_2) which inhibits macrophage proliferation and clonal expansion (6). It is particularly interesting that when promonocytes or monocytes are removed from the bone marrow (BM) and cultured in vitro, they develop phenotypic and functional characteristics which resemble resident tissue macrophages (6).

Receptors and Surface Antigens

Macrophages are known to bear over 30 distinct receptors (5). Changes in the receptors being expressed can be used to characterize their state of differentiation.

As previously mentioned, the macrophage expresses receptors involved with phagocytosis: i.e. the Fc receptors, receptors for complement, and a receptor for mannose/fructose/N-acetylglucosamine terminated glycoproteins (56,97). Peritoneal macrophages express two major Fc receptors. FcRI recognizes γ2a while FcRII recognizes the γ1 and γ2b IgG isotypes (5,127). The binding of these receptors reportedly results in the enhancement of phospholipase A_2, the arachidonic acid cascade and increased intracellular levels of cyclic AMP (5). The number of FcRI and FcRII copies has been shown to vary according to the maturational stage and this variation can be induced by exposure to β or γ-interferon (130). The number of FcRI and FcRII receptors are higher in inflammatory than in resident peritoneal macrophages (5). Immunologically mediated inflammation (BCG-elicited macrophage) yields macrophages with increased FcRI but decreased FcRII receptors (5, 27).

Macrophages in all stages of differentiation have been shown to express receptors for complement components. The two major receptors, CR1 and CR3,

recognize and bind different portions of the third component of complement (C3) (5). It appears that CR1 and CR3 are functionally altered during development. Resident macrophages bind but can not ingest complement-coated RBC's, while inflammatory macrophages will bind and phagocytize antigen by these receptors (5). Griffin (34) has shown that in resident macrophages complement receptors are not freely mobile in the phospholipid bilayer of the plasma membrane and therefore do not gather around a bound particle to permit endocytosis. In inflammatory macrophages this is not the case as complement receptors are fully mobile (5,34).

Peritoneal macrophages also have receptors for glycoproteins ending in mannose, fucose, or N-acetylglucosamine residues (45,118). This receptor probably aids in the recognition and phagocytosis of bacteria and other microorganisms. As is the case with complement receptors, macrophage activation leads to a decrease in the number of these receptors (27).

Expression of class II major histocompatibility antigens (Ia antigens) are important in antigen presentation by macrophage to lymphocytes. Rosenthal and Shevach (98) first noted that the macrophage and T cells must be histocompatible in order to cooperate together in the immune response. Only Ia positive macrophages can function as accessory cells for the presentation of antigen and removal of these cells eliminates the immune response (139). The presence of Ia antigens is characteristic of the activated macrophage. Resident and inflammatory macrophages show low levels of Ia antigen; however, immune elicited macrophages contain a large percentage of Ia-positive cells (125,126). The expression of Ia is induced <u>in vitro</u> by lymphokines (MAF or γ-IFN), while it is down regulated by prostaglandin E_2(PGE_2) (5).

Recently, Hamilton et al. (37) has correlated the expression of receptors for the iron-binding glycoprotein, transferrin with macrophage differentiation.

Inflammatory macrophages and monocytes express large numbers of receptors for this molecule, while resident macrophages and immune-elicited macrophages express significantly fewer receptors (37). Interestingly, the transferrin receptor is down regulated by the addition of macrophage activating factor (MAF) or recombinant γ-IFN (37) and is therefore a useful marker for examining the mechanism of action of these LK's on macrophages.

Many investigators have associated the expression of membrane ectoenzymes with macrophage development, the most well characterized of which is the 5'nucleotidase (5'N). This enzyme hydrolyzes the phosphoester linkage in 5'-mononucleotides, liberating a nucleotide and inorganic phosphate (23). Resident peritoneal cells have been shown to contain high levels of this enzyme. In contrast, macrophages elicited by sterile or immune inflammatory reagents exhibit dramatically reduced levels (22). This decrease has been shown to result from increased membrane recycling and decreased synthesis of the enzyme (5). Because of the inverse relationship between expression of 5'N and the inducibility of Ia antigen by MAF, this activity is thought to be regulated by a signal(s) which leads to increased responsiveness to MAF (5) or perhaps macrophages with low 5'N express receptors for γ-IFN.

The ectoenzymes, alkaline phosphodiesterase I (APD-I) and leucine aminopeptidase (LAP), have also been used to phenotypically characterize macrophage populations. APD-I functions to sequentially remove terminal monophosphate nucleotides from the 5' end of polyribonucleotides or oligodeoxyribonucleotides starting at a nucleotide with a free 3'-OH group at the tail of the chain (24). Leucine aminopeptidase sequentially cleaves amino acids from the N-terminal end of peptides in a nonspecific manner (71). Both of these enzymes have been found to increase several fold in inflammatory macrophages as compared to resident macrophages. In activated macrophage, LAP levels remain high;

however, the level of APD-I appears to be even lower than that seen in resident macrophages (22).

Peritoneal macrophages express receptors for regulatory molecules which may act to induce or suppress macrophage functions. Many of these receptors are constitutively expressed and are not altered as the macrophage proceeds through the various stages of activation. The receptor for acetylated proteins and lipoproteins are of this type. The triggering of this receptor stimulates secretions of neutral proteases and in primed macrophages results in secretion of plasminogen activator and cytolytic proteinase (5). The receptors for $\alpha 2$ macroglobulin are also present throughout the development of tumoricidal and microbicidal functions. Binding of this receptor inhibits neutral protease secretion and therefore acts in conjunction with the receptor for acetylated proteins and lipoproteins to autoregulate the secretion of plasminogen activator and cytolytic proteinase (5).

The receptor for colony-stimulating factor (CSF) is expressed early on in development and all macrophage populations respond to it, although the magnitude of proliferation may vary. Inflammatory macrophages show the greatest proliferation in response to CSF, while resident and activated macrophages are much less responsive (5). In addition to cellular proliferation, engagement of this receptor results in increased spreading in mature macrophages and differentiation of pre-monocytes and monocytes into macrophages (130).

Many receptors for LK are expressed only as a result of humoral signals received earlier in differentiation. Identifying these receptors has proven difficult until recently due to the lack of purified factors. The migration inhibitory factor (MIF) receptor has been identified as a L-fucose containing glycoprotein (41). Receptors for macrophage activating factor (MAF) have been

much more difficult to define because this molecule has been identified functionally in Con A stimulated lymphocyte supernatants. It is now thought that activity once attributed to this one factor may indeed be do to several lymphokines (30,31). Gamma interferon is believed to be one form of MAF (5) and receptors appear to be present in inflammatory (TG-elicited) macrophages but lacking in resident macrophages (37). The binding of γ-IFN to its receptor leads to increased cytolytic activity (89,95).

Peritoneal macrophages express many other receptors too numerous to mention in detail. Some of these include receptors for lactoferrin, chemotactic factors, fibronectin, high density lipoproteins, fibrinogen, and insulin (5,97).

Secretory Functions

If any one characteristic could possibly begin to explain the diverse functions of the macrophage perhaps it is the secretory function. The macrophage is able to manufacture, secrete, and/or store a vast array of biologically active products, which include hydrolytic enzymes, cell stimulatory proteins and a vast array of other molecules. On exposure to a variety of inflammatory-inducing substances the macrophage is triggered to release a variety of acid hydrolases (cathepsins, hyaluronidase, β-glucuronidase, acid phosphatase, aryl sulfatase) (5). These preformed enzymes are released within 4-6 hours from lysozomes (97) and release has been shown to be specific for potentially pathogenic substances since the phagocytosis of biologically unreactive substances does not induce their release (91). Although their exact extracellular function is not known these enzymes are believed to participate in the initiation and perturbation of tissue destruction seen in chronic inflammatory diseases (91,97).

Another major secretory product of the macrophage is lysozyme, a 14,000 molecular weight protein which hydrolyzes N-acetylmuramic-β-1,4-N-acetyl-

glucosamine linkages of peptidoglycans in bacterial cell walls (5,91,97). Unlike the acid hydrolases, lysozyme is a constituitive product of the macrophage (91,97) and is secreted in large quantities by all macrophage populations (5).

A third group of hydrolytic enzymes produced by the macrophage are the neutral proteases. This group includes the enzymes elastase, collagenase, plasminogen activator, and a cytolytic proteinase (91,97). Unlike the acid hydrolases, these enzymes are synthesized following a stimulatory event and their production can be blocked by protein synthesis inhibitors (91,97).

While resident macrophages secrete low levels of neutral proteases, inflammatory macrophages secrete substantial amounts. Werb and Gordon (133) have reported that TG-elicited macrophages secrete high levels of collagenase while resident macrophages produce barely detectable amounts. Resident macrophages however, can be triggered to secrete slightly higher levels by phagocytic stimuli. These findings appear to be true for elastase, plasminogen activator, and cytolytic proteinase enzymes as well (5,91,97).

The macrophage also secretes a variety of reactive oxygen intermediates. These include superoxide anion, hydrogen peroxide, and hydroxyl radical (5,6,50,78,97). Evidence suggests that these oxygen metabolites play a role in the tumoricidal and microbicidal functions of the macrophage . Murray et al. (75) has shown that these highly reactive molecules are involved in killing of the intracellular parasite Toxoplasma gondii. Others have demonstrated that the release of H_2O_2 by activated macrophages leads to the rapid lysis of certain tumor cell targets (78,79,80,81). Inflammatory macrophages have been shown to have a 10-fold greater secretory capacity of reactive oxygen intermediates than resident macrophages (100). However, macrophages immunologically activated in vivo by BGG or P. acnes secrete the largest amounts of these oxygen intermediates (6).

Macrophages serve as a major source of arachidonic acid metabolites such as prostaglandins of the E series, leukotriene C_4 and B_4, and thromboxane B_2 (5,92,97,107). Prostaglandin E_1, is known to greatly enhance collagenase production in stimulated macrophages. They have also been shown to exhibit antiproliferative and immunosuppressive effects (5,97) on T cells and to inhibit generation of many lymphokines (68). Thromboxane B_2 is a principle metabolite of the macrophage as a result of phagocytic stimuli (92) and is a potent mediator of vasoconstriction and platelet aggregation (36). Two major leukotrienes are also secreted. Leukotriene C_4 (slow-reacting substance of anaphylaxis) and leukotriene B_4, a potent chemotactic factor are generated by the lipooxygenase pathway (97).

Macrophages produces a variety of compounds with stimulatory activity on cells involved in immune regulation. Macrophages produce CSF similar to that produced by the fibroblast L929. This factor is produced by all macrophages and acts to regulate macrophage morphology, differentiation, and proliferation (5).

Macrophages also secrete Class I (β) interferon. Secretion of β-IFN has been shown to be induced by CSF (130). It has been suggested that this enables the macrophage to autoregulate its function by providing the signals necessary for differentiation into a responsive macrophage (130). Warren and Vogel (130) have recently shown β-IFN can increase Fc-mediated phagocytosis in the presence of highly purified, β-IFN depleted CSF. This increased activity is eliminated with the addition of anti-β-IFN. Furthermore, β-IFN can provide the appropriate signal necessary for activation for tumoricidal activity although it is quantitatively less effective than γ-IFN (90).

Interleukin 1 (IL-1) or lymphocyte activating factor is a major signal for lymphocyte stimulation. It is released by macrophages in response to viral, bacterial, and fungal infection (5). IL-1 appears to be identical to endogenous

pyrogen which induces the febrile response and regulates several phenomena associated with inflammation (97,110).

Activated and inflammatory macrophages secrete components of both the classical and alternative complement pathways. These include C1q, C2, C3, C4, C5, factor B, factor D, properdin, C3b-inactivator, and B1H (5,11,62). Supernatants from antigen stimulated lymphocytes have been shown to enhance production of these complement components (62). Resident and inflammatory macrophages secrete the largest quantities and produce the largest number of individual products compared to activated macrophages (5). Scott et al. (107) have shown that the overall secretory capacity of these products is down regulated as a consequence of the activated state.

Macrophage Regulatory and Effector Functions

Macrophages exhibit three major functional roles in the immune response. First, they act as scavengers of extracellular debris (5,97,134). Second, they play a regulatory role in the induction of the immune response, having been shown to display both activational and suppressive controls (5,97,125). Third, macrophages serve as effector cells capable of destroying viruses (5), bacterial (50), intracellular and extracellular parasites (12,50,86), and tumor cells (2,4,5,6,80).

The phagocytic role of the macrophage has long since been established (15,134). Although earlier investigators had demonstrated the phagocytic capability of the macrophage (15), Metchnikoff was the first to establish their importance and demonstrated that animals resistant to certain bacterial infections had an increased capacity for phagocytosis of microorganisms (5,15).

Virtually all macrophage populations in various stages of differentiation express some phagocytic ability (97). Cells from the promonocyte stage onward have been demonstrated to phagocytose latex beads nonspecifically and IgG

coated particles specifically by Fc receptors (5,97,130). Opsonized particles coated with the C3 component of complement can also be ingested via a complement receptor (25,34).

Phagocytosis can be divided into three stages: attachment to the macrophage, ingestion of the particle, and digestion within a phagolysosome (15,134). The initial attachment and recognition occurs by the receptors previously mentioned. Normally, complement and IgG coated particles are phagocytized by a zipper-like mechanism in which F_c and C3 receptors on the surface of the macrophage direct movement of the membrane over the surface of the particle (134). However, particle attachment does not always indicate phagocytic recognition (93). At low receptor densities particles may bind macrophage surface receptors without being ingested (93,134). If receptors on the macrophage surface are not mobile enought in the fluid phase of the membrane to allow zippering then phagocytosis is also interrupted at binding (5,134).

When zippering is triggered, the particle is surrounded by the macrophage membrane. Once this circumferential zippering is completed, an endocytic vacuole known as a phagosome is formed. This phagosome fuses with a primary or secondary lysosome to form a phagolysosome (134). Within this compartment, the phagocytosed material is digested at low pH by a barrage of hydrolytic enzymes (134).

Phagocytosis is greatly enhanced in inflammatory macrophages as compared to resident peritoneal macrophages (5,112). This increased efficiency is partly due to an increase in the number of FcRI and FcRII in inflammatory macrophages (5). In contrast, resident macrophages have higher numbers of complement receptors but because they are not freely mobile in the membrane, bound particles are not ingested (5,34). Inflammatory macrophages also have

increased reserves of phosphocreatine which is a major energy source during phagocytosis (5).

The positive immunogenic role of the macrophage is a result of two well defined steps. First, they have been shown to remove extracellular debris (antigen); and second, they can retain degraded peptides (antigen) in lymphoid tissue for presentation to immunospecific lymphocytes. Mosier (73) first demonstrated the requirement for adherent cells in the immune response to sheep red blood cells (SRBC). He cultured murine spleen cells in plastic dishes for 30 min and removed the adherent cells. Neither fraction (adherent or nonadherent cells) could respond in vitro to SRBC. However, when the fractions were mixed together anti-SRBC antibodies were produced. Nossal et al. (87) were the first to show that in vivo, injected antigen localizes in the lymph node medulla and persists for long periods of time within the macrophages.

A third step, which until recently was somewhat obscure, is the actual presentation of antigen to the lymphocytes. In order for an accessory cell to perform this function, it must be able to express Class II glycoproteins (Ia antigens) on its surface, process antigen, and synthesize and release interleukin 1 (126).

The importance of Ia antigens was first described by Rosenthal and Shevach (109) who demonstrated that macrophages and T cell must be histocompatible in order to result in lymphocytic proliferation. Using strain 2 and strain 13 guinea pigs, they found that antigen-pulsed macrophages could activate syngeneic lymphocytes while allogeneic macrophages failed to activate T cell proliferation. Because the MHC's of strain 2 and strain 13 guinea pigs differ only at the I-region, they proposed that identity between I-region antigens is required for effective T-cell-macrophage interactions. In an accompanying paper, (109) they showed that antigenic non-responders were a result of a

macrophage defect in which antigen could not be presented to T lymphocytes in an effective way.

Further studies have shown that it is the macrophage and not the T cell Ia antigens that are important in these interactions. Thomas and Shevach (120) demonstrated that the receptor on the helper T cell recognizes antigen in association with the H-2I region product and this product does not need to be expressed by both the macrophage and the T cell. In addition, the killing of Ia-positive macrophage with anti-Ia and complement was shown to impair the immune response to antigen (139) while removal of Ia-positive T cells had no effect (121).

Macrophages have been demonstrated to internalize antigenic proteins where they are catabolized to smaller fragments and later found either associated with the cell surface or released into the culture supernatant (126). Using lysosomotropic agents, such as chloroquine or ammonium chloride, to raise the pH of lysosomes results in the inhibition of antigen-presenting functions. This indicates the need for antigen to be internalized and processed in an acidic vesicle before it can be expressed on the cell surface for presentation (126).

Unanue (126) has shown that untreated macrophages can present hen egg lysozyme (HEL) to T cell hybridoms, paraformaldehyde-fixed cells could not. However, trypsin treated HEL could be presented by live macrophages or prefixed macrophages. Amino acid analysis of the immunogenic peptide revealed a very nonpolar portion and an area of predominantly polar amino acids.

It has been suggested that globular proteins or proteins of microbial origin must go through a processing stage in order to be expressed on the macrophage surface membrane. The fragment expressed must contain an immunogenic determinant, distinct from those of self-proteins, in association with a nonpolar stretch of amino acids for anchorage in the membrane (126). These immunogenic

fragments then either complex directly with Ia molecules or are located in close proximity with Ia due to random collisions in the membrane (126).

When a T helper cell precursor recognizes antigen in association with Ia antigens, the macrophage is stimulated to secrete IL-1. IL-1 in turn, serves as a stimulus for T cell activation which signals T helper cell precursors to differentiate into helper effector cells. These cells express receptors for and secrete IL-2 (T cell growth factor) (42).

Several investigators have demonstrated the need for IL-1 for efficient antigen presentation. Kramer and Unanue (58) indicated that while B cells are 10-100 times less effective at presenting KLH, the addition of macrophage conditioned media was able to increase their accessory function. Scala and Oppenheim (101) showed that monocytes pulsed with antigen and treated with paraformaldehyde could only present antigen to T lymphocytes in the presence of IL-1. Monocytes fixed prior to the antigen pulse could not present antigen even when IL-1 was added to the culture.

Thus the macrophage appears to be perfectly suited for its accessory role in antigen presentation. It is capable of interacting with a variety of antigenic proteins, processing them for expression on the cell membrane in association with Ia antigens, and secretes IL-1. While the role of other Ia positive cells in antigen presentation is unclear, the macrophage appears to be of major importance.

In addition to its immunostimulatory role, the macrophage also plays a vital role in suppression of the immune response. Macrophages <u>in vitro</u> have been shown to inhibit lymphocyte functions, particularly at high numbers or if activated for tumoricidal or microbicidal functions (125). It is this suppressive role of the activated macrophage that may prevent immune responses from becoming exceedingly destructive (97).

The suppressive effects of the macrophage are best realized by the secretion of prostaglandins, thymidine, oxygen intermediated and other factors (97,125). Prostaglandins have been shown in vivo to decrease antibody formation and in vitro to lower antigen and mitogen induced lymphocyte stimulation (97). Cyclooxygenase inhibitors such as indomethacin have been shown to reverse the inhibitory effects of macrophages in macrophage -lymphocyte interactions (125); however, it reportedly has no effect on macrophage tumoricidal activity (111). Thymidine appears to be a potent inhibitor of the immune response by interfering with the conversion of cytidylate to deoxycytidylate and blocking DNA synthesis (116). High levels of thymidine have been shown to inhibit the proliferation of rapidly dividing lymphocytes and thymocytes (117).

Macrophages have also been found to secrete large quantities of the enzyme arginase which catabolize the essential amino acid arginine and reportedly suppresses lymphocyte proliferation (18,59,97). Oxygen metabolites, particularly hydrogen peroxide may also inhibit lymphocyte proliferation in response to antigen (69). Given that the addition of reducing agents such as 2-mercaptoethanol enhances the proliferative response of lymphocytes to antigen (125), a proposed immunosuppressive role for oxygen intermediates is not unfounded.

The term "activated" in reference to large, nonspecifically microbicidal macrophages was first introduced by Mackaness (63). Later, when the tumoricidal activity of these macrophages was realized (40) the definition was expanded.

Development of the activated macrophage appears to be a complex, multistep process. As monocytes leave the circulation and enter into the tissues, the macrophage becomes exposed to a variety of positive and negative effector molecules that induce morphological, metabolic, and physiological changes

activating them for a variety of functions (5). It should be strongly emphasized that macrophage activation for destruction of tumor cells is not always synonymous with activation for the destruction of microorganisms (30,31,51,52,135). Because this term has been operationally defined, investigators should take care to indicate the function for which the macrophage has been activated.

It is now well established that the macrophage plays a central role in protection against facultative and obligate bacterial pathogens (51,63,108). Activated macrophages are also important in the elimination of viruses and extracellular parasites and protozoans (30,56,85,136).

Metchnikoff first noted that macrophages from immune animals showed increased resistance to bacterial challenge due to their increased phagocytosis and microbicidal activity (5). It was later determined that macrophages responding to infection with one organism could nonspecifically resist infection against antigenically unrelated bacteria (9). Fowles et al. (28) demonstrated the link between specific cell-mediated immunity and the nonspecific functions of the activated macrophage when they demonstrated that culture supernatants from antigen specific T lymphocytes could activate macrophages for nonspecific protective functions.

The mechanism by which macrophages recognize such a wide range of microorganisms is not well known; however, the ability to distinguish and respond to unrelated microorganisms has been demonstrated to occur by several mechanisms. Multicellular parasites (e.g. Schistosoma, Trichinella) are killed extracellularly by an IgE-dependent mechanism (26,20). Capron et al. (14) demonstrated that serum IgE acted on the macrophage because preincubation of schistosomula with immune serum did not induce killing while treated

macrophages were activated within one hour.

Fc-mediated phagocytosis is probably the primary immune-mediated mechanism for microbial attachment to macrophages. The protozon parasite Toxoplasma gondii is bound to the macrophage plasma membrane via the FcII receptors when opsonized with serum IgG (74). Phagocytosis directed by these receptors is particularly important with encapsulated bacteria such as Steptococcus pneumoniae, Hemophilus influenzae, and Neisseria meningitidis (74).

Complement receptors (CR1, CR3) also play a role in the uptake of microorganisms and is believed to play an important role when a nonimmune host is first confronted with an organism. Lipopolysaccharide from bacterial cell walls has been shown to bind complement thereby promoting the uptake of the organism (74). Nonimmune receptors may also play a role in the binding and uptake of some microbes. These receptors have been suggested to play an important role in the defense against microorganisms which fail to stimulate a strong humoral response and in nonimmune animals (74). Receptors recognizing glycoproteins ending in mannose or frucose have been reported to play a role in the phagocytosis of Salmonella typhimurium, Klebsiella aerogenes, and Corynebacterium parvum (132).

Attempts to demonstrate activation of macrophages for microbicidal activity in vitro were initially futile due to the extracellular replication of target microbes (77). The replacement of facultative intracellular bacteria by obligate intracellular parasites avoids this problem and allows an accurate assessment of the role macrophage in protection against pathogenic microorganisms.

In nonimmune macrophages, Toxoplasma, Leishmania, and Trypanosoma sp. enter by endocytosis but then reside in different locations within the cell. The uptake of these protozoans fails to trigger an oxidative respiratory burst or

synthesis of lysosomal enzymes. Toxoplasma gondii has been demonstrated to prevent fusion of the phagosome with lysosomes by altering the phagosomal membrane by an unknown mechanism. Leshmania amastigotes replicate within the phagolysosome by inactivating lysosomal enzymes and Trypanosoma cruzi lyse the phagosomal membrane and replicate in the cytosol (52).

When exposed to antigen and ConA-stimulated splenic supernatants, microbicidal activity can be induced in both resident and inflammatory macrophages (52,85). This acquisition of microbicidal activity against Leshmania, Toxoplasma, Trypanosomes, and Candida has been demonstrated to be closely linked with increased production of H_2O_2 and other reactive oxygen intermediates (52,74,75,76,85).

Murray et al. (75) has reported the augmented oxidative capacity of T. gondii activated macrophages to be antigen specific since boosting T. gondii immune mice (i.p.) with the same organism resulted in increased H_2O_2 and superoxide anion (O_2^-) generation. Boosting with BCG or PPD (purified protein derivative) did not increase these oxygen intermediates. These differences are thought to result from increased lymphokine production in antigen-stimulated T cells (76).

Microbicidal activity may possibly be enhanced by other nonoxidative mechanisms; however, their importance here remains to be established. Some of these mechanisms include lowering the pH of the phagosomes, secretion of lysozymal hydrolases, and the production of cationic proteins and peptides (5,60).

The macrophage has also been found to play an important role in the destruction of neoplastic cells. Alexander and Evans (7) first reported that exposure of peritoneal macrophage to LPS or double standard RNA rendered them cytotoxic to both syngeneic and allogeneic lymphoma cells. Hibbs et al. (38,39) found that macrophages activated for microbicidal functions using agents such as BCG, C. parvum, B. pertussis, and T. gondii were also cytotoxic to

tumor cells but not to normal untransformed cells. Consequently, because these macrophages were obtained from mice responding to microbial infections, tumoricidal macrophages were considered to be "activated" by definition.

Macrophage-mediated tumor cell cytotoxicity is a multistep process which involves: specific binding of neoplastic target cells; prolonged contact with the tumor cell target; and secretion of lytic products which appear to alter the integrity of the target membrane (5). Resident and thioglycollate-elicited macrophages have been shown to interact very little with adherent or nonadherent tumor cell targets in vitro. In contrast, macrophages activated by macrophage activating factor (MAF) and LPS have been shown to come into intimate contact with tumor cell targets (5,6). Using electron microscopy, activated macrophages can be observed to spread out over their target in clusters with their cytoplasmic membranes closely molded to it for several hours (6). After 6 hr of contact with activated macrophages, tumor cell division is almost completely abrogated (6). This cytostatic activity is followed by swelling of the cytoplasmic membrane (6) and complete lysis of the neoplastic cell within 16-18 hours (4).

The activated macrophage appears to be highly efficient and selective in destruction of neoplastic cells in vitro. One macrophage appears capable of lysing several targets; and when added to mixed cultures of neoplastic and nonneoplastic cells, they are not cytolytic for the normal cells (6).

Specific binding of tumor cell targets can be augmented by the treatment of inflammatory, but not resident, macrophages with LK (6,65). Binding capacity appears to be maximal after 8-12 hours of LK treatment (48). Augmented binding requires metabolically active macrophages; however, dead targets are bound (6). Intact microtubules and microfilaments are also necessary as demonstrated by the inhibition of tumor cell binding by the colchicine treatment (48). Specific

binding has also been shown to require the presence of a trypsin sensitive membrane structure and the presence of divalent cations (6). Examination of tumor cell binding by macrophages using electron microscopy reveals a small space or cleft between the points of contact. It has been proposed that this space functions to concentrate and protect lytic mediators (5,6,48).

While the activated macrophage has been reported to secrete many factors cytotoxic to other cells (4), few have been well characterized. The most extensively studied of these factors involved with macrophage-mediated tumor cytolysis includes arginase, hydrogen peroxide, and a novel cytolytic protease.

Neoplastic cells have been demonstrated to require large amounts of arginine and because macrophages are known to secrete high concentrations of arginase it has been considered a possible mediator of macrophage-mediated tumor cytotoxicity. The major support for arginase in this role is the finding that arginine can block tumor cytolysis when included in macrophage cytotoxicity assays (4). Arginase probably does not play a major role because it is secreted by inflammatory as well as activated macrophages and therefore does not correlate with tumor cell cytotoxicity (4,5,6).

Hydrogen peroxide has been implicated in microbicidal function of the macrophage and may also play a role in tumoricidal activities. It has been reported to lyse a variety of tumor cells; however, the effective concentration varies widely (79,80). Inhibition of H_2O_2 activity by catalase and anaerobiosis do not inhibit macrophage-mediated cytolysis. Therefore, while H_2O_2 may play a role in tumor destruction, apparently it is not a sufficient mediator on its own.

Adams et al. (1) first reported the presence of a novel serine protease present in the supernatants of BCG-activated macrophages but not in the supernatants from resident and inflammatory macrophages. This cytolytic protease (CP) is quite potent, having a 50% cytolytic concentration of around 1.0

x 10^{-9}M (4). Much evidence has accumulated indicating the importance of CP in macrophage-mediated lysis of neoplastic cells. First, there is a strong correlation between the secretion of CP and the development and expression of tumoricidal activity. Resident and inflammatory macrophages do not display tumoricidal activity nor do they secrete CP. Immune-elicited (BCG, C. parvum) or inflammatory macrophage treated with LK and LPS in vitro are cytolytic and secrete CP spontaneously (4,48). Second, low molecular weight protease inhibitors block CP activity and inhibit tumoricidal activity of activated macrophage and do so by acting at the target injury stage (47). Third, binding of viable tumor cells or plasma membranes by BCG-elicited macrophage results in secretion of CP while normal cells do not trigger CP release (4). Furthermore, addition of tumor cells to inflammatory macrophage does not stimulate CP release (48). Finally, the kinetics for the destruction of neoplastic cells in vitro with CP is similar to that of macrophage-mediated tumor cell cytotoxicity (4,6). Taken together, these findings indicate that CP is a major effector of macrophage-mediated tumor cytotoxicity.

Adams et al. (2) have shown that while H_2O_2 is unable to mediate tumor cytolysis, it appears to act synergistically with CP. Exposure of tumor cells to non-lytic concentrations of H_2O_2 (10^{-6}M or less) along with non-lytic concentrations of CP results in target cell lysis. It is possible that other secretory products of activated macrophages may act in conjunction with each other in a similar manner to mediate tumor cell killing.

Another lytic mechanism for the destruction of tumor cells by activated macrophages involves antibody-dependent cellular cytotoxicity (ADCC). Antibody-dependent cellular cytotoxicity appears to be a multistep event similar to direct tumor cytotoxicity, requiring binding of tumor targets and release of cytolytic mediators. However, the method of target recognition and

lysis is different. This mechanism kills neoplastic cells indirectly by recognition of the Fc portion of immunoglobulin (IgG) specific for determinants of the target cell (3). Direct contact-mediated cytolysis can require 1-3 days, in contrast, ADCC can result in tumor cell lysis within 4-6 hours (3,6).

Other findings have indicated that activation of macrophages for direct tumor cytolysis is distinct from activation for ADCC. Peritoneal macrophages elicited by BCG in C3H/HeJ and A/J mice, which are genetically deficient for the ability to recognize and lyse neoplastic cells respectively, can be activated for ADCC (3). This suggests that these two mechanisms differ in both the recognition and effector steps (3).

Nathan and Cohn (81) have suggested that H_2O_2 may be the lytic mediator of ADCC. It has been shown that anaerobiosis, deprivation of glucose, or addition of H_2O_2 scavengers such as thioglycollate inhibit ADCC activity (4). Binding of macrophage Fc receptors has also been shown to trigger the release of large quantities of H_2O_2 (4,5). These findings seem to indicate a major role for oxygen metabolites in ADCC.

A third function mediated by macrophages against neoplastic cells is target cytostasis. When tumor cells are cocultured in the presence of activated macrophages they show a reduced rate of cell division (6,30). This cytostatic activity can result by blocking DNA synthesis (S phase) or mitosis in the target cell (5,6).

Keller (54) demonstrated that whether an activated macrophage will be tumoricidal or cytostatic is dependent upon the tumor target, as certain tumor cells which are more resistant to cytolysis may still be vulnerable to the cytostatic mechanism of the activated macrophage. The inhibition of division in neoplastic cells can occur as a result of a reversible process or an irreversible process which leads to destruction of the cell. The mouse adenosarcoma cell line

EMT-6 shows a reduction in cell division in the presence of activated macrophages. This effect is reversible upon removal of the effector cells from culture (5,6). Cytostasis is also manifested in target cells before they are lysed by activated macrophages (6). Although the results are similar, the mechanism from which these two events arise are quite different.

The mechanism by which activated macrophages mediate their cytostatic effect is not well known. Of the possible cytotoxic substances secreted by the activated macrophage thymidine, arginase, prostaglandin (PGE), and interferon have all demonstrated anti-proliferative effects on neoplastic cells in vitro (6,35,97).

Signal Requirements for Macrophage Activation

Activation of inflammatory or responsive macrophages for cytotoxic activity directed towards tumor cell or microbial targets requires a two stage reaction sequence: a priming signal which induces a responsive, noncytotoxic state, and a triggering signal which stimulates the macrophage to become functionally activated (77). Resident macrophages which are unresponsive to activating signals become responsive when cultured for several days (6).

The priming signal for responsive macrophages has been demonstrated in vitro to be supplied by T cell-derived LK(s) known functionally as macrophage activating factor (MAF) (6,8). The primed macrophages can then be activated by nanogram quantities of endotoxin. Other products such as tumor cell supernatant, a low molecular weight serum factor, maleylated protein, and high doses of LK can also provide the triggering signal (6).

While there are obvious similarities in the regulation of tumoricidal and microbicidal functions, they may be dissociated according to the regulating signals required. T cell LK's appear to have an omnipotent role in functional regulation and activation of macrophages (31,89,114,138). It has been shown that

T cells can produce a variety of LK's with distinct physiological influences on the macrophage (31). Macrophages have also been shown to be heterogeneous in their response to the various LK's (114). Therefore, the use of crude supernatants from mitogen- and antigen-stimulated lymphocytes is of limited value in determining the signals and events leading to functional activation.

Determining the relationship between soluble T cell mediators and the regulation of macrophage functions must be approached at a single cell level; however, obtaining enough material from a single T-lymphocyte is impossible. When Kohler and Milstein (57) announced that they had immortalized an antigen-specific, IgG secreting plasmacytoma (P3-X63Ag8) with spleen cells from immunized mice, they opened the door for more in-depth studies concerning the precise nature of T cell-macrophage interactions.

The techniques for preparing T cell hybridomas is essentially a modification of the Kohler-Milstein method for the production of monoclonal antibody secreting cell lines but in which helper or suppressor T cells are fused with BW5147, a Thy 1.1 positive cell line of AKR ($H\text{-}2^k$) origin. Hybrid cells are then cultured in selective HAT medium (DMEM, 10% FCS, 100 µm hypoxanthine, 10 µM aminopterin, 30 µM thymidine). The BW5147 is deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) and cannot survive for longer than 24-48 hours in this medium (57). All cells living at day 14 can be assumed successful fusions since survival requires the HGPRT gene from the spleen cell parent and the genetic information for continuous growth from the lymphoma parent (99). Once the hybridomas become established they can be screened for the production of the desired lymphokines. Hybridoma technology offers a straightforward approach for determining the number and nature of the LK's produced by different T cell subsets. T cell hybridomas are a great advantage as they provide a defined source of material for functional,

biochemical and genetic analysis.

Hypothesis

It has been proposed that macrophages in various states of functional activation (i.e. resident, responsive, primed, and fully activated) are in different stages of the cell cycle. During these stages, the macrophages differ markedly in their capacity to respond to LK (112,113,114). In order to look more precisely into the process of macrophage activation and the role of T cell derived LK's we have constructed T cell hybridomas. The purpose of this study was to determine the role of T cell hybridoma LK's and recombinant γ -interferon in inducing different protective mechanisms in various macrophage populations. This was done by challenging in vitro with tumor cells, live opportunistic amoebae and amoebic or bacterial lysates. Supernatants from T cell hybridoma lines T-3 and T-9 were both capable of protecting resident macrophages from Naegleria lysates but are unable to elicit a similar response in TG macrophages. Gamma interferon treatment did not protect either macrophage population. It was further demonstrated that γ-interferon does not protect TG macrophages from the destructive effects of the adenylate cyclase enzyme of Bordetella. We have, therefore, concluded that protection from the cytopathic amoeba Naegleria fowleri and the bacterium Bordetella pertussis is a non-γ-interferon induced mechanism distinct from the mechanism involved in the destruction of tumor cells.

MATERIALS AND METHODS

Animals. Eight to ten week old, female C3H/HeN mice (Charles River Breeding Laboratories, Kingston, NY) were used in this study as the source of resident and thioglycollate (TG) macrophages.

Peritoneal macrophages. Resident and TG-macrophages were harvested by reflecting the skin over the abdomen and injecting 5.0 mls of Hank's balanced salt solution (HBSS); Ca^{++}/Mg^{++} free) into the peritoneal cavity. After agitating the abdomen, cells were drawn off and suspended in HBSS. Cells were centrifuged at 500 x g at 25°C, washed twice, and resuspended in 10 ml of Dulbecco's modified minimum essential medium (DMEM) (Flow Laboratories) + 10% FCS. Red blood cells were lysed with 5.0 ml tris-buffered NH_4Cl (pH 7.2). TG-macrophages were obtained from mice receiving 1.0 ml (i.p.) of 10% Brewer's thioglycollate 4 days prior.

Tissue culture media. T-cell hybridomas were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, penicillin and streptomycin (100 µg/ml), 4% sodium bicarbonate, and 15 mM HEPES (pH 7.2). Hybridoma T-cell lines were also maintained in HB101 serum-free medium specifically for culturing murine myeloma and hybridoma cell lines (Hana Biologics, Inc., Berkeley, Calif.). Hybridomas were adapted and maintained in this medium to facilitate the isolation and purification of T cell derived LK's.

T-cell hybridoma culture supernatants. T-cell hybridomas were prepared as described by Niederhuber et al. (84). Briefly, $1x10^7$ HAT sensitive BW5147 thymic lymphosarcoma cells were fused with $1x10^8$ nylon wool purified, ConA stimulated splenic T-cells (Fig. 1). Hybrid cells were recloned by limiting dilution and screened for their ability to protect adherent resident peritoneal cells against the effect of Naegleria cytotoxic factor(s), their ability to increase tumor cell cytolysis by TG-elicited macrophages, and induction of Ia antigen

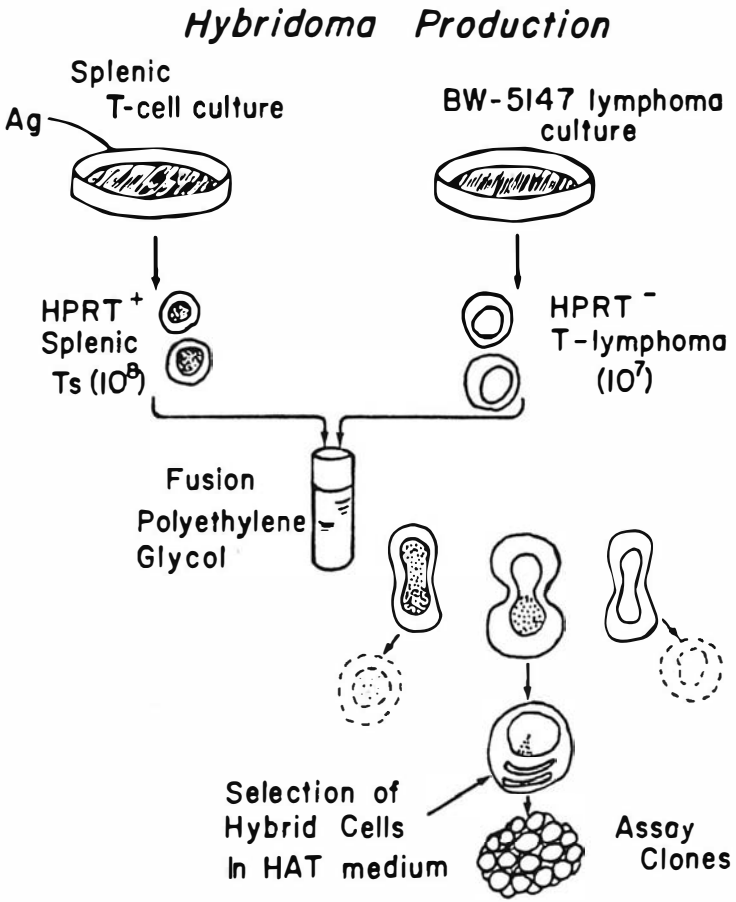


FIG. 1. Production of T cell hybridomas.

expression in TG-elicited macrophages after 24 hr of treatment. When used for lymphokine production, T cell hybridomas T-3 and T-9 were seeded at either $1x10^6$ cells/ml in DMEM + 10% FCS or $6x10^4$ cells/ml in HB101 serum free media. Supernatants were collected after 24-48 hr by centrifugation at 500 x g for 10 min at 4°C.

Determination of hybridoma division time. T-cell hybridomas were maintained at $1x10^6$ cells/ml in DMEM + 10% FCS. Total cell number per flask was determined at 24, 48, and 72 hr.

IL-2 assay. IL-2 dependent, C10 cells ($1x10^4$ cells) were cultured for 24 hour along with serial dilutions of T-3 and T-9 supernatants in flat bottom tissue culture plates. C10 were also cultured in TCGF conditioned medium or DMEM as positive and negative controls. The cells were then pulsed with 1μCi ^{3}H-TdR for an additional 24 hour. Wells were harvested using a MASH cell harvester and assayed using a liquid scintillation counter.

Induction of hybridoma derived LK. In an attempt to maximize the production of LK from hybridomas T-3 and T-9, cells were incubated at various densities ($2.5x10^4$, $5x10^4$, $1x10^5$m $5x10^5$) with or without 5μg ConA. At 24 and 48 hr, 5.0 ml of supernatant was collected and the ConA was absorbed out of the supernatants by adding G-25 sepharose beads and centrifuging at 1000 x G for 10 min. ConA-free supernatants were then collected. The amount of LK produced was reflected as the percent cytostasis induced by the supernatants in TG-macrophage (cytostasis assay was performed as described earlier).

Macrophage-mediated destruction of Naegleria fowleri. Resident macrophages (2×10^5) were incubated along with diluted T-3 or T-9 supernatants (1:1, 1:2, 1:8). After 24 hrs, 2×10^4 ^{3}H -uridine labelled N. fowleri target cells (which had been cultured with 5μ Ci ^{3}H -uridine 24 hr prior) were added. After an additional 24 hr, 100 μl of each culture supernatant was

removed and added to 3 ml of Beckman redi-solve scintillation fluid. All samples were run in triplicate and assayed using a liquid scintillation counter (2 min, 2% error).

Assay for macrophage protection against Naegleria fowleri lysate. Naegleria lysates were provided by Dr. Francine Marciano-Cabral (Department of Microbiology and Immunology, Medical College of Virginia) and were prepared by freeze-thawing using a dry ice - EtOH bath, followed by submersion in a hot water bath for 30 sec. After freeze-thawing 4 times, the amoebae were sonicated for 3, one min intervals. Resident and thioglycollate-recruited macrophages were cultured at $3x10^5$, $1.5x10^5$, and $3x10^4$ cells/ml along with $3x10^4$ equivalents of crude N. fowleri lysate (lysate equal to $3x10^4$ live Naegleria), and either recombinant γ-IFN, T-3, or T-9 at 37°C and 5% CO_2. At the end of 18 hr, the macrophages were pulsed for 2 hr with 50 µl (5 µCi) of $Na_2{}^{51}CrO_4$. The wells were washed 3 times to remove unbound ^{51}Cr and then 100 µl of 1N NaOH was added and incubated for 5 min at room temperature. This was repeated once again and then samples were radioassayed using a LKB gamma counter (all samples were run in triplicate).

Assay for macrophage protection against Bordetella pertussis lysates. Resident and TG-macrophages were cultured at $3x10^5$, $1.5x10^5$, and $3x10^4$ cells/ml along with lysates from Bordetella strain BP338 (wild type) (45 µg protein/ml) and recombinant γ-IFN. After 18 hr, macrophages were pulsed for 2 hr with 50 µl (5µCi) of $Na_2{}^{51}Cr0_4$. The wells were washed three times to remove unbound ^{51}Cr and then 100 µl of 1NaOH was added and incubated for 5 min at room temperature. This was repeated and samples were radioassayed using a LKB gamma counter (all samples were run in triplicate). Bordetella lysates were a gift from Alison Weiss (Department of Microbiology, University of Virginia, Charlottesville).

Tumoricidal assay. The activation of resident or TG-elicited macrophages for tumoricidal activity was determined using the C3H/HeJ fibrosarcoma, L929, as target cells (67). Adherent peritoneal exudate cells ($5x10^5$) were placed into 24 well tissue culture plates with dilutions of T-3, T-9 or γ-IFN. After 24 hr the wells were washed twice with warmed media and cultured with $5x10^4$ ^3H-TdR labelled L929 target cells. After an additional 24 hrs, 5 µg DNase was added 20 min prior to harvesting the supernatants to facilitate the release of radioactivity from killed target cells (129) and 0.5 ml of each culture supernatant was removed and then placed in 10 ml of Beckman redi-solve scintillation cocktail. All samples were run in triplicate and assayed using a liquid scintillation counter (2 min, 2% error).

Induction of macrophage-mediated tumor cell cytostasis. Diluted supernatants from T-3 and T-9, or cloned γ-IFN were compared for their ability to activate resident or TG-elicited macrophages for tumor cell cytostasis. Adherent PEC's ($1x10^5$) were either incubated for 24 hrs with LK or LK was added simultaneously with the addition of $1x10^4$ B16F10 melanoma target cells. After an additional 24 hrs, the cells were washed twice with warm DMEM and the cells were pulsed for 24 hours with 1 µCi ^3H-TdR. Wells were harvested using a MASH cell harvester and assayed using a liquid scintillation counter (2 min, 2% error) (53). Percent cytolysis was calculated by the formula:

$$\text{Cytostasis (\%)} = \frac{\text{experimental CPM}}{\text{control CPM}} \times 100$$

Experimental CPM: B16F10 target cells and macrophage; control CPM: B16F10 tumor cells in the absence of macrophage.

Kinetics for the induction of tumor cell cytostatic activity by LK. Adherent, thioglycollate-elicited macrophages ($1x10^5$) were added in 100µl to 96 well plates. Macrophages were treated with 1:10 dilutions of T cell hybridoma

supernatants (T-3 or T-9) or 10U of cloned γ-IFN for 0, 6, 12, 18, and 24 hr. At the end of each incubation period, 1 x 10^4 B16F10 melanoma target cells were added. After an additional 24 hr incubation, the cells were given 24 hr pulse with ^{3}H-TdR (1μCi/well) and assayed as described.

Induction of Ia-antigen expression. The T-cell hybridoma supernatants were screened for their ability to induce Ia antigen expression using immunofluorescence. TG elicited PEC's ($5x10^5$ cells/100 μl) were adhered on glass slides and fixed for 15 min using 1% paraformaldehyde. Macrophages were then washed in DMEM (5% rabbit serum) and after 20 min fresh DMEM (5% rabbit serum) containing a 1:80 dilution of the monoclonal antibody 10-2.16 (anti-I-A^k) was added for 40 min at 4^{o}C. Unbound antibody was removed by washing twice with cold media. Next, media containing a 1:30 dilution of fluorescein-conjugated goat-anti-mouse antibody (heavy and light chain specific) was added at 4^{o}C for an additional 40 min. Cells were again washed twice to remove any unbound antibody and analyzed using epiilumination.

Determination of ectoenzyme profiles. Resident or TG-LK-treated macrophages ($1x10^6$) were lysed in 1.0 ml of 0.05% triton X-100 and then frozen at -20^{o}C. Alkaline phosphodiesterase I (APD-I) activity was determined by taking 100 μl of the macrophage lysates and aliquoting into disposable glass 12x75 mm tubes along with the substrate, 1.5 mM p-nitrophenyl thymidine-5'-monophosphate (prewarmed to 37^{o}C). The samples were mixed well, covered with aluminum foil to protect from light, and then incubated at 37^{o}C water bath. After 30 min the samples were placed on ice and the reaction stopped by the addition of 1.0 m of 0.1 N NaOH and vortexed. Absorbances were determined using a Beckman spectrophotometer at 400 nm (24). To determine 5' nucleotidase activity (5'N), 500 μl of 0.018 mM ^{3}H-AMP substrate was added to 100 μl of macrophage lysate and incubated at 37^{o}C in a water bath, shaking every 10 min. After 30

min the samples were placed on ice and the reaction stopped by adding 200 µl of 0.25 M $ZnSO_4$ and vortexed, followed by the addition of 200 µl 0.25 M Ba $(OH)_2$ and vortexing once again. Samples were then centrifuged at 1000 x g for 30 min after which 500 µl of the supernatant was added to 10 mls of Beckman redi-solve scintillation fluid and each sample was counted in a Beckman scintillation counter (23). Resident and TG-macrophages were then compared for changes in API and 5'N activity.

Transferrin binding. Transferrin was iodinated using enzymobead reagents (Biorad). Briefly, transferrin was radioiodinated with lactoperoxidase and glucose oxidase coupled to agarose beads according to the manufacturer's instructions. $Na^{125}I$ (1.0m Ci) was added to the reaction along with the protein to be labelled. The reaction was allowed to proceed for 25 minutes at RT and then the contents of the vial were placed on a Sephadex G-25 column and centrifuged at 100 x G for 2 minutes. The effluent was collected in a polystyrene test tube and diluted with 1.0 ml of PBS and 2µl was counted in a LBK gamma counter to determine the CPM/µg protein. A TCA precipitation was done to determine the efficiency of labelling. To assay for transferrin binding, 1 x 10^6 resident or TG-macrophages were cultured in 24 well plates in DMEM alone or in the presence of γ-IFN, T-3 or T-9. After 24 hr wells were washed twice with Hanks (BSA 1 mg/ml). ^{125}I-labelled transferrin (2 nmoles) was then added to 1.0 ml Hanks buffer along with a 1000 fold excess of unlabelled transferrin. Plates were incubated at 4^oC for 1 hr and then washed twice with buffer. This was followed by the addition of 0.1 N NaOH (0.5 ml) which was allowed to stand overnight at 4^oC. Results were determined using a LKB gamma counter. All samples were run in triplicate.

Recombinant γ-interferon. The recombinant mouse γ-IFN used in this study was generously supplied by Patrick W. Gray (Genetech, South San Francisco,

CA), and was produced from monkey COS-7 cells transfected with a plasmid containing the murine γ-IFN gene expressed under control of the SV40 promoter. Supernatants contained 19,000 units/ml as determined in a cytopathic effect inhibition assay using encephalomyocarditis virus on L929 murine fibrosarcoma cells (33).

Anti-γ-interferon. The rabbit anti-mouse γ-IFN was a gift from Howard M. Johnson (Dept. of Comparative and Expt. Pathology, Univ. of Florida, Gainesville) and was produced by stimulating spleen cells with staphylococcal enterotoxin A as described earlier. The serum titer used in this study contained antibody sufficient to neutralize 1,000 units of γ-IFN/ml (88).

Neutralization studies. The effects of anti-γ-IFN on LK activation of TG-elicited macrophages for B16F10 tumor cell cytostasis was determined. Diluted supernatants from T-3 and T-9 (1:5, 1:10, 1:20) were added to $1x10^5$ adherent PEC's along with antibody sufficient to neutralize 10 U of γ-IFN activity. After 24 hr $1x10^4$ B16F10 melanoma target cells were added and the ability of anti-γ-IFN to inhibit macrophage cytostatic activity was determined.

Molecular characterization of T-3 and T-9. In order to establish the protein nature of the LK's, supernatants were incubated at 56°C, for 3 min, 80°C or 100°C for 5 min. Supernatants were also treated with 6.5 units of immobilized α-chymotrypsin (Sigma) for 1 hr. Treated supernatants were then assayed for cytostatic activity. The molecular weight range of T-9 was determined using an Amicon filtration system. Using a ym30 (mw. cut off 30K) and a ym10 (mw. cut off 10K) filter, three fractions were collected: (a) $\geq$ 30,000, (b) $\geq$ 10,000 $\leq$ 30,000, (c) $\leq$ 10,000. These fractions along with unfractionated supernatant were then assayed for cytostatic activity.

RESULTS

Screening of T-cell hybridomas. Supernatants from the T-cell hybridomas were collected and initially screened for their ability to protect against the cytotoxic effects of Naegleria lysate, increase tumoricidal activity, and induce Ia-antigen expression in resident and TG-macrophages.

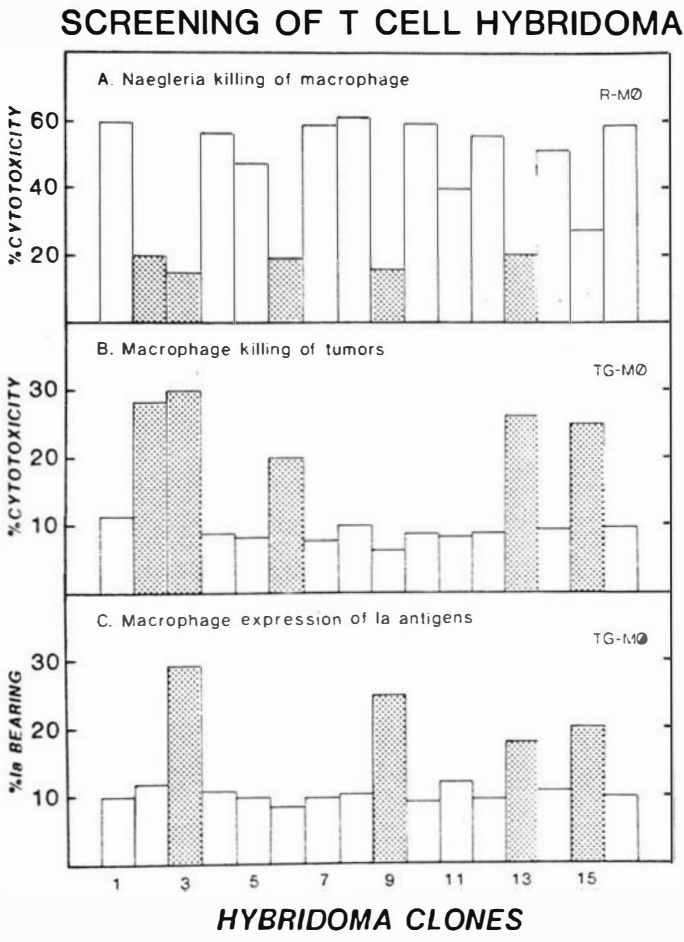
Supernatants from 5 of the 16 cloned cell lines (T-2, T-3, T-6, T-9, T-13) were able to significantly lower Naegleria lysate induced cytotoxicity in resident macrophages (Fig. 2A). Supernatants from clones T-2, T-3, T-6, and T-13 induced tumoricidal activity in TG macrophages, while this population appeared to be unresponsive to T-9 supernatants (Fig. 2B). Only three (T-3, T-9, T-13) of the original clones with activity protecting against Naegleria lysates also induced Ia antigen expression in TG-macrophages (Fig. 2C). Based on thse three activity patterns were selected T-3 and T-9 for further characterization.

T-3 and T-9 were both maintained in DMEM and 10% FCS and gradually adapted to HB101 serum free medium. Both clones continued to produced sufficient LK in either media (serum-free supernatants were designated 3HB and 9HB).

T-cell hybridoma division times. Both hybridomas T-3 and T-9 were observed to double in number every 24 hr. Cell number increased from the initial $1x10^6$ cells/ml to $1.8x10^6$ after 24 hr, $4.1x10^6$ cells/ml at 48 hr, and $12.8x10^6$ at 72 hr. (Fig. 3). The growth of both T-3 and T-9 was particularly density dependent which may explain why cellular division proceeded at a slightly increased rate between 48 and 72 hr.

IL-2 determination. None of the culture supernatants screened contained any IL-2 activity relative to the positive controls containing TCGF (Table 1). At all three dilutions, one of the T cell supernatants were able to induce a proliferative response as determined by the uptake of ^{3}H-TdR.

Fig. 2. Screening of cloned T cell hybridoma supernatants. A. Protection of resident macrophage against destruction crude Naegleria fowleri lysate. Adherent peritoneal macrophage (3×10^5) were co-cultured with 3×10^4 equivalents of crude lysate and a 1:2 dilution of crude hybridoma supernatants. After 18 hr, macrophage were given a 2 hr pulse with $Na_2{}^{51}CrO_4$. Unbound ^{51}Cr was washed free and viable macrophage were lysed in 1N NaOH. Triplicate samples were assayed using a LKB gamma counter. B. Activation of TG-elicited peritoneal macrophage for tumoricidal function. TG-macrophage were cultured 24 hr with 1:10 dilution of the hybridoma supernatants. Supernatants were removed and 5×10^4 ^3H -TdR labelled L929 cells were added for an additional 24 hr. Prior to harvesting 5 μg DNase was added and then 0.5 ml of culture supernatant was assayed. Results are presented as percent ^3H -TdR released. C. Induction of Ia-antigen expression. TG-macrophage (5×10^5/100 μl) were treated for 24 hr with hybridoma supernatants and then adhered to glass slides and fixed in 1% paraformaldehyde. Macrophage were washed and a 1:30 dilution of a monoclonal antibody 10-2.16 (anti-$I\text{-}A^k$) was added, followed by the addition of fluorescein-conjugated goat-anti-mouse antibody (diluted 1:30). Cells were analyzed using epiilumination and expressed as % of total population expressing Ia antigen.


SCREENING OF T CELL HYBRIDOMA
A. Naegleria killing of macrophage
R-MØ
%CYTOTOXICITY
60
40
20
B. Macrophage killing of tumors
TG-MØ
%CYTOTOXICITY
30
20
10
C. Macrophage expression of Ia antigens
TG-MØ
%Ia BEARING
30
20
10
1
3
5
7
9
11
13
15
HYBRIDOMA CLONES

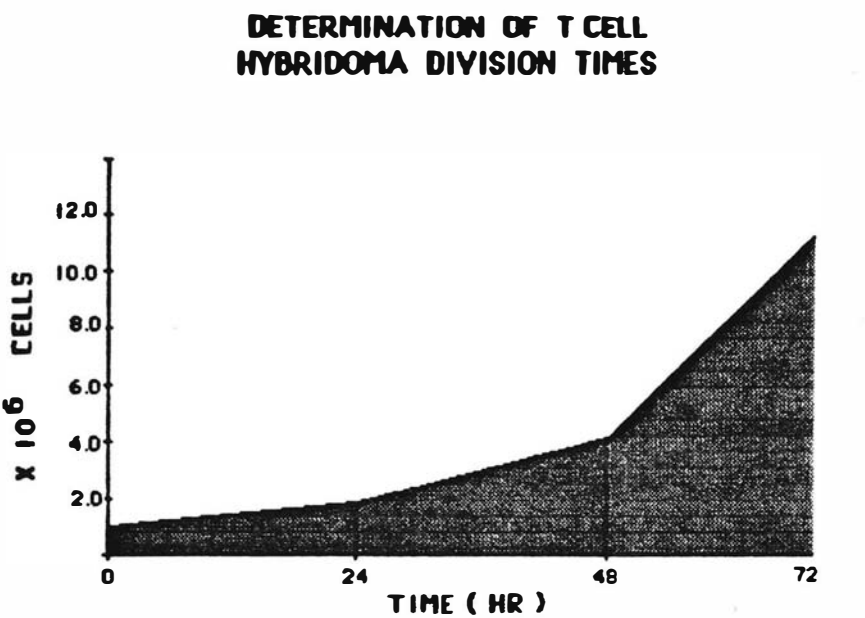


Fig. 3. T cell hybridoma division time. T cell hybridomas T-3 and T-9 were initially cultured at 1 X 10^6 cells / ml. Cell numbers were determined at 24, 48, and 72 hr.

Table 1. Assay for IL-2 activity in T cell hybridoma supernatants[a]

SAMPLES	MEDIA	CPM +/-S.D. (% v/v condition media) 50	25	12.5	6.25
1-4	3 S[b]	334 ± 99	585 ± 195	798 ± 301	391 ± 70
5-8	3 HB[c]	348 ± 84	357 ± 19	405 ± 73	51 ± 290
9-12	9 S	240 ± 48	429 ± 108	474 ± 55	518 ± 155
13-16	9 HB	868 ± 46	912 ± 150	943 ± 218	887 ± 159
17-20	TCGF	12355 ± 238	15315 ± 736	19372 ± 1808	13656 ± 1027
21	none	279 ± 109			

[a]IL-2-dependent C10 cells were cultured at $1X10^4$ along with serial dilutions of T-3, T-9, or TCGF. Cells were pulsed with 1 μCi ^3H -TdR for an additional 24 hr and then harvested on a MASH cell harvester and assayed using a liquid scintillation counter.

[b]Supernatants were obtained from cultures grown in DMEM + 10% FCS.

[c]Supernatants wre obtained from cultures grown in HB101 serum-free media.

Induction of hybridoma-derived LK. While it has been demonstrated that the addition of ConA to hybridoma cultures can increase production of LK (84), this was not the case for T-3 or T-9. The addition of ConA did not result in any significant differences when compared to untreated cultures. Dilution of the supernatants did not show any differences in the cytostatic activity induced in the treated macrophages. Both the treated and untreated supernatants produced maximum cytostatic activity at 1:10 and 1:20 dilutions (Fig. 4). The length of exposure to ConA did not effect LK production as treatment for 24 hr (data not shown) or 48 hr yielded similar results. Varying the cell number also did not produce a differential response to ConA (data not shown).

Macrophage protection against Naegleria fowleri. The ability of T-3 and T-9 supernatants to protect resident macrophages against the cytopathic effects of live Naegleria and Naegleria lysates were compared. Resident macrophages resulted in the specific release of less than 10% of the ^{3}H -uridine labelled Naegleria in the absence of LK. When cultured in the presence of T-3 or T-9, resident macrophages mediated the specific release of 42% and 43% of the ^{3}H -uridine label respectively (Fig. 5) (spontaneous release was les than 6%). T-3 and T-9 LK's also protected resident macrophages from destruction by Naegleria lysates. Resident macrophage destruction was decreased by 15% and 24% upon the addition of T-3 or T-9 supernatants (Fig. 6). The cytostatic activity (anti-proliferative effect as determined by ^{3}H -TdR uptake) in the same population of resident macrophages towards B16F10 target cells however was low. This activity could not be increased by the addition of T-3 or T-9 supernatants (data not shown).

Cloned γ-IFN was unable to offer similar protective mechanism to resident macrophages. Ten units of γ-IFN had no effect on cytotoxicity in lysate-treated resident macrophages. Regardless of the target/effector ratio (resident

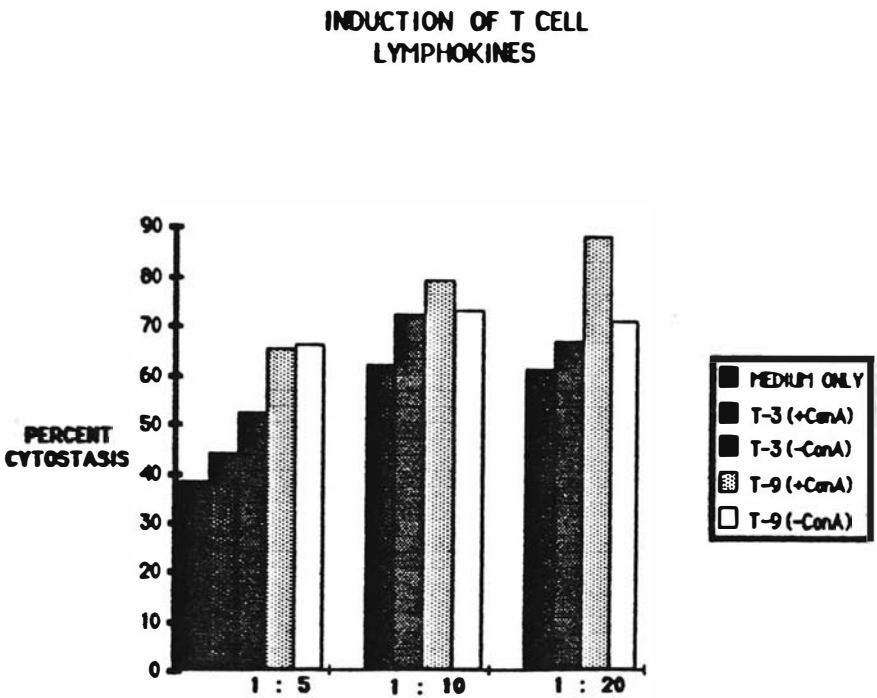


Fig. 4. Induction of hybridoma derived LK. Hybridomas T-3 and T-9 (5×10^5) were incubated with or without 5 µg Con A. After 48 hr, 5.0 ml of supernatant was collected and Con A was absorbed out of the supernatant using G-25 sepharose beads and centrifuging at 1000 X G for 10 min. ConA - free supernatants were then collected and assayed for the induction of cytostatic activity in TG-M0.

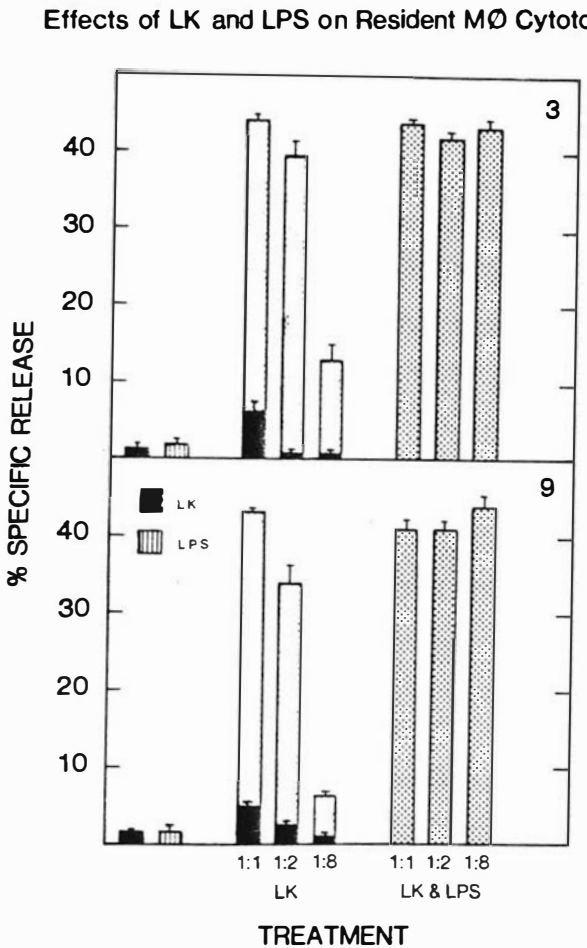


Fig. 5. Macrophage-destruction of Naegleria fowleri. Resident macrophage were incubated for 24 hr with diluted T-3 and T-9 supernatants (1:1, 1:2, 1:8) followed by the addition of 2 X 10^4 ^3H - uridine labelled N. fowleri target cells. After an additional 24 hr, 100 µl of each cultured supernatant was removed and added to 3 ml scintillation cocktail and assayed using a scintillation counter (all samples were run in triplicate).

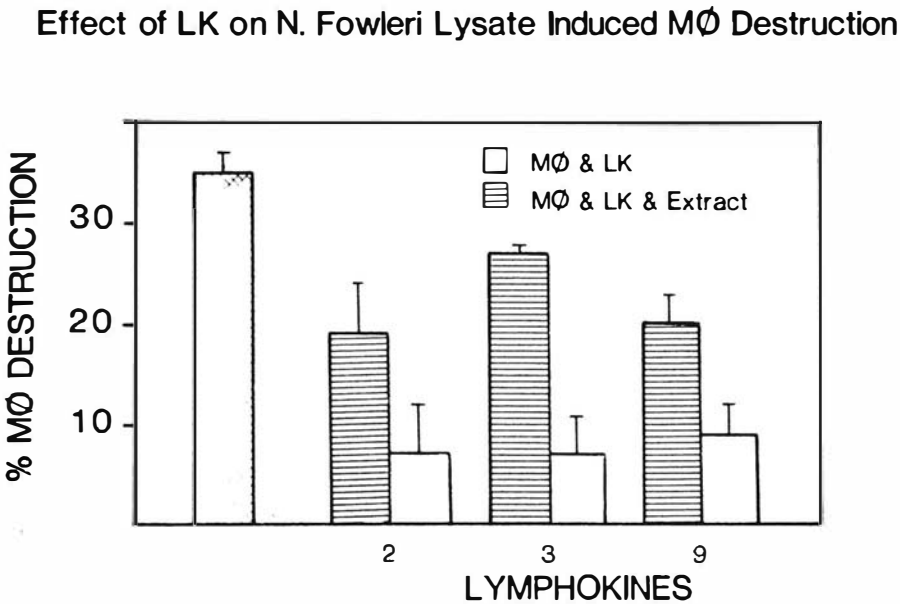


Fig. 6. Effect of T cell hybridoma-derived LK on the destruction of macrophage by Naegleria fowleri lysates. Resident macrophage were cultured at 3 X 10^5/well with a 1:2 dilution of T-2, T-3, or T-9 LK. After 24 hr 3 X 10^4 Naegleria equivalents were added for an additional 18 hr. Macrophage were pulsed with 2 hr with 54Ci ^{51}Cr/well. Unbound ^{51}Cr was washed free and viable cells lysed in 1N NaOH. Triplicate samples were assayed using a LKB gamma counter.

macrophages: Naegleria equivalents), γ-IFN did not appear to influence their vulnerability to Naegleria lysates (Table 2). Thioglycollate-elicited macrophages were also sensitive to the cytotoxic effect of lysate and as seen with resident macrophages, the addition of γ-IFN was unable to prevent their destruction. (Table 2). Unlike resident macrophages, which were not activated for tumor cell cytostasis by LK (T-3, T-9, γ-IFN), γ-IFN treatment of TG-macrophages resulted in increased activity directed against B16F10 melanoma cells (Table 3).

Macrophage protection against Bordetella pertussis. The ability of recombinant γ-IFN to protect TG-macrophages against the cytopathic effects of Bordetella pertussis (strain BP338) lysates was also determined. At all target (TG-macrophages)/effector (BP338 lysates) ratios tested, γ-IFN was unable to offer protection (Fig. 7). Both γ-IFN-treated and untreated macrophages were equally susceptible to the cytopathic effects of lysates. This observed destruction of TG-macrophages by BP338 lysates is density dependent. Using $3x10^5$ macrophages, 57% of the untreated macrophages and 51% of the γ-IFN-treated macrophages were killed. This increased to 69% and 66% respectively with $1.5x10^5$ macrophages and 90% and 92% using $3x10^4$ cells.

Kinetics for the induction of cytostatic activity by T cell LK. In order to determine the exposure time to T-3 and T-9 required to produce maximal cytostatic activity, TG-macrophages were exposed to LK for various time periods between 24 and 48 hr. All macrophages were exposed to target cells for the same period of time (24 hr). Exposure of macrophages to LK for 24 hr induced cytostatic activity in TG-macrophages (Fig. 8). This activity remained constant over the next 48 hr, as further exposure was unable to increase activity.

Alteration of ectoenzyme profiles. Several investigators have associated plasma membrane ectoenzyme levels with differentiation or activational changes in

Table 2. Effect of recombinant γ-IFN on the destruction of macrophage by Naegleria fowleri Lysates

Macrophage/ Naegleria Equivalents[a]	% Macrophage Destruction (^{51}Cr Uptake)			
	Resident Macrophage		Thioglycollate Macrophage	
	medium	γ-IFN (10 U)	medium	γ-IFN (10 U)
$3X10^5/3X10^4$	21 ± 15[b]	17 ± 12	20 ± 4	14 ± 3
$1.5X10^5/3X10^4$	22 ± 12	29 ± 8	22 ± 8	23 ± 3
$3X10^4/3X10^4$	26 ± 6	15 ± 7	30 ± 12	26 ± 7

[a]Macrophage were cocultured with Naegleria lysates for 18 hr, pulsed for 2 hr with 5μCi of ^{51}Cr, and lysed with 1N NaOH.

[b]Mean ± standard deviation.

Table 3. Enhancement of tumor cell cytostasis by recombinant γ-IFN

	% Cytostasis (^{3}H -Tdr Release)			
	Resident Macrophage		Thioglycollate Macrophage	
Macrophage/B16F10[a]	medium	γ-IFN (10 U)	medium	γ-IFN (10 U)
$1X10^{5}/1X10^{4}$	11 ± 5[b]	0	66 ± 9	76 ± 3
$5X10^{4}/1X10^{4}$	0	18 ± 1	64 ± 1	80 ± 1
$1X10^{4}/1X10^{4}$	0	0	18 ± 10	78 ± 2

[a]Macrophage were treated with recombinant γ-IFN for 24 hr and wells were washed 3X to remove excess LK prior to the addition of B16F10 target cells. Cultures were incubated an additional 24 hr followed by a 24 hr pulse with 1μCi ^{3}H -TdR. Wells were harvested using a MASH cell harvester and asayed using a liquid scintillation counter.

[b]Mean ± standard deviation.

CYTOTOXIC EFFECT OF BORDETELLA
LYSATES ON THIOGLYCOLLATE MØ

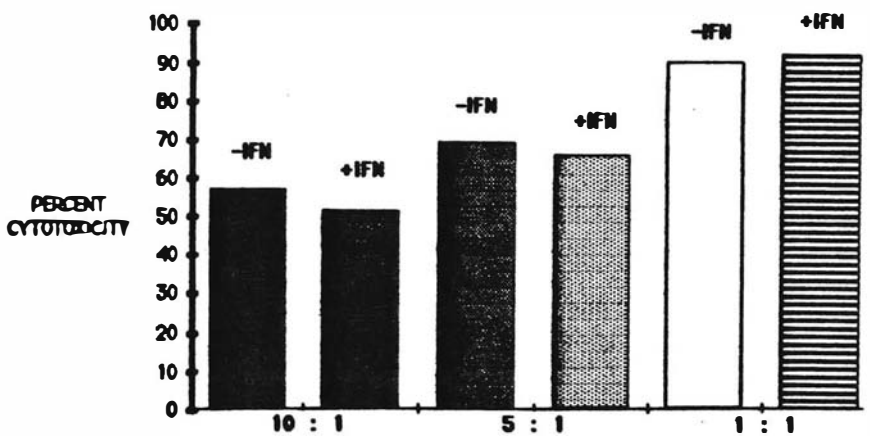


Fig .7 . Effect of cloned gamma - interferon on the destruction of T6-MØ by Bordetella pertussis (BP338, wild type) lysates. T6-elicited MØ were cultured with 10 units gamma- interferon After 24 hr 100µl ofBordetella lysate (45µg/ml protein) was added for an additional 18 hr.MØ were pulsed for 2 hr with 5 µCi ^{51}Cr / well. Unbound ^{51}Cr was washed free and viable cells lysed in 1N NaOH. Triplicate samples were assayed using a LBK gamma counter.

KINETICS FOR THE INDUCTION OF CYTOSTATIC ACTIVITY IN TG-ELICITED MØ

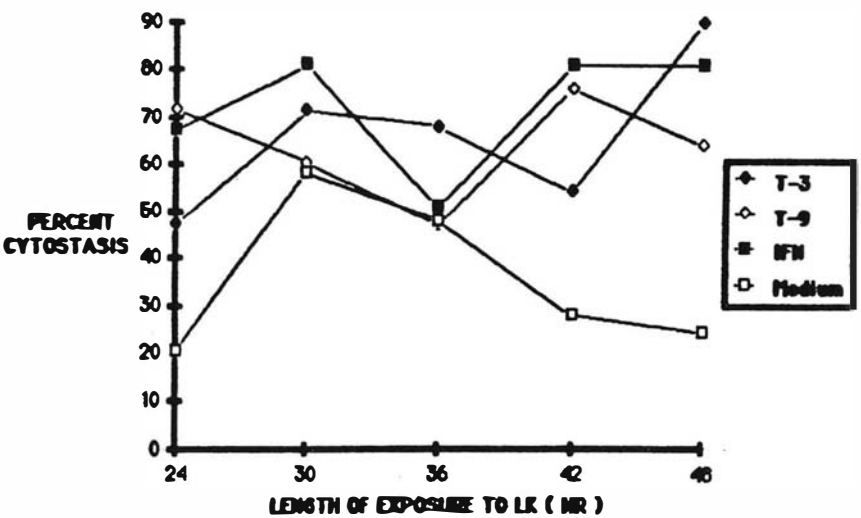


Fig. 8. Kinetics for the induction of cytostatic activity by T cell LK. Thioglycollate - elicited MØ ($1X10^5$) were added to 96 well plates and treated with T cell hybridoma supernatants or cloned gamma-interferon for 0,6,12,18,and 24 hr. At the end of each incubation period, $1X10^4$ B16F10 melanoma target cells were added. After an additional 24 hr, cells were pulsed with 1µCi [^{3}H]-TdR and harvested and assayed as described.

macrophage populations and have demonstrated changes in the ectoenzyme levels in tumoricidal macrophages (19,21,70). We therefore chose to determine the effects of T-3 and T-9 on APD-I and 5'N activity. Resident macrophages initially had lower APD-I activity but higher 5'N activity as compared with TG-macrophages. After treatment with T-3 or T-9 for 24 hr there were no significant changes in APD-I or 5'N levels in either macrophage population (Fig. 9, 10).

Because T-3 exhibited the capability to induce tumor cell cytotoxicity and cytostasis, protect against the cytopathic effects of Naegleria fowleri lysates, and induced Ia expression, but did not alter ectoenzyme profiles at 24 hrs, we decided to observe the kinetics of T-3 modulation of ectoenzyme levels in resident-macrophages (Fig. 11, 12). Alkaline phosphodiesterase I activity was observed to decrease during the first 4 hrs. Between 4-12 hrs there was an increase in T-3 treated cells, while activity continued to decrease in untreated resident-macrophages. At 24 hrs, activity levels were not significantly higher than the untreated controls.

The 5'N activity was also observed to decrease rapidly in T-3 treated and untreated resident macrophages during the first 4 hrs. Activity leveled from 4-12 hrs, but continued to decrease in both treated and untreated cells.

Expression of transferrin receptors. Recent findings have shown that the expression of transferrin receptors may be a useful marker of the responsive stage for macrophage functional activation (37,131). For this reason we wanted to determine the effects of T-3 and T-9 supernatants on the number of transferrin receptors. We found TG-elicited macrophages initially bound significantly higher quantities of ^{125}I-transferrin compared to resident macrophages (Table 4). Untreated resident macrophages bound 17 pico moles of transferrin. When treated with T-3 or T-9 the number of pico moles bound

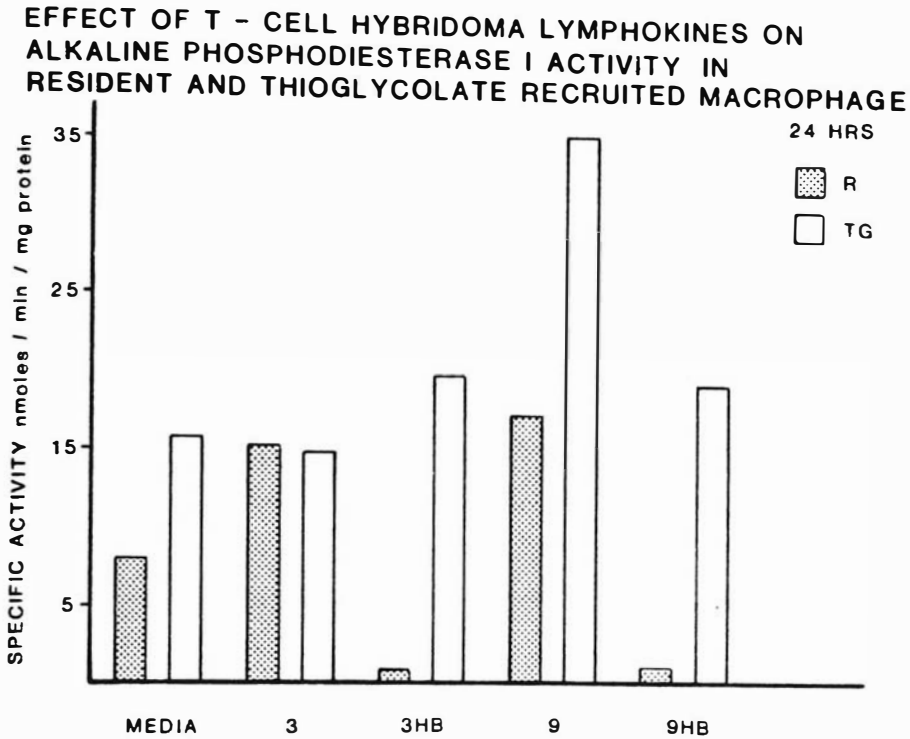


Fig. 9. Effect of T cell hybridoma lymphokines on alkaline phosphodiesterase I activity in resident and thioglycollate-recruited macrophages. Resident and TG macrophages were treated with 1:10 dilutions of T-3 or T-9 hybridoma supernatants containing serum (3,9) or without serum (3HB, 9HB). After 24 hr, $1X10^6$ cells were lysed in 1.0 ml of 0.05% triton X-100. Alkaline phosphodiesterase I levels were determined as described in Materials and Methods.

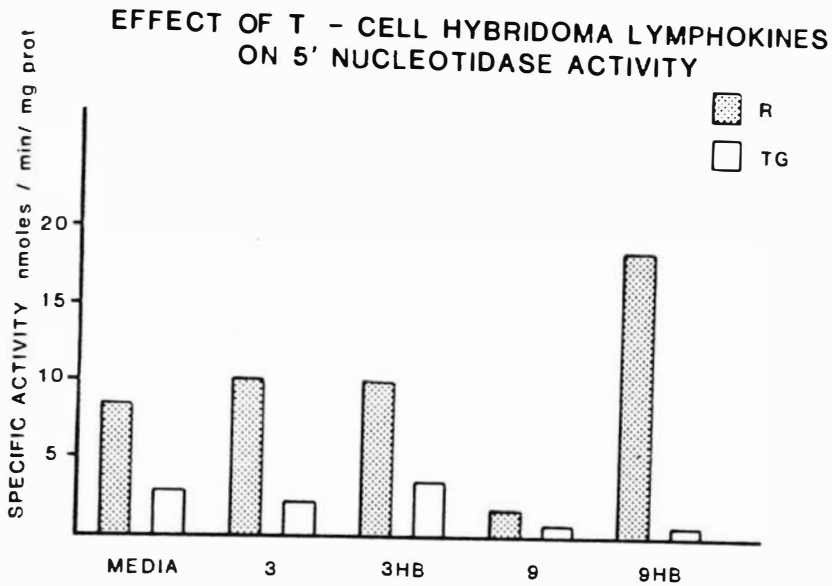


Fig. 10. Effect of T cell hybridoma lymphokines on 5'nucleotidase activity. Resident and TG-recruited macrophages were treated with 1:10 dilutions of T-3 or T-9 hybridoma supernatants containing serum (3,9) or without serum (3HB, 9HB). After 24 hr, $1X10^6$ cells were lysed in 1.0 ml of 5% triton X-100. 5'nucleotidase levels were determined as described in Materials and Methods.

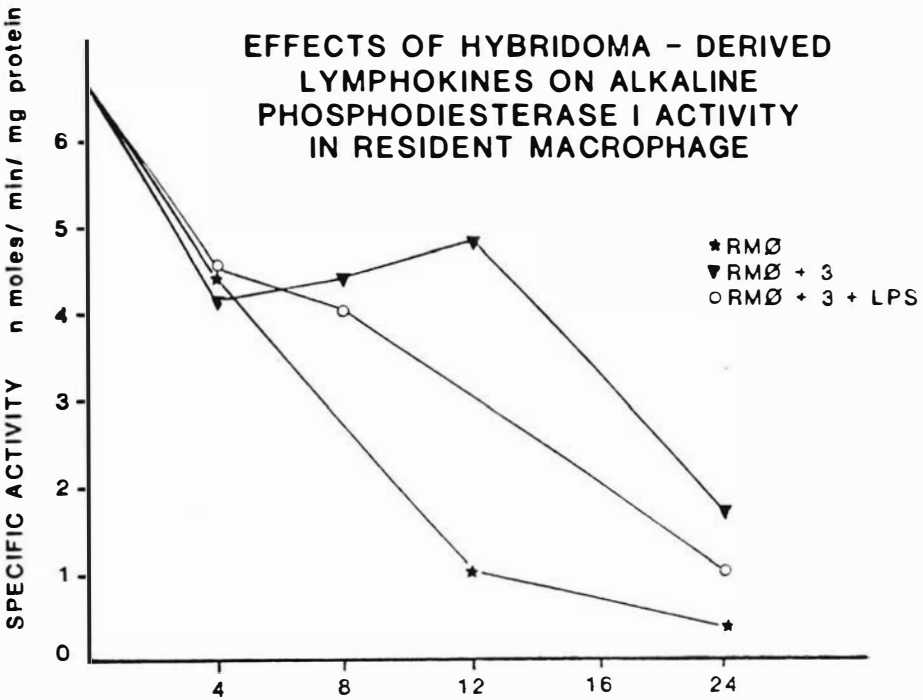


Fig. 11. Effects of hybridoma-derived lymphokines on alkaline phosphodiesterase I activity in resident macrophages over 24 hr. Resident macrophages were treated for 4, 8, 12, 16, and 24 hr with 1:10 dilutions of T-3 supernatant (with or without 5 µg LPS). After the specified periods, $1X10^6$ cells were lysed in 1.0 ml of 0.05% triton X-100 and assayed as described in Materials and Methods.

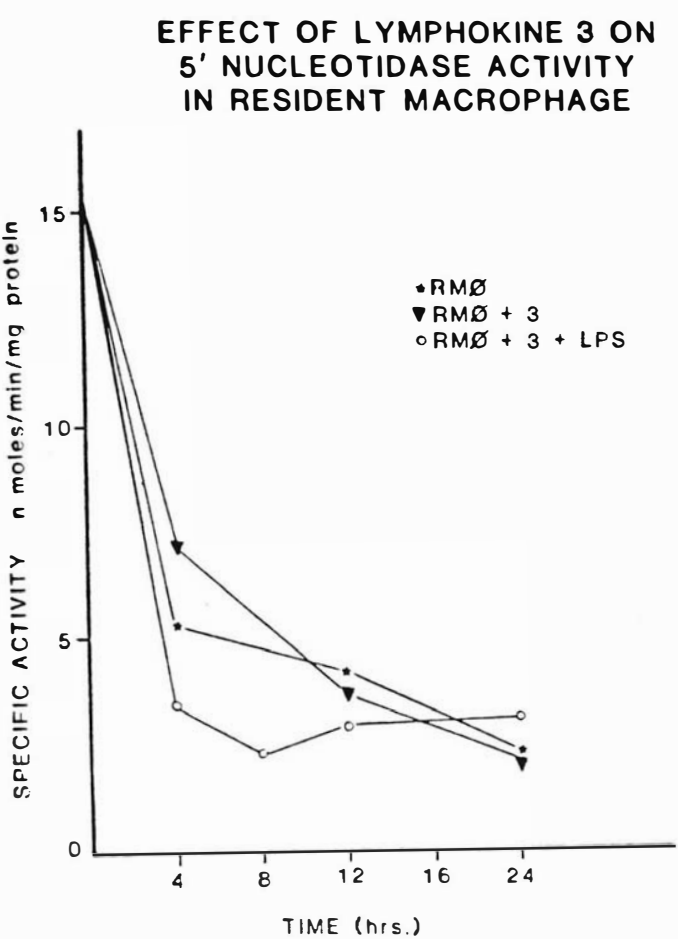


Fig. 12. Effect of T-3 derived lymphokine on 5'nucleotidase activity in resident macrophage over 24 hr. Resident macrophage were treated for 4, 8, 12, 16, 24 hr with 1:10 dilutions of T-3 supernatant (with or without 5μg LPS). After the specified periods, $1X10^6$ cells were lysed in 1.0 ml of 0.05% triton X-100 and assayed as described in Materials and Methods.

Table 4. Transferrin binding by resident and thioglycollate-elicited peritoneal macrophage [a]

Treatment	Transferrin Bound (pico moles)	
	Resident Macrophage	Thioglycollate Macrophage
medium	17.4 ± 0.5	26.1 ± 1.6
T-3	24.6 ± 0.7	27.1 ± 1.8
T-9	21.0 ± 2.4	31.3 ± 0.1
γ-IFN	13.4 ± 0.9	23.4 ± 1.3

[a]Transferrin binding was determined in the presence of 2.0 n moles ^{125}I-labelled and 2000 nmoles of unlabelled transferrin after 1 hr cells were washed 3 times and lysed in 0.1N NaOH.

increased to 25 and 21 respectively. Gamma-IFN induced a slight decrease in the number of moles bound by resident macrophages. When TG-macrophages were treated with T-3, no significant increase was observed in transferrin binding. However, elicited macrophages treated with T-9 showed an increase in the number of transferrin receptors and bound 31 pica moles. As seen with resident macrophages, γ-IFN produced a slight decrease in the number of moles of transferrin bound.

Serological characterization. Antibody capable of neutralizing the anti-viral activity of 10 units of γ-IFN was able to neutralize a 1:5 dilution of T-9 supernatant and prevent the induction of cytostatic activity in TG-macrophage populations against B16F10 melanoma cells. While inducing a 37% increase in activity over the media control, T-9 activity was reduced to less than 20% in the presence of anti-γ-interferon (Fig. 13). T-3 supernatants appeared relatively unaffected by antibody, maintaining high levels of activity (55%), demonstrating that the activity contained in T-3 supernatants is due to a unique, non-gamma-IFN molecule.

Characterization of lymphokine activity. A standard cytostatic assay, utilizing TG-macrophages and B16F10 melanoma target cells, was used to determine the heat stability and molecular nature of T-3 and T-9 LK. Treatment of supernatants at 56^{o}C for 30 min did not effect T-3, but eliminated the ability of T-9 to induce cytostatic activity in TG-macrophages completely. Incubation at 80^{o}C for 5 min lowered T-3 activity to less than 10% and at 100^{o}C for 5 mins all activity was lost (Fig 14A). Both T-3 and T-9 were totally inactivated by α-chymotrypsin (Fig. 14B). T-3 and T-9 increased tumor cytostatic activity in TG-macrophages by approximately 38-40% as compared to the medium control. After proteolytic treatment neither supernatants showed any activity. The cytostatic activity of T-9 LK was found in two amicon filter separated fractions.

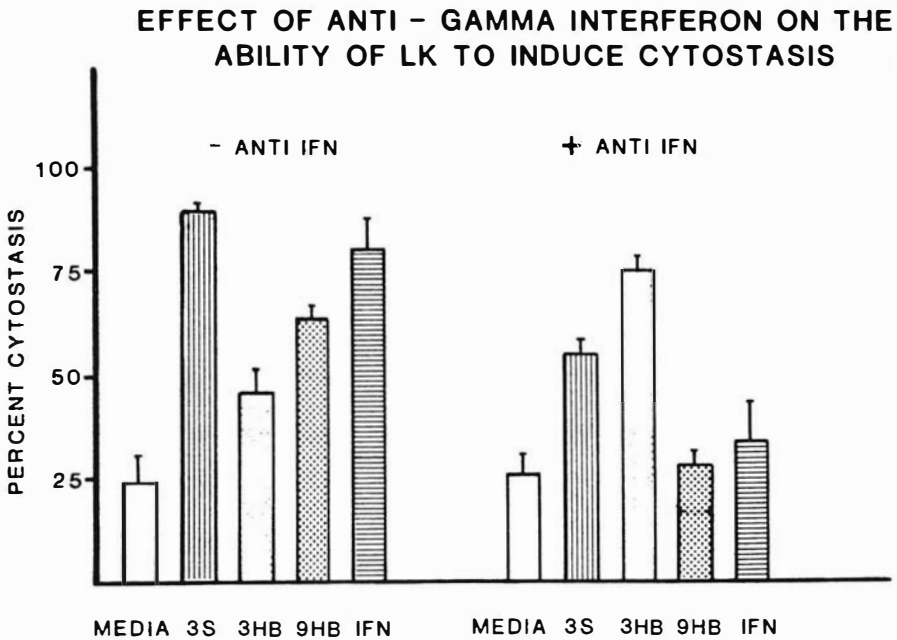
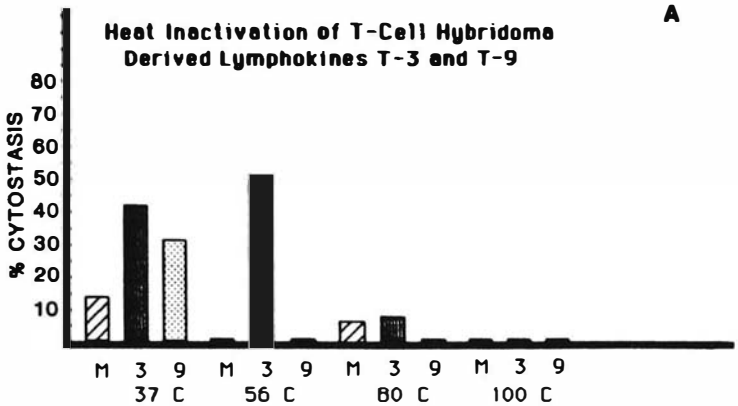


Fig. 13. Neutralization of T cell hybridoma derived lymphokines by gamma interferon specific antibody. TG-macrophage (1×10^5) were cultured in the presence of T-3 and T-9 LK (diluted 1:5) and antibody sufficient to neutralize 10μ of -IFN. After 24 hr wells were washed and 1×10^4 B16F10 melanoma target cells were added and cytostatic activity determined. Bars represent mean percentages ($\pm$ standard deviation).

Fig. 14. Characterization of T cell hybridoma derived lymphokines. A. Heat inactivation of T cell hybridoma derived lymphokines T-3 and T-9. Supernatants from T-3 and T-9 were incubated at 56°C for 30 min, 80°C or 100°C for 5 min. After heat treatment supernatants were assayed for their ability to induce tumor cell cytostasis in TG-elicited macrophages as described in Materials and Methods. B. Proteolytic inactivation of T cell hybridoma derived lymphokines T-3 and T-9. Supernatants were treated with 6.5 units of immobilized α-chymotrypsin for 1 hr. Carboxymethyl cellulose beads were removed by centrifugation at 400 x g for 30 min. Treated supernatants were then assayed for the induction of cytostatic activity in TG-macrophages. C. Molecular weight determination of T-9 derived lymphokine. Supernatant from T-9 hybrids grown in HB101 serum-free medium were fractionated using an Amicom filtration system and a ym 30 (mw. cut off 30K) and a ym10 (mw. cut off 10K). Three fractions were collected: (a) $\geq$ 30,000 (b) $\geq$ 10,000 $\leq$ 30,000, and (c) $\leq$ 10,000. These fractions were screened for the induction of cytostatic activity in TG-macrophage.


A
Heat Inactivation of T-Cell Hybridoma
Derived Lymphokines T-3 and T-9
% CYTOSTASIS
80
70
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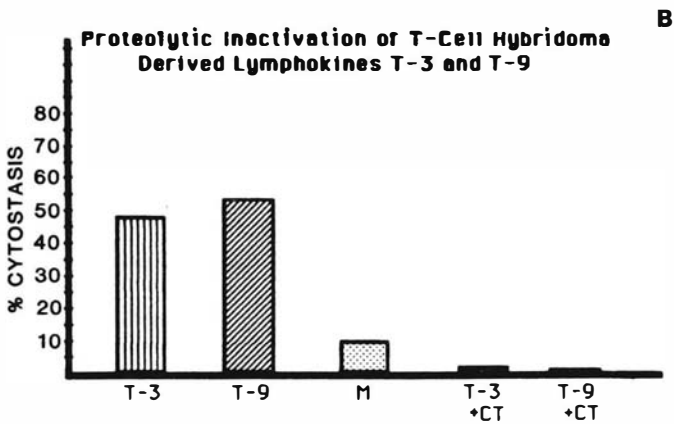
M 3 9 M 3 9 M 3 9 M 3 9
37 C 56 C 80 C 100 C


B
Proteolytic Inactivation of T-Cell Hybridoma
Derived Lymphokines T-3 and T-9
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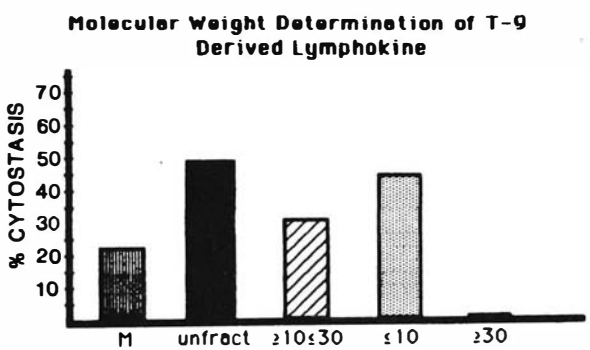
Molecular Weight Determination of T-9
Derived Lymphokine
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% CYTOSTASIS
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60
50
40
30
20
10
M
unfract
≥10≤30
≤10
≥30

Both the 10-30,000 molecular weight fraction and the less than 10,000 fraction contained activity comparable to the unfractionated sample. The greater than 30,000 fraction showed no activity (Fig. 14C).

DISCUSSION

The role of T cell derived lymphokines as modulators of macrophage phenotype and function is well documented (13,103,113). We chose to screen our hybridoma supernatants based on known γ-IFN activities: the ability to increase tumoricidal activity and the expression of Ia antigens in peritoneal exudate macrophages. Purified γ-IFN has been shown to increase Ia expression in a concentration dependent fashion (10,105,119,128), while α-IFN and β-IFN result in minimal increased Ia expression (96,105,137). Cloned or hybridoma-derived γ-IFN has also been well documented to prime murine macrophages for tumoricidal activity (16,33,89). While α-IFN/β-IFN also show tumoricidal activity, γ-IFN has been reported to be approximately 850-1,000 times more effective at activating macrophages (89,95). Earlier findings, that mouse peritoneal macrophages co-cultured with the opportunistic pathogen, Naegleria fowleri were killed within 18 hours, (Marciano-Cabral, unpublished data) lead us to screen our supernatants for LK's that would offer protection.

Macrophage activation as determined by screening, appeared to correlate with the dilution of supernatant; however, it was important to maintain the grown of T-3 or T-9 in log phase and with high viability (80%) to insure maximal and consistent production of LK. Both hybridoma cell lines demonstrated doubling times of 18-24 hr and were constituitive producers of lymphokines as indicated by the inability to increase LK production by stimulation with Con A. Supernatants were also screened for IL-2 (TCGF) production. Neither T-3 or T-9 demonstrated any IL-2 activity as determined by their inability to induce a proliferative response in the IL-2 dependent C10 cell line (Table 1).

Functionally, the activation of macrophages has been defined in terms of microbicidal and tumoricidal activity (31,37,47,61). Recent attempts have been made to define discrete stages of activation. The terms resident, responsive,

primed and fully activated have been employed to describe the level of activation with regards to tumoricidal activity, and the method of elicitation (37,47).

These macrophage have been shown to exhibit biochemical markers justifying this categorization (13,37,47,70,102). We have found that resident and TG-elicited peritoneal macrophages responded quite differently to T-3 and T-9 supernatants when examined for cytostatic and cytotoxic activity directed towards tumor cell targets. Both T-3 and T-9 were capable of inducing tumor cytostatic activity in TG-macrophages but not in resident macrophages populations. While T-3 activated TG-macrophages for tumoricidal functions (but not resident macrophages), T-9 was inactive in both macrophage populations. Kinetic studies using B16F10 melanoma target cells show that development of cytostatic function in TG-elicited macrophages requires approximately 24 hr of exposure to LK (T-3, T-9, or γ-IFN). While the level of activity achieved appears to vary according to the LK employed and the length of exposure, it is difficult to determine whether this is due to the intrinsic nature of the molecules (as seen between α-, β-, and γ-IFN) or because of variability in LK secretion by the hybridoma cultures. However, we attempted to avoid the latter possibility by pooling supernatants.

The finding that both T-3 and T-9 were capable of inducing protective mechanisms against Naegleria lysates in resident but not in TG-macrophages offers more evidence of functional differences between these populations. The inability of cloned γ-IFN to protect either resident or TG macrophage supports the finding that MAF (γ-IFN) inducing tumoricidal activities are different from those inducing anti-parasitic activities (51,135).

A dissociation between tumor cytostatic and bacteriocidal mechanisms has also been shown (31,61). We, therefore, decided to look at the ability of γ-IFN to

protect TG-macrophages from Bordetella lysates. B. pertussis, strain BP338 (wild type), contains high levels of adenylate cyclase. This enzyme is thought to impair host defense mechanisms by entering phagocytic cells and converting ATP into cyclic AMP, a known inhibitor of phagocytosis (17). Results were similar to those obtained in the Naegleria model with γ-IFN unable to prevent TG-macrophage destruction.

A recent report indicates that human T cell clones, specific for a purified Mycobacterium tuberculosis antigen, release a form of MAF which is not neutralized by monoclonal antibody against γ-IFN (8). Others have shown that at high concentrations, recombinant γ-IFN failed to prevent the intramacrophage replication of M. tuberculosis and actually caused increased mycobacterial replication and macrophage destruction (21).

The cytopathogenicity of Naegleria fowleri for a variety of mammalian cells in vitro has been demonstrated (64); however, the mechanism by which the amoeba exerts this effect remains an enigma. It has been suggested that N. fowleri ingests target cells by phagocytosis or by drawing in small portions of the target cell plasma membrane until the integrity of the membrane is disrupted (64). Other investigators have suggested that a cytopathic toxin or enzyme (phospholipases) are released (19,122). Most investigators are in agreement that some type of cell contact is required for target cell injury (64,122).

We have found that live Naegleria and lysates were able to mediate a cytopathic effect in both resident and TG-macrophages in an 18-hr assay. These findings suggest the presence of a cytotoxic factor(s) which is either present on the membrane surface or is secreted from lysosomes or vesicles upon contact with target cells. Curson et al. (19) has shown that phospholipase 2-lysophospholipase enzymes are found in the supernatants of axenically grown N. fowleri and that hyperimmune rabbit antisera prepared

against these enzymes could prohibit or delay the cytopathic effects of live Naegleria in Vero cell cultures.

It is particularly interesting that a unique non-gamma interferon LK (derived from T-3 and T-9) is capable of protecting resident macrophages from Naegleria lysates but does not alter the effects on TG-macrophages. The observed change in susceptibility does not involve APD-I or 5'N ectoenzyme expression since we did not detect any significant changes in the levels of these ectoenzymes in treated and untreated macrophage populations over 24 hr. It is possible that T-3 and T-9 induce the modulation of a receptor for the cytolytic substance from the surface of resident macrophages while not affecting TG-macrophages.

This difference in responsiveness to LK could also be due to the particular stage of the cell cycle in which TG-macrophages and resident macrophages are found. Sorg et al. (112,113,114) has suggested that as macrophages differentiate from bone marrow precursors in the presence of CSF (colony stimulating factor) there is an intense phase of proliferation. Some macrophages continue to cycle and accumulate in late G_1. These macrophages are functionally capable of responding to a wide variety of LK and chemotactic factors as well as producing interferon (113). As proliferation begins to slow the cells differentiate in G_1 and lose most of these functions as they enter into G_0. Resident macrophages are thought to be in G_0, while TG-macrophages are found in late G_1 (112,113,114). According to this model, resident macrophages must pass from G_0 into late G_1 in order to become activated for tumoricidal or bactericidal functions (112,113,114). Lymphokine (T-3, T-9 or γ-IFN) alone is not capable of pushing resident macrophages from G_0 to late G_1 and therefore they do not display tumoricidal functions. Thioglycollate macrophages, already present in late G_1, are induced to kill tumors with the addition of LK.

It has been shown that the number of transferrin receptors in TG-macrophages is higher than in resident macrophages. After treatment with γ-IFN the number of transferrin receptors is markedly reduced; however, in resident macrophages levels remain unaffected (131). We obtained similar results in TG-macrophages, and in resident macrophages we saw only a slight decrease in transferrin binding after exposure to γ-IFN. These findings indicate that resident macrophages are unresponsive to γ-IFN because they lack or express very few receptors for this molecule. The failure of γ-IFN to alter the cytopathic effect of crude Naegleria lysates on resident macrophages is in line with this theory. Both T-3 and T-9 supernatants induce unique transferrin binding patterns in resident and TG-macrophage, distinct from γ-IFN and appear to act differentially on these macrophage populations. Supernatant from T-9 increased transferrin binding in both resident and TG-macrophage populations, while T-3 increased binding in resident cells only and had no effect on TG-macrophages.

The protective mechanism induced by T-3 or T-9 in resident macrophage populations could not be demonstrated in TG-macrophages using either LK. We speculate that macrophage in G_0 express receptors for the LK present in T-3 and T-9 supernatants. As these resting cells receive signals moving them sequentially from early to late G_1 they begin to express receptors for γ-IFN and lose receptors for T-3 and T-9 supernatants.

We have shown that TG-macrophages can effectively destroy tumor cells after LK treatment (T-3, T-9, or γ-IFN). The addition of LPS was not necessary as a second signal but we can not rule out the possibility of low levels of endotoxin in the culture media. These macrophages were unable to demonstrate any lytic effect or protection against Naegleria fowleri. Resident macrophages, which are thought to be functionally down-regulated and unresponsive to LK

signals (37,112,131) did not demonstrate any tumoricidal activities. However, treatment with T-3 or T-9 was able to protect resident cells from Naegleria lysates and induce amoebicidal activity, demonstrating a capacity to respond to LK. This finding therefore demonstrates that resident macrophages may play a role in effector functions.

The data in this paper supports earlier findings that lymphokines from different T-cell clones can activate distinct macrophage functions (31). T-3 and T-9 supernatants were shown to differ both serologically and biologically. The molecular moiety in T-3 and T-9 supernatants responsible for the induction of cytostatic activity is heat labile and sensitive to α-chymotrypsin and therefore is not endotoxin, but more likely a protein or glycoprotein. Furthermore, T-9 LK appears to have a molecular weight approximately that of γ-IFN.

It has been argued that MAF and γ-IFN are one and the same (23,55,89,106); however, there have been previously reported findings of cloned hybridoma cell lines exhibiting MAF activity and no γ-IFN activity (31). While it is apparent that γ-IFN induces many of the functional characteristics of activation in macrophages, we offer supporting evidence that it is not the only form of macrophage activating factor. While both T-3 and T-9 supernatants induce some macrophage functions associated with γ-IFN (89,138) they also contain activities not described to γ-IFN. Anti-γ-interferon is able to block tumor cell cytostasis induced by T-9 supernatants but not by T-3 supernatants. It can be concluded that this activity may be shared by both γ-IFN and a non-γ-IFN LK. This non-γ-IFN molecule is responsible for protection against the cytopathic effects of Naegleria lysate.

Our data supports the fact that distinct T cell subsets produce different lymphokines under various physiological conditions and that the effect these lymphokines have on macrophages depends upon the biochemical nature of the

responding macrophage population. We have introduced evidence that the signals for activation of macrophage populations in response to the protozoan, Naegleria fowleri and the bacterium, Bordetella pertussis are distinct from activation for tumoricidal activity, and appear to be more closely associated with the activation of bacteriocidal and anti-parasitic mechanisms which are not γ-IFN induced. Furthermore, we have proposed an association between the cell cycle and the responsiveness to specific LK.

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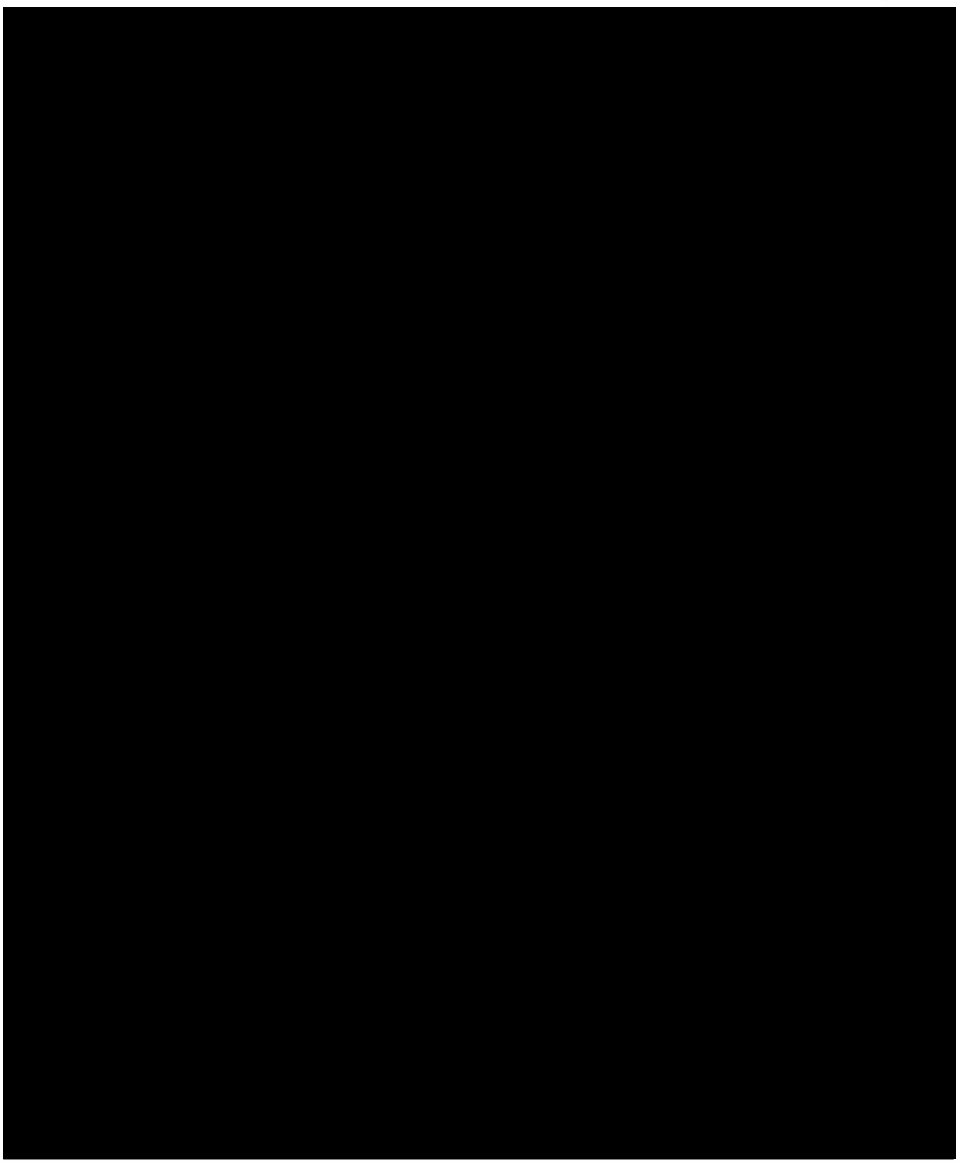
Vita



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