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Interactions Between Murine Peritoneal Macrophages and Naegleria Fowleri

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Interactions Between Murine Peritoneal Macrophages and Naegleria fowleri

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This is to certify that the dissertation prepared by Leigh Ann Belcher entitled Interactions Between Murine Peritoneal Macrophages and Naegleria fowleri has been approved by her committee as satisfactory completion of the dissertation requirement for the degree of Master of Science.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

INTERACTIONS BETWEEN MURINE PERITONEAL MACROPHAGES AND NAEGLERIA FOWLERI

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In the present study, we have examined the differences among Corynebacterium parvum-activated, thioglycollate-elicited, and resident macrophages from (C57BL/6 X $C_3H)F_1$ mice utilizing two microcytotoxicity assays. Macrophage and Naegleria fowleri were interchangeably employed as effector and target cells. To assess the ability of the macrophage to lyse N. fowleri, the amoebae were labeled with 5 μCi of ^{3}H-uridine per 5 x 10^5 cells for 18 h prior to co-culturing with adherent macrophage monolayers. Although the macrophage-induced lysis was density and time dependent, C. parvum macrophages consistently demonstrated an increased cytotoxic response to N. fowleri after 24 h co-incubation at a 10:1 effector-to-target ratio. In contrast, thioglycollate and resident macrophages were unable to elicit a cytotoxic response to N. fowleri.

To ascertain the ability of N. fowleri to destroy the three macrophage populations, a ^{51}Cr post-labeling technique was employed. After 24 h of co-incubation, uptake of ^{51}Cr into remaining C. parvum-activated and thioglycollate-elicited macrophages exceeded that for resident macrophages. Parallel electron microscopic studies confirmed simultaneous destruction of both amoebae and macrophages. Concurrent studies utilizing cell-free lysates of N. fowleri displayed the same divergent spectrum of macrophage destruction for the different macrophage populations as was seen with N. fowleri trophozoites.

Catalase is an enzyme which breaks down hydrogen peroxide, a chemical released from activated macrophages, which could contribute to macrophage-induced cytotoxicity. Leupeptin is an inhibitor of some serine and cysteine proteases. Activated macrophages also secrete proteases which may contribute to macrophage-induced cytotoxicity. Catalase, leupeptin, or hydrogen peroxide were added to macrophage cultures or N. fowleri lysate prior to co-incubation or during co-incubation to determine possible roles of hydrogen peroxide and proteases in macrophage susceptibility to N. fowleri lytic substances. Exogenously added hydrogen peroxide had no effect on resident macrophage survival. Catalase and leupeptin produced effects unique for the macrophage cell type, with survival closely

correlating to the types and quantities of cytotoxic substances associated with the macrophage. Results suggest that macrophage susceptibility to N. fowleri lysate may be due to the absence or presence of two or more macrophage components.

INTRODUCTION

The Macrophage

The macrophage is a predominant cell of the reticulo-endothelial system (RES) derived from mesodermal precursors in the bone marrow, which is capable of phagocytizing particulate matter, adheres to a variety of surfaces, and participates in the immune response by processing and presenting antigen (30). Macrophages differ morphologically depending on their location and mobility. In addition, it has been demonstrated by Metchnikoff as early as 1905 that macrophages undergo morphological modification as well as functional modification in the presence of foreign invaders to the human body such as bacteria (67). The term "activated macrophage" has been used extensively to describe a cell which has undergone such a metamorphosis. Activated macrophages exhibit enhanced microbiocidal and tumoricidal activity as well as biochemical alterations such as increased lysosomal enzyme activities, greater respiratory activity, including increased hydrogen peroxide production, induction of the secretion of neutral proteases, and increased transport and metabolism of glucose (43). Cohn (19) has proposed a theoretical sequence for this activation process which includes cell spreading, increased

cellular metabolism, stimulation of IgM and C3b-coated particle ingestion, proteinase secretion, stimulated pinocytosis and plasma membrane ectoenzyme modification. The consequences of these sequential changes are H_2O_2 production as well as microbiocidal and tumoricidal activity.

There appears not to be a single method by which an activated macrophage destroys a target cell. Four mechanisms described in the literature are: 1) exocytosis of lysosomal enzymes, 2) peroxidative attack, 3) phagocytosis (93), and 4) cytolysis due to a unique protease or cytolytic factor (CF) (1). These mechanisms may function in concert to bring about target cytotoxicity. A "cytolytic synergy" between two or more toxic components has been proposed as a mechanism of tumor cell damage (1). Mauel (49) has proposed that different effector molecules from activated macrophages may be responsible for microbiocidal activity against various types of target microorganisms.

While it has been well documented that the amount of lysosomal enzymes produced and released increases in the activated macrophage, Bucana et al. (13) have described the passive uptake of activated macrophage lysosomes into tumor cell targets as a mechanism of cytolysis. Peroxidative attack, or a mechanism which facilitates the transfer of oxygen from hydrogen peroxide to a tissue requiring oxygen, seems to be a second mechanism in macrophage-induced microbiocidal and tumoricidal activity. Indeed, macrophages

have been shown to possess a similar "respiratory burst" system to that of polymorphonuclear leukocytes. Mobilization of membrane bound Ca^{2+} and changes in intracellular Ca^{2+} concentration appear to stimulate macrophages, leading to activation of membrane-bound NADPH oxidase (78). This enzyme in turn sets up a cascade of reactions leading to H_2O_2 production and release as well as the release of other intermediate products of oxygen reduction such as superoxide anions (O^-_2), hydroxyl radicals (OH·) and singlet oxygen ($\cdot O_2$) (30). These intermediates are highly reactive agents, which are toxic to all cells. Phagocytosis, a third mechanism for target cell cytotoxicity, is an energy and temperature-dependent process, with Ca^{2+} and Mg^{2+} playing roles in the ingestion of particles. The movement of the plasma membrane in preparation for engulfment is closely associated with the polymerization of actin filaments (4). Once the target cell is internalized, the phagosome unites with the lysosome which contains numerous hydrolytic enzymes. The fourth mechanism by which activated macrophages kill target cells has been presented by Adams et al. (2). They proposed that target cell lysis was induced by a cytolytic factor known to be a special neutral protease. They have shown that activated macrophages secrete CF which subsequently lyses tumor target cells.

Macrophage Altering Agents

Biological and chemical agents with macrophage activating potential include: microorganisms, lipopolysaccharides (LPS) (5); poly rI:rC (5); glucan, dextran sulfate (80); pyran (41), and lymphokines (60). Both a freeze-dried preparation of an attenuated strain of Mycobacterium tuberculosis (Bacillus Calmette-Guerin or BCG) (86) and a formalin killed preparation of Corynebacterium parvum have been shown to be potent activators of macrophages by measuring tumor growth in vitro (60). In addition to agents which activate macrophages, there is a second group of substances which attract macrophages to the site of their inoculation, referred to as elicited macrophages. Such agents include glycogen, starch, proteose peptone, mineral oil, liquid paraffin, Freunds complete adjuvant, concanavalin A, pokeweed mitogen, zymosan, and thioglycollate (22). The exact origin and nature of macrophages elicited by these agents are vague. It is possible that these elicited cells represent 1) resident macrophages recruited from other areas, 2) a more advanced maturational phase of macrophages, 3) a population of macrophages unique to the eliciting agent (81), or 4) a combination of different macrophage populations or developing stages of macrophages.

Thioglycollate Elicited Macrophages

Ectoenzyme studies have indicated the uniqueness of a thioglycollate-induced macrophage (TG) from an activated or

resident macrophage; however, its exact nature and where it lies with respect to activation remains to be defined. A distinctive feature of the TG macrophages consists of a significant increase in size and a change in morphology. Macrophages derived from TG elicitation are unusually large, vacuolated, and spread freely. Many of the lysosomal hydrolases as well as the secretory enzyme levels increase dramatically in macrophages elicited by thioglycollate. Other properties of the TG macrophage which are associated with an activated state include increased phagocytosis, ATP levels, and superoxide anion production (19). Despite all these signs of activation the TG macrophage has little if any bactericidal or tumoricidal activity. In vivo studies suggest that thioglycollate medium may itself enhance bacterial growth (54). The capacity of the TG macrophage to kill has varied according to the host, the amount and kind of TG injected, and at what time following injection macrophages are collected. However, there is little question that this type of cell which is obtained following TG elicitation is different from an activated macrophage and possibly cells elicited by other means.

Corynebacterium parvum Activated Macrophages

Intraperitoneal administration of C. parvum results in splenomegaly, hepatomegaly (14), stimulation of the reticuloendothelial system, an increase in antibody production, induction of delayed hypersensitivity to unrelated antigens

(63), an increase in the threshold for tolerance induction (73), induction of autoimmunity (51), and a depression of lymphocyte function (14). The morphology and function of a C. parvum macrophage resembles that described earlier for the activated macrophage.

There have been many investigators seeking explanations or theories for the mechanism(s) of activation with C. parvum. Christie and Bomford (18) have proposed a direct contact mechanism between macrophages and C. parvum, and a concomitant immune response mechanism where T lymphocyte products are necessary for activation. Ogmundsdottir and Weir (68) confirmed the former proposal by demonstrating direct binding of C. parvum sugar determinants in the cell wall to glass-adherent mouse peritoneal exudate cells. In addition, Chapes and Haskill (16) demonstrated that macrophage-induced cytotoxicity is closely associated with intracellular C. parvum. Activation in vivo with C. parvum does not appear to depend on intact thymus function (95). In contrast, in vitro reactivation with C. parvum of cytotoxicity induced by macrophages is thymus dependent (32). Although much research is still required before we develop a firm understanding of the mechanism of activation by C. parvum, the fact remains that macrophages consistently demonstrate characteristics of activation including their enhanced ability to kill. Presently, there is no satisfactory single assay which can discriminate among different populations of macrophages. Morahan et al. (57) have

described characteristic ectoenzyme profiles for resident, thioglycollate elicited, and C. parvum activated macrophages using 5'Nucleotidase (5'N), leucine aminopeptidase (LAP), and alkaline phosphodiesterase (APD) as markers for activity. Thioglycollate macrophages differ from resident and C. parvum macrophages by the greater expression of APD. C. parvum retains little if any 5'N activity and expresses more LAP activity as compared to resident macrophages. Thioglycollate macrophages display similar 5'N and LAP activities as C. parvum. Therefore, to distinguish among the populations, the profile of at least three ectoenzymes is necessary, and for positive identification, biochemical and functional markers are beneficial.

Macrophage-Microorganism Interactions

Macrophages have been examined quite extensively for their ability to phagocytize and destroy microorganisms. Some organisms, however, are capable of parasitizing and replicating within macrophages. Such organisms include legionella, listeria, salmonella, brucella, mycobacterium, clamydia, and rickettsia representing the bacteria, and protozoans such as leishmania, toxoplasma, and trypanosoma (56). Intracellular replication of these pathogens can be inhibited by activation of macrophages. Many of these pathogens are lysed by activated macrophages, although some organisms have evolved the capacity to evade effector mechanisms elicited by activated macrophages. The evasive

mechanisms which Leishmania, Toxoplasma, and Trypanosoma utilize to sustain life provide important information with regard to the killing capacity of the activated macrophage.

Upon entrance to the macrophage, Toxoplasma gondii prevents the fusion of the phagosomal membrane and lysosome and replicates within the converted phagosome (39). Trypanosoma cruzi, on the other hand, enters the macrophage and lyses the phagosomal membrane to ultimately replicate in the cell cytoplasm (64). Finally, the Leishmania amastigote replicates within the fused, active phagolysosome, presumably protected by a leishmanial excreted factor (82). It is not clear what factor(s) or process(es) of the activated macrophage is(are) capable of altering the previously described mechanisms of intracellular survival; therefore, it appears that the balance of this intimate relationship is critical to infection of the host. Borges et al. (8) showed that inhibition of multiplication of T. gondii was induced by macrophages activated in the presence of T lymphocyte products, and trypanocidal activity was demonstrated using Bacille Calmette-Guerin (BCG) activated macrophages (65). Mauel et al. (50) have demonstrated destruction of Leishmania enrietti in guinea pig activated macrophages.

Previously described macrophage-microorganism interactions have dealt with intracellular pathogens; therefore, it may be useful to characterize the interaction with

extracellular microorganisms to further understand the mechanisms of macrophage-induced cytotoxicity. Landolfo et al. (45) demonstrated macrophage-induced cytotoxicity of Trichomonas vaginalis, a protozoan responsible for urogenital tract infections, utilizing ^{3}H-thymidine labeled T. vaginalis as the target cell and murine peritoneal macrophages as the effector cells. Cytotoxicity due to the macrophage was determined by the amount of label released from the protozoan. Bout et al. (9) used a more complex system for examining macrophage-induced cytotoxicity by employing a multicellular helminth parasite, Schistosoma mansoni as the target cell, while lymphokine-activated murine peritoneal macrophages served as the effector cells. Mortality of schistosomula was determined in 48 h by direct visual observation. Killing of the schistosomula occurred with activated macrophages but not with resident macrophages. In vitro killing of schistosomula by C. parvum activated macrophages has been suggested to be due partly to arginase originating from the macrophage (67).

Similar cytotoxicity studies have been performed using polymorphonuclear leukocytes (PMN) as effectors against two amoebae, Entamoeba histolytica and Naegleria fowleri. The conditions under which E. histolytica and PMN's interacted were described by Guerrant et al. (33). Amoebae-induced PMN destruction, as well as PMN-induced amoebae destruction were observed. E. histolytica trophozoite destruction by

sensitized lymphocytes and macrophages also has been demonstrated (31). Ferrante and Thong (28) reported that neutrophils were capable of killing N. fowleri. Neutrophil accumulation in the mouse peritoneal cavity was induced by an intraperitoneal (i.p.) inoculation of thioglycollate. Subsequent injection of N. fowleri i.p., and collection of peritoneal exudate provided samples for phase contrast microscopic examination. Neutrophils killed N. fowleri by either "group attack," extracellular release of enzymes, or by a pinching off process with subsequent phagocytosis.

Naegleria fowleri

N. fowleri, an amoeboflagellate, is the causative agent of the fatal disease primary amoebic meningoencephalitis (PAME) (29). Although N. fowleri is a ubiquitous soil and water amoeba there have been relatively few cases (100-200) of PAME recognized (20). Experimental evidence implicates an intranasal route of infection with subsequent invasion of the brain causing eventual coma and death.

Most investigators have concentrated on in vivo systems to study the ability of N. fowleri to cause PAME; however, several investigators have demonstrated Naegleria-induced cytopathogenicity on a variety of target tissue culture cells (11,21,89). Presently, it appears that N. fowleri is capable of efficient destruction of all co-cultured mammalian cells, except the mouse peritoneal PMN. In all cases the amoeba has been the effector and all

targets lacked aggressiveness; therefore, a system with the incorporation of two effector cells might provide insights to the function of either cell type. In the present study, the interaction between various macrophage populations and N. fowleri has been investigated.

Biochemical characteristics and effector functions for macrophage populations exposed to various agents remain highly diverse and complex. In addition to differences shown between macrophages treated with different agents, there may occur within each population a heterogeneity of its own. Consequently, development of techniques that would provide consistent information concerning the differences in macrophage populations are of prime concern.

Study Objectives

The principal objective of this study was to observe the interactions between N. fowleri and various murine peritoneal macrophages. In doing so, we hoped to develop a system whereby activated macrophages could destroy N. fowleri amoebae, and determine the mechanism of macrophage-induced N. fowleri cytotoxicity. In addition, by using microcytotoxicity studies we believed that a functional difference could be observed among TG, CP, and resident macrophages with respect to their ability to kill and resist being killed. N. fowleri as well as the macrophage are effector cells; therefore, it was necessary to develop

two assay systems, one which could measure *N. fowleri* destruction, and the other designed to quantitate macrophage destruction. The various profiles of cytotoxicity should help identify an activated macrophage from a resident macrophage. To correlate the activation state with other indicators of activation, ectoenzyme data were collected to correspond to the degree of cytotoxicity. In addition to defining separate macrophage populations, a better understanding of the mechanisms of cytotoxicity was to be defined by the use of specific blocking agents of cytolytic substances. The role of cell-to-cell contact and adherence in cytotoxicity has been addressed using cell-free *N. fowleri* lysates. Preliminary *in vivo* studies of macrophage-*Naegleria* interactions as measured by survival were explored to correlate with *in vitro* studies. Finally, a comparison of *in vitro* lymphokine activation of resident macrophages to *in vivo C. parvum* activation of macrophages was performed to determine whether *C. parvum* macrophage-induced *N. fowleri* cytotoxicity was unique or characteristic to the "activated" macrophage.

MATERIALS AND METHODS

Amoeba Strain, Cultivation, and Maintenance

Naegleria fowleri (LEE) was used throughout this study. *N. fowleri* (LEE) was isolated in 1968 from the brain of a patient with primary amoebic meningoencephalitis (PAME) (23).

Amoebae were grown in Nelson medium consisting of Page amoeba saline (0.12 g NaCl, 0.004 g $MgSO_4 \cdot 7H_2O$, 0.004 g $CaCl_2 \cdot 2H_2O$, 0.142 g Na_2HPO_4 and 0.13 g KH_2PO_4 per liter of distilled water) supplemented with 0.1% (w/v) Panmede liver digest (Panmede, Paines and Byrne Limited, Greenford, England), 0.1% (w/v) glucose and 2% (v/v) calf serum (Grand Island Biological Co., Grand Island, NY).

Stock cultures were maintained in 75 cm^2 tissue culture flasks (Corning) and fed every 4 d with fresh Nelson medium. Amoebae were grown and maintained at 37°C and doubled in numbers every 7 h. *N. fowleri* amoebae used in the following experiments were in the stationary phase of growth.

Amoebae Harvesting and Counting

Amoebae were harvested by mechanical perturbation of flasks, collected in plastic conical test tubes, and centrifuged at 200 x g for 5 min in a Beckman model J-6 centrifuge. Amoebae were suspended in fresh Nelson medium and

counted with an electronic particle counter (Coulter Counter, Z_{B1}, Coulter Electronics, Inc., Hialeah, FL) by adding 0.2 ml of amoeba suspension to 9.8 ml of electrolyte solution consisting of 0.5% (v/v) formalin and 0.4% (w/v) NaCl in distilled water. Cuvettes containing amoebae were vortexed at setting 7 for 4 sec to disperse cell aggregates. Of the 3 consecutive counts taken the 2 counts closest to one another were averaged multiplied by 100 to provide cells/ml. Coulter settings for counting amoebae were: gain 0, matching switch 20 k, band-with selector extended, 1/amplification 4, 1/aperature current 1, lower threshold 10, and upper threshold maximum (91). When counts were 10^4 or greater, a correction chart was utilized to adjust to the true count which corrected for simultaneous passage of 2 or more amoebae through the aperature.

Preparation of Amoebic Lysate

Amoebae in the stationary phase of growth were removed by mechanical perturbation, centrifuged, and counted according to the protocol for amoebae harvesting and counting. Cells were suspended in complete endotoxin-free RPMI-1640 medium (GIBCO, Grand Island, NY) at a cell density of 1 x 10^7 amoebae/ml and freeze-thawed 4 times at -70°C using an ethanol-dry ice slush. Suspensions were thawed quickly in hot tap water. Centrifugation of the lysate at 200 x g for 5 min was done prior to filtration through a 0.45 μm acrodisc filter (Gelman, Ann Arbor, MI). Lysates were

diluted so that 3 x 10^5 amoebae cell equivalents were added to 2 x 10^5 adherent macrophages. Lysates were prepared and kept on ice for 1 h prior to their distribution into microtiter wells.

Mouse Strain

Male and female (C57BL/6 X C_3H)F_1 ($B_6C_3F_1$) mice obtained from the Frederick Cancer Research Center were used as the source of peritoneal exudate cells. Mice were housed 4 per cage and were fed ad libitum.

Peritoneal Cell Preparations

$B_6C_3F_1$ mice were used at 8 to 10 w of age. Mice were sacrificed by cervical dislocation. The skin over the abdomen was reflected and 8 ml of endotoxin-free Hanks' balanced salt solution (HBSS) (GIBCO) supplemented with 200 µg/ml streptomycin and 200 U/ml penicillin were injected into the peritoneal cavity. After agitation of the abdomen, peritoneal cell suspensions were removed with the same needle and syringe used to dispense the HBSS. Peritoneal cells were centrifuged at 200 x g for 5 min. Following the removal of supernatant, red blood cells were lysed with 5 ml 0.83% Tris-buffered NH_4Cl (Trizma base - Trizma HCl, Sigma Chemical Co., St. Louis, MO) and subsequently washed twice to remove NH_4Cl with 10 ml HBSS, and suspended in endotoxin-free RPMI-1640 medium (GIBCO, Grand Island, NY) containing 100 U/ml penicillin and 100 µg/ml streptomycin,

2 mM glutamine, and 10% heat-inactivated fetal calf serum (Sterile Systems, Inc.). Peritoneal cells were kept on ice, counted, and the percent macrophages determined. A cell differential analysis was done to determine percent macrophages by spinning 2 x 10^5 peritoneal exudate cells (PEC) with 0.2 ml fetal calf serum in a cytocentrifuge at 1000 rpm for 7 min. Slides were air dried prior to staining 1 min with Wright's Camco Quik Stain (Scientific Products) and subsequently counterstained for 3 min with an overlay of Geimsa stain (GIBCO). Slides were rinsed in tap water and air dried prior to evaluating 100 cells under oil immersion for the percent of PEC's which were macrophages.

In Vivo Treatment Protocol

Peritoneal cells from untreated mice were used as resident macrophages (R). Mice were injected intraperitoneally (i.p.) with 0.2 ml (70 mg/kg) of a formalin killed preparation of Corynebacterium parvum (CP) (Propionibacterium acnes) (Burroughs-Wellcome Research, Triangle Park, NC) 7 d prior to harvesting the peritoneal cells. Pyran copolymer (P) Lot# (XA 124-177) (pyran was kindly provided by Dr. Albert E. Munson, Department of Pharmacology and Toxicology, MCV/VCU) (Hercules Chemical Co., Wilmington, DE) was administered i.p., 0.2 ml (25 mg/kg) per mouse, 7 d prior to harvesting peritoneal cells. To obtain elicited macrophages, mice were inoculated i.p. with 1 ml of a 10%

solution of Brewer's thioglycollate medium (TG) (Difco Laboratories, Detroit, MI) 4 d prior to harvesting the cells.

Morphological Assays

Peritoneal cell suspensions containing 3 x 10^5 macrophages were seeded into 8-chambered Lab-Tek tissue culture chambers (Lab-Tek Products, Naperville, IL) to perform morphologic assays. Cells were plated in complete media (RPMI) at macrophage-to-amoeba ratios (E:T) corresponding to 20:1, 10:1, 5:1, and 1:1, and allowed to adhere for 2 h in a humidified atmosphere at 37°C containing 5% CO_2. Medium was aspirated and the chambers were washed 3 times to remove nonadherent cells. Identification of adherent cells as macrophages was based on morphological and cytochemical characteristics. Assays such as staining for nonspecific esterase (α-Naphthyl Acetate Esterase Histozyme Kit, Sigma Chemical Co.) (96) and acid phosphatase (Sigma Chemical Co.) (42) were performed. Following incubation and phase contrast microscopic examination, Lab-Tek contents were fixed in 2% paraformaldehyde at 27°C for 20 min. A modified hematoxylin-eosin staining procedure was used:

1. Remove plastic chamber and seal from Lab-Tek.
2. Wash 3 times in tap water for 2-3 min.
3. Stain with hematoxylin in copeland jar for 30 min [Papanicoloau Hematoxylin Stain (Harris) Fisher Scientific Co.].

4. Wash 2 times in tap water for 2-3 min.
5. Place in Scott's solution (2 g sodium bicarbonate, 20 g $MgSO_4$ in 1 liter distilled water) for 2-3 min (37).
6. Wash 3 times in tap water for 2-3 min.
7. Counterstain in alcoholic eosin (Allied Chemical) for 2 min.
8. Destain in 95% ethanol for 1 min.
9. Destain in 100% ethanol for 1 min.
10. Clarify in xylene for 3 min.
11. Mount coverslip with Permount (Fisher Scientific Co.).
12. Allow to dry 2 d before observing under-oil immersion.

Electron Microscopic Studies

Cultures of N. fowleri amoebae and macrophages for electron microscopy were fixed in situ by removing the culture medium and adding 2.5% glutaraldehyde to the cell cultures. The cultures were post-fixed with 2% OsO_4 and routine electron microscopy techniques were performed (48). Ultrathin sections were cut and stained with uranyl acetate followed by lead citrate. Grids were examined in an RCA EMU-3F electron microscope operating at 100 KV.

Ectoenzyme Assays

Ectoenzyme data were kindly provided by Dr. L. Schook, Department of Microbiology and Immunology, MCV/VCU. The state of activation of adherent cells was assessed by utilizing ectoenzyme analysis. The enzyme activity present in cell lysates of resident, thioglycollate elicited, C. parvum activated and pyran activated macrophages was determined.

Approximately 2 x 10^6 macrophages were seeded into 35 x 10 mm tissue culture dishes (Costar, Cambridge, MA) and allowed to adhere for 2 h. Dishes were washed 3 times with HBSS prior to the addition of 1 ml 0.05% Triton X-100 solution in distilled water (Fisher Scientific Co.). Lysates were incubated at room temperature for 15 min, followed by trituration with a 1 ml serological pipet, and placed in tubes to be centrifuged at 850 x g for 20 min. Supernatants were collected and frozen at -20°C prior to protein determination, 5'Nucleotidase analysis (5'N), leucine aminopeptidase (LAP) analysis, and alkaline phosphodiesterase (ADP) analysis.

Determination of 5'Nucleotidase Activity

The 5'Nucleotidase assay was performed according to Edelson and Duncan (26). Cell lysates obtained by treatment with Triton X-100 (0.1 ml) were placed in 12 x 75 mm glass test tubes (Curtin, Matheson Scientific, Inc.). The substrate was made immediately prior to the assay and kept

on ice, and was added to the lysates in 0.5 ml quantities, shaken, and incubated at 37°C in a water bath for 30 min. The substrate contained 25 nCi 5'-[^{3}H]-AMP/ml (Amersham Corp., Arlington Heights, IL), 0.15 mM adenosine-5'-monophosphoric acid (Sigma Chemical Co.) and 6 mM p-nitrophenyl phosphate (Sigma) in 100 ml Tris-HCl buffer (54 mM Tris, 13 mM $MgCl_2$ in 1 liter distilled water, pH 9.0).

The reaction was stopped by placing tubes on ice and adding 0.2 ml, 0.25 M $ZnSO_4$ and subsequently vortexing (100 ml distilled water added to 7.19 g $ZnSO_4$ $7H_2O$ and stored indefinitely). Freshly filtered 0.25 M $Ba(OH)_2$ was added in 0.2 ml quantities and subsequently vortexed [100 ml distilled water added to 7.89 g $Ba(OH)_2$ $8H_2O$].

The tubes were centrifuged at 1500 x g for 20 min at room temperature. The supernatant was added in 0.5 ml quantities to 5 ml Aquassure (New England Nuclear, Boston, MA) in 6 ml plastic minivials (plastic sample liquid scintillation vials, Wheaton Scientific, Millville, NJ). Counting standards (maximal release) were prepared by diluting 1.0 ml substrate with 1 ml water and added to vials. Samples were counted 10 min in liquid scintillation counter (Beckman). Calculations were as follows:

$$\text{Specific Activity} = \text{nM 5'-AMP hydrolyzed/min/mg protein} = \frac{(\underline{CPM}_{exp.} - \underline{CPM}_{BL}) \times 75 \text{ nM}}{(\underline{CPM}_{STD.} - \underline{CPM}_{BL})}$$

$$\frac{}{(30 \text{ min}) \times (\text{mg protein}/0.1 \text{ ml})}$$

Determination of Leucine Aminopeptidase

The leucine amino peptidase assay was performed according to Morahan (56). Macrophage lysate samples of 200 µl were combined with 1600 µl of 0.1 M, filter sterilized, PBS (500 ml, 0.1 M Na_2HPO_4 and 80 ml, 0.1 M KH_2PO_4, pH 7.5) and warmed at 37°C for 20 min. Leucine p-nitroanilide substrate (10 nM in absolute methanol, Sigma Chemical Co.) was added rapidly in 100 µl quantities to macrophage lysates to produce 1 mM concentration, and reincubated at 37°C for 30 min. Substrate was stored at 4°C for no longer than 1 w. The reaction was stopped by placing the tubes on ice following vortexing. The enzyme standard contained 25 µl of the 1:100 dilution of stock enzyme [diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin] (Sigma Chemical Co.) and 75 µl of 0.05% Triton X-100 in distilled water. Blank tubes contained 100 µl of 0.05% Triton X-100 in distilled water. The concentration of p-nitroaniline was read at 405 nm within 1 h. The following formula was used to determine specific activity in nmoles/mg protein/min at 37°C:

$$SA = \frac{\text{O.D. for 200 µl cell lysate}}{\text{mg protein in vol.}} \times \frac{1000}{\text{min. of rxn.} \times 9.6}$$

Determination of Alkaline Phosphodiesterase Activity

The alkaline phosphodiesterase assay was performed according to Edelson and Gass (27). Macrophage lysate sample (100 µl) was added to 500 µl of substrate. Substrate was prepared by mixing 8.1 mg of p-nitrophenyl thymidine-5'-monophosphate with 10 ml of buffer, and protected by

aluminum foil from light. The substrate was prepared fresh for each assay. To prepare buffer 73.2 ml of Solution A and 26.8 ml of Solution B were mixed and adjusted to pH 9.6 prior to the addition of 44 mg of zinc acetate. Solution A contained a mixture of 7.5 g glycine and 5.85 g NaCl in one liter of distilled water and was stored at 4°C. Solution B consisted of a mixture of NaOH (2 g) in 500 ml of distilled water and was stored at room temperature. Samples were shaken and incubated in a 37°C water bath for 30 min and protected from light with aluminum foil. The reaction was stopped by adding 1.0 ml of 0.1 N NaOH and vortexing. Blank tubes were prepared by diluting 500 µl of substrate with 100 µl 0.05% Triton X-100. The optical density was measured at 400 nm using the blank for zero setting. The specific activity of each sample was calculated by the following formula:

$$SA = \text{nmoles product/min/mg protein}$$

$$= \frac{\text{O.D.}}{\text{mg progein}} \times \frac{\text{vol (1.55 mls) } 1.6}{\text{time (30 min) x E } \left(0.012 \frac{\text{ml}}{\text{nmole}}\right)}$$

Cytotoxicity Assays

Macrophage as Effector Cells (^{3}H-Uridine Release Assay)

The ^{3}H-uridine release cytotoxicity assay was performed similarly to the method described by Meltzer (53). Modifications are described in the following three sections.

Labeling of Target Cells (N. fowleri Amoebae)

N. fowleri amoebae were harvested and counted with an electronic particle counter (Coulter Electronic, Inc., Hialeah, FL) according to the method of Weik and John (91). Amoebae (5×10^5 cells) were seeded in 2 ml of Nelson medium in 25 cm^2 tissue culture flasks 2 d prior to the assay. Amoebae were labeled with 2 μCi/ml of ^{3}H-uridine (New England Nuclear, Boston, MA) 18 h before co-culturing with different macrophage populations. Pilot studies employed 10 μCi/ml ^{3}H-thymidine, 10 μCi/ml ^{75}Selenomethionine, and 2 μCi/ml ^{125}IUdR. Labeled amoebae were washed twice in Nelson media and allowed to incubate 4 h in 37°C in RPMI media before a final wash with RPMI to remove spontaneously released ^{3}H-uridine.

Effector Cells

Peritoneal macrophages from various sources were collected as previously described.

Assay

Effector cells (2×10^5 macrophages/well) were plated in 96-well U-bottom microtiter plates (Costar, Cambridge, MA) at E:T ratios of 10:1, 5:1, 2:1, or 1:1 in endotoxin-free complete RPMI-1640 medium and were allowed to adhere for 2 h under standard conditions. Nonadherent cells were removed with 3 washes of HBSS. Amoebae were aliquoted, in the appropriate numbers to obtain the specified E:T ratios, using a multipipeter, onto the adherent peritoneal cells

and centrifuged for 2 min at 200 x g to assure cell-to-cell contact. Macrophage-amoebae cultures were incubated for 12, 24, 48, and 72 h under standard conditions. Wells containing macrophages and N. fowleri amoebae were plated in groups of 5, while spontaneous and maximum release controls were repeated in 10 wells. Wells containing ^{3}H-uridine labeled Naegleria provided for assessment of spontaneous release while labeled Naegleria amoebae treated with 1% sodium dodecyl sulfate (SDS) were used to assess maximum release.

Following co-incubation of macrophage and amoebae cultures, microtiter plates were centrifuged 300 x g for 10 min and culture supernatant from each well was placed in mini-vials with subsequent addition of scintillation cocktail to be radioassayed in an LKB 1218 Rackbeta liquid scintillation counter. Spontaneous release wells and maximum release wells were prepared in the same manner with the exception that maximum release well contents were removed prior to centrifuging and washed once with 150 µl of 1% SDS. The % specific release was determined by the following equation:

$$\% \text{ specific release} = \left(\frac{\text{exp. cpm} - \bar{X} \text{ cpm spon. release}}{\bar{X} \text{ cpm max. release} - \bar{X} \text{ cpm spon. release}}\right) \times 100$$

Macrophage as Target Cells (^{51}Cr Post-labeling Assay of Macrophage)

Adherent peritoneal cells were co-cultured in 96 U-bottom microtiter wells with N. fowleri amoebae or N. fowleri freeze-thawed, cell-free lysate for 6 to 72 h at 37°C and 5% CO_2. Macrophage cultures containing amoebae were refrigerated for 20 min and washed 3 times at the end of the incubation period with HBSS to remove amoebae. Macrophage cultures exposed to N. fowleri lysate were washed 3 times to remove residual amoebic lysate. Incorporation of radiochromium by residual adherent macrophages was assessed (87). Remaining adherent macrophages were labeled for 2 h with 50 µl/well of 5 µCi of $Na_2{}^{51}CrO_4$ (sodium chromate, New England Nuclear, Boston, MA). Each well was washed 3 times with HBSS to remove unbound ^{51}Cr and 100 µl of 1N NaOH were added and allowed to incubate at room temperature for 5 min before aliquoting into Titertek supernatant collection system tubes (Flow Laboratories, McLean, VA). An additional 100 µl of NaOH were placed in the wells to remove residual ^{51}Cr. N. fowleri amoebae or lysate were co-incubated with macrophages and repeated in 5 wells, whereas controls were repeated in 10 wells. Samples were radioassayed in an LKB gamma counter and percent macrophage destruction was determined by the following equation:

$$\% \text{ destruction of macrophages} = \left[1-\left(\frac{\text{exp. cpm} - \bar{X}\ \text{media control cpm}}{\bar{X}\ \text{macrop. control cpm} - \bar{X}\ \text{media control cpm}}\right)\right] \times 100$$

Preparation of Antiserum

Antiserum was provided by the laboratory of Dr. F. Marciano-Cabral, Department of Microbiology and Immunology, MCV/VCU. N. fowleri were maintained in Nelson medium at 37°C for 5 d. Cultures containing 4 x 10^7 amoebae/ml were washed 3 times in phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde. The fixed antigen preparation was mixed with an equal volume of complete Freund's adjuvant (GIBCO, Grand Island NY). New Zealand white rabbits (male 5-10 pounds) were immunized with fixed amoebae at weekly intervals. One w after the 4th immunization, the rabbits were bled via cardiac puncture and the serum was collected. Serum was head inactivated at 56°C for 30 min and stored at -20°C.

Immunization schedule was as follows:

1. Injection day 0 - rear footpads
2. Injection day 14 - rear flanks (intramuscular)
3. Injection day 21 - rear flanks
4. Injection day 28 - rear flanks
5. First bleed day 31-32
6. Injection day 36 - rear flanks
7. Second bleed day 40

Antiserum was checked for agglutinating titer using the agglutination assay described by Reilly et al. (76).

Preparation of Lymphokines

Lymphokine preparations were kindly provided by Dr. L. Schook, Department of Microbiology and Immunology, MCV/VCU.

Lymphokines were prepared according to Niederhuber et al. (62). Spleens from mice were minced using frosted end microscope slides and cells were pooled and pelleted. Cells were suspended in 0.83% Tris-NH_4Cl (0.1 ml packed cells/ml Tris-NH_4Cl) and held at 25°C for 2-5 min. Dulbecco minimal essential media (DMEM) (GIBCO) was added (10 ml) and cells were centrifuged at 300 x g for 10 min, washed twice with DMEM, and resuspended in a final volume of 10 ml. Cells were subsequently counted using trypan blue to check for viability, and adjusted to 2 x 10^6 cells/ml before seeding into flasks containing DMEM and 10% FCS (GIBCO). Spleen cells were stimulated with 2.5 µg concanavalin A (Con A)/ml of cells for 48 h. Fusion of the Con A stimulated spleen cells and the BW5147 (HGPRT-) T cell lymphoma obtained from American Type Culture Collection (ATCC, Rockville, MD) was performed 48 h following spleen cell collection. The BW5147 cells were grown in DMEM and 10% FCS and split 1:2. A nylon wool column was prepared according to Henry (34), placed in a 50 ml test tube, rinsed with DMEM and 15% FCS warmed to 37°C and incubated 1 h at 37°C. The nylon wool was rinsed with 10 ml of media and allowed to run dry. Stimulated spleen cells were

adjusted so that 1 x 10^8 cells were added to the column and incubated at 37°C for 45 min. Cells were collected in 15 ml tubes by washing the column with DMEM and eluting at 1 drop/min. Cells were centrifuged (8 min, 150 x g), counted and resuspended in media. Lymphoma cells and spleen cells were mixed together at a 1:2 ratio, respectively, in a 50 ml centrifuge tube and centrifuged at 400 x g for 10 min at 25°C. The supernatant was removed prior to the gentle addition of 1 ml of warm 50% polyethylene glycol (PEG) (MW of 1000) over a 1 min period. Supernatant was stired gently for 1 additional min before adding and stiring 1 ml of warm DMEM (serum-free) to dilute PEG without lysis of cells. An additional 1 ml of DMEM was added before adding 7 ml of serum-free DMEM over a 2-3 min period. The cells were centrifuged at 400 x g for 10 min at 25°C and the supernatant was removed. Cells were resuspended in 35 ml DMEM + 15% FCS + HAT (hypoxanthine-aminopterin-thymidine medium), dispensed in 0.2 ml aliquot per well in 96-well microtiter plates (flat bottom) and placed in a 7% CO_2 incubator at 37°C. HAT medium was added on day 5 and day 10, and every 3 d following day 10. After 14 and 21 d supernatants were removed from positive wells containing growth and stored at 4°C for subsequent use in cytotoxicity assay.

In Vivo Macrophage and Naegleria Interactions

Female $B_6C_3F_1$ mice were treated with pyran copolymer (50 mg/kg, i.v.) or Corynebacterium parvum (70 mg/kg, i.v.) on day 4 and day 3, followed by challenge with a low virulent strain of Naegleria fowleri (i.n.) in numbers ranging from 5 x 10^4 amoebae/mouse to 1 x 10^6 amoebae/mouse. Mortality data were recorded daily for 21 d.

In Vitro Macrophage or N. fowleri Treatments

Leupeptin (Acetyl-L-leucyl-L-leucyl-L-argininal) (Sigma Chemical Co., St. Louis, MO) Synthetic

Leupeptin was preincubated with different macrophage populations at concentrations of 1500 µg/ml, 1000 µg/ml, and 500 µg/ml for 30 min prior to the co-culture of lysate and macrophage.

Catalase (Sigma Chemical Co.) (from bovine liver 17,600 U/mg prot.)

A stock solution was made in HBSS and stored at 4°C in a vial covered with aluminum foil to protect from light. Macrophages were preincubated 30 min with catalase before adding N. fowleri lysate for 18 h.

Hydrogen Peroxide (H_2O_2) (Sigma Chemical Co.)

N. fowleri lysate was preincubated with hydrogen peroxide 30 min before adding to washed adherent peritoneal exudate cells.

Antibody to N. fowleri

Dilutions of 1:10, 1:500, and 1:1000 of anti-N. fowleri heat inactivated serum were added to co-cultures of N. fowleri cells and macrophages.

Lymphokines

Lymphokines prepared from hybridoma T cells as previously described were preincubated 24 h with adhered resident macrophages prior to a 6 h co-incubation of macrophages and N. fowleri lysate.

Statistical Methods

The level of confidence for all experiments was set at 95%. Increases and decreases for percent-specific release or macrophage destruction were statistically significant based upon this level of confidence.

Homogeneity of variance was determined using the Barlett test (83). If the data were homogeneous, a parametric analysis of variance (ANOVA) was used. Dunnett's multi-range test was employed to examine significant differences between the control group and each individual experimental group. When a non-homogeneous population was analyzed, a non-parametric ANOVA was employed followed by a Wilcoxon's rank test (92) to determine significant differences between a control group and experimental group. When determining differences among all experimental and control groups at one time, Duncan's test (24) was utilized.

Jonckheere's test against ordered alternatives (36) provided significance levels for determination of dose-response relationships.

RESULTS

Morphological Studies

To determine whether CP-activated macrophages were capable of killing N. fowleri amoebae in vitro, the two cells were combined in tissue culture chambers and examined by inverted phase contrast microscopy for movement, change in morphology, lysis, and phagocytosis.

N. fowleri cells were co-cultured in Lab-Tek chambers with adherent CP, TG, and resident macrophage monolayers. After 24 h and a 20:1 macrophage-to-amoeba ratio, there were very few TG or resident macrophages remaining. Amoebae were usually round with pseudopods extending in several directions. The CP macrophages were present and reduction in their numbers was not evident at the same time period and ratio. Lysis of N. fowleri was apparent in CP macrophage wells and phagocytosis of N. fowleri was demonstrated by light microscopy at 6 h following co-cultivation. With the exception of TG macrophages, both amoeba and macrophage had a tendency to round up when co-cultured and distinguishing the two cell types was often difficult; therefore, differences were often observed based upon movement of N. fowleri.

Normal peritoneal macrophages from experimental animals vary greatly in size and morphology. These cells have a diameter ranging from 10-30 μm (30). In contrast, N. fowleri amoebae range from 15-30 μm x 6-9 μm in diameter (17). Samples of media from chambers indicating no movement, yet containing obvious amoeboid structures were removed and placed in Nelson medium, resulting in no amoebae attachment nor movement. Transmission electron microscopy (Fig. 1) demonstrated amoebae as well as macrophage destruction.

To check for activation of resident cells, or an extremely altered TG or CP population, the number of PEC's per mouse were recorded, ectoenzyme profiles were determined, and cell differentials were performed. Characteristic cell numbers for $B_6C_3F_1$ mice exposed to CP, TG, and untreated mice were 5.0×10^6, 2×10^7, and 3.0×10^6 PEC's/mouse, respectively. Percents of macrophages were approximately 50, 70, and 35%, respectively. Significant deviations from the normal expected values for PEC number and percent of macrophages were important when comparing experiments and interpreting results.

Ectoenzymes

Resident, TG, and CP macrophages possess unique ectoenzyme profiles for leucine aminopeptidase (LAP), alkaline phosphodiesterase (APD), and 5'Nucleotidase (5'N). These

enzymes were monitored for each macrophage population to provide insight for changes in a population treated with one agent.

Ectoenzyme-specific activities reported in Table 1 and shown in Figure 2 illustrate the expected ectoenzyme profiles for each macrophage population (CP, TG, and resident). Ectoenzyme analysis was performed in order to correlate the state of activation to cytotoxicity. Unless indicated, ectoenzyme profiles were generally in agreement with those reported in the literature (54). Alkaline phosphodiesterase (APD) activity for TG macrophages remained the same or increased slightly when compared to the activity for resident macrophages. Corynebacterium parvum (CP) macrophages demonstrated decreased levels of APD activity. The activity of 5'Nucleotidase (5'N) was consistently lower in TG-elicited and CP-activated macrophages when compared to the activity of the ectoenzyme in resident macrophage populations. Both CP and TG macrophages showed an increase in leucine aminopeptidase (LAP) over the presence of the ectoenzyme in resident macrophages.

Labeling of Naegleria

Preliminary labeling experiments for N. fowleri were performed using ^{3}H-thymidine, ^{75}Selenomethionine (10), and ^{125}IUdR (Iododeoxyuridine) (35). ^{3}H-thymidine was added at 10 μCi/ml to approximately 1 x 10^{6} amoebae in 2 ml.

Approximately 0.1 cpm per amoeba was incorporated and spontaneous release ranged from 25 to 49% in 72 h on three separate occasions. A 10 μCi/ml quantity of ^{75}Selenomethionine was added to 5 x 10^6 amoebae in 2 ml. Approximately 0.13 cpm of ^{75}Se per amoeba was incorporated and spontaneous release was as high as 50% in 24 h and 70% or greater by 72 h. ^{125}IUdR (2 μCi/ml) was not sufficiently incorporated into N. fowleri following treatment with FUdR (fluorodeoxyuridine). FUdR was used to inhibit thymidilate synthetase and increase ^{125}IUdR incorporation (35). N. fowleri amoebae (5 x 10^5 cells in 2 ml) were labeled with 2 μCi/ml ^{3}H-uridine yielding approximately 0.13 cpm to 0.35 cpm per amoeba. Spontaneous release ranged from 20 to 70% in 12 to 72 h, respectively. Based upon these results, ^{3}H-uridine was chosen for further study to determine macrophage-induced N. fowleri lysis or release of label.

Macrophage as Effector (^{3}H-Uridine Release Assays)

Morphological studies using Lab-Tek chambers under the inverted phase contrast microscope suggested that CP macrophages were capable of killing N. fowleri at 20:1 and 10:1 E:T ratios. Therefore, several E:T ratios (10:1, 5:1, 2:1, and 1:1) were investigated by measuring the amount of specific release of ^{3}H-uridine from N. fowleri. This technique provided for a more quantitative and accurate measure of killing (Table 2, Fig. 3). Resident macrophages

did not show a significant increase in release of label from N. fowleri as compared to spontaneous release for any of the E:T ratios tested. Macrophages derived from mice injected with CP demonstrated no significant effect on the release of ^{3}H-uridine from N. fowleri at a 2:1 or 1:1 E:T ratio; however, at a 5:1 and 10:1 E:T ratio, there was a significant increase in release of label over spontaneous release.

The kinetics of CP, TG, pyran, and resident macrophage-induced release of label were investigated (Table 3, Fig. 4). After 24, 48, or 72 h of co-cultivation, TG and resident macrophages caused a similar low level release of N. fowleri incorporated label. In contrast, CP-activated macrophages produced a significantly increased cytotoxic response at 24 and 48 h. The release of label disappears at 72 h and falls to the TG and resident macrophage level. The release of ^{3}H-uridine from N. fowleri induced by pyran-activated macrophages is higher than that elicited by TG and resident macrophages. However, these values are significantly lower than that for CP macrophages at 24 and 48 h.

Antibody produced against N. fowleri amoebae was added to co-cultures of N. fowleri amoebae and adherent macrophages to determine whether the amoebae could be immobilized or altered otherwise to render them more susceptible to macrophages.

The % specific release of ^{3}H-uridine from N. fowleri amoebae after the addition of various concentrations of amoeba-specific antibody is illustrated in Table 4. The addition of specific antibody to N. fowleri amoebae in the absence of macrophages caused an 11.0 $\pm$ 1, 29.6 $\pm$ 1.4, and a 13.2 $\pm$ 1.3 % release of ^{3}H-uridine at a 1:10, 1:500, and 1:1000 antibody dilution, respectively. Macrophages activated with CP caused the greatest release of label from N. fowleri at all three concentrations of antibody. At 1:10 and 1:500 antibody concentrations, ^{3}H-uridine release was significantly greater than macrophage and amoeba in the absence of antibody. There was no such increase using a 1:1000 antibody concentration. At a 1:10 antibody dilution neither TG nor resident macrophage-induced N. fowleri lysis was significantly altered. Release of label remained the same for TG and resident macrophages with and without antibody (1:500) despite the evident increase in release caused by antibody with amoebae. The effect of antibody on the macrophage populations could not be directly measured in this assay; however, further studies using a post-labeling technique for remaining macrophages suggested that the antibody enhanced macrophage adherence or size. At a 1:1000 antibody concentration release of N. fowleri incorporated ^{3}H-uridine in the presence of TG and resident macrophages displayed significant decreases illustrating the same macrophage protection as described above. According

to Jonckheere's test against ordered alternatives, there was a significant dose response due to the three antibody concentrations for CP macrophage destruction.

Macrophage as Target ($Na_2{}^{51}CrO_4$ Post-Labeling of Macrophage)

N. fowleri Whole Cells

To determine the effect of N. fowleri amoebae on the three macrophage populations (CP, TG, and resident), macrophages were labeled with $Na_2{}^{51}CrO_4$ at various time periods after co-cultivation with N. fowleri. The ^{51}Cr labeling of a particular well was taken as a relative measure of well cell density (87). The amount of ^{51}Cr incorporated by macrophages cultured in the presence of N. fowleri was compared to the amount of ^{51}Cr incorporated into macrophages cultured in the absence of N. fowleri to determine the percent of remaining macrophage. This was subsequently expressed as % macrophage destruction. Effector-to-target cell ratios of 1:5, 1:10, and 1:20 for CP, TG, and resident macrophages were investigated (Table 5, Fig. 6). At all three E:T ratios, the majority of the resident macrophages was destroyed in 24 h. Macrophages obtained from TG and CP treated animals demonstrated a significantly lower profile of N. fowleri-induced destruction and demonstrated significant resistance at 24 h with a 1:20 amoeba to macrophage (E:T) ratio. There was a significant difference in release

of label from TG and CP macrophages at 1:5 and 1:10 but not for a 1:20 E:T ratio.

A kinetic study of differential macrophage destruction was performed using a 1:20 E:T ratio. As shown in Table 6, Figure 7, CP macrophages were not significantly destroyed at 4, 8, 18, and 24 h; however, by 48 h 52.8 $\pm$ 2.6 % destruction had been attained. Macrophages derived from TG-treated and naive mice followed a similar progression of destruction with time. At 18 h a 44.5 $\pm$ 2.5 % destruction of resident macrophages and 15.3 $\pm$ 1.7 percent destruction of TG macrophages was observed. The three macrophage populations showed distinct patterns of destruction. Resident macrophages were destroyed more readily than TG-elicited and CP-activated macrophages. Consistent results were obtained at 24 h with respect to unique destruction patterns. The amount of macrophage destruction at each time period varied, causing kinetics to shift from experiment to experiment. The variation may be due to a change in mouse age and immune status.

N. fowleri Lysates

To eliminate the complexity of a cell-cell interaction, N. fowleri cells were replaced by N. fowleri lysates. Lysates of N. fowleri were incubated on macrophage monolayers to determine their effect on CP, TG, and resident macrophages (Table 7, Fig. 8). At 18 h of co-incubation,

the N. fowleri lysate (equivalent of 3:1 E:T ratio) destroyed 85 ± 1.2% of the resident macrophage population, while destruction of CP and TG macrophages was minimal, at 22 and 16%, respectively. The ability of the N. fowleri lysate to kill macrophages correlated closely with that observed in 24 h for N. fowleri amoebae. This study prompted an investigation of the kinetics of lysate-induced macrophage destruction (results not shown). The N. fowleri lysate was shown to destroy CP, TG, and resident macrophages as early as 4 h following cultivation at an equivalent amoeba-to-macrophage ratio of 1.5:1. The amount of destruction was unique to the type of macrophage co-cultured with the lysate (Fig. 8). The three macrophage populations were destroyed equally by 48 h.

To determine whether the production and release of lytic substances from N. fowleri amoebae depended on the macrophage as a stimulus, lysates and conditioned media from N. fowleri and N. fowleri exposed to macrophages were employed.

Lysates or conditioned media from N. fowleri or N. fowleri previously exposed to TG macrophages for 24 h were co-incubated with resident macrophages for 18 h (Table 8, Fig. 9). Conditioned media from either source demonstrated minimal macrophage destruction of 22%. Both N. fowleri lysate (3:1 E:T equivalent) and macrophage-primed N. fowleri lysate displayed similar elevated levels of destruction around 80%.

Catalase

Activated macrophages possess increased production and secretion of hydrogen peroxide (H_2O_2). Several investigators have suggested macrophage release of H_2O_2 as a major killing mechanism. Catalase, an enzyme which can break down H_2O_2, has been found in most cells and abundantly in N. fowleri amoebae. In an attempt to discover whether H_2O_2 is a major mechanism used by CP macrophages to abolish N. fowleri lysate activity, catalase was employed to break down macrophage-derived hydrogen peroxide. Catalase was pre-incubated with CP, TG, and resident macrophage monolayers for 30 min prior to the addition of N. fowleri lysate (1.5:1 E:T equivalent) for 18 h of co-incubation (Table 9, Fig. 10). The addition of catalase to TG macrophage cultures showed little destruction, as measured by remaining macrophages at 2500 U/ml but did not destroy at lower concentrations. Lower concentrations (1000, 500, and 250 U/ml) of catalase were employed for resident macrophages because concentrations of 2500 U/ml to 1500 U/ml were extremely toxic. Lower concentrations displayed toxicity of less than 20.3 $\pm$ 8%. Catalase destroyed some CP macrophages at 2500, 2000, and 1500 U/ml. Unlike the effect of different catalase concentrations on TG and resident macrophages, there was no dose response of catalase for CP macrophages. Resident macrophages displayed a

significant dose reponse to catalase and N. fowleri lysate. N. fowleri lysate-induced destruction of CP-activated macrophages was unusually high at 18 h, which was consistent with an elevation (30%) in polymorphonuclear leukocytes (PMN) observed in the PEC differential. No significant differences were detected in N. fowleri lysate-induced destruction of resident or CP macrophages in the presence of added catalase. However, a significant increase in TG macrophage destruction was noted following catalase exposure at all concentrations tested.

Hydrogen Peroxide

An additional method to determine whether macrophage-derived H_2O_2 was capable of neutralizing or destroying the lytic activity of N. fowleri lysate was to incubate H_2O_2 with the lysate prior to the application on resident macrophage monolayers.

N. fowleri lysate was incubated with hydrogen peroxide (H_2O_2) 30 min prior to addition to a resident macrophage monolayer for 6 h. Concentrations of 10, 50, 100, 500, 1000, and 5000 nm of H_2O_2 were initially employed, and did not change the destructive capability of N. fowleri lysate on resident macrophages (data not shown). Further investigation utilizing 5, 10, 50, 100, and 500 μM of H_2O_2 to pretreat lysate confirmed the ineffectiveness of H_2O_2 on N. fowleri lysate (Table 10, Fig. 11). Toxicity was

minimal up to 10 μM H_2O_2. A 96.8 ± 0.2 % destruction of resident macrophages was observed when 100 or 500 μM H_2O_2 were added. Similar H_2O_2 concentrations were incubated with labeled N. fowleri amoebae to determine the effective concentration needed to kill N. fowleri, as measured by the release of ^{3}H-uridine (results not shown). At H_2O_2 concentrations ranging from 10 nm to 500 μM, there was little, if any, release of ^{3}H-uridine from N. fowleri when compared to labeled N. fowleri amoebae incubated in the absence of H_2O_2.

Leupeptin

Activated macrophages produce and secrete a variety of proteases which may be instrumental in producing target cytotoxicity. Leupeptin is an inhibitor of cysteine and serine proteases. The addition of this inhibitor to macrophage monolayers prior to incubation with N. fowleri lysate was used to abolish the resistance to lysate shown by CP macrophages. The use of leupeptin was employed to determine whether protease activity may be a second major mechanism of CP macrophages protecting them from N. fowleri lysate.

The effect of leupeptin was investigated by preincubating macrophages 30 min with 1500, 1000, and 500 μg/ml of leupeptin prior to the addition of N. fowleri lysate for 6 h (Table 11, Fig. 12). Leupeptin-induced destruction of TG as well as CP macrophages remained below 10%, while

leupeptin-induced macrophage destruction of resident macrophages was less than 18%. This destruction due to leupeptin in the absence of N. fowleri lysate was dose responsive for resident and TG macrophages, but not for CP macrophages.

Leupeptin did not change N. fowleri lysate-induced CP or resident macrophage destruction at any of the concentrations. At all leupeptin concentrations, there was a significant increase in TG macrophage destruction due to N. fowleri lysate.

Lymphokines

Lympholines in vitro were used as an alternative method of activating macrophages to that shown in vivo with C. parvum. Several lymphokine preparations from T cell hybridomas were added to resident macrophage monolayers for 24 h prior to assessing N. fowleri lysate-induced macrophage destruction (Table 12, Fig. 13). The three lymphokine samples tested evoked less than 10% destruction of resident macrophages in the absence of N. fowleri lysate. In the presence of N. fowleri lysate and lymphokines, there was a significant decrease in destruction of resident macrophages.

In Vivo Macrophage and N. fowleri Interactions

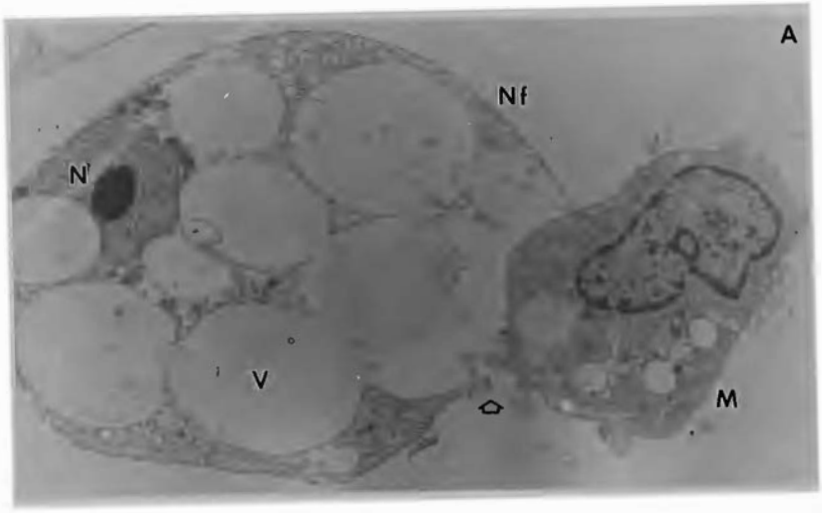
In vivo correlates to the previous in vitro work were performed using C. parvum and pyran copolymer (i.p.) as activating agents and N. fowleri (LEE) as infecting agent at 5×10^4, 5×10^5, and 1×10^6 amoebae/mouse (i.n.) (Table 13). Neither activating agent delayed death significantly or caused less deaths overall in the mice exposed to three numbers of N. fowleri. In fact, it appears that more deaths occurred with the administration of C. parvum than without, and death occurred slightly earlier than the control with N. fowleri exposure. Pyran did not show as dramatic a difference as did C. parvum; however, it did cause a few more deaths with the high and low doses compared to control. At necropsy amoebae were recovered from the brain. The lungs of mice treated with C. parvum or pyran were free of amoebae. In contrast, amoebae were recovered from brain and lungs in untreated mice.

Figure 1. Transmission electron micrograph (x4000) of 19-h co-cultures (1:10 amoeba:macrophage ratio) of peritoneal macrophages and Naegleria fowleri.

A. Evident breakdown (⇦) of the N. fowleri amoeba membrane is shown at the point of contact with CP-activated macrophage (M). The amoeba is highly vacuolated (V) and the nucleus (N) remains intact.

B. A CP-activated macrophage (M) has phagocytized an N. fowleri amoeba (⇦), whereas other surrounding macrophages (M*) appear to be lysed (⇦).

Transmission Electron Microscopy



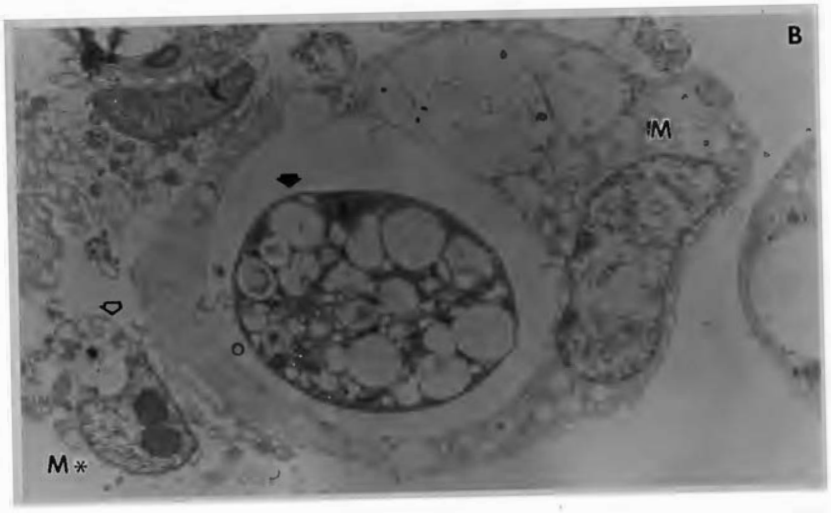


Table 1

Macrophage Ectoenzyme Activity

Macrophage Population	Alkaline Phosphodiesterase (APD)	Leucine Aminopeptidase (LAP)	5'Nucleotidase (5'N)
CP	0.389 ± 0.073	1.034 ± 0.064	0.044 ± 0.027
TG	1.798 ± 0.147	4.492 ± 0.600	0.018 ± 0.011
R	0.814 ± 0.257	0.345 ± 0.156	1.354 ± 0.177

Specific activities are expressed in nmoles/mg protein/minute ± standard error derived from 5 experiments.

CP - Corynebacterium parvum macrophages

TG - Thioglycollate macrophages

R - Resident macrophages

Figure 2. Plasma membrane ectoenzyme activity of alkaline phosphodiesterase (APD), leucine aminopeptidase (LAP) and 5'Nucleotidase (5'N), obtained from Corynebacterium parvum (CP), thioglycollate (TG), and resident (R) macrophages.

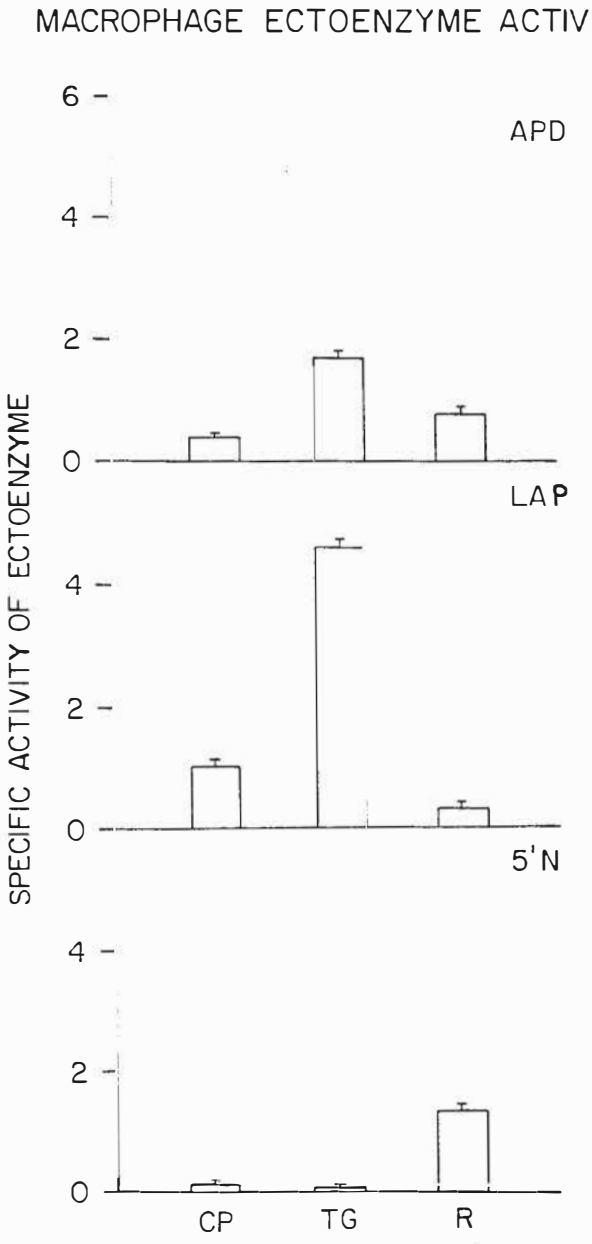

MACROPHAGE ECTOENZYME ACTIVITY
SPECIFIC ACTIVITY OF ECTOENZYME
APD
LAP
5'N
6
4
2
0
CP
TG
R

Table 2

Release of ^{3}H-Uridine From N. fowleri at Various E:T Ratios[a]

	Effector-to-Target Ratios[b]			
	10:1	5:1	2:1	1:1
CP	3286.9 ± 73.0[c]	2533.3 ± 242.5[c]	1125.0 ± 49.5	1107.2 ± 46.0
R	1184.5 ± 68.0	1146.4 ± 34.5	1114.5 ± 49.5	1181.9 ± 26.0

Spontaneous release - 1009.6 ± 49.5

[a] 24-h co-culture time, macrophage to amoeba (E:T)

[b] Values are expressed in mean counts per min ± SE from 5 wells per group.

[c] $p<0.01$ when compared to spontaneous release.

Figure 3. Release of ^{3}H-uridine from N. fowleri at 24 h using various macrophage to amoeba (E:T) ratios (counts per minute $\pm$ SE for 5 wells). Mean spontaneous release was 1009.6 $\pm$ 49.5.

RELEASE OF ^{3}H-URIDINE FROM N. FOWLERI AT VARIOUS E:T RATIOS

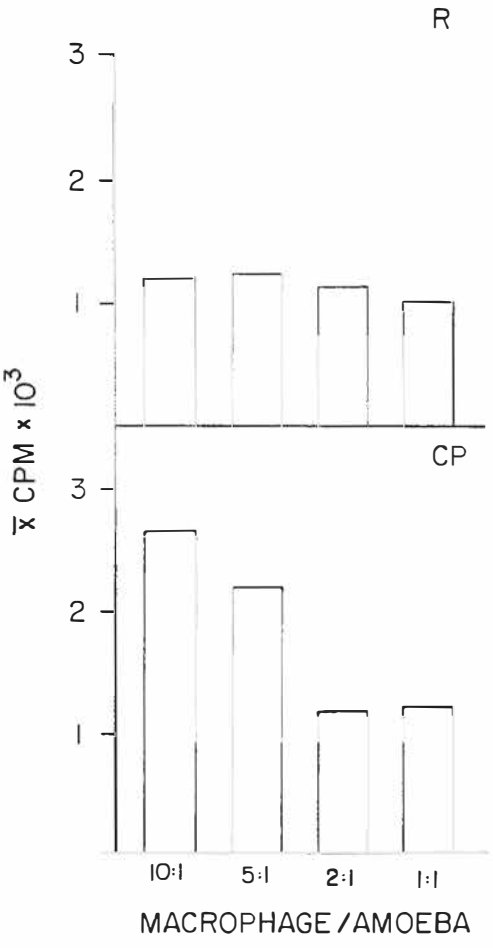


Table 3

Release of ^{3}H-Uridine From N. fowleri

Co-incubated With Macrophages[a]

Macrophage Population	Time[b] 24 H[c]	48 H[c]	72 H
CP[g]	38.8 ± 2.1	55.6 ± 2.3	11.8 ± 6.5
P[f]	13.2 ± 0.7	15.6 ± 2.1	25.6 ± 2.2
TG[d]	3.3 ± 1.1	10.5 ± 1.6	8.4 ± 1.8
R[e]	5.6 ± 0.9	15.3 ± 1.7	17.3 ± 2.9

[a]10:1 effector-to-target ratio.

[b]Values are expressed in % specific release ± SE and 5 wells per group.

[c]CP (Corynebacterium parvum) differs from P (pyran), TG (thioglycollate), and R (resident), $p<0.01$.

[d]TG at 24 h differs from 48 h (72 h, $p<0.05$), $p<0.01$.

[e]R at 24 h differs from 48 and 72 h, $p<0.01$.

[f]P at 24 h differs from 72 h (not 48 h), $p<0.01$.

[g]CP at 24 h differs from 72 h (48 h, $p<0.05$), $p<0.01$.

Figure 4. Release of ^{3}H-uridine from N. fowleri trophozoites, following co-incubation with macrophages: (●) C. parvum, (▲) thioglycollate, (□) pyran, (○) resident. Cells were co-incubated at a macrophage-to-amoeba (E:T) ratio of 10:1. Data were reported as mean % specific ^{3}H-uridine release into the supernatant ± SE from 10 wells.

KINETICS OF THE RELEASE OF ^{3}H-URIDINE
FROM N. FOWLERI COINCUBATED WITH MACROPHAGES

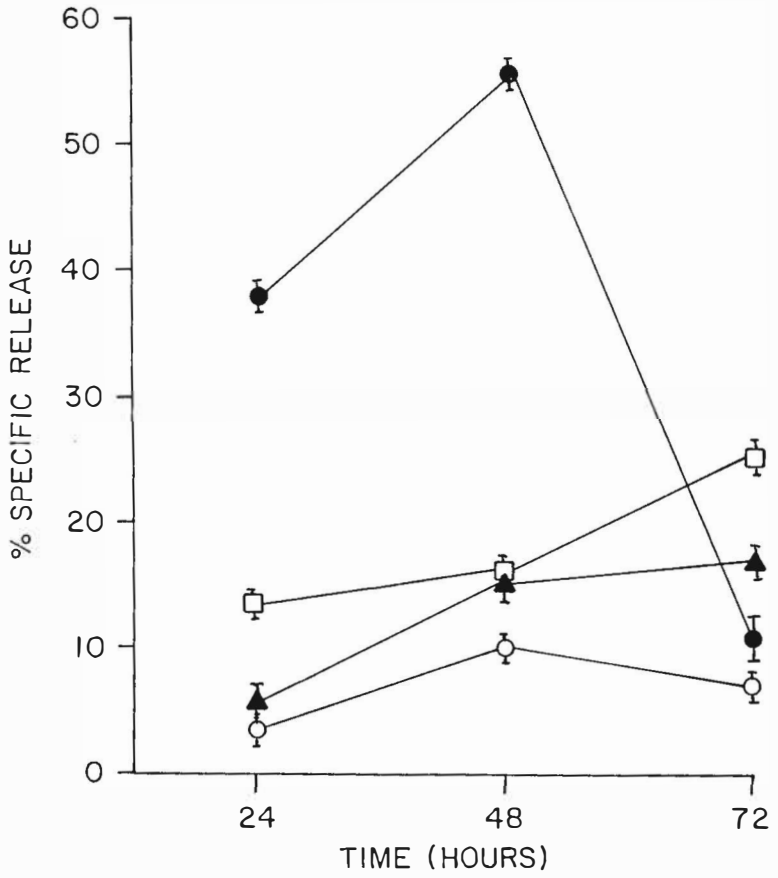


Table 4

The Effect of Antibody on Macrophage-Induced ^{3}H-Uridine Release From *N. fowleri*[a]

Macrophage Population	*N. fowleri* Cells (10:1 E:T Ratio) and Antibody Concentration[b]			
	1:10	1:500	1:1000	No Antibody
CP[c]	65.4 ± 2.3	84.6 ± 2.1	53.0 ± 3.5	30.0 ± 2.4
TG[d]	23.4 ± 0.9	15.4 ± 1.9	9.2 ± 1.3	11.8 ± 0.4
R	22.2 ± 1.9	12.2 ± 1.1	2.2 ± 1.1	5.25 ± 0.9
No Macrophage	11.0 ± 1.1	29.6 ± 1.4	13.2 ± 1.3	

[a] 24 h co-incubation; antibody present for 24 h.

[b] Values represent mean % specific release ± SE from 5 wells.

[c] CP + antibody (1:10 and 1:500) increased release, $p<0.01$.

[d] TG + antibody (1:500 and 1:1000) decreased release, $p<0.01$.

Figure 5. Antibody produced in the rabbit against N. fowleri was added to the co-culture at a macrophage-to-amoeba (E:T) ratio of 10:1 (□) and added to Naegleria (▨) for an 18-h incubation period. Release of ^{3}H-uridine from N. fowleri was compared to the release from N. fowleri and macrophage (▣). Data were expressed as the mean % specific release $\pm$ SE derived from 5 wells per group.

THE EFFECT OF ANTIBODY ON MACROPHAGE-INDUCED ^{3}H-URIDINE RELEASE FROM N. FOWLERI

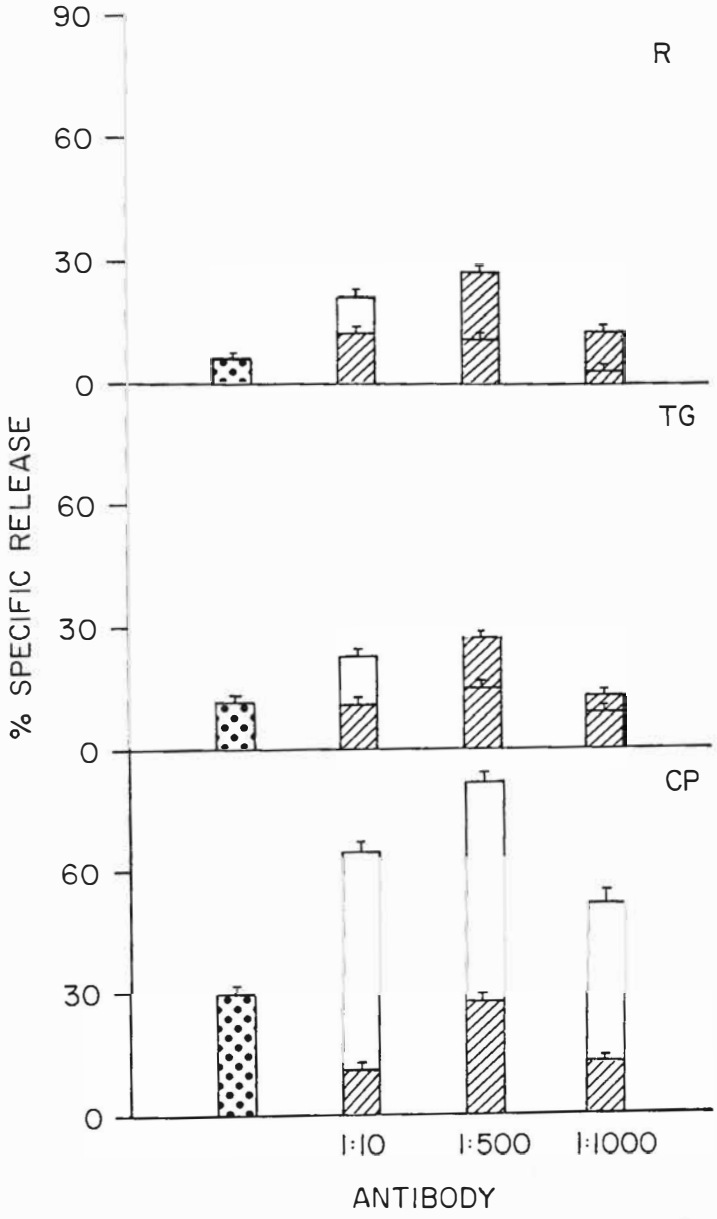


Table 5

N. fowleri-Induced Macrophage Destruction at Different E:T Ratios[a]

Macrophage Population	Effector:Target Ratios[b] 1:5[c]	1:10[c]	1:20[d]
CP	60.8 ± 2.3	51.4 ± 2.7	22.8 ± 3.2
TG	48.0 ± 1.5	31.2 ± 2.5	22.0 ± 2.8
R	90.2 ± 0.7	80.0 ± 0.8	72.6 ± 1.0

[a] 24-h co-incubation, ^{51}Cr post-labeling study.

[b] Data were reported as mean % destruction of macrophage ± SE from 5 wells.

[c] CP differed from R and TG, $p<0.01$.

[d] CP differed from R, $p<0.01$.

Figure 6. N. fowleri cells were overlaid on different macrophage monolayers and incubated for 24 hours at three amoeba-to-macrophage (E:T) ratios. Percent destruction of macrophages was determined by post-labeling remaining macrophages and was reported as mean $\pm$ SE with 5 wells per group.

N. FOWLERI-INDUCED MACROPHAGE DESTRUCTION AT DIFFERENT E:T RATIOS

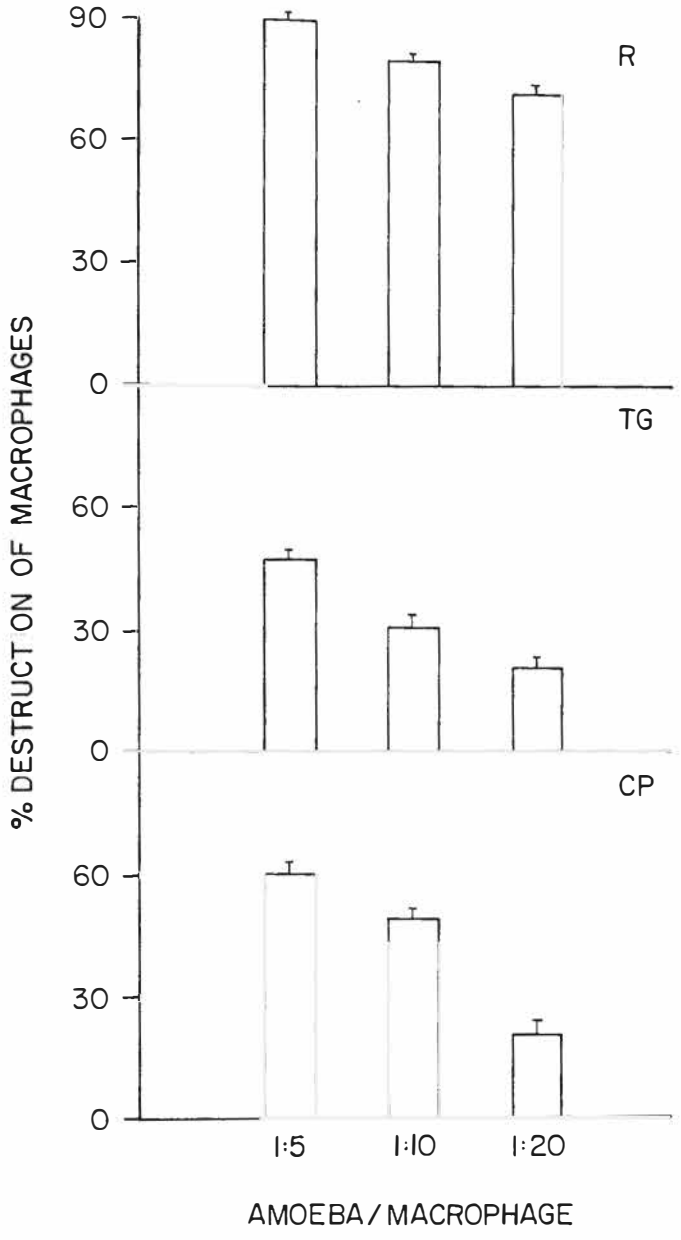


Table 6

Kinetics of N. fowleri-Induced Macrophage Destruction[a]

Macrophage Population	Time[b] 4 H	8 H	18 H	24 H	48 H
CP[c,d]	-1.7 ± 3.3	-2.8 ± 3.0	-10.3 ± 4.0	-26.5 ± 2.3	52.8 ± 2.6
TG[e]	0.4 ± 2.4	-7.1 ± 3.2	15.3 ± 1.7	36.4 ± 2.4	76.5 ± 2.1
R[f]	-0.2 ± 5.4	-4.3 ± 2.8	44.5 ± 2.5	72.5 ± 1.9	97.7 ± 0.1

[a] ^{51}Cr post-labeling study; 1:20 effector-to-target ratio.

[b] Values were presented as mean % destruction of macrophages ± SE from 10 wells.

[c] CP was different from TG and R at 18, 24, 48 h, $p<0.01$.

[d] CP at 4 h differs from 24 and 48 h, $p<0.01$.

[e] TG at 4 h differs from 18, 24, and 48 h, $p<0.01$.

[f] R at 4 h differs from 18, 24, and 48 h, $p<0.01$.

Figure 7. N. fowleri cells were co-incubated with an adherent monolayer of macrophages; CP (○), TG (△), or R (□) at an amoeba-to-macrophage (E:T) ratio of 1:20. Macrophage destruction was assessed by post-labeling remaining macrophages with $Na_2{}^{51}CrO_4$. Mean % macrophage destruction $\pm$ SE was obtained from 5 wells per group.

KINETICS OF N. FOWLERI-INDUCED MACROPHAGE DESTRUCTION

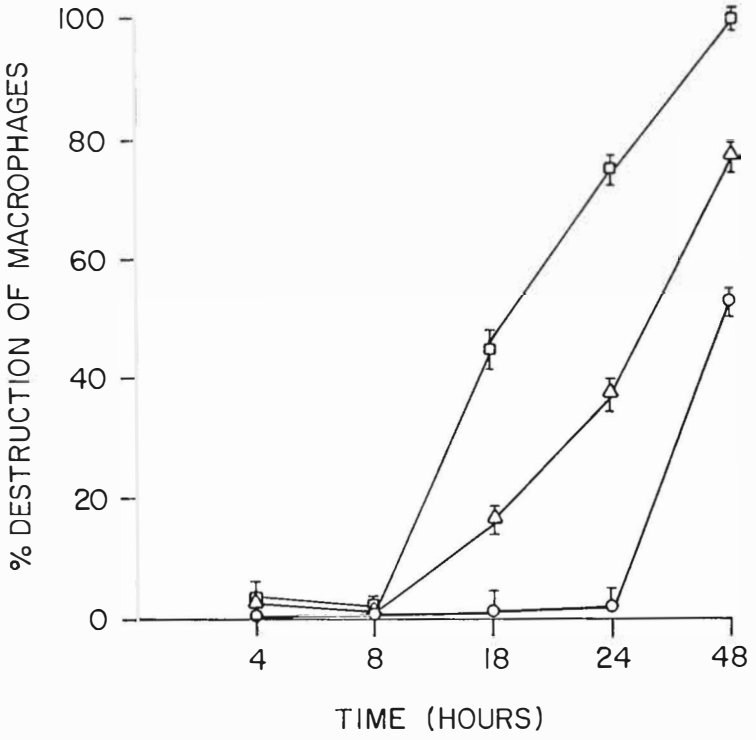


Table 7

N. fowleri Cell or Lysate-Induced Macrophage Destruction[a]

Macrophage Population	*N. fowleri* Lysate[b]	*N. fowleri* Cells 20:1
CP[c]	22.0 ± 3.8	22.8 ± 3.2
TG[c]	16.0 ± 2.4	22.0 ± 2.8
R[c]	85.0 ± 1.2	72.6 ± 1.0

[a] ^{51}Cr post-labeling study; lysate was a 3:1 E:T equivalent; cells were a 20:1 E:T ratio; 24 h co-incubation.

[b] Data represent mean % destruction of macrophages ± SE from 5 wells.

[c] CP and TG lysate and cells did not differ; R lysate differs from cells, $p<0.01$.

Figure 8. Three macrophage populations were co-incubated for 24 h with N. fowleri cells at an amoeba-to-macrophage (E:T) ratio of 1:20, or 18 h with N. fowleri lysate (equivalent E:T ratio of 3:1). Percent destruction of macrophages was determined by post-labeling remaining macrophages with $Na_2{}^{51}CrO_4$ and reported as mean $\pm$ SE from 5 wells.

N. FOWLERI CELL OR LYSATE-INDUCED MACROPHAGE DESTRUCTION

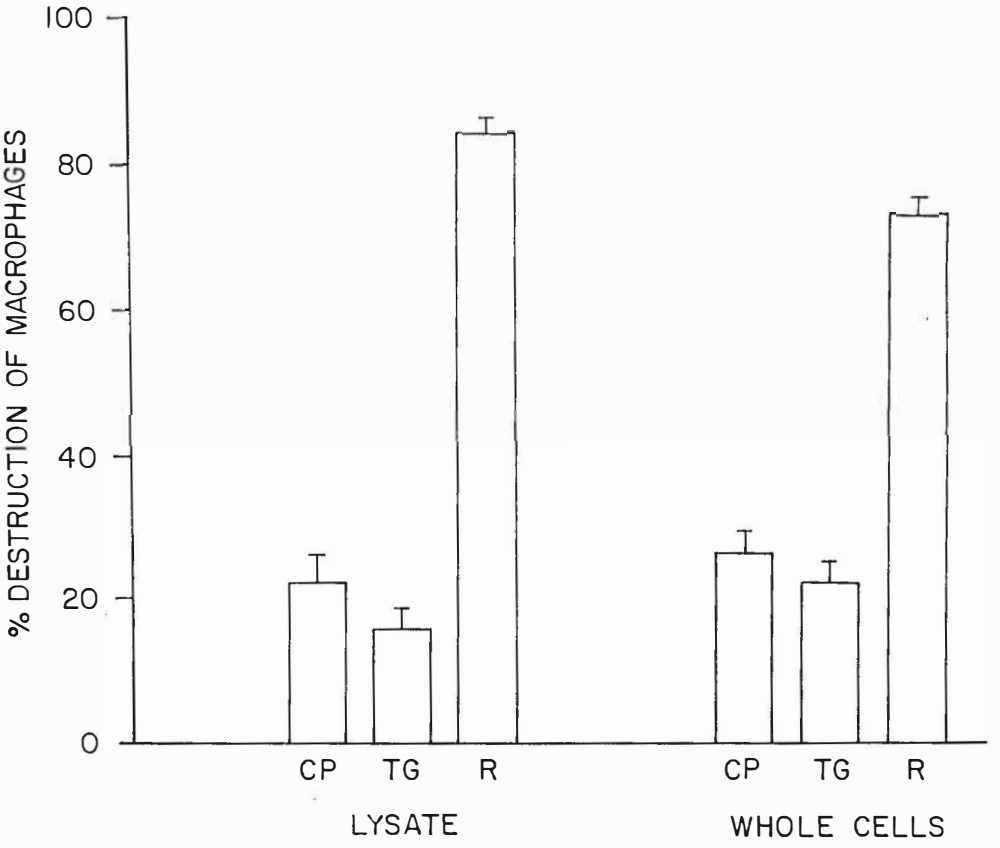


Table 8

The Effect of N. fowleri Lysate or Conditioned Media on Resident Macrophages[a,b]

	Lysate	Conditioned Media
N. fowleri[c] Alone	82.0 ± 1.4	23.0 ± 1.3
MØ Primed N. fowleri[c]	87.0 ± 1.6	22.0 ± 2.3

[a] ^{51}Cr post-labeling study; lysate 3:1 E:T ratio equivalent; conditioned media from 1 x 10^7 cells; 18 h co-incubation.

[b] Values represent the mean % destruction of macrophages ± SE from 5 wells.

[c] Lysate and conditioned media from both types of N. fowleri differ, $p<0.01$.

Figure 9. Resident macrophage monolayers were incubated for 18 h with lysates (▨) or conditioned media (⊡) obtained from N. fowleri or from N. fowleri exposed to TG-elicited macrophages for 24 h prior to assay. The lysates represent the equivalent of a 3:1 amoeba-to-macrophage ratio. The undiluted, conditioned media was obtained from N. fowleri cultures having a cell density of 1×10^7 amoebae/ml. Percent destruction of macrophages was determined by post-labeling the monolayers with $Na_2{}^{51}CrO_4$ and reported as the mean $\pm$ SE derived from 5 wells per group.

THE EFFECT OF N. FOWLERI LYSATE OR CONDITIONED MEDIA ON MACROPHAGES

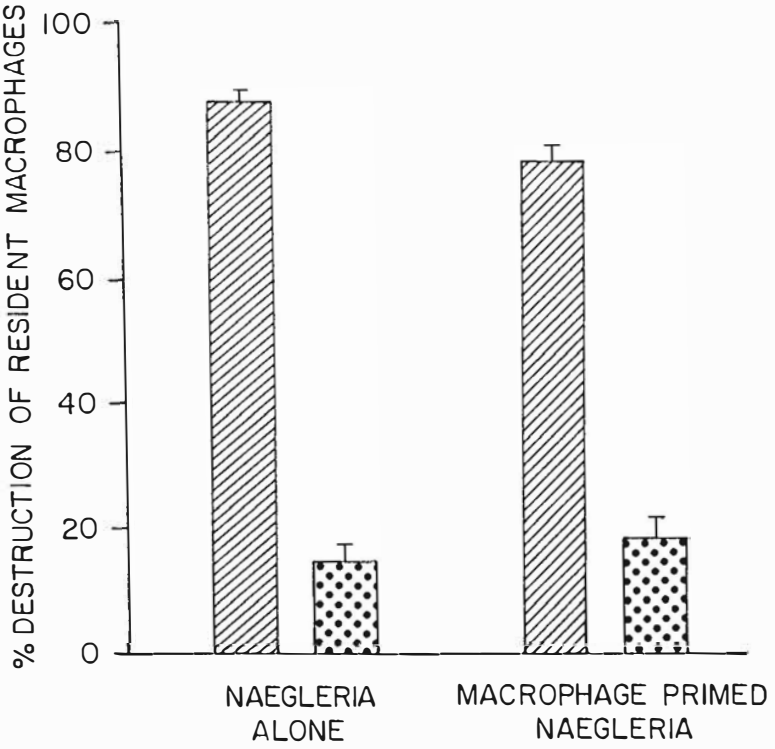


Table 9

The Effect of Catalase on N. fowleri Lysate-Induced Macrophage Destruction[a]

Macrophage Population	Catalase + N. fowleri Lysate[b]			Catalase			N. fowleri Lysate Alone
	2500[d]	2000	1500	2500	2000	1500	
CP	81.4 ± 0.9	77.4 ± 2.3	78.8 ± 1.2	14.8 ± 0.9	10.8 ± 0.8	14.2 ± 1.1	68.6 ± 1.2
TG[c]	55.8 ± 1.3	49.8 ± 1.7	42.4 ± 1.7	5.2 ± 4.3	-3.0 ± 2.4	-6.4 ± 1.2	33.6 ± 1.9
	1000[d]	500	250	1000	500	250	Lysate Alone
R	99.4 ± 0.4	96.4 ± 1.1	96.6 ± 0.7	20.3 ± 8.0	17.4 ± 5.0	7.3 ± 5.0	96.6 ± 0.24

[a] ^{51}Cr post-labeling study; catalase was pre-incubated 30 min with macrophages and lysate was added for 18 h.

[b] Data were reported as mean % destruction of macrophages ± SE from 5 wells.

[c] By subtracting out toxicity it was clear that the addition of catalase significantly increased destruction of TG macrophages, $p<0.01$.

[d] U/ml.

Figure 10. Varying concentrations of catalase were added to adherent macrophages for 30 min prior to the addition of N. fowleri lysate at the equivalent of a 1.5:1 amoeba-to-macrophage (E:T) ratio and co-cultured for 18 h (▭). These values were compared to macrophage and N. fowleri lysate wells (▩) and macrophage and catalase wells (▨). Destruction of macrophages was assessed by post-labeling macrophages remaining, with $Na_2{}^{51}CrO_4$ and reporting means of % macrophage destruction $\pm$ SE from 5 wells.

THE EFFECT OF CATALASE ON N. FOWLERI LYSATE-INDUCED MACROPHAGE DESTRUCTION

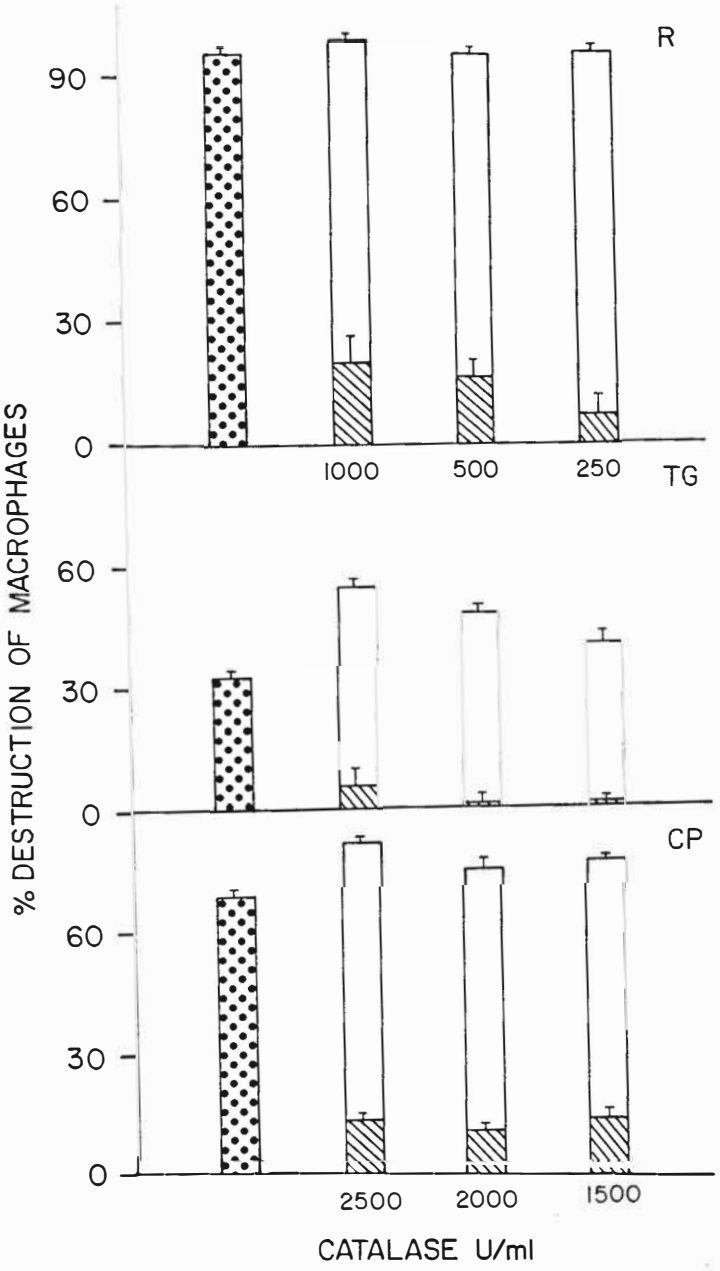


Table 10

The Effect of Hydrogen Peroxide on N. fowleri Lysate-Induced Macrophage Destruction[a]

	H_2O_2 Concentration (μM)					
Macrophage	5	10	50	100	500	No H_2O_2
R + N. fowleri Lysate	33.2 ± 4.2	31.4 ± 5.3	31.4 ± 5.9	30.4 ± 4.0	37.0 ± 6.8	35.9 ± 6.4
R Alone	18.5 ± 0.6	13.0 ± 3.6	46.4 ± 5.2	96.6 ± 0.3	96.8 ± 0.2	

[a] ^{51}Cr post-labeling study; N. fowleri lysate and H_2O_2 pre-incubated 30 min and co-incubated 6 h with macrophages.

[b] Values were represented as mean % destruction of macrophages ± SE from 5 wells.

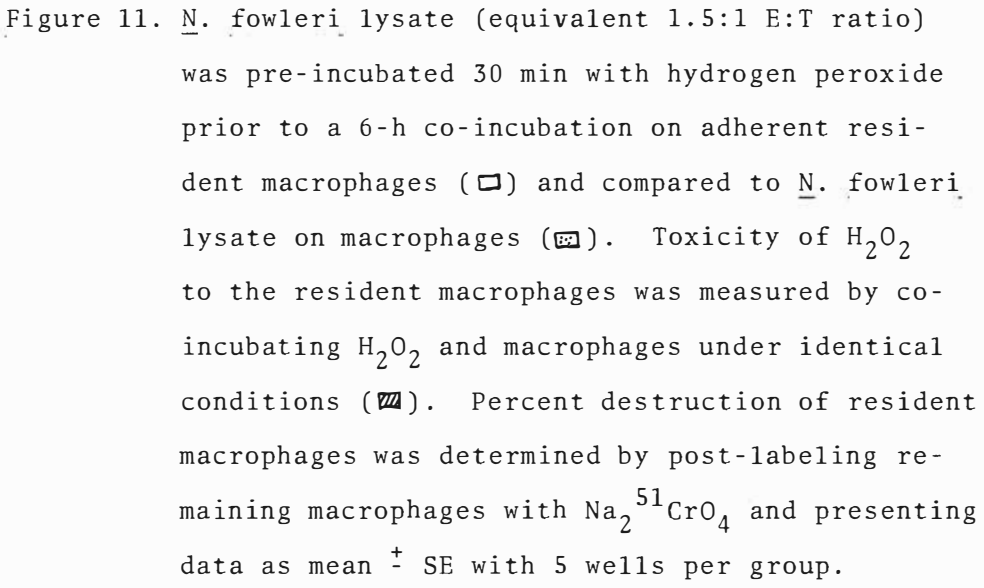


Figure 11. N. fowleri lysate (equivalent 1.5:1 E:T ratio) was pre-incubated 30 min with hydrogen peroxide prior to a 6-h co-incubation on adherent resident macrophages (□) and compared to N. fowleri lysate on macrophages (▨). Toxicity of H_2O_2 to the resident macrophages was measured by co-incubating H_2O_2 and macrophages under identical conditions (▩). Percent destruction of resident macrophages was determined by post-labeling remaining macrophages with $Na_2{}^{51}CrO_4$ and presenting data as mean $\pm$ SE with 5 wells per group.

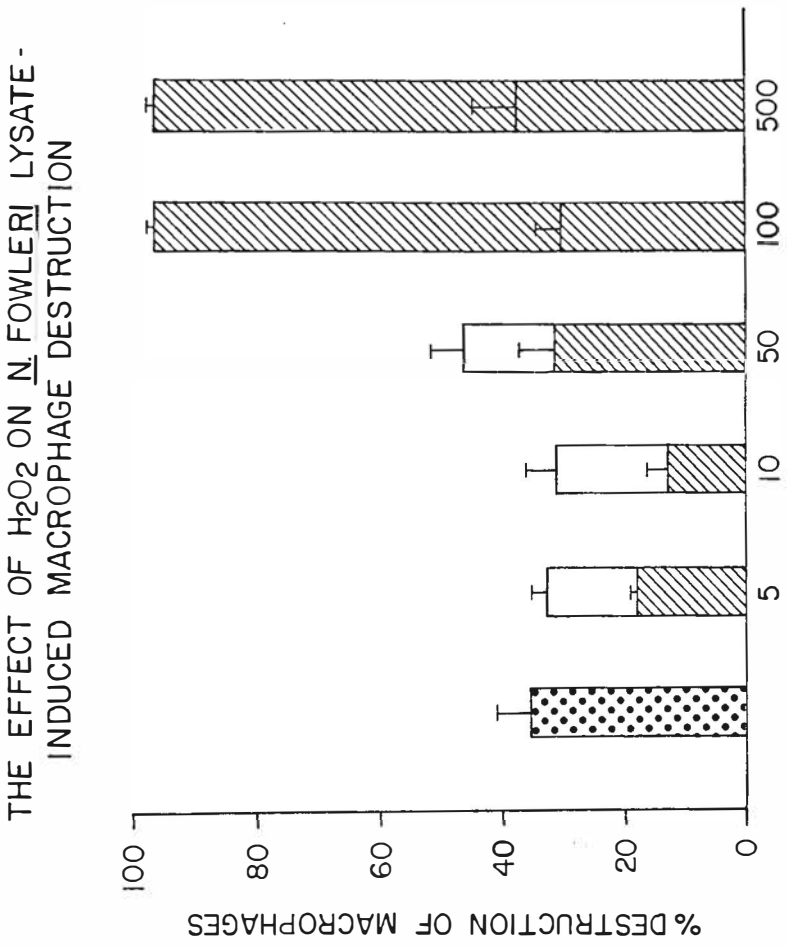

THE EFFECT OF H_2O_2 ON N. FOWLERI LYSATE-INDUCED MACROPHAGE DESTRUCTION
% DESTRUCTION OF MACROPHAGES
0
20
40
60
80
100
5
10
50
100
500
H_2O_2 (μM)

Table 11

The Effect of Leupeptin on Macrophage Destruction[a]

Macrophage Population	Leupeptin + N. fowleri Lysate[b]			Leupeptin Alone			N. fowleri Lysate Alone
	1500[c]	1000	500	1500	1000	500	
CP	30.4 ± 1.4	29.5 ± 1.2	31.4 ± 2.9	6.6 ± 1.2	9.0 ± 2.8	9.6 ± 1.7	23.4 ± 1.2
TG[d]	41.6 ± 3.5	42.6 ± 2.4	48.6 ± 1.2	7.6 ± 5.1	3.8 ± 2.3	2.6 ± 2.6	17.8 ± 2.4
R	57.8 ± 3.6	56.2 ± 6.5	45.4 ± 3.8	17.6 ± 2.4	10.6 ± 4.3	6.7 ± 2.4	46.0 ± 4.8

[a] ^{51}Cr post-labeling study; 30-min pre-incubation of leupeptin and N. fowleri lysate; 6-h co-incubation with macrophages.

[b] Values were reported as % destruction of macrophages ± SE from 5 wells.

[c] μg/ml.

[d] Leupeptin increased lysate-induced TG destruction, $p<0.01$.

Figure 12. Adherent macrophages were pre-incubated for 30 min with leupeptin prior to the addition of N. fowleri lysate (equivalent of a 1.5:1 E:T ratio) for 6 h (□) and compared to macrophage and N. fowleri lysate (▨). Macrophage toxicity due to leupeptin was taken into consideration (▧). Percent destruction of macrophages was assessed by post-labeling remaining macrophages with $Na_2{}^{51}CrO_4$ and reported as the mean ± SE with 5 wells per group.

THE EFFECT OF LEUPEPTIN ON MACROPHAGE DESTRUCTION

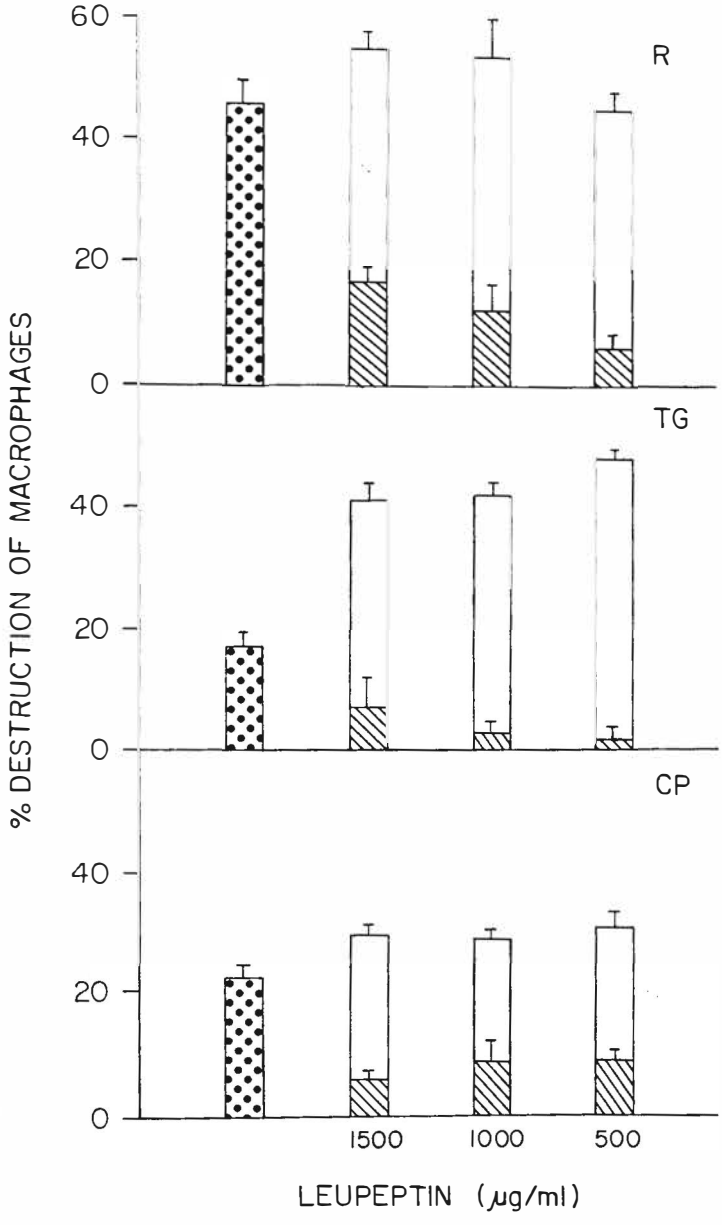


Table 12

The Effect of Lymphokines on N. fowleri Lysate-Induced Macrophage Destruction[a]

Macrophage Population	Lymphokine + N. fowleri Lysate[b]			Lymphokine Alone			N. fowleri Lysate Alone
	1	2	3	1	2	3	
R[c]	19.7 ± 5.0	27.5 ± 1.6	20.5 ± 2.9	7.5 ± 4.8	7.7 ± 4.3	9.2 ± 3.9	35.9 ± 2.8

[a] ^{51}Cr post-labeling study; lymphokine and macrophage pre-incubated 24 h; macrophage and N. fowleri lysate co-incubated 6 h.

[b] Values were presented as % destruction of macrophages ± SE from 4 wells.

[c] Lymphokines decreased destruction of macrophages, $p<0.01$.

Figure 13. Lymphokines obtained from the supernatants of hybridoma T cells were pre-incubated undiluted for 24 h on adherent resident macrophages prior to the addition of N. fowleri lysate (equivalent of 1.5:1 E:T ratio) for 6 h (□). These values were compared to the effect of lysate on macrophages (▨), and the effect of lymphokine on macrophages (▩). Percent destruction of macrophages was determined by post-labeling remaining macrophages with $Na_2{}^{51}CrO_4$ and results were expressed as mean ± SE with 4 wells per group.

THE EFFECT OF LYMPHOKINES ON N. FOWLERI LYSATE-INDUCED MACROPHAGE DESTRUCTION

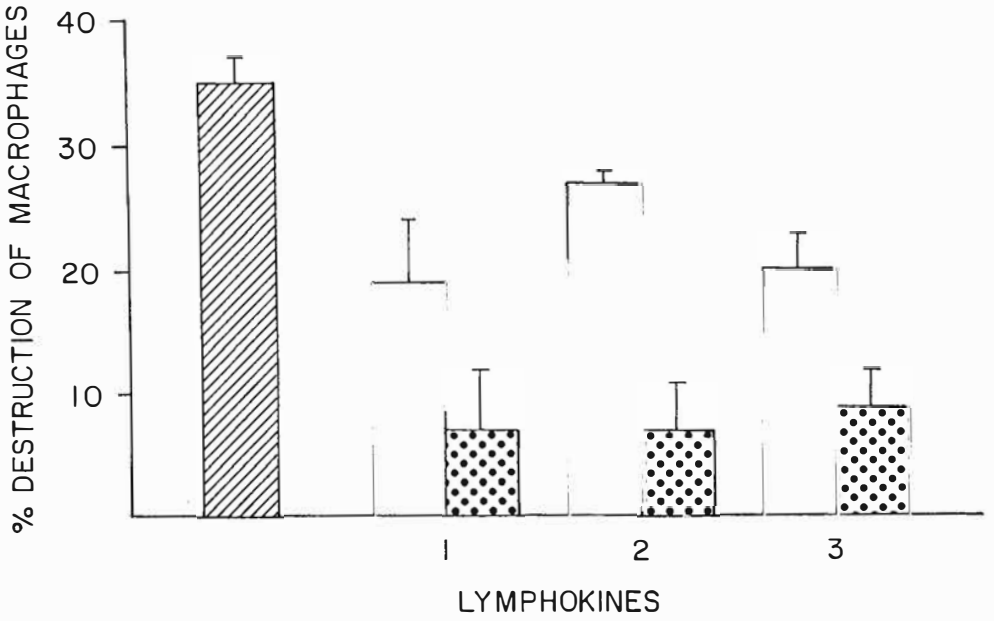


Table 13

Effect of Pyran Copolymer or Corynebacterium parvum on Lethality of Naegleria fowleri for Female B6C3F1 Mice

	Group	Dead/Total	% Mortality[b]	Days of Death
A.	1×10^6 amoebae/mouse i.n. - low virulent strain			
	Naive	2/6	33	15,27
	C. parvum[a]	4/6	67	10,14,14,15
	Pyran[a]	3/6	50	14,16,21
B.	5×10^5 amoebae/mouse i.n. - low virulent strain			
	Naive	2/6	33	10,15
	C. parvum	6/6	100	10,11,14,15,16,19
	Pyran	2/6	33	19,20
C.	5×10^4 amoebae/mouse i.n. - low virulent strain			
	Naive	2/6	33	16,16
	C. parvum	4/5	80	12,17,18,21
	Pyran	3/6	50	15,19,21

[a]Female B6C3F1 mice were treated with pyran copolymer (50 mg/kg, i.v.) or Corynebacterium parvum (70 mg/kg, i.v.) on day -4 and day -3 and then challenged with a low virulent strain of Naegleria fowleri on day 0.

[b]Mortality data were recorded daily for 21 d.

DISCUSSION

Macrophages are important effector cells in host resistance to numerous infections. The importance of activated macrophages in resistance to intracellular parasites has been investigated (39). Other than Schistosomes (9), Trichomonas (45), and Trypanosoma musculi (88), little information is available on the interaction of macrophage populations with extracellular parasites. In the present study, we have examined the differences among Corynebacterium parvum-activated, thioglycollate-elicited, and resident macrophages from $B_6C_3F_1$ mice utilizing two microcytotoxicity assays. Macrophages and N. fowleri have been interchangeably employed as effector and target cells. N. fowleri amoebae were the effectors when macrophage destruction was measured, whereas, macrophages were the effectors when release of amoeba label was measured.

Examination of mixed cultures by light microscopy revealed that C. parvum-activated macrophages were capable of killing the amoebae up to 48 h post-incubation. At a 20:1 macrophage-to-amoebae ratio, CP macrophages showed macrophage-induced lysis as well as phagocytosis of amoebae, suggesting that more than one mechanism is responsible for

killing. In contrast, TG-elicited and resident macrophages were highly susceptible to killing by N. fowleri amoebae.

To assess the ability of the macrophage to lyse N. fowleri, the amoebae were labeled with ^{3}H-uridine prior to co-culturing with adherent macrophage cultures. The amount of ^{3}H-uridine released by N. fowleri in the presence of macrophages was density and time dependent. This study clearly indicates that CP-activated macrophages kill N. fowleri amoebae effectively at a 10:1 E:T ratio in 24 h. Under identical conditions, TG and resident macrophages did not produce significant killing.

Macrophages activated in vivo with immunomodulators such as pyran and C. parvum have been shown to exhibit tumoricidal or microbiocidal activity (60). Both TG-elicited and normal resting macrophages lack enhanced anti-tumor and anti-microbial activity. The increased cytotoxic response of CP macrophages for N. fowleri may be due to release of amoebicidal factors from the macrophage upon contact with the amoebae. Tumor cell cytotoxicity mediated by activated macrophages requires cell-to-cell contact (3).

The cytotoxic activity of CP-activated macrophages against N. fowleri amoebae is lost after in vitro incubation for 24 h (31,47). This could explain the inability of CP macrophages to continue killing N. fowleri amoebae after prolonged incubation in vitro. Alternatively, the absence of continued CP macrophage-induced killing may be the

result of a reduction in macrophage numbers due to lytic substances from lysed N. fowleri amoebae. The inability to measure ^{3}H-uridine release from N. fowleri induced by CP macrophages after 48 h could be the result of macrophage phagocytosis. Pyran-activated macrophages appear to have a much lower killing capacity for N. fowleri amoebae than CP macrophages. This is in contrast to tumoricidal activity (57).

CP macrophages demonstrated a much higher capacity for mediating a cytolytic response against N. fowleri amoebae in the presence of anti-N. fowleri antibodies (in the absence of complement). N. fowleri specific antibody diluted 1:10 strongly agglutinates amoebae while antibody diluted 1:500 to 1:1000 immobilizes the amoebae. Since contact between macrophages and N. fowleri may be important, the addition of immobilizing amounts of antibody would ensure that the amoebae remained closely in contact with the macrophages. An alternative explanation for the increased killing of N. fowleri amoebae by CP macrophages in the presence of specific antibody would be antibody-dependent cell-mediated cytotoxicity (ADCC) (85). BCG-activated macrophages are more effective in mediating ADCC against tumor targets than macrophages elicited by TG (44). Koren et al. (44) have suggested that the augmentation of an ADCC response by BCG-activated macrophages may arise from a) alterations in the number or affinity of Fc

receptors on the activated macrophage and/or b) enhanced secretion of cytolytic effector molecules by the activated macrophage. In addition, engagement of Fc receptors on macrophages results in extensive secretion of oxygen metabolites including H_2O_2 (60). Nathan et al. (60) proposed that H_2O_2 plays a role in mediating lysis of antibody-coated tumor targets by activated macrophages. Furthermore, Johnson et al. (38) demonstrated that increased binding of tumor targets to activated macrophages augments the secretion of cytolytic factor (CF). Recent evidence indicates that CF and H_2O_2 can act synergistically in producing lysis of neoplastic targets (1).

The role of phagocytosis following the addition of amoebae-specific antibody to N. fowleri and macrophages is unclear. Although coating of antibody on the amoeba surface followed by phagocytosis via Fc binding is possible, measurement of such a mechanism is difficult using the release assay. The reduced cytolytic activity observed for TG macrophages in the presence of specific antibody might be explained by increased phagocytosis which would make less targets available for lysis; however, preliminary light microscopic observations cannot presently confirm this assumption. In contrast, light microscopic observations demonstrated no increase in antibody-independent TG macrophage phagocytosis. This would eliminate phagocytosis as an explanation for reduced release of label from N.

fowleri as compared to release of label induced by CP macrophages in the absence of antibody.

The present study suggests that specific antibody not only immobilized the amoebae but also increased contact of the amoebae to activated macrophages and may have stimulated release of both oxygen metabolites and cytolytic factor.

The inability to measure an adequate cytolytic response of activated macrophages after 48 h prompted further studies on the fate of the activated macrophage following loss of activation.

The ^{51}Cr post-labeling assay demonstrated that destruction of macrophages by N. fowleri was also concentration and time dependent. Greater destruction of resident macrophage populations may correspond to absence of cellular defense mechanisms. The ability of N. fowleri to readily destroy resident macrophages probably resembles the ability of the amoebae to destroy a variety of cultured mammalian cells (48). In contrast, CP and TG macrophages were capable of resisting considerable destruction. This TG macrophage resistance was not observed using light microscopy. CP macrophages possess increased amounts and secretion of lytic substances such as plasminogen activator, collagenase, and elastase which may reduce the number of viable amoebae (78). In addition, inhibitory agents may be released which are capable of neutralizing N. fowleri lytic factors. The increase in susceptibility of CP macrophages

to N. fowleri after 48 h of incubation is consistent with the loss of activation. Although TG macrophages were not capable of an increased cytolytic response they too were capable of resisting destruction by amoebae for several hours.

N. fowleri destroys a variety of mammalian cells in culture. The proposed mechanism is thought to be a combination of trogocytosis (repeated nibbling) and a release of cytotoxic substances (12,15,89). The resistance mechanism to N. fowleri destruction observed for TG macrophages could be attributed to 1) the membrane of TG macrophage may not favor binding of N. fowleri amoebae to allow immediate destruction; 2) the microenvironment may alter the amoebae in such a way as to prevent it from feeding; 3) the size of the TG macrophage is larger; therefore, it would take longer to destroy.

The differential destruction of TG, CP, and resident macrophages by N. fowleri lysate is possibly due to a distinctive macrophage membrane sensitivity or the ability to neutralize N. fowleri lysate lytic factors.

Using lysates and conditioned media of N. fowleri results indicated N. fowleri previously exposed to macrophages may not be capable of producing or secreting more lytic substances with the capacity to destroy macrophages than N. fowleri in the absence of priming macrophages. This would suggest that N. fowleri lytic elements are prepackaged and not inducible by macrophages.

Normal peritoneal macrophages and macrophages elicited with thioglycollate both release very little H_2O_2 in response to a triggering agent and are non-cytotoxic (61). Hydrogen peroxide or other oxygen intermediates might be responsible for CP macrophages' increased resistance to N. fowleri. N. fowleri amoebae or lysates are likely to possess mechanisms for disposal of H_2O_2. Pathogenic N. fowleri are known to contain abundant catalase (94). In an attempt to increase the susceptibility of CP macrophages to N. fowleri lysate, exogenous catalase was added. However, no significant difference in destruction of activated macrophages was noted. A significant increase in TG macrophage destruction detected may be explained by: 1) the catalase may change the membrane of the TG macrophage altering adherence or 2) what little H_2O_2 is present in the TG macrophage may be destroyed and leave the macrophage vulnerable to attack. Presumably, the CP and TG macrophage would lose comparable amounts of H_2O_2; however, cytolytic factor which is absent in the TG macrophage would still be active in the CP macrophage. According to Nathan and Root (61), macrophages produce H_2O_2 upon activation and release is dependent upon a triggering stimulus. CP macrophages show apparent need for a lysate or cellular stimulus to release enough H_2O_2 to be sufficiently disarmed by the addition of catalase. An increase in resident macrophage destruction cannot be confirmed due to the limitations of

the assay. The assay can measure a maximum of 100% macrophage destruction, but theoretically lysate and catalase may have attained values greater than 100%. These results do not dismiss H_2O_2 as a defense mechanism but suggest that H_2O_2 alone is probably not responsible for CP resistance to N. fowleri lysate.

An alternative procedure for determining the role of H_2O_2 in macrophage defense against N. fowleri lysate was to add exogenous H_2O_2 to the lysate and subsequently measure lysate-induced resident macrophage destruction. Nathan et al. (58) demonstrated the release of H_2O_2 at approximately 0.5 nmol/5 min for 10^6 BCG-induced peritoneal cells. Using this estimate and making the assumption that CP macrophages might show similar release of H_2O_2, the amounts initially employed exceeded the expected release by greater than 10-fold (30 min exposure of N. fowleri lysate to H_2O_2 theoretically derived from 2×10^5 macrophages). In spite of excess H_2O_2, lysate-induced macrophage damage was unchanged, suggesting that these H_2O_2 concentrations were unable to alter the killing ability of N. fowleri lysate for the most susceptible target in this assay, the resident macrophage. The H_2O_2 did not demonstrate any detrimental effects on N. fowleri cells at the same concentrations. These results further support the assertion that H_2O_2 cannot be the sole mechanism of CP macrophage resistance to N. fowleri lysate, and more than one factor may be involved.

Neutral proteases are among the most important secretory substances of macrophages (60). Leupeptin, an inhibitor of proteases containing cysteine and serine groups, was utilized in an attempt to block a critical killing mechanism of macrophages (66).

N. fowleri lysate-induced CP macrophage destruction demonstrated no change when macrophages were previously treated with leupeptin. Two explanations for leupeptin's ineffectiveness are 1) leupeptin may be an inappropriate inhibitor for cytolytic factor (protease) and 2) there may have been little to no release of proteases prior to the addition of N. fowleri lysate. Adams demonstrated inhibition of cytolysis of neoplastic targets due to cytolytic factor by activated macrophages using bovine pancreatic trypsin inhibitor and diisopropylfluorophosphate (2). The absence of a change in resident macrophage destruction might be attributed to their lack of cytolytic factor as well as other proteases. On the other hand, TG macrophage destruction showed a pronounced increase in destruction when macrophages were pre-incubated with leupeptin. Cytolytic factor is absent from TG macrophages, but other proteases are present. Therefore, this increase might be accounted for by 1) a TG macrophage membrane sensitivity to leupeptin or 2) the lack of a combination of neutralizing substances following inhibition of one or more proteases. It seems that leupeptin is incapable of abolishing CP

macrophage resistance to N. fowleri lysate. The concept of two or more defense mechanisms involved in these interactions remains a reasonable hypothesis. It is evident from the catalase and leupeptin studies that these macrophage populations are indeed different and respond uniquely to natural stimuli as well as exogenous agents.

Resident macrophages were exposed to lymphokines to achieve macrophage activation. An increase in time-related resistance to N. fowleri lysate was demonstrated. Lymphokine mediators are the only agents capable of inducing the full spectrum of activity associated with macrophage activation (60). Adams (1) demonstrated that two separate lymphokines induced anti-leishmanial and anti-ricketsial activity while a third lymphokine achieved the above in addition to anti-tumor and anti-schistosomal activity. Resident macrophage destruction by N. fowleri lysate decreased following lymphokine treatment for 24 h. Whether these resident macrophages attained a true state of activation or one similar to that of CP macrophages has not been established. Shultz (79) described a sequence of steps in macrophage activation for tumor cytotoxicity consisting of two basic signals. The first signal presented by the lymphokine primes the macrophage, and a second triggering signal or interaction with a microenvironmental signal provided cytolytic activity. In the present study, the second signal is probably a component of

the N. fowleri lysate that results in a fully activated and secreting macrophage.

To determine whether previously reported in vitro findings of macrophage and N. fowleri amoeba interactions in this study were related to that found in vivo, mice were exposed to macrophage activating agents and challenged with N. fowleri amoebae.

In vivo activation of macrophages by i.v. and i.p. injections of C. parvum and subsequent challenge intranasally with N. fowleri demonstrated no increase of host survival to N. fowleri infection. Although there was no increase in the survival rate, a decrease in amoebae in the lung could represent an increase in host resistance. This decrease in the number of amoebae could be the result of activated alveolar macrophage-induced killing or the mobilization of the amoebae away from the lung and towards the brain. N. fowleri appeared to reach the brain quicker when given C. parvum or pyran, suggesting that those reaching the lung traveled away from the activated macrophage site upon recognition. Whether these immunomodulating agents are capable of altering the blood-brain barrier has not been established, but may explain why controls had a lower level of survival. In addition, C. parvum has been shown to depress lymphocyte function, which would be critical to host resistance to N. fowleri (14). These results suggest also that close contact may be important for macrophage-induced N. fowleri killing to occur.

The results of the present study demonstrate that the macrophage populations are indeed unique. Should the TG and CP macrophage populations overlap, this might explain similar resistance to N. fowleri lysate; whereas, if the resident and TG macrophages share common cell subpopulations, the reason for similar low levels of N. fowleri destruction may be elucidated.

Regardless of the nature of these three macrophage populations, CP-activated macrophages have been shown to kill N. fowleri amoebae. In contrast, TG and resident macrophages lack this ability to kill. Mechanisms for killing may include phagocytosis, lysis, and peroxidative attack, but it is difficult at this time to select one mechanism responsible for macrophage-induced killing of N. fowleri. This study has provided partial characterization of N. fowleri-macrophage interactions and will serve as a solid foundation upon which future investigations will be based.

SUMMARY

In the present study we have demonstrated that activated macrophages can kill N. fowleri optimally at a 10:1 effector-to-target cell ratio, 24 h following co-culture. It was apparent from light and transmission electron microscopic observations, and post-labeling studies that N. fowleri display their own killing capabilities through phagocytosis and a decreased number of macrophages. The CP-activated macrophage and N. fowleri have played effector cell as well as target cell roles. If the macrophage could be continuously activated, the surviving cell population might feasibly be the macrophages. In the systems utilized in this study, N. fowleri amoebae ultimately dominate.

The different macrophage populations kill differentially and can be killed at different rates. The use of lysates has simplified the interaction and demonstrates that the differential killing does not originate from an N. fowleri cellular property, but rather the differential time-related susceptibility of CP, TG, and resident macrophages is due to membrane sensitivity, neutralization capabilities, or some other property unique to that particular macrophage population. The active property in N. fowleri lysate has not been determined; however, if purified could

be a readily obtainable substance useful in distinguishing between an activated and resident macrophage. In addition, it may distinguish between two macrophage populations activated by different substances (i.e., BCG, C. parvum, pyran).

The second part of this study was directed at investigating the possible mechanisms each macrophage population utilized to deal with N. fowleri-induced destruction. Antibody, leupeptin, or catalase interacted with each macrophage population differently. Leupeptin did abolish some TG macrophage activity. Hydrogen peroxide alone showed little evidence of playing a role in CP macrophage defense against N. fowleri; however, synergistic reactions with other lytic substances must not be dismissed and probably provides a better explanation for the ability of a CP macrophage to kill and resist destruction. The presence of antibody illustrated the ability of the macrophages to take advantage of the presumably immobilized N. fowleri cells.

Information gained from this study has provided insight into the complexity of the interactions between N. fowleri amoebae and murine peritoneal macrophages. Although CP macrophage-induced N. fowleri cytotoxicity has been established, a mechanism responsible for this killing remains to be defined. In contrast, CP, TG, and resident macrophages display distinctive spectrums of activity following exposure to N. fowleri amoebae and N. fowleri lysate. These

differential susceptibilities may also be a key to the undefined macrophage-amoeba relationship.

Further investigation is necessary to determine the exact nature and quantity of substances released from the amoebae as well as macrophages. The results presented in this study provide answers to some questions which should serve as a basis for future investigations.

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