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Research Article

Capable effector mechanisms in the human immune response against the cytolytic, protozoan parasite Entamoeba histolytica have not been described. To identify a competent human effector cell, we studied the in vitro interactions of normal human polymorphonuclear neutrophils, peripheral blood mononuclear cells (PBMC), monocytes (MC), and MC-derived macrophages with virulent axenic amebae (strain HMI-IMSS). Amebae killed neutrophils, PBMC, MC, and MC-derived macrophages (P less than 0.001), without loss of parasite viability. The addition of heat-inactivated immune serum did not enable leukocytes to kill amebae, nor did it protect these host cells from amebae. MC-derived macrophages, activated with lymphokine elicited by the mitogens conconavalin A, phytohemagglutinin, or an amebic soluble protein preparation (strain HK9), killed 55% of amebae by 3 h in a trypan blue exclusion assay (P less than 0.001); during this time, 40% of the activated macrophages died. Lysis of amebae was confirmed using 111Indium oxine radiolabeled parasites and was antibody independent. Macrophage death appeared to be due to the deleterious effect of lysed amebae rather than the contact-dependent effector mechanisms of E. histolytica. Adherence between activated macrophages and amebae was greater than that between other leukocytes and amebae (P less than 0.001). Microscopic observations, kinetic analysis of the killing of amebae by activated macrophages, and suspension of amebae with adherent activated macrophages in a 10% dextran solution indicated that […]

Interaction of Human Leukocytes and *Entamoeba histolytica*

Killing of Virulent Amebae by the Activated Macrophage

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Abstract

Capable effector mechanisms in the human immune response against the cytolytic, protozoan parasite *Entamoeba histolytica* have not been described. To identify a competent human effector cell, we studied the in vitro interactions of normal human polymorphonuclear neutrophils, peripheral blood mononuclear cells (PBMC), monocytes (MC), and MC-derived macrophages with virulent axenic amebae (strain HMI-IMSS).

Amebae killed neutrophils, PBMC, MC, and MC-derived macrophages ($P < 0.001$), without loss of parasite viability. The addition of heat-inactivated immune serum did not enable leukocytes to kill amebae, nor did it protect these host cells from amebae. MC-derived macrophages, activated with lymphokine elicited by the mitogens conconavalin A, phytohemagglutinin, or an amebic soluble protein preparation (strain HK9), killed 55% of amebae by 3 h in a trypan blue exclusion assay ($P < 0.001$); during this time, 40% of the activated macrophages died. Lysis of amebae was confirmed using ^{111}Indium oxine radiolabeled parasites and was antibody independent. Macrophage death appeared to be due to the deleterious effect of lysed amebae rather than the contact-dependent effector mechanisms of *E. histolytica*. Adherence between activated macrophages and amebae was greater than that between other leukocytes and amebae ($P < 0.001$). Microscopic observations, kinetic analysis of the killing of amebae by activated macrophages, and suspension of amebae with adherent activated macrophages in a 10% dextran solution indicated that contact by activated macrophages was necessary to initiate the killing of amebae. Catalase but not superoxide dismutase inhibited the amebicidal capacity of activated macrophages ($P < 0.001$). However, activated macrophages from an individual with chronic granulomatous disease were able to kill amebae, but not as effectively as normal cells ($P < 0.01$).

In summary, activated MC-derived macrophages killed virulent *E. histolytica* trophozoites through a contact-dependent, antibody-independent mechanism involving oxidative-dependent and -independent processes.

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Introduction

Entamoeba histolytica is an invasive, destructive, protozoan parasite that causes substantial worldwide morbidity and mortality (1). Clinical studies indicate that recurrence of invasive disease is unusual, which suggests that protective immunity develops (2). At present, it is unclear which human immune effector mechanisms are involved in preventing recurrent invasive amebiasis.

Humoral immune mechanisms do not appear to be responsible for acquisition of effective immunity to invasive disease. Systemic antibody against *E. histolytica* develops after invasive disease (3, 4), but does not appear to protect against recurrences (5, 6). Calderon et al. (7) and Aust-Kettis et al. (8) have shown that amebae in vitro can aggregate, ingest, and shed attached specific antiamebic antibody while remaining viable, a process that may be protective for the parasite. Normal and immune sera have been shown to be amebicidal via the alternate and classical pathways of complement (9–11); however, Reed et al. (12) reported that amebae isolated from patients with liver abscess were resistant to complement-mediated lysis. *E. histotyica* trophozoites have been characterized to be potent effector cells. Axenic amebae kill target mammalian tissue culture cells and human neutrophils by sequential adherence, cytolytic, and phagocytic events (13–15). The effector functions of the ameba add to the complexity of the interaction with host cellular immune mechanisms.

Jarumilinta and Kradolfer (16), Guerrant et al. (17), and Ravdin et al. (15) have demonstrated that virulent amebae are cytolethal to human neutrophils. Neutrophils were only able to kill less virulent strains of amebae using a contact-dependent, nonoxidative mechanism independent of serum factors (15, 17).

In humans, depression of cell-mediated immunity may occur during acute invasive disease. Cell-mediated immunity was studied by delayed hypersensitivity skin testing to amebic antigen (18), T lymphocyte numbers (19, 20), and T lymphocyte function as reflected by mitogen- and antigen-induced blastogenesis (19, 21) or macrophage inhibition factor production (18). In animal models, increased invasive disease occurs with depression of cell-mediated immunity that is induced by steroid treatment, thymectomy, radiation, splenectomy, and antilymphocyte globulin (22–24). Protection against invasive disease after immunization with amebic antigen or before *Trichinella spiralis* infection is mediated by cell-mediated immune mechanisms (25, 26). In animals, peripheral lymphocytes, spleen cells, and particularly macrophages, have been reported to be competent effector cells in vitro (27–30).

The purposes of the present studies were to characterize the in vitro interactions between virulent axenic *E. histolytica* trophozoites and human leukocytes in order to identify potential

host effector cells and to characterize the mechanisms by which human effector cells killed amebae. This study demonstrates that the normal human monocyte-derived macrophage (MO),[1] activated by lymphokine elicited with mitogens, or by an amebic soluble protein preparation (SPP), is effective in vitro in killing virulent axenic *E. histolytica* trophozoites. Activated macrophages appear to kill amebae extracellularly, through a contact and partly oxidative-dependent mechanism; studies with cells from a patient with chronic granulomatous disease (CGD) confirm the presence of alternative nonoxidative macrophage amebicidal mechanisms.

Methods

Cultivation and harvesting of E. histolytica

Axenic *E. histolytica* trophozoites, strain HM1/IMSS, was kindly provided by Dr. L. Diamond of the National Institutes of Health and maintained in our laboratory. The amebae were grown in TYI-S-33 medium (trypticase, yeast extract, iron, and serum), as developed by Diamond et al. (31), containing 100 U/ml penicillin and 100 μg/ml of streptomycin sulfate (Gibco Laboratories, Grand Island, NY).

Maintenance of amebic cultures and harvesting of amebae was as described previously (13). The amebae used in experiments were in the logarithmic phase of growth within 72 h after subculture. After harvesting, amebae were washed twice in medium 199 (M.A. Bioproducts, Walkersville, MD) that was supplemented with 5.7 mM L-cysteine (Sigma Chemical Co., St. Louis, MO) and 0.5% bovine serum albumin (BSA) (Sigma Chemical Co.), adjusted to pH 6.8, and sterilely filtered using a 0.22-μm filter (Nalge Co., Rochester, NY). After removal of the final supernatant, the amebic pellet was resuspended in 3 ml of the medium, counted in a hemacytometer, then further diluted to the desired concentration before interaction with the leukocytes.

Subjects

All subjects were healthy University of Virginia employees between the ages of 23 and 40 who had no history of amebic infection. Blood was also obtained from an individual who was previously characterized to have typical CGD of childhood (32). This 30-yr-old male has X-linked CGD that was diagnosed at age 20, and has been shown to have no neutrophil or monocyte (MC) respiratory burst activity (32). His clinical course has been characterized by recurrent skin infections, pneumonia, and hepatic abscess, which were primarily due to *Staphylococcus aureus.*

Preparation of leukocytes

Polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque (Sigma Chemical Co.) gradient centrifugation (33). PMN were further purified by sedimentation in 3% dextran, followed by hypotonic saline lysis of erythrocytes (34), and yielded >98% PMN by morphologic criteria. Adherent, MC-enriched cells were prepared by incubation in plastic petri dishes (100 × 20 mm, Falcon Laboratories, Oxnard, CA) for 2 h at 37°C, followed by gentle scraping with a rubber policeman (35). MC enrichment was reflected by 80–90% nonspecific esterase staining cells and over 75% staining with OKM1 monoclonal antibody. MO were further prepared by incubation of MC in teflon-coated vials (Scientific Specialties Service, Inc., Randallstown, MD), which prevented MC reattachment. All cellular preparations had >95% viability by trypan blue exclusion criteria.

Preparation of lymphokine. PBMC, at 3–5 × 10^6 cells/ml in medium 199, plus 10% heat inactivated pooled AB serum (Gibco Laboratories), were incubated at 37°C with 5% CO_2 for 3 d with the mitogens conconavalin A (Con A) at 20 μg/ml, or phytohemagglutinin (PHA) at 10 μg/ml (Sigma Chemical Co.). Alternatively, PBMC were stimulated over 5 d with an SPP of HK9 amebae (HK9 SPP) (ICN Medical Diagnostics Products, Covina, CA; Lot 4684) at 100 μg/ml. HK9 SPP has been previously shown by Diamanstein et al. (36) to be mitogenic for normal human peripheral T lymphocytes. Supernatants of the stimulated PBMC were recovered as lymphokine after the described incubation, sterilely filtered with a 0.22-μm filter, and stored at −70°C before use. Control lymphokines were generated as the supernatants of unstimulated PBMC or from PBMC to which Con A or HK9 SPP was added at the end of the incubation period.

Activation of macrophages. MO were incubated in teflon-coated vials at 1 × 10^6/ml in medium 199 with 13% fresh autologous serum. Lymphokines were added as 10–15% of the total volume. Cells were fed each day with fresh medium containing lymphokine (10–15% of the total volume). After 3 d incubation, the cells, now referred to as activated macrophages, were recovered from the vials, counted, and resuspended at the desired concentration.

Studies of the interaction of human leukocytes and amebae: cell survival. Host cells and amebae were suspended in medium 199 that contained 5.7 mM L-cysteine, 0.5% BSA, and 10% heat inactivated autologous serum, (total volume 1 ml), centrifuged at 150 *g* for 5 min at 4°C in plastic test tubes (12 × 75 mm, Falcon Laboratories), then incubated at 37°C with 5% CO_2 for time periods varying from 1 to 18 h. In certain experiments, 10% heat inactivated immune serum from a patient with amebic liver abscess (anti-amebic antibody titer of 1:1,024 by indirect hemagglutination) was used. After incubation, the pellets were resuspended by vortexing and the tubes placed in ice. 0.1 ml of an aqueous 0.4% trypan blue solution was added; the cells were mixed by repetitive pasteur pipetting and a drop of the cellular suspension was placed into a hemacytometer. The viability of both host cells and amebae was assessed by trypan blue exclusion criteria (37) and related to the initial concentration of cells placed in the assay. The data were expressed as the percentage viability of cells remaining after the period of incubation or as percentage of control viability at each time point studied. Control viability of host cells or amebae incubated alone was always ≥95%. Initial studies were carried out at a host cell to parasite ratio of 200:1, as this ratio was previously found to be optimal in the killing of a less virulent strain of amebae by PMN (16). Similar ratios of effector to target cells have been used in vitro with macrophages and tumor cells (40).

Release of ^{111}Indium oxine (In Ox) radiolabel from amebae was also studied to verify killing and the trypan blue exclusion method (13, 38, 39). Amebae were harvested as described, washed in growth medium (TYI-S-33), then in RPMI-1640 (M.A. Bioproducts) plus 10% heat inactivated fetal calf serum. Amebae were incubated with In Ox (150–200 μCi; Med-Physics, Inc., Emeryville, CA) in 0.5 ml of RPMI-1640 plus 10% heat activated fetal calf serum at 37°C for 15–20 min. The amebae were washed twice in TYI medium and incubated for a further 2–3 h at 37°C to allow for release of endocytosed radiolabel. Spontaneous release after this time period was 7–20% measured serially from 1 to 24 h. For the In Ox release assay, amebae were washed twice and resuspended at 1 × 10^5/ml in medium 199 that contained L-cysteine (5.7 mM), BSA (0.5%), and 10% heat inactivated autologous serum. A suspension of either nonactivated or activated macrophages (1–2 × 10^6/ml in 0.1 ml) was added. The tubes were centrifugated at 150 *g* for 5 min at room temperature and allowed to incubate for 6 h at 37°C with 5% CO_2 in a total volume of 1 ml. After incubation, 0.5 ml of the supernatant was carefully removed and counted with a gamma counter to assess release of radiolabel. Visual

1. *Abbreviations used in this paper:* AcMO, activated monocyte-derived macrophage(s); CAT, catalase; CGD, chronic granulomatous disease; Con A, concanavalin A; In Ox, ^{111}Indium oxine; MC, monocyte(s); MO, monocyte-derived macrophage; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SPP, soluble protein preparation; SOD, superoxide dismutase.

inspection of the supernatants revealed no cells, and filtering supernatants with a 0.22-μm filter did not change results. Spontaneous release was determined by measurement of released radiolabel in supernatants from amebae incubated alone. Total releasable label was determined by lysis of amebae in distilled water. Percentage of specific release was calculated as: [(Experimental release − spontaneous release)/(Total release − spontaneous release)] × 100.

After removal of 0.5 ml of supernatant and counting of In Ox activity, the amebae and macrophages were also assessed for viability by the trypan blue exclusion method.

In some experiments, In Ox-labeled amebae were added to monolayers of macrophages. Monolayers were prepared by placing 5×10^6 PBMC (0.5 ml of a 1×10^7/ml-suspension) in medium 199, plus 10% heat inactivated fetal calf serum onto 22 × 22 sterile glass coverslips (40). After 2 h incubation at 37°C with 5% CO_2 the monolayers were washed and 1 ml of medium 199 plus 13% autologous serum was added. The macrophage monolayers were then incubated for 7–10 d before use in the In Ox release assay. Monolayers contained 1×10^6 macrophages by visual count. To activate macrophages, Con A-elicited lymphokine was added as 10–15% of the total volume of the culture medium 3 d before use. 1×10^4 In Ox-labeled amebae in 1 ml total volume were added to the monolayers for the assay. After a 6-h incubation, the supernatant was aspirated, and centrifuged at 150 *g* for 10 min at 4°C to pellet the amebae. The supernatant was recovered and 0.5 ml used for measurement of In Ox activity in a gamma counter. Visual inspection demonstrated no cells in these supernatants; filtering supernatants did not change the results. Spontaneous and total release was determined as previously described.

Kinetic analysis of the killing of amebae by activated macrophages using the Poisson distribution. The complete derivation of the Poisson distribution as applied to T lymphocyte contact-dependent cytopathogenicity has been detailed (13, 41). The Poisson distribution can be applied to ascertain the probability of occurrence of a random event (42). The random event we are studying is contact between effector and target cells resulting in death of the target cell. The following assumptions were made: (*a*) target cell death occurs when one or more effector cells are in contact with the target cell; no target cell death occurs if effector cells are not in contact with the target cell; (*b*) contact of one macrophage (effector cell) with a target does not affect the probability of a second macrophage making contact (applicability of the Poisson distribution).

Let t equal the initial number of target cells (amebae), x the number of activated macrophages added, n the fraction of activated macrophages that are effector cells, and y the fraction of target cells (amebae) killed or corrected specific killing.

The fraction of target cells that survived $(1 - y)$ was equal to the probability of a target cell not being contacted by an effector cell. This probability was given by the first term of the Poisson distribution, therefore: $1 - y = \exp(-nx/t)$; $\ln(1 - y) = -nx/t$; $\ln(1/1 - y) = nx/t$; and $\log \ln(1/1 - y) = \log(nx/t)$. Thus, a linear relationship between $\log \ln(1/1 - y)$ and $\log(nx/t)$ would fulfill the Poisson distribution and suggest that target cell death is a contact-dependent event. The amebae were kept at a constant number (1.0×10^4), and the number of activated macrophages was varied from 5.0×10^4 to 2.0×10^6/ml.

Studies of adherence between amebae and host cells

Amebae and host cells were harvested and washed as described, and suspended at a host cell to amebic ratio of 20:1 (2×10^5 host cells and 1×10^4 amebae in a total volume of 1 ml). The cells were centrifuged at 150 *g* for 5 min and then incubated for 2 h at 4°C. After incubation, 0.8 ml of the supernatant was removed and the pellet was broken up by five gentle rotations of the tube. A drop of the cell suspension was placed on a hemacytometer and the coverslip applied. The number of amebae with at least three adherent host cells (a rosette) and the nonrosetted amebae were counted; the data was expressed as the percentage of amebae that formed rosettes.

Dextran suspension studies. Activated macrophages and amebae (ratio of 100:1) were centrifuged at 150 *g* for 5 min and then incubated for 1 h at 4°C to establish adherence. The supernatant was removed and the cells were resuspended in 1 ml of a 10% solution of dextran (500,000 mol wt, Sigma Chemical Co.) (43) in medium 199 with 5.7 mM L-cysteine, 0.5% BSA, and 10% heat inactivated autologous serum. Controls consisted of activated macrophages and amebae suspended in the dextran solution alone or together (but without prior centrifugation to allow for adherence between cells). The cells were incubated in dextran for 3 h at 37°C with 5% CO_2; the viability of activated macrophages and amebae was assessed by trypan blue exclusion criteria. Previous studies have established that cytolytic capacity of amebae and human cells, particularly T lymphocytes, is unaltered in 10% high molecular weight dextran solutions (14, 43).

Studies examining the role of oxidative mechanisms in the killing of amebae by activated macrophages

Activated macrophages were preincubated for at least 30 min with catalase (CAT) (3,000 U/ml or 0.25 mg/ml) or superoxide dismutase (SOD) (300 U/ml or 0.11 mg/ml) (Sigma Chemical Co.), and the CAT and SOD were included in the 3-h incubation with amebae. Controls of heat-inactivated (70°C for 2 h) CAT and SOD were performed. MC obtained from an individual with typical CGD of childhood were activated with Con A-elicited lymphokine (from normal PBMC), and the cells were incubated with amebae for 3 h at 37°C.

Exogenous H_2O_2 was added to amebae in concentrations ranging from 10^{-1} to 10^{-7} M that were in medium 199 with 5.7 mM L-cysteine and 10% heat inactivated fetal calf serum. Cytotoxic effect on the amebae after 1 to 2 h incubation at 37°C was assessed by both the trypan blue exclusion method as well as with measurement of In Ox release from amebae.

Statistics

All results were expressed as the mean±SEM. The t test for unpaired samples was used to determine significance.

Results

Interaction of neutrophils, PBMC, and MC with amebae. In the presence of virulent amebae (strain HM1), the viability of PMN, PBMC, and MC was significantly decreased ($P < 0.001$) (Table I). The decrease in PMN viability was greater than that seen in any of the other cell types studied ($P < 0.001$). At the same time, no decrease in amebic viability was observed. The addition of 10% heat inactivated immune serum did not enable these host cells to kill amebae, nor increase their survival with amebae present (Table I).

Interaction of MC-derived macrophages and amebae. MC-derived macrophages treated with control medium or with control lymphokines were not effective in killing amebae (Table II). Macrophages demonstrated decreased viability due to amebic cytolytic activity ($P < 0.001$). The concentrations of mitogens and amebic protein that produced maximal [^{3}H]thymidine incorporation into DNA of cultured lymphocytes (Con A, 110, 193.2 cpm at 20 μg/ml; PHA, 143, 163.7 cpm at 10 μg/ml; and HK9 SPP, 120, 961.0 cpm at 100 μg/ml) were used to elicit lymphokines. Activation of MC-derived macrophages by lymphokines was indicated by their increased adherence to glass, increased size on observation, increased protein content per 10^6 cells ($P < 0.05$), and increased phagocytic activity for latex and opsonised zymosan particles ($P < 0.01$) compared with nonactivated cells. Macrophages activated with Con A-elicited lymphokine were able to kill amebae;

*Table I. Interaction of PMN, PBMC, and MC with Amebae**

Interaction	Viability‡				
	Autologous serum		Immune serum		
	Host cell§	Amebae‖	Host cell§	Amebae‖	
					n
PMN + amebae	27.85±1.37¶	99.70±3.06	22.77±1.50¶	101.83±4.15	9
PBMC + amebae	53.91±3.25	100.40±7.87	49.87±3.67	99.50±3.68	9
MC + amebae	53.90±2.50	102.00±9.08	55.20±5.20	97.90±8.15	9

* At 37°C for 3 h. ‡ Expressed as percentage viability of paired controls of host cells or amebae; viability of controls of host cells or amebea alone were >92%. Trypan blue exclusion criteria for viability of cells was used. § Host cell viability in all instances was reduced from control values, $P < 0.001$. ‖ Amebic viability, in all instances, was not altered from control values. ¶ PMN viability was significantly decreased vs. PBMC or MC, $P < 0.01$.

amebic viability decreased to 43% after 3 h (Table II) ($P < 0.001$). Lymphokines elicited with PHA or an amebic soluble protein preparation (strain HK9) were equally effective in activating macrophages to kill amebae ($P < 0.001$). Con A- and PHA-elicited lymphokines, when added at the time of the assay, did not alter viability of amebae. The viability of activated macrophages decreased with amebae present ($P < 0.001$).

The addition of 10% heat inactivated immune serum did not potentiate the killing of amebae by activated macrophages (Table II). Activated macrophages were able to kill amebae in TYI-S-33, the growth medium for amebae in axenic culture. As an additional control to assess amebic cytolytic capacity in defined medium, amebae killed 75% of target Chinese hamster ovary cells in medium 199 supplemented with cysteine, BSA, and fetal calf serum after 3 h at 37°C ($P < 0.001$), which was identical to results seen in TYI-S-33 medium.

To corroborate the trypan blue exclusion method, In Ox released from labeled amebae was measured during interaction with macrophages. Labeling amebae with In Ox had no effect on amebic viability or cytolytic function. Activated macrophages killed virulent amebae with 33.8% specific In Ox release by 6 h ($P < 0.001$) (Table III); In Ox released from amebae was equal to control values when amebae were incubated with nonactivated macrophages. Monolayers of activated macrophages were also able to kill In Ox-labeled amebae ($P < 0.001$) (Table III). Paired studies assessing amebic viability by the trypan blue exclusion criteria gave similar results.

The killing of amebae by activated macrophages during longer incubation periods is seen in Fig. 1. As the duration of incubation increased from 1 to 18 h, there was a progressive decline in amebic viability from 62 to 9% ($P < 0.01$). Parasite viability was decreased even after a 1-h incubation ($P < 0.05$). Macrophage viability decreased to 42 and 25% at 6 and 18 h, respectively ($P < 0.001$).

The effect of varying cell ratios on the interaction of activated macrophages with amebae is represented in Fig. 2. Amebic viability decreased as the ratio of activated macrophage to amebae increased ($P < 0.001$). Amebae were killed by activated macrophages at ratios as low as 10:1 ($P = 0.042$). Ratios of activated macrophages to amebae >100:1 did not result in increased killing of amebae.

There was a reduction in the viability of activated macrophages as the ratio of activated macrophages to amebae

*Table II. Interaction of MO and Amebae, and Effect of Various PBMC Supernatants**

Macrophages treated with:	Viability‡			
	Autologous serum		Immune serum	
	Macrophage§	Amebae	Macrophage§	Amebae
Control medium ($n = 10$)	57.24±4.71	107.10±9.41	52.50±2.00	97.00±4.54
Control PBMC supernatant ($n = 6$)	51.23±3.19	101.35±8.05	ND‖	ND
Control PBMC supernatant plus Con A ($n = 6$)	48.45±3.08	91.53±10.05	ND	ND
Control PBMC supernatant plus HK9 SPP ($n = 3$)	50.03±5.11	98.99±5.67	ND	ND
Con A-elicited lymphokine ($n = 12$)	59.74±1.48	42.98±3.36¶	48.38±3.61	56.83±7.19¶
PHA-elicited lymphokine ($n = 19$)	48.21±3.94	44.16±3.46¶	45.12±4.11	45.23±5.17¶
HK9 SPP-elicited lymphokine ($n = 6$)	59.12±6.19	38.81±9.05¶	ND	ND

* At 37°C for 3 h. ‡ Expressed as percentage of viability of paired controls of macrophages or amebae incubated alone in identical test conditions; viability of all controls was ≥90%. Trypan blue exclusion criteria for viability was used. § Macrophage viability, in all instances, was decreased from control of macrophages alone, $P < 0.001$. ‖ ND indicates not done. ¶ Amebic viability was decreased vs. all other controls, $P < 0.001$.

*Table III. Amebecidal Activity of Activated Macrophages in Pellets or Monolayers as Measured by In Ox Release from Amebae or the Trypan Blue Exclusion Method**

	Corrected specific In Ox release from amebae		Percentage of dead amebae by trypan blue‡ exclusion criteria	
	MO plus amebae	AcMO plus amebae	MO plus amebae	AcMO plus amebae
Pellet	0.28±0.28 (n = 6)	33.50±2.10§ (n = 10)	4.67±3.66 (n = 6)	45.20±1.51§ (n = 10)
Monolayers	0.35±0.17 (n = 9)	42.44±1.61§ (n = 9)	—	—

* All studies performed at MO ameba ratios of 100:1, and incubated over 6 h at 37°C.
‡ Calculated as 100% viable amebae.
§ Percentage of amebae killed was increased vs. nonactivated MO plus amebae, $P < 0.01$.

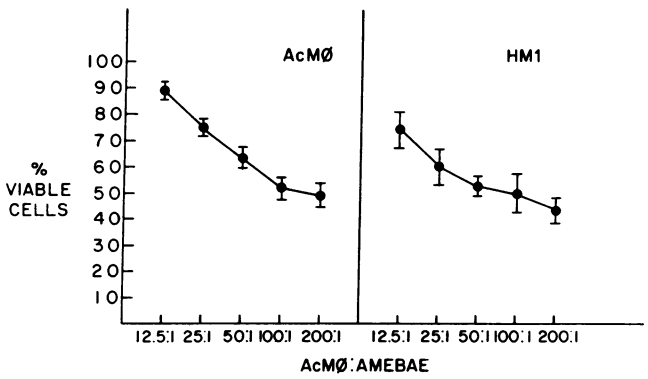

Figure 2. The interaction of activated macrophages and HM1 amebae: the effect of varying cell ratios at 37°C for 3 h. Data represents the mean±SEM of 12 studies. As the ratio of AcMO to amebae increased from 12.5:1 to 200:1, AcMO viability (*left*) decreased from 90 to 49% and HM1 amebic viability (*right*) decreased from 76 to 43% ($P < 0.001$ for each).

increased ($P < 0.001$). The high level of activated macrophage viability at low macrophage to amebae ratios suggested that macrophages were resistant to the parasite's cytolytic effector mechanisms. To determine the mechanisms responsible for the death of activated macrophages at higher macrophage to amebae ratios, we studied the interaction of activated macrophages with the Entamoeba-like Laredo strain, which is nonpathogenic in humans and nonvirulent in vitro (15). At a 100:1 ratio, activated macrophages killed all of the Laredo amebae after 6 h at 37°C without any loss in macrophage viability, suggesting that macrophage autolysis is not required for amebicidal activity. Activated macrophages were incubated for 3 h at 37°C with lysates of HM1 amebae prepared by distilled water lysis, lyophyllization, and resuspension in supplemented medium 199. Amebic lysates were toxic to activated macrophages (19.2, 38.2, and 48.9% macrophages killed by lysates of 10^3, 10^4, and 10^5 amebae/ml, respectively, $P < 0.05$), suggesting that lysis of amebae at higher macrophage to amebae ratios could result in the release of amebic products harmful to macrophages. The *N*-acetyl-D-galactosamine inhibitable amebic lectin has been demonstrated to be involved in amebic cytolysis of mammalian tissue culture cells and human neutrophils (14, 15). The addition of *N*-acetyl-D-galactosamine (45 mM) did not prevent the death of activated macrophages incubated with amebae at a 100:1 ratio for 3 h at 37°C (n = 6), further indicating that amebic cytolytic activity is not responsible for the reduced survival of macrophages observed at high macrophage to amebae ratios.

Activated macrophages have been previously reported to kill target cells by contact-dependent mechanisms (44). We evaluated the kinetics of the amebicidal activity of activated macrophage by varying the concentration of activated macrophages over a one and one-half log range with the number of amebae kept constant. A plog of log ln (1/1 − fraction of amebae killed) vs. log of activated macrophage concentration was a linear function (r = 0.9749, slope = 0.45, $P < 0.001$) (Fig. 3). This linear correlation, fulfilling the Poisson distribu-

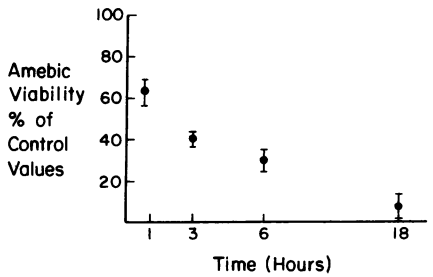

Figure 1. Time course of activated macrophage killing of amebae. Data represents the mean±SEM of from 6 to 12 studies. AcMO and amebae were incubated at a 200:1 ratio for the indicated time periods at 37°C. As the time of incubation increased from 1 to 18 h, amebic viability decreased from 62 to 9% ($P < 0.001$). Amebic viability at 1 h was decreased from control ($P < 0.05$).

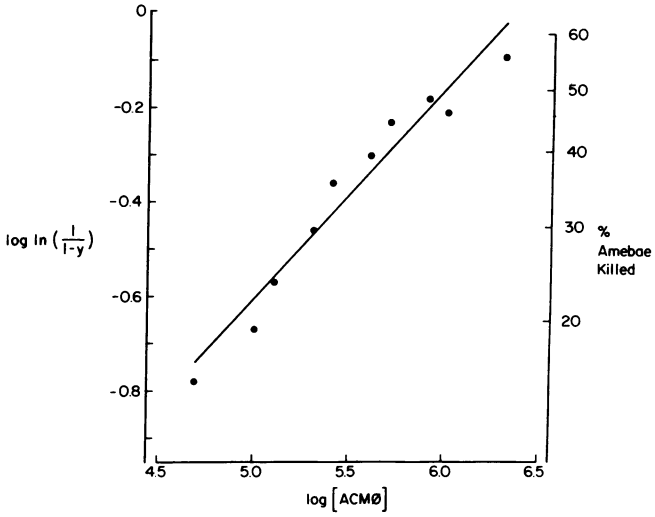

Figure 3. Contact-dependent killing of amebae by activated macrophages, as demonstrated by the Poisson distribution. The percentage of amebae killed (y, mean of 12 studies) was determined at each AcMO quantity; the log ln (1/1-mean percentage of amebae killed) was then calculated. The log of the number of AcMO plotted against log ln (1/1-mean percentage of amebae killed) was linear (r = 0.9749, $P < 0.001$), which indicated a contact-dependent event.

tion, indicates that activated macrophages kill amebae through a contact-dependent process (13, 41).

Studies of adherence between amebae and host cells

Microscopically, we observed intimate contact between activated macrophages and amebae. Amebae that had been killed possessed adherent-activated macrophages. It was suggested by morphologic observations and the preceeding kinetic analysis that adherence events between host cells and amebae were important. At 4°C, in the same defined test medium used in viability studies, 55% of amebae formed rosettes with PMN; amebic rosette formation with MC was significantly less at 33% ($P < 0.001$ vs. PMN). Rosette formation between amebae and nonactivated macrophages was 32%, which was also lower when compared with PMN ($P < 0.005$). Activated macrophages had greater adherence to amebae than did PMN, MC, or nonactivated macrophages, as 73% of amebae formed rosettes with activated macrophages ($P < 0.001$ vs. all other cells).

Dextran suspension studies. To study the role of adherence in macrophage amebicidal activity, we used a dextran suspension method described by Martz (43) to study the contact-dependent cytolytic mechanism of T-lymphocytes. When activated macrophages and amebae were suspended in a 10% dextran solution without prior centrifugation to allow for adherence, macrophage and amebic viability was unaltered (Table IV). However, if activated macrophages and amebae were first centrifuged and incubated at 4°C for 1 h to establish adherence, and then suspended in the dextran solution, activated macrophages were able to kill amebae ($P < 0.001$, Table IV). When activated macrophages and amebae were added to the dextran solution without prior centrifugation (at a ratio of 100:1), only 3.9 and 5.2% of amebae had ≥3 adherent macrophages at time zero and 3 h, respectively. After centrifugation and incubation at 4°C for 1 h, 94.2 and 70.0% of amebae present had ≥3 adherent macrophages after zero and 3 h in the dextran solution. After 3 h incubation at 37°C in the dextran, 89% of dead and only 10% of viable amebae had three or more adherent-activated macrophages ($P < 0.01$).

Studies examining the role of activated macrophages oxidative mechanisms in the killing of amebae

Catalase but not superoxide dismutase significantly inhibited the killing of amebae by activated macrophages ($P < 0.001$, Table V). In the presence of CAT, but not SOD, macrophage survival decreased ($P < 0.01$). Heat-inactivated CAT or SOD had no effect on the interaction of activated macrophages and amebae (Table V). CAT (3,000 U/ml), SOD (300 U/ml), or heat-inactivated enzymes had no effect on the viability of controls of macrophages or amebae incubated alone. We studied the effect of exogenously added H_2O_2 on amebic trophozoites. 50% reduction in viability, as measured by the trypan blue exclusion method or In Ox release, was observed at an H_2O_2 concentration of 10^{-2} M ($P < 0.001$).

Macrophages from an individual with typical CGD (32) were activated with lymphokine elicited by Con A from PBMC obtained from a normal individual. Activated CGD macrophages were capable of killing amebae ($P < 0.001$), but not as effectively as activated macrophages from normal individuals ($P < 0.01$, Table V).

*Table IV. Dextran Suspension of Cells Demonstrating Contact-dependent Killing of Amebae by Activated Macrophages**

Activated macrophages and amebae	Viability‡	
	Activated macrophages	Amebae
Without prior centrifugation; nonadherent cells ($n = 6$)	103.30±2.50	99.96±5.53
Suspended after centrifugation; adherent cells ($n = 6$)	69.85±5.19§	50.74±5.68§

* At 37°C for 3 h.

‡ Expressed as percentage viability of paired controls; viability of controls was ≥99%. Trypan blue exclusion criteria for viability of cells was used.

§ Viability of activated macrophage and amebae suspended after centrifugation was decreased, vs. no prior centrifugation or controls, $P < 0.001$.

Discussion

Evidence from in vivo models indicate that cell-mediated mechanisms are operative in the acquisition of immunity to invasive amebiasis (6, 18–30). This study established that the human activated monocyte-derived macrophage (AcMO) is capable of an effector role in the cell-mediated immune response against the cytolytic protozoan parasite *E. histolytica.*

We have found that neutrophils, PBMC, MC, and nonactivated MO are all ineffective in killing virulent axenic amebae and are in fact readily killed by the ameba (15). The mechanism by which amebae kill human leukocytes appears to be sequential adherence and cytolytic mechanisms (13–15). Lysis of leuko-

*Table V. Role of Oxidative Mechanisms in the Killing of Amebae by Activated Macrophages**

Interaction	Percentage of viability‡	
	AcMO	Amebae
AcMO + amebae ($n = 16$)	53.30±2.21§	50.11±3.52‖
AcMO + amebae + CAT (3,000 U/ml) ($n = 11$)	40.80±3.83¶	79.13±4.04**
AcMO + amebae + heat-inactivated CAT (3,000 U/ml) ($n = 4$)	44.75±3.60§	50.50±2.86‖
AcMO + amebae + SOD (300 U/ml) ($n = 7$)	44.76±4.28§	51.90±4.16‖
AcMO + amebae + heat-inactivated SOD (300 U/ml) ($n = 4$)	50.15±1.29§	48.52±12.20‖
AcMO (CGD) + amebae ($n = 4$)	52.63±1.54§	70.60±4.49‡‡

* At 37°C for 3 h.

‡ Expressed as percentage viability of paired controls; viability of all controls was ≥95%. Trypan blue exclusion criteria was used in assessing viability of cells.

§ AcMO viability decreased vs. control of AcMO alone, $P < 0.001$.

‖ Amebic viability decreased vs. control of amebae alone, $P < 0.001$.

¶ AcMO viability decreased vs. control of AcMO alone, $P < 0.001$, and decreased vs. AcMO + amebae, $P < 0.01$.

** Amebic viability increased vs. AcMO plus amebae, $P < 0.001$.

‡‡ Amebic viability decreased vs. control of amebae alone, $P < 0.001$, and increased vs. normal AcMO plus or minus amebae, $P < 0.01$.

cytes by amebic trophozoites could result in the release of toxic leukocyte products that may be harmful to host tissues, thus contributing to the pathogenesis of disease (45). In the presence of amebae, survival of PBMC, MC, and MO is superior to that seen with neutrophils. This inherently lower susceptibility of mononuclear cells to amebic cytolysis may relate to the ability of activated macrophages to kill this protozoan pathogen.

Macrophages play a prominent role in combating both intracellular organisms and extracellular targets, such as tumor cells (46). Treatment of mononuclear phagocytes in vitro with supernatants of mitogen or antigen-stimulated T lymphocytes enhances their capacity to inhibit the intracellular replication of *Toxoplasma gondii* (47), *Trypanosoma cruzi* (48), *Mycobacterium tuberculosis* (49), *Leishmania donovani* (50), and also increases extracellular tumoricidal activity (41, 51). We found that both classic T cell mitogens and an amebic SPP, demonstrated to have mitogenic activity (36), were effective in activating macrophages to kill this extracellular pathogen.

The killing of amebae by activated macrophages appeared to be a serum independent, immunologically nonspecific event that was fairly complete by 18 h incubation. Activated macrophage viability also progressively decreased over the 18 h incubation, yet at 18 h the ratio of viable macrophages to amebae was still >200:1, which is more than sufficient to initiate killing of amebae. That this is a contact-mediated process is supported by our microscopic observations, mathematical application of the Poisson distribution, and studies in high molecular weight dextran solutions. Adherence between activated macrophages and amebae was by a serum-independent mechanism, with activated macrophages exhibiting greater adherence to amebae than other host cells studied. However, given the high background of rosette formation with MC or nonactivated macrophages, increased activated macrophage adherence may be contributory but not sufficient to explain the ability of activated macrophages to kill amebae. At present, it is unclear whether this adherence event is mediated by a specific macrophage receptor or is initiated by the amebic GalNAc inhibitable adhesin (14, 15). Kinetic studies applying the Poisson distribution supported our microscopic observation that activated macrophages kill amebae by a contact-mediated mechanism. If contact was not required, or macrophages killed amebae in a concentration-independent manner, then this study would not have resulted in a linear function (41). Suspension of amebae and activated macrophages in dextran demonstrated conclusively that adherence between activated macrophages and amebae is required to initiate the cytolethal effect of these host cells on the amebae. Similar contact dependence in extracellular killing of tumor cells has been observed with macrophages or T lymphocytes (41, 43, 44).

In the process of killing *E. histolytica* trophozoites, activated macrophage viability significantly decreased. The *N*-acetyl-D-galactosamine inhibitable amebic lectin has been shown to be involved in the cytolytic capacity of amebae for mammalian tissue culture cells or human neutrophils (14, 15). In the presence of *N*-acetyl-D-galactosamine, neutrophils become capable of killing virulent amebic trophozoites while neutrophil survival increases (15). *N*-acetyl-D-galactosamine had no effect on the interaction of activated macrophages and amebae, which suggested that activated macrophages are more resistant to amebic effector mechanisms. In addition, at low macrophage to amebae ratios, amebae did not kill activated macrophages, as has been seen with other mammalian target cells or neutrophils (13–15). The toxic effect of amebic lysates suggests that upon being killed by activated macrophages, amebae release toxic products that are harmful to macrophages. This is consistent with the high levels of activated macrophage viability at low macrophage to ameba ratios when amebae remained viable as well. We cannot completely exclude a component of macrophage autolysis in the interaction of activated macrophages and amebae. However, our studies with the nonpathogenic Laredo strain would not support a macrophage autolytic process, as macrophage death was not observed during killing of Laredo amebae. In addition, catalase resulted in a further decrease in macrophage viability, arguing against an oxidative-mediated macrophage autolytic event. Loss of activated macrophage oxidative activity in the presence of catalase may have increased their susceptibility to amebic cytolytic activity.

These studies indicated that both oxidative-dependent and -independent mechanisms are important for the killing of amebae by the activated macrophage. Studies with mouse peritoneal macrophages (52–56) and human MC-derived macrophages (57, 58) have revealed a close correlation between the capacity of these cells to release reactive oxygen intermediates and their ability to either inhibit the growth of certain intracellular pathogens or kill tumor cells. Incubation of murine peritoneal macrophages with mitogen or antigen-elicited lymphokine has enhanced the secretion of H_2O_2 and the subsequent killing of *T. gondii* (53), *T. cruzi* (54), *Leishmania enrietti* (55), *Mycobacterium microti* (56), and *L. donovani* (59). Nakagawara et al. (60) have recently shown that in vitro stimulation of human MC-derived macrophages with Con A-elicited lymphokine enhanced their capacity to secrete reactive oxygen intermediates (60). Murray et al. (61), in a phagocyte-free system, reported that HM1 amebic trophozoites were susceptible to oxidative products, particularly H_2O_2 or H_2O_2 in combination with halide and myeloperoxidase (61). We confirmed the susceptibility of virulent amebic trophozoites to exogenous H_2O_2. The concentration of H_2O_2 effective against *E. histolytica* has been associated with 50% death of an intracellular pathogen, *T. cruzi* (62). The inhibition of activated macrophage amebicidal activity by catalase, the susceptibility of amebae to H_2O_2, and the lack of effect by SOD are compatible with the findings of Murray et al. (61), who showed the sensitivity of amebae to H_2O_2 but not superoxide anion. That alternative nonoxidative macrophages' amebicidal mechanisms exist is suggested by the partial inhibition with catalase and studies with cells from an individual with CGD. Diamond et al. (63) demonstrated that the extracellular killing of *Aspergillus fumigatus* hyphae by normal human MC was oxidatively dependent. However, hyphal damage was also evident with whole MC or MC granule-enriched fractions from patients with CGD. Fungicidal activity was also inhibited by polyanions, suggesting that cationic proteins may be involved (63). Recently, Murray et al. (64) provided evidence that the killing of the intracellular parasite, *Leishmania donovani*, by human activated macrophages, was both dependent and independent of oxidative mechanism. Neutrophils have highly active oxidative cytolethal mechanisms, yet were unable to kill amebae, indicating that there are important characteristics of activated macrophages,

other than oxidative metabolism, contributing to their antiprotozoal effector mechanisms.

Ghadirian and Meerovitch (28) recently found in an in vivo model that macrophages were largely responsible for host resistance against invasive amebic liver abscess and metastatic dissemination (29). Our studies indicate that the activated macrophage is a competent human effector cell against *E. histolytica* trophozoites and may be operative in preventing recurrent invasive amebiasis. In these studies, macrophages were nonspecifically activated with mitogen-elicited lymphokines to kill amebae. In preliminary studies we have found that an SPP from axenic (strain HM1) amebae can elicit lymphokine from lymphocytes of patients treated for amebic liver abscess that effectively activated macrophages of normal uninfected individuals to kill virulent amebae. In addition, macrophages from these patients could be activated with Con A-elicited lymphokines to kill amebae.

In summary, for the first time the human AcMO was shown to be capable of killing the virulent, extracellular, protozoal parasite, *E. histolytica.* After appropriate activation and establishment of contact, both macrophage oxygen-dependent and oxygen-independent mechanisms are involved in killing the parasite. Further work to define the mechanisms of adherence between macrophages and amebae and the macrophage constituents responsible for the nonoxidative killing of amebae will need to be pursued. These observations begin to elucidate a potentially effective human defense mechanism against invasive amebiasis, and suggest the possibilities for future immunoprophylaxis against this disease.

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