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The Arginine-Dependent Cytolytic Mechanism Plays a Role in Destruction of *Naegleria fowleri* Amoebae by Activated Macrophages

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Mouse peritoneal macrophages activated by different immunomodulators (*Mycobacterium bovis* bacillus Calmette-Guérin or *Propionibacterium acnes*) destroy *Naegleria fowleri* amoebae by a contact-dependent process and by soluble cytolytic molecules secreted by macrophages in response to lipopolysaccharide. The goal of this study was to determine whether the arginine-dependent cytolytic mechanism which results in the production of nitric oxide from arginine by activated macrophages destroys the amoebae. Amoebicidal activity of activated macrophages was determined by coculturing macrophages with *N. fowleri* amoebae radiolabeled with ^{3}H-uridine. The percent specific release of radiolabel was used as an index of cytolysis of the amoebae. The inhibitors N^G-monomethyl-L-arginine and arginase were used to determine whether the arginine pathway was a major effector mechanism responsible for amoebicidal activity of activated macrophages. Both the arginine analog N^G-monomethyl-L-arginine and arginase, which breaks down arginine, decreased macrophage amoebicidal activity. Addition of arginine to arginine-free medium restores amoebicidal activity to activated macrophage cultures. These results demonstrate that the arginine pathway is an important mechanism for the destruction of susceptible *N. fowleri* amoebae.

Activated macrophages are efficient cytotoxic cells as well as the producers of over 100 secretory molecules (32). Effector functions of activated macrophages are tightly regulated by a complex network of cytokines. Mouse peritoneal macrophages may be activated in vivo by infection with microorganisms or in vitro by exposure to lymphokines. Macrophages activated for cytolysis of one target cell are not necessarily cytolytic for other target cells (25, 31, 38). It is unclear whether individual macrophage populations are capable of eliciting several cytolytic mechanisms which act on the appropriate target cell or whether subpopulations of macrophages exhibit cytolytic mechanisms effective against specific target cells.

Current studies have focused on identifying the effector molecules that mediate the direct, nonspecific cytotoxicity of activated macrophages. Reactive oxygen intermediates produced during the respiratory burst have been implicated as one of the major mechanisms of macrophage microbicidal effector functions (9, 34). Also, nonoxidative mediators of cytolysis are produced by activated macrophages, including tumor necrosis factor alpha (TNF-α), interleukin-1 alone or in combination with TNF-α, and cytolytic protease (1, 2, 20, 23, 24, 33). TNF-α effects tumoricidal activity but has no direct cytolytic activity for a number of parasitic protozoa (5, 8). More recently, the cytotoxic capacity of nitric oxide produced by nitrogen oxidation of L-arginine has been demonstrated for parasites (3, 16, 22, 26, 27, 29, 37). Macrophage cytolytic activity correlates with the production of L-arginine-derived nitrite (NO_2^-), which is a stable degradation product of the freely diffusible, short-lived, and highly toxic effector molecule nitric oxide (13, 17, 18, 35). The toxicity of nitric oxide is a result of the inhibition of selected iron-dependent enzymatic pathways involved in cellular respiration, energy production, and DNA synthesis of target cells (10, 18, 21, 35). Recently, it has been demonstrated that TNF-α may indirectly effect cytolysis of parasitic organisms by serving as an inducer of the L-arginine-dependent cytolytic pathway (15, 26). TNF-α has also been shown to synergize with gamma interferon in the presence of lipopolysaccharide (LPS) and induce the production of nitric oxide in the target cells themselves (4).

The purpose of the present study was to determine whether nitric oxide produced by activated macrophages is an effective mediator in the cytolysis of *Naegleria fowleri* amoebae. Activated macrophages as well as soluble factors released from macrophages effect direct cytolysis of *N. fowleri* (6, 7). In this study, the arginine analog N^G-monomethyl-L-arginine (N^GMMA) and the enzyme arginase were used to inhibit the production of nitric oxide by activated macrophages. The results demonstrate that amoebicidal activity of activated macrophages is decreased by arginine pathway inhibitors. The L-arginine-dependent cytolytic pathway appears to be responsible for the majority of the contact-dependent amoebicidal activity of activated macrophages; however, additional macrophage effector molecules play a role in macrophage-mediated killing of *N. fowleri* amoebae.

MATERIALS AND METHODS

Animals. Eight- to ten-week-old $B_6C_3F_1$ (C57BL/6 × C3H) female mice (Frederick Cancer Research Center) were used as the source of peritoneal exudate cells.

Macrophages. Resident peritoneal macrophages were obtained from untreated mice. Peritoneal macrophages were elicited with thioglycolate (TG) by injecting 10% TG into the peritoneal cavity 3 days prior to harvesting cells. TG-elicited macrophages were used for scanning electron microscopy (SEM) studies. *Mycobacterium bovis* bacillus Calmette-

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Guérin (BCG)-activated macrophages were obtained from mice inoculated intraperitoneally with 5×10^6 viable bacillus organisms 14 and 7 days prior to harvest. *Propionibacterium acnes* (formerly *Corynebacterium parvum*)-activated macrophages were obtained from mice injected intraperitoneally with 0.2 ml (70 mg/kg of body weight) of a formalin-fixed preparation of *P. acnes* organisms (Burroughs-Wellcome, Research Triangle Park, N.C.) 7 days prior to the harvest of peritoneal exudate cells.

Peritoneal exudate cells were obtained as described previously (6). Briefly, mouse peritoneal cavities were lavaged with cold Hanks' balanced salt solution (GIBCO, Grand Island, N.Y.) supplemented with antibiotics. Cells were depleted of erythrocytes with distilled H_2O, washed three times with Hanks' balanced salt solution, and suspended in RPMI 1640 medium (GIBCO) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 1% glutamine, 1% minimum essential medium, vitamins, 1% nonessential amino acids, 1.5% sodium bicarbonate (7.5% solution), 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, Va.). Macrophages were counted on a hemacytometer and plated in sterile, 96-well, round-bottom microtiter plates at a concentration of 3×10^5 macrophages per well. Plates containing macrophages were centrifuged at $500 \times g$ for 2 min and incubated at 37°C in the presence of 5% CO_2 for 2 h to allow for cell adherence. Following the incubation period, the medium was removed and the cultures were washed with warm RPMI 1640 medium without fetal calf serum to remove nonadherent cells. Cultures consisted of macrophage monolayers, as determined by nonspecific esterase staining (30). For assays requiring arginine-free medium, macrophages were washed twice with warm RPMI 1640 medium lacking fetal calf serum and arginine.

Amoebae. *N. fowleri* LEE (ATCC 30894), originally isolated in 1968 from the brain of a patient with fatal primary amoebic meningoencephalitis, was grown axenically in Nelson medium in tissue culture flasks at 37°C (11).

SEM. Cocultures of macrophages and amoebae were prepared for scanning electron microscopy (SEM) as described previously (28). Briefly, *N. fowleri* amoebae were added to coverslips containing TG-elicited, BCG- or *P. acnes*-activated macrophages. Coverslip cultures were incubated at 37°C for 24 h, fixed for 1 h with warm 2% glutaraldehyde in 0.1 M sodium-cacodylate-hydrochloride buffer (pH 7.2), and postfixed in 1% OsO_4 containing 0.15 M cacodylate buffer. After a rinse in 0.15 M cacodylate buffer, cultures were dehydrated in a graded series of ethanol, subjected to critical-point drying, and sputter coated with gold (28). Coverslips were examined in a Hitachi HS-500 scanning electron microscope operating at an accelerating voltage of 20 kV.

Amoeba labeling. *N. fowleri* amoebae were radiolabeled with 10 μCi of [^{3}H]uridine (New England Nuclear Corp., Boston, Mass.) per ml as described previously (6). Briefly, amoebae were radiolabeled for 24 h in a 75-cm^2 tissue culture flask. The amoebae were washed, counted, and diluted in RPMI 1640 medium or arginine-free RPMI 1640 medium to obtain a concentration of 3×10^5 amoebae per ml.

Reagents. N^GMMA, an analog of L-arginine, and arginase (2,500 U), an enzyme which eliminates extracellular arginine, were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Measurement of NO_2^- production. Culture fluids were assayed for NO_2^- with the Griess reagent (14). Briefly, macrophage-conditioned medium (MφCM) was prepared by plating the macrophages in the absence or presence of N^GMMA. The macrophage cultures were triggered with 10 ng of LPS per ml and incubated for 20 h to allow for the production and secretion of cytolytic factors into the MφCM. After 20 h of incubation, 50 μl of MφCM was incubated with 50 μl of 1% sulfanilamide and 50 μl of 1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4 (Sigma) at room temperature for 5 min. The optical density at 570 nm was measured. NO_2^- was quantified by comparison to $Na(NO_2)$ as a standard.

Cytotoxicity assays. Cytotoxicity assays were performed in sterile, U-bottom, 96-well microtiter plates (Costar, Cambridge, Mass.) (6). Plates contained monolayers of macrophages in RPMI 1640 medium with or without arginine. Various concentrations of the arginine analog N^GMMA, arginase, or arginine were added to the macrophage monolayers. Radiolabeled amoebae were added at a macrophage effector-to-target cell ratio of 10:1 (3×10^4 targets per well). Microtiter plates containing the cocultures of macrophages and amoebae were centrifuged at $500 \times g$ for 2 min and incubated at 37°C in the presence of 5% CO_2 for 20 h. Following the incubation period, the plates were centrifuged at $1{,}000 \times g$ for 10 min. Supernatant fluid was harvested individually, and the radioactivity present was determined by liquid scintillation spectrometry. Total counts of [^{3}H]uridine-labeled amoebae were determined by incubating amoebae with 1% sodium dodecyl sulfate. The percent specific release (percent amoebicidal activity) of radiolabel correlating to cytolysis of amoebae was calculated according to the following formula: % specific release = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

Statistical methods. Statistical analysis was performed on all data by using Student's *t* test. A 95% confidence level was chosen for all experiments to determine the statistical significance for differences between paired values of percent specific release.

RESULTS

SEM of macrophage-amoeba cultures. SEM was performed to define the interaction of *N. fowleri* amoebae with different macrophage populations (Fig. 1). Amoebae were capable of destroying resident and inflammatory macrophages when cocultured in vitro (6). In the presence of TG-elicited macrophages, amoebae remained amoeboid and used food cups to ingest the macrophages (Fig. 1A). In contrast, when they were cocultured with activated macrophages, *Naegleria* organisms were no longer amoeboid. Rounded amoebae were seen bound to the *P. acnes*- and BCG-activated macrophages. Although both populations of activated macrophages were capable of destroying the amoebae, the morphological appearances of the amoebae were different. Cellular debris from injured amoebae was apparent in cocultures of *P. acnes*-activated macrophages (Fig. 1B). Injured amoebae were observed attached to the surface of BCG-activated macrophages (Fig. 1C).

Effect of N^GMMA on activated-macrophage amoebicidal activity. Radiolabeled *N. fowleri* was added to monolayers of activated macrophages at a macrophage effector-to-target cell ratio of 10:1. To determine whether macrophage cytolytic activity was due to the production of nitric oxide, various doses of the arginine analog N^GMMA were added to cocultures of macrophages and amoebae to inhibit the metabolism of arginine during the 20-h cytotoxicity assays.

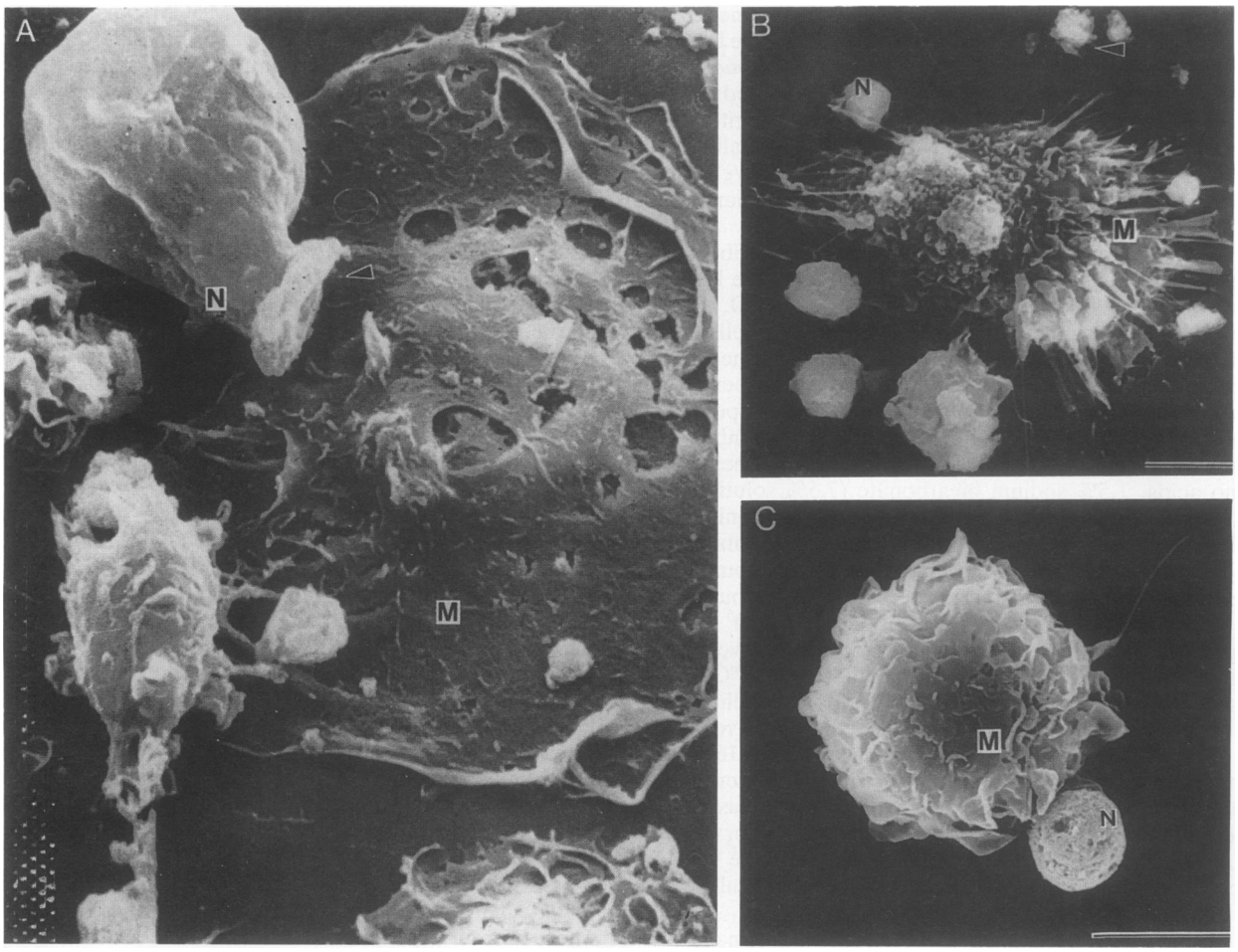

FIG. 1. SEM of macrophage-amoeba cultures. *N. fowleri* amoebae (N) were cultured with macrophages (M) for 24 h at 37°C. (A) *N. fowleri* amoebae use food cups (arrowhead) to ingest TG-elicited macrophages. (B) Cellular debris (arrowhead) from injured amoebae was present in cocultures of *P. acnes*-activated macrophages. (C) Injured amoebae were attached to the surface of BCG-activated macrophages. Bars, 5 μm.

Peritoneal macrophages activated in vivo with BCG or *P. acnes* mediate direct cytolysis of *N. fowleri* amoebae. N^GMMA decreased amoebicidal activity in a dose-related manner, but the arginine analog did not completely inhibit amoebicidal activity at the doses tested (Fig. 2).

NO_2^- production. The cytolytic molecule nitric oxide is unstable and rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-). Nitrite concentration can be measured in MϕCM and used as an indicator of nitric oxide production (17). Therefore, in order to determine whether there was a correlation between NO_2^- production and the cytolytic activity of activated macrophages, the concentration of nitrite produced by activated macrophages was determined in the absence or presence of N^GMMA. When N^GMMA is absent, BCG-activated macrophages are cytolytic for *N. fowleri* amoebae and nitrite (38.0 μM) can be detected in the MϕCM produced by these macrophages. However, in the presence of 12.5 and 50 μM N^GMMA, amoebicidal activity is reduced or eliminated and reduced amounts of nitrite (3.3 and 2.6 μM, respectively) are detected in the MϕCM.

Effect of depletion of arginine by arginase on activated macrophage amoebicidal activity. To confirm that amoebicidal activity of activated macrophages was due in part to nitric oxide produced from the metabolism of L-arginine, arginase was added to the cocultures to deplete L-arginine from the culture medium during the cytotoxicity assays. Figure 3 demonstrates that the addition of arginase to the culture system reduces or inhibits activated-macrophage amoebicidal activity.

Amoebicidal activity of activated macrophages in arginine-free medium. To determine whether activated macrophages are capable of exhibiting amoebicidal activity in the absence of arginine, macrophages were washed with arginine-free medium prior to the addition of amoebae in arginine-free medium. Various concentrations of arginine were added back to the cocultures during the cytotoxicity assays. Figure 4 demonstrates that activated macrophages exhibited decreased amoebicidal activity in the absence of arginine. However, the addition of the optimal concentration of arginine to the medium increases the cytolytic activity of the macrophages at least twofold.

DISCUSSION

Macrophages activated in vivo with BCG or *P. acnes* mediate cytolysis of a number of protozoa, other microorganisms, and tumor cells in vitro (6, 7, 12, 31). We have shown previously using *N. fowleri* amoebae and L929 tumor

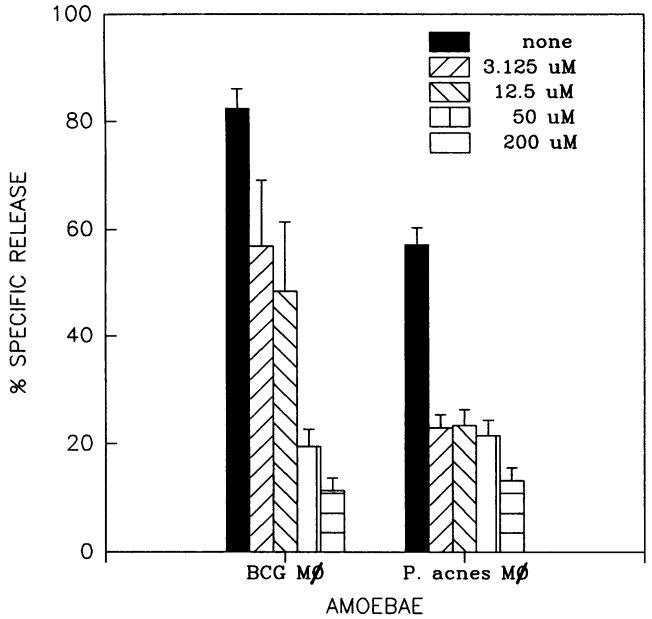

FIG. 2. Effect of N^GMMA on activated-macrophage amoebicidal activity. Bars represent percent specific release of radiolabel from amoebae in the absence or presence of N^GMMA. N^GMMA decreased but did not eliminate amoebicidal activity by activated macrophages.

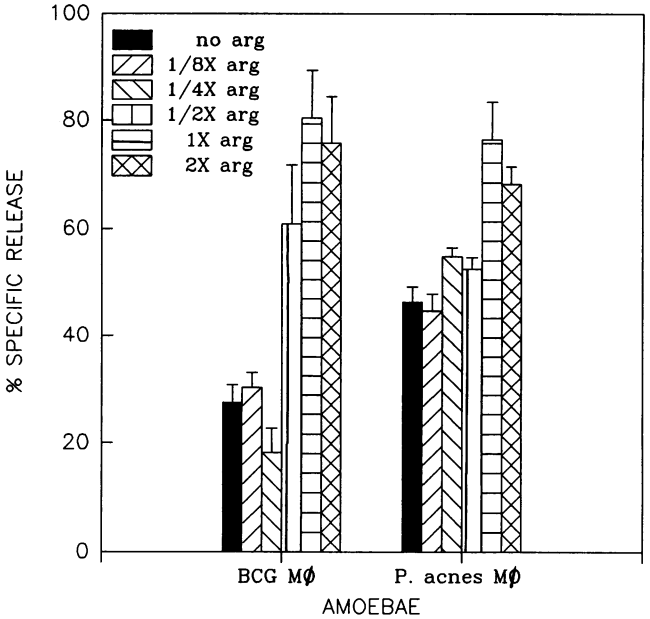

FIG. 4. Amoebicidal activity of activated macrophages in arginine-free medium. Bars represent percent specific release of radiolabel from amoebae in the absence or presence of various fractions of the optimal concentration of arginine. In the presence of the optimal concentration of arginine, activated macrophages exhibited an approximately twofold increase in amoebicidal activity.

cells as targets that different macrophage cytolytic mechanisms are responsible for amoebicidal and tumoricidal activities (12). Our studies confirmed that TNF-α is the major tumoricidal factor produced by activated macrophages for certain tumor cells but TNF-α alone has no direct effect on cytostasis nor cytolysis of *N. fowleri* amoebae. Evidence for the existence of separate tumoricidal and amoebicidal entities includes the following: recombinant TNF-α or recombinant interleukin-1α or interleukin-1β alone did not elicit amoebicidal activity, and preparative electrofocusing of BCG-activated MϕCM resulted in acidic fractions which contain tumoricidal activity, whereas fractions with a basic isoelectric point contained peak amoebicidal activity (12).

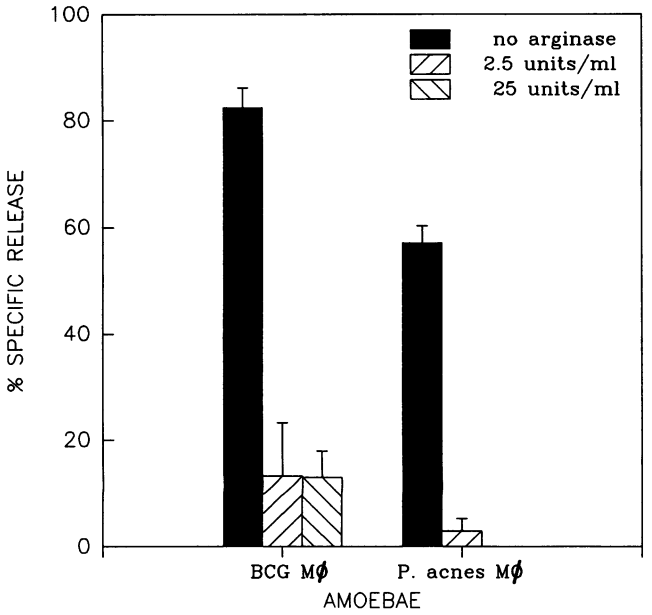

FIG. 3. Effect of depletion of arginine by arginase on activated-macrophage amoebicidal activity. Bars represent percent specific release of radiolabel from amoebae in the absence or presence of arginase. Arginase decreased or completely eliminated amoebicidal activity by activated macrophages.

The cytolytic molecules that activated macrophages use to destroy pathogens are not clearly defined. Nitric oxide produced by nitrogen oxidation of L-arginine reportedly plays an important role as a cytolytic effector molecule of activated macrophages (9, 10, 13, 16–19, 35). Resident tissue macrophages produce very little nitric oxide de novo; however, activated macrophages produce high levels of nitric oxide (13).

In the present study, the role of the L-arginine-dependent cytolytic pathway in activated macrophages was investigated as the source of amoebicidal activity. Cytotoxicity assays revealed that the arginine analog N^GMMA, which acts as a selective inhibitor of L-arginine-dependent nitrogen oxidation, decreased or inhibited the macrophage cytolytic activity for *N. fowleri* amoebae.

Although the pathway for nitrogen oxidation of L-arginine is not fully elucidated, it is known that nitric oxide, the cytolytic effector molecule, is a short-lived intermediate product of the pathway (13, 17, 18, 35). Nitric oxide is quickly degraded into nitrite (NO_2^-) and nitrate (NO_3^-). NO_2^- can be detected in the culture medium and used as a measurement of nitrogen oxidation of L-arginine and as a quantitative index of macrophage activation and function (13, 16, 35). The amoebicidal activity of activated macrophages correlates with an increase in NO_2^- concentration in medium harvested from activated macrophage cultures.

To determine whether nitric oxide is cytolytic for *N. fowleri*, cytotoxicity assays were performed in the absence of extracellular arginine. Exogenously added arginase depletes the culture medium of arginine and inhibits macrophage-mediated cytolysis since macrophages require arginine to produce effector molecules. Thus, arginase was added to the cocultures of activated macrophages and *N. fowleri* amoebae to eliminate arginine. Removal of arginine from the macrophage culture system with arginase results in a significant decrease in amoebicidal activity. In addition, macrophages and radiolabeled amoebae were cocultured in arginine-free medium. In the absence of extracellular arginine, activated macrophages exhibited reduced amoebicidal activity. Addition of arginine significantly increases macrophage amoebicidal activity, indicating the importance of arginine in macrophage-mediated killing of *N. fowleri* amoebae. Recently, it has been demonstrated also that the arginine-dependent cytolytic pathway is an important mechanism for the destruction of the enteric parasite *Entamoeba histolytica* (27).

The results of this investigation indicate that the L-arginine pathway plays a major role in contact-dependent macrophage amoebicidal activity. However, our studies do not rule out the possibility that other cytolytic mechanisms are important or act indirectly, since activated macrophages exhibit amoebicidal activity in arginine-free medium. In the absence of arginine, other cytolytic mechanisms contribute to macrophage amoebicidal activity.

Cytolytic activity of activated macrophages is distinct for different target cells (12, 25, 31). Studies using two-dimensional gel electrophoretic analysis of macrophage cultures activated by different immunomodulators demonstrate unique protein profiles from macrophage populations depending on the activation process (36). In this context, our SEM studies suggest that macrophages activated in vivo with BCG or *P. acnes* use different cytolytic mechanisms in vitro to lyse *N. fowleri* amoebae. The morphological appearance of injured amoebae in contact with activated macrophages differs depending on the immunomodulator used to activate the macrophages in vivo. Cellular debris from lysed amoebae was visible when the amoebae were cocultured with *P. acnes*-activated macrophages, and injured amoebae were observed attached to BCG-activated macrophages. In addition, we have shown previously that antiserum to TNF-α decreases amoebicidal activity of conditioned culture medium from BCG-activated macrophages but anti-TNF-α has no effect on amoebicidal activity from *P. acnes*-activated MϕCM. Furthermore, protease inhibitors decrease amoebicidal activity present in *P. acnes*-activated MϕCM but have no effect on amoebicidal activity present in BCG-activated MϕCM (12). BCG-activated macrophages appear to use a combination of cytolytic molecules to lyse *N. fowleri* amoebae, including a basic protein and nitric oxide. TNF-α or an immunologically related molecule may act in an indirect manner. *P. acnes*-activated macrophages do not appear to use TNF-α or a related cytotoxin in a direct or indirect manner but rather appear to use a combination of nitric oxide and a cytolytic molecule susceptible to protease inhibitors.

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