Cytokine Activation of Murine Macrophages for In Vitro Killing of *Entamoeba histolytica* Trophozoites

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Received 21 November 1988/Accepted 28 February 1989

Macrophage-mediated effector mechanisms against the protozoan parasite *Entamoeba histolytica* were studied. Unstimulated macrophages were inefficient at killing *E. histolytica* trophozoites in vitro and were killed by the trophozoites. Conversely, immature cells of the mononuclear phagocyte lineage (promonocytes) were shown to display a strong spontaneous amebicidal activity. The acquisition of macrophage amebicidal activity following cytokine treatment was investigated. Gamma interferon, tumor necrosis factor alpha, and macrophage colony-stimulating factor 1, or combinations thereof, were shown to endow murine bone marrow-derived macrophages with significant amebicidal activity. Low doses of gamma interferon and tumor necrosis factor alpha and of gamma interferon and colony-stimulating factor 1 were shown to act synergistically in this phenomenon. This enhancement of amebicidal activity was shown to operate on bone marrow-derived macrophages, elicited peritoneal macrophages, and, to a much lesser extent, spleen macrophages. Although acquisition of amebicidal activity was associated with a strong respiratory burst, the addition of oxygen-free radical scavengers showed that the killing activity was approximately 45% 

**MATERIALS AND METHODS**

**Mice.** Inbred BALB/c mice (8 to 12 weeks of age) were obtained from Charles River Breeding Laboratories, St. Constant, Quebec Canada. They were housed in plastic cages in pathogen-free conditions and fed water and Purina chow ad libitum.

**Parasites.** The pathogenic strain of *E. histolytica* used was HM1-IMSS grown axenically in TYI-S-33 medium as described previously (4). Trophozoites were harvested during log-phase growth after 48 h to 72 h of subculture by chilling the cultures in ice water (5 min) and sedimentation (300 × g, 10 min).

**Macrophage populations.** Peritoneal exudate cells were isolated by peritoneal lavage of mice injected intraperitoneally 3 days previously with 1.5 ml of 10% proteose peptone (Difco Laboratories, Detroit, Mich.). Cells were washed twice in complete medium (RPMI 1640 supplemented with 10% fetal calf serum and antibiotics) and plated in 35-mm petri dishes (5 × 10^6 per dish) for 2 h at 37°C in 5% CO₂–95% air, followed by removal of nonadherent cells by suction. Spleen macrophages were obtained as described previously (7). BM-derived macrophages were obtained after in vitro culture of BM cells from murine femurs in the presence of L-cell supernatant (8).

**Macrophage-mediated killing.** Macrophage amebicidal activity was assessed by a method described in detail elsewhere (6). Adherent macrophages were dislodged by EDTA treatment as described previously (7). In some cases, macrophages were preactivated by 18 h of incubation with the indicated concentrations of cytokines. Macrophages (2 ×

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* Corresponding author.
10⁶/ml) and amebae (10⁴/ml) were suspended in complete medium supplemented with 5.7 mM cysteine in plastic test tubes. Cells were centrifuged at 150 × g for 5 min and incubated at 37°C in 5% CO₂ for appropriate periods of time. Tubes were placed on ice, and viability of the cells was assessed by trypan blue exclusion. The data are expressed as percent viability of controls of amebae or macrophages incubated alone.

**Cytokines.** Mouse recombinant TNF-α was purchased from Intermedico, Markham, Ontario, Canada. It had an activity of 4 × 10⁷ U/mg. Macrophage CSF was also purchased from Intermedico; its activity was >10⁶ CFU/mg of protein. IFN-γ was purchased from Amgen, Thousand Oaks, Calif. All cytokines were dissolved in RPMI 1640 with 10% fetal calf serum.

**Antibodies.** Rabbit anti-mouse TNF-α was purchased from Intermedico. It is a polyvalent rabbit antiserum prepared with sera from hyperimmune New Zealand rabbits. Normal rabbit serum was used as a negative control. Purified monclonal rat immunoglobulin G subclass 1 against mouse IFN-γ was purchased from Lee Biomolecular, San Diego, Calif.

**H₂O₂ production.** Release of H₂O₂ by macrophages was determined quantitatively by the H₂O₂-mediated and horseradish peroxidase-dependent oxidation of phenol red to a product whose absorbance is read at 610 nm (18). Macrophages were established in 1.6-cm wells and covered with 1 ml of phenol red solution per well; the solution contained 140 mM NaCl, 10 mM phosphate buffer (pH 7.0), 5.5 M dextrose, 0.28 mM phenol red (Sigma Chemical Co., St. Louis, Mo.), and 10 U of horseradish peroxidase (Sigma) per ml. The stimulants were added, and the plates were incubated for 60 min at 37°C. Controls included wells with horseradish peroxidase but no stimulants and wells with horseradish peroxidase but no cells. After incubation, the cell-free supernatants were transferred to tubes and rendered alkaline by the addition of 10 µl of 1 N NaOH per tube. The A₆₁₀ was read against a blank to which 10 µl of 1 N NaOH was added. Standard curves were made by using the same batch of horseradish peroxidase with H₂O₂ solutions ranging in concentrations from 1 to 60 µM.

**Promonocytes.** BM cultures were established by the method described by Baccarini et al. (2). The narrow of two femurs of a single mouse was collected, and 5 × 10⁶ cells were plated in 10 ml of RPMI 1640 supplemented with 15% fetal calf serum and antibiotics in petri dishes. After 18 h of incubation, 20% conditioned medium of L-929 cells (4-day supernatant) was added to the cultures. Non-adherent BM cells were collected after 3 days in culture and subjected to discontinuous Percoll gradients as described previously (2). The first three fractions were pooled and used in subsequent assays. The processing of these effector cells for the assay of amebic killing was similar to that described for macrophages.

**LK-rich fluid.** Unfractionated splenocytes (1.5 × 10⁶/ml) from normal BALB/c mice were incubated with 5 µg of concanavalin A per ml for 48 h at 37°C in 5% CO₂. The cells were incubated in RPMI 1640 with 10% fetal calf serum and antibiotics. Control supernatants were prepared from normal splenocytes to which concanavalin A was added at the end of the incubation period. Supernatants were collected after 48 h and centrifuged at 500 × g for 15 min. Residual concanavalin A was neutralized by the addition of α-methylmannoside (Sigma). LK-containing fluid was then concentrated to 20 times, using polyacrylamide absorbent gel (Sigma). LK-rich fluid was supplemented with 1 µg of lipopolysaccharide (LPS) per ml in selected experiments, sterilized by filtration through a 0.45-µm membrane filter (Millipore Corp., Bedford, Mass.), and stored at -20°C. Before use, the supernatants were thawed and diluted in RPMI 1640 containing 15% fetal calf serum and antibiotics. Macrophages were activated by 18-h incubation with LK-LPS fluid (20 to 30%, vol/vol) before interaction with amebae.

**Assessment of requirement for contact.** To assess the importance of cell-target contact in the assay, chambers with 0.45-µm-pore-sized membrane filters (Millipore) were purchased. Cells were placed in the wells, whereas amebae were placed in the upper chamber. Following incubation in different conditions, cell viability was assessed as described previously.

**Scavengers and inhibitors.** For all experiments in which scavengers and inhibitors were used, elicited peritoneal macrophages were treated with crude LK-rich fluid supplemented with LPS (see above) for 18 h before interaction with amebae.

 Tosyl lysyl chloromethyl ketone (TLCK), aprotinin, bovine pancreatic trypsin inhibitor, α-1-antitrypsin, actinomycin D, and cycloheximide were purchased from Sigma and used at concentrations indicated. Controls consisted of cells and amebae incubated with inhibitor but without LK, amebae incubated with LK and inhibitor but without amebae, and cells incubated with LK and inhibitor but without amebae. When reagents were dissolved in dimethyl sulfoxide (Sigma), controls were added to demonstrate that the final concentration of dimethyl sulfoxide in the cultures was innocuous to macrophages and trophozoites. In all cases, viability of amebae or macrophages or both was assessed by trypan blue exclusion.

**RESULTS**

**Interaction between promonocytes and E. histolytica trophozoites.** Macrophage precursors have been shown to be potent natural killer cells against some tumor cells and Leishmania donovani (2, 3). These cells mediating natural cytotoxic effector mechanisms have also been shown to exert strong candidicidal activity (2). Therefore, it was of interest to clarify the capacity of this cell population to exert cytolytic activity against E. histolytica. Different numbers of BM-derived macrophage precursors were obtained and challenged in vitro with E. histolytica to assess their killing efficiency.

A strong amebicidal activity was displayed by the effector cells at a variety of effector/target ratios in a 6-h assay (Fig. 1). To determine the kinetics of events leading to destruction of amebae by the effector cells, a series of experiments was conducted in which effector and target cells were incubated at a 50:1 ratio for various time periods. Figure 2 shows that the killing of the amebae was already detectable after a 1-h cocultivation (18%) and increased significantly (P < 0.01 to 0.001) over the next 8 h, reaching plateaus after 8 to 18 h (about 55% amebic killing). These results thus establish the promonocyte as highly amebicidal.

**Activation of amebicidal activity by macrophages.** As noted previously by other investigators (9), we observed that unstimulated murine peritoneal macrophages or BM-derived macrophages were inefficient as killing E. histolytica, regardless of the macrophage/ameba ratio (10:1 to 1,000:1). Whereas ameba viability stayed constant, the percent viability of the macrophage decreased steadily during the interaction. In three separate experiments, at an effector/target ratio of 100:1, after 6 h of interaction, viability of
amebae was 93 ± 3%, whereas viability of macrophages was 63 ± 12%.

It has been shown before that acquisition of amebicidal activity occurred in monocyte-derived human macrophages following activation with LKs; this activation was due, in part, to IFN-γ as shown by depletion studies with anti-IFN-γ antibodies (21). Therefore, it was decided to probe further the potential of various cytokines to activate macrophages to kill ameba. Preliminary experiments showed that LK-rich fluid could activate peritoneal macrophages to kill amebae in a standard assay system (68 ± 13% of killing; effector/target ratio of 100:1 after a 6-h incubation time). This acquisition of killing activity was neutralized by the addition of anti-IFN-γ antibodies (1,500 neutralizing units/ml; 21 ± 16% killing of amebae) and to a lesser extent by anti-TNF antibodies (3,000 neutralizing units/ml; 51 ± 4% killing) as compared with 71 ± 6% in control cultures in the LK-rich fluid during the activation of effector macrophages.

In another set of experiments, recombinant IFN-γ was used to activate BM-derived macrophages in the presence or absence of LPS. Table 1 shows that treatment of macrophage monolayers with 100 to 1,000 U of IFN-γ 18 h before assessment of amebicidal activity led to significant killing of E. histolytica trophozoites in the absence of LPS (22 to 42% killing). The addition of LPS (20 to 50 ng/ml)−IFN-γ preparations led to a significant increase in the percentage of amebicidal activity, indicating that two signals are required to express high levels of cytotoxicity against E. histolytica, as described in macrophage killing of amebae, tumor cells, and L. enrietti (9, 16, 17). LPS (20 to 50 μg/ml) alone did not activate macrophages to kill amebae. Regardless of the macrophage activation regimen used, a high proportion (35 to 51%) of macrophages were killed during the interaction with amebae, whereas macrophages not in contact with amebae (i.e., incubated alone) consistently maintained >95% viability. Contrary to the findings of Salata et al. (22), we could not see a correlation between levels of macrophage survival and amebicidal activity. The reason(s) for this discrepancy remains unclear. Control studies also showed that IFN-γ with or without LPS at the doses used was not cytotoxic by itself against E. histolytica trophozoites in the absence of macrophages.

Other cytokines were tested, notably TNF-α, to determine their potential to activate macrophages to express amebicidal activity. TNF-α, used at 10 and 102 U, was ineffective at enhancing killing of E. histolytica trophozoites by BM-derived macrophages (Table 2). Addition of higher doses of TNF-α (103 to 105 U) led to significant enhancement of killing (15 to 43%) of E. histolytica trophozoites.

By using different cytokine combinations, it could be shown that TNF-α could synergize with IFN-γ. This pheno-

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**TABLE 1. Cytokine regulation of macrophage- E. histolytica interaction**

<table>
<thead>
<tr>
<th>Macrophage treatment</th>
<th>% Amebic viability (± SD)</th>
<th>% Dead macrophages (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>97 ± 2</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>IFN-γ (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>76 ± 7*</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>300</td>
<td>72 ± 6*</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>500</td>
<td>68 ± 9*</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>750</td>
<td>69 ± 11*</td>
<td>31 ± 8</td>
</tr>
<tr>
<td>1,000</td>
<td>56 ± 9**</td>
<td>51 ± 13</td>
</tr>
<tr>
<td>IFN-γ (300 U/ml) + LPS (20 ng/ml)</td>
<td>57 ± 7**</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>IFN-γ (100 U/ml) + LPS (50 ng/ml)</td>
<td>52 ± 11**</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>IFN-γ (300 U/ml) + LPS (50 ng/ml)</td>
<td>40 ± 10**</td>
<td>43 ± 6</td>
</tr>
</tbody>
</table>

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a All experiments were performed with BM-derived macrophages in a standard killing assay (effector/target ratio of 100:1 and 6-h incubation). b Macrophages were incubated for 18 h with cytokines before interaction with ameba. c Means ± standard deviations of one set of experiments repeated twice with similar results. *P < 0.05 compared with untreated cells; **P < 0.01 compared with untreated cells.

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**FIG. 1. Interaction between E. histolytica trophozoites and promonocytes at different effector/target ratios. Viability of E. histolytica trophozoites was assessed after 6 h by trypan blue exclusion. Viability of control amebae was 96 ± 2% in three separate experiments. Results are expressed as means ± standard errors of three separate experiments. By Student’s t test, compared with control, * = P < 0.01, ** = P < 0.001, and *** = P < 0.0001.

**FIG. 2. Interaction between promonocyte and E. histolytica trophozoites (50:1 ratio). Viability of the E. histolytica trophozoites was assessed by trypan blue exclusion. Representative results are from three experiments which gave similar results. By Student’s t test, * = P < 0.05 and ** = P < 0.001, compared with control. Viability of control amebae varied from 97 to 92% (0 to 8 h of incubation) to 85 to 76% (10 to 18 h of incubation).
nomenon was seen in BM-derived macrophages treated with low doses of IFN-γ (10² U/ml) and TNF-α (10² to 10³ U/ml). These doses of cytokines were by themselves inefficient at enhancing amebicidal activity; however, in combination, they endowed BM-derived macrophages with significant (73 to 87%) killing activity. Treatment of macrophages with CSF-1 (10² and 10⁴ U/ml) rendered BM-derived macrophages marginally amebicidal (P < 0.05). However, incubation of BM-derived macrophages with CSF-1 and IFN-γ (10³ U/ml) rendered macrophages highly cytotoxic (82% killing) for *E. histolytica* trophozoites in a synergistic fashion.

**Lack of amebicidal activity by IFN-γ and TNF-α in the absence of macrophages.** The above experiments did not distinguish between the possible role of TNF-α as a stimulus of macrophage effector functions or as cytokytic molecules by itself, although the latter possibility was considered unlikely. Furthermore, synergy between IFN-γ and TNF-α for direct tumor cell cytotoxicity has been described previously (26). Therefore, experiments were designed to probe the ability of IFN-γ and TNF-α to kill amebae in the absence of viable macrophages. In three separate experiments, amebae in TYI-S-33 complete medium were supplemented with IFN-γ or TNF-α or both (100 to 1,000 U/ml), and after 20 to 72 h, the number of viable cells was measured. The cytokines did not kill amebae or prevent parasite multiplication at the incubation times. In addition, macrophages treated with IFN-γ and TNF-α at doses that induce amebicidal activity, which were subsequently fixed with formaldehyde, did not kill amebic trophozoites (0 to 4% killing in three different experiments). These results suggest that killing is performed only by viable activated cells and does not result from absorption of TNF-α on the cell surface.

**Treatment of different populations of macrophages with active LKS.** Having demonstrated that cytokines are involved in the acquisition of amebicidal activity by BM-derived macrophages, we decided to investigate the effect of these cytokines on activation of other populations of macrophages to kill virulent amebae. Table 2 shows that the most potent combination, IFN-γ and TNF-α, failed to endow spleen macrophages with full amebicidal activity (about 20% amebic killing), whereas peritoneal macrophages were fully activated to kill up to 88% of amebae (Table 2).

**Respiratory burst in macrophages activated with various cytokines.** To seek a possible link between respiratory burst activity and amebicidal activity, the release of H₂O₂ by activated macrophages was assessed following phorbol myristate acetate (PMA) triggering. Table 2 shows that BM-derived macrophages demonstrated enhanced burst after activation with TNF-α and IFN-γ or combinations thereof. Since these preparations also endowed macrophages with high levels of amebicidal activity, this seemed to suggest a correlation between secretion of H₂O₂ and killing. However, some IFN-γ and cytokine combinations which were relatively inactive at endowing cells with amebicidal activity were very efficient at priming macrophages for enhanced H₂O₂ release, indicative of a certain dissociation between H₂O₂ release and amebic killing. This was notable in the case for spleen macrophages pretreated with TNF-α and IFN-γ, which, although endowed with high H₂O₂ release potential, killed amebae to a marginal (20%), although significant, extent. This is in contrast to BM-derived macrophages treated with IFN-γ and TNF-α that had the same level of respiratory burst activity but in which up to 73% amebicidal activity was observed (Table 2).

**Dissection of the mechanism(s) of amebicidal activity in LK-LPS-activated macrophages.**

(i) **Importance of macrophage-amebae contact.** It was next decided to investigate further the mechanisms involved in the acquisition of amebicidal activity by macrophages. In the killing of most extracellular targets, binding to targets is a necessary step in the cytosis (13, 15). To assess the requirement for cell contact in the described amebicidal activity, LK-LPS-activated peritoneal macrophages were separated from amebic trophozoites by using a membrane filter (0.45 μm) permeated with pores that allowed movement of fluid, but not cells. In four experiments, the level of amebicidal activity decreased from 68 ± 4% when cells and amebae were not separated to 12 ± 3% when they were separated by the filter, suggesting a requirement for cell contact.

(ii) **Effect of protease inhibitors and inhibitors of protein synthesis.** The effect of adding protease inhibitors to prevent amebicidal activity by activated peritoneal macrophages was tested. Only TLCK at 2 × 10⁻⁴ M significantly (62%) inhibited amebicidal activity. In contrast, bovine pancreatic trypsin inhibitor, α-1-antitrypsin, and aprotonin were wholly inactive at reversing amebicidal activity. None of the agents at the doses used were toxic for macrophages or amebae (Table 3).

In addition, the effect of inhibiting protein synthesis on the ability of activated macrophages to kill amebic targets was probed. Results shown in Table 3 suggest that prevention of protein synthesis by cycloheximide and actinomycin D was highly detrimental to LK-activated macrophages in terms of amebicidal activity.

(iii) **Effect of reactive oxygen intermediate scavengers.** In a last set of experiments, it was decided to investigate the involvement of reactive oxygen intermediates in the killing of *E. histolytica* by activated macrophages. Accordingly, LK-LPS-activated macrophages were incubated with amebae in the presence or absence of various inhibitors of reactive oxygen intermediates (Table 4). It was found that catalase inhibited killing by 45%, whereas superoxide dis-

### TABLE 2. Cytokine regulation of macrophage-<em>E. histolytica</em> interaction

<table>
<thead>
<tr>
<th>Macrophage source</th>
<th>Treatment (U/ml)</th>
<th>% Amebic viability (± SD)</th>
<th>H₂O₂ production (nmol/mg per h)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMDM</td>
<td>None</td>
<td>97 ± 2</td>
<td>91 ± 11</td>
<td>5</td>
</tr>
<tr>
<td>BMDM</td>
<td>TNF-α (10)</td>
<td>91 ± 6</td>
<td>89 ± 8</td>
<td>8</td>
</tr>
<tr>
<td>BMDM</td>
<td>TNF-α (10⁴)</td>
<td>94 ± 5</td>
<td>109 ± 12</td>
<td>7</td>
</tr>
<tr>
<td>BMDM</td>
<td>TNF-α (10³)</td>
<td>83 ± 8</td>
<td>156 ± 24</td>
<td>8</td>
</tr>
<tr>
<td>BMDM</td>
<td>TNF-α (10⁶)</td>
<td>71 ± 6*</td>
<td>209 ± 15</td>
<td>7</td>
</tr>
<tr>
<td>BMDM</td>
<td>TNF-α (10⁴)</td>
<td>56 ± 8*</td>
<td>279 ± 38</td>
<td>6</td>
</tr>
<tr>
<td>BMDM</td>
<td>IFN-γ (10³) + TNF-α (10³)</td>
<td>26 ± 9**</td>
<td>295 ± 32</td>
<td>7</td>
</tr>
<tr>
<td>BMDM</td>
<td>IFN-γ (10³) + TNF-α (10⁴)</td>
<td>11 ± 3***</td>
<td>436 ± 77</td>
<td>5</td>
</tr>
<tr>
<td>BMDM</td>
<td>CSF-1 (10³)</td>
<td>89 ± 8</td>
<td>112 ± 9</td>
<td>7</td>
</tr>
<tr>
<td>BMDM</td>
<td>CSF-1 (10⁴)</td>
<td>71 ± 12*</td>
<td>136 ± 25</td>
<td>5</td>
</tr>
<tr>
<td>BMDM</td>
<td>CSF-1 (10⁴) + IFN-γ (10⁴)</td>
<td>17 ± 11***</td>
<td>211 ± 32</td>
<td>7</td>
</tr>
<tr>
<td>Spleen</td>
<td>TNF-α (10³) + IFN-γ (10³)</td>
<td>79 ± 8*</td>
<td>286 ± 79</td>
<td>8</td>
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<tr>
<td>PEC</td>
<td>TNF-α (10³)</td>
<td>77 ± 6*</td>
<td>149 ± 19</td>
<td>8</td>
</tr>
<tr>
<td>PEC</td>
<td>IFN-γ (10³)</td>
<td>51 ± 10**</td>
<td>208 ± 33</td>
<td>8</td>
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<tr>
<td>PEC</td>
<td>TNF-α (10³) + IFN-γ (10³)</td>
<td>11 ± 6**</td>
<td>387 ± 81</td>
<td>8</td>
</tr>
</tbody>
</table>

*Macrophages were treated for 18 h with the respective cytokine(s) before assay of amebic activity. BMDM, Bone marrow-derived macrophages; PEC, peritoneal exudate cells.

*Compared with untreated controls: *P < 0.05; **P < 0.01; ***P < 0.001.

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TABLE 3. Effect of various treatments on amebicidal activity of activated macrophages

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Concentration (µg/ml)</th>
<th>% Amebic Viability ± SD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPTI</td>
<td>10^3–10^5 KIU/ml</td>
<td>41 ± 8</td>
</tr>
<tr>
<td>α-1-Antitrypsin</td>
<td>100–400 µg/ml</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>TLCK</td>
<td>2 × 10^4 M</td>
<td>78 ± 8*</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>10^3–10^4 KIU/ml</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>10 µg/ml</td>
<td>69 ± 3*</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>50 µg/ml</td>
<td>81 ± 7*</td>
</tr>
</tbody>
</table>

*LK-LPS-activated peritoneal exudate cell macrophages (see Materials and Methods) were used in a standard killing assay. BPTI, Bovine pancreatic trypsin inhibitor. Shown are representative results of one experiment repeated twice with similar results.

†KIU, Kallikrein inactivation units.

‡ Concentrations of scavengers were selected based on previous studies. Inhibitor activity was expressed as percentage of amebic viability compared to control, which is defined as 100%.

The results presented show that potent amebicidal activity can be spontaneously performed by mouse promonocytes and murine macrophages activated with a variety of cytokines, following treatment with recombinant TNF-α, recombinant IFN-γ, and CSF-1. Furthermore, we identified some of the parameters involved in this killing activity.

Innate resistance represents the first defense of every organism against microbial, viral, and parasitic insults, especially in cases in which a quick drastic reduction of the pathogenic burden is decisive. Natural cytotoxic effectors have been described in a variety of systems, notably mediated by cytolytic activities of immature cells obtained from BM cultures (2). This cytolytic activity encompasses a broad range of targets, from YAC-1 lymphoma cells to Leishmania promastigotes and amastigotes and Candida albicans (2, 3).

The effector cells mediating spontaneous cytotoxicity are heterogeneous. Much attention has been given to natural killer cells, large granular lymphocytes, and mature macrophages. Baccarini et al. (2) have recently described strong spontaneous cytotoxicity exerted by nonadherent nonphagocytic cells in the earlier stages of macrophage differentiation. Using virulent E. histolytica trophozoites, we demonstrated that the nonphagocytic macrophage precursors are highly amebicidal. Of interest is that this cell type can be found in the spleen and liver of normal mice and in infected mice in much greater numbers. It thus remains to be seen whether these promonocytes can be of importance in relevant in vivo situations in invasive amebiasis.

Our study used an purification procedure described by others which led to >95% pure macrophage precursors. Of importance in our study was the finding that macrophage precursors were highly resistant to killing by E. histolytica, which is a very potent effector cell in its own right (2). Accordingly, macrophage precursors proved to possess an overall amebicidal activity much more elevated than unstimulated mouse or human macrophages. The BM-derived macrophage precursor activity was lost during in vitro maturation to mature macrophages, as described previously for the competence to kill YAC-1 lymphoma cells and C. albicans (2).

Another aspect studied in this report is the ability of various cytokines to endow macrophages with amebicidal activity as well as the mechanism(s) involved in this phenomenon. A first finding was the efficiency of recombinant IFN-γ in mediating acquisition of amebicidal activity in both systems. In the mouse macrophage-ameba interaction, the addition of trace amounts of endotoxin (LPS) rendered IFN-γ-activated murine macrophages more cytolytic for E. histolytica trophozoites, as had been demonstrated previously (9).

Cytokines other than IFN-γ were also used to activate murine macrophages to kill E. histolytica trophozoites in vitro. TNF-α was found to be capable of endowing macrophages with significant amebicidal activity. Synergy between IFN-γ and TNF-α was seen when both cytokines were used at doses too low to activate killing by themselves. This synergy between IFN-γ and TNF-α has already been observed in macrophage killing of schistosomula and P. brasiliense targets (8). CSF-1 also was shown to be quite active at endowing BM-derived macrophages with amebicidal activity. This is in keeping with recent reports describing the ability of CSF-1 to activate macrophages to perform superior effector functions (14, 30).

The importance of oxidative intermediates in macrophage-mediated killing of E. histolytica trophozoites was shown to be about 43% in our assay. It was decided to investigate the role of nonoxidative mechanisms in this phenomenon.

Studies with tumor cells as targets have indicated that contact between activated macrophages and tumor cells plays a role in target killing by stimulating the release of toxic factors, as well as in protecting these molecules from inhibitors potentially present in the extracellular surroundings. Our studies indicate that contact is essential for effective amebic killing, since separation of the effector cells from targets by a permeable membrane (0.45-µm pores) significantly prevented killing activity by LK-activated macrophages.

Interaction of cells with E. histolytica does not seem to require divalent cations, as is the case with tumor cell binding, since EDTA did not inhibit amebic killing activity in our system. The importance of the galactose-α1,3N-acetyld-galactosamininhibitable adherence lectin of E. histolytica remains to be assessed in this system. Attempts to inhibit amebic adherence to macrophages in the presence of galactose (55 to 100 mM) gave inconclusive results, presumably due to rapid degradation of galactose by amebic enzymes. Inhibitors of protein synthesis, cycloheximide (10 µg/ml) and actinomycin D (50 µg/ml), prevented amebic killing by LK-treated macrophages, indicating that macrophage-killing molecules are synthesized de novo after cytokine treatment or that the release of cytolytic molecules requires protein.

TABLE 4. Effect of reactive oxygen intermediate scavengers on amebicidal activity of activated macrophages

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Concentration (µg/ml)</th>
<th>% Amebic Viability ± SD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>31 ± 8</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>1 mg/ml</td>
<td>37 ± 14</td>
</tr>
<tr>
<td>Histidine</td>
<td>10 mM</td>
<td>22 ± 15</td>
</tr>
<tr>
<td>Mannitol</td>
<td>50 mM</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>Catalase</td>
<td>1 mg/ml</td>
<td>62 ± 9*</td>
</tr>
<tr>
<td>Boiled catalase</td>
<td>1 mg/ml</td>
<td>34 ± 9</td>
</tr>
</tbody>
</table>

*Peritoneal exudated cell macrophages were incubated in LK-LPS-rich supernatant fluid, as described previously in a standard killing assay. Values are representative results of one experiment repeated twice with similar results. *P < 0.002 by Student’s t test compared with controls.
synthesis. Similar findings were reported in the killing activity of LK-activated macrophages against Schistosoma mansoni larvae (13).

Soluble cytolsins produced by activated macrophages, e.g., neutral serine protease (1), have been described previously by several groups (1, 28). Various groups have reported inhibition of macrophage killing ability in the presence of the protease inhibitors bovine pancreatic trypsin inhibitor and TLCK (1) or in the presence of TLCK only (12, 13). Of the tryptic and chymotryptic protease inhibitors used in our assay system, TLCK prevented parasite killing by up to 61%. This, together with the 45% inhibition of amebic killing with catalase, which scavenges H2O2, appears to be the mechanism by which macrophages exert a direct amebicidal effect, i.e., a combination of oxidative (H2O2) and nonoxidative (proteases) mechanisms.

Patients treated for amebic liver abscess develop a cell-mediated immune response against E. histolytica. This includes a specific T-lymphocyte proliferative response to amebic antigen production of LKs that activate macrophages in vitro and antigen-specific cytotoxic T-cell activity (20, 23, 24). This led to the widely accepted belief that invasive amebiasis triggers an efficient immune response in humans and other animals. Macrophages are among the cells which express this acquired resistance. This study indicates that cells of the macrophage compartment can either exert spontaneous cytotoxicity (promonocytes) or acquire this potential after appropriate cytokine treatment (macrophages) and can have important implications in host defense mechanisms against amebiasis.

ACKNOWLEDGMENTS

K. Chadee is a Natural Sciences and Engineering Research Council of Canada (NSERC) University Research Fellow. This work was supported by grants from NSERC and the Medical Research Council of Canada (MA 10187) to K. Chadee. M. Denis is the recipient of a postdoctoral fellowship from Fonds de Recherche en Santé du Québec. Research at the Institute of Parasitology is supported by NSERC and the Fonds FCAR pour l’Aide à la Recherche.

We thank Kathy Keller for technical assistance and Shirley Mongeau for secretarial assistance.

LITERATURE CITED


