SUSCEPTIBILITY OF *LEISHMANIA* TO OXYGEN INTERMEDIATES AND KILLING BY NORMAL MACROPHAGES*

By HENRY W. MURRAY‡

From The Cornell University Medical College, New York 10021

In previous reports, we demonstrated that the enhanced capacity of immunologically activated macrophages to generate toxic oxygen intermediates beyond superoxide anion (O_2^-)[1] and hydrogen peroxide (H_2O_2) contributes importantly to the killing and inhibition of growth of *Toxoplasma gondii* (1, 2). In contrast, unperturbed resident peritoneal cells from normal mice and those elicited by inflammatory agents display comparatively little oxidative activity and fail to restrict the replication of this intracellular parasite (1, 2). The ability of *T. gondii* to successfully parasitize resident and inflammatory macrophages also appears to result from two other related observations: ingested toxoplasmas avoid effective triggering of the oxidative burst of nonactivated cells (2, 3), and the parasite itself is intrinsically resistant to the toxicity of either O_2^- or H_2O_2 alone (4). The latter finding may reflect particularly rich endowment of *T. gondii* with enzymatic scavengers of O_2^- and H_2O_2 including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPO) (5).

This study extends such an analysis to an additional intracellular protozoan, *Leishmania*, which also readily parasitizes mononuclear phagocytes (6). These observations indicate that virulent parasites such as *Leishmania* and *T. gondii* vary widely in their susceptibility to reactive oxygen products, and that such differences are reflected in the fate of these pathogens within both cell-free oxidative environments and the macrophage cytoplasm.

Materials and Methods

Parasites. *Leishmania donovani* (LD) and *Leishmania tropica* (LT) promastigotes were maintained by standard culture techniques at 25°C, and were passed weekly in Schneider's Drosophilia medium (Grand Island Biological Co., Grand Island, N. Y.) for LD, and in Medium 199 (Grand Island Biological Co.) for LT. Each medium contained 20% heat-inactivated fetal bovine serum (HIFBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

* Supported by U. S. Public Health Service grant AI-16963-01 and Rockefeller Foundation grant RF 78021.

‡ Recipient of the Andrew W. Mellon Teacher-Scientist Award, a Rockefeller Foundation grant, and a Research Career Development Award in Geographic Medicine.

[1] *Abbreviations used in this paper:* Con-A, concanavalin A; D_{10}HIFBS, Dulbecco's medium containing 10% HIFBS, penicillin, and streptomycin; DABCO, diazabicyclo-octane; GO, glucose oxidase; GPO, glutathione peroxidase; HIFBS, heat-inactivated fetal bovine serum; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose; LD, *Leishmania donovani*; LD_{50}, LD_{100}, flux of H_2O_2 which killed 50 and 100% of promastigotes, respectively; LPO, lactoperoxidase; LT, *Leishmania tropica*; NBT, nitroblue tetrazolium; O_2^-, superoxide anion; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase; and XO, xanthine oxidase.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/05/1302/14 $1.00
Volume 153 May 1981 1302–1315

The 1 S (Sudan) strain of LD (7) and LT strain 252 (from Iran), which were originally isolated from human infections, were generously provided by Dr. J. Keithly and Dr. A. Ebrahimzadeh, respectively, of The Cornell University Medical College, New York. Throughout this study, both LD and LT promastigotes remained virulent; LD caused fatal infections in golden hamsters (7), and LT produced characteristic footpad lesions accompanied by fatal visceralization of infection in susceptible BALB/c mice (A. Ebrahimzadeh, unpublished observations).

During the log phase of growth, promastigotes were harvested from the culture medium by centrifugation, washed in phosphate-buffered saline (PBS), pH 7.4, and were resuspended in PBS for sonication, in PBS or Krebs-Ringer phosphate buffer with 5.5 mM glucose (KRPG), pH 7.4, for cell-free microbicidal experiments, or in Dulbecco's modified Eagle's medium containing 10% HIFBS, penicillin, and streptomycin (D_{10}HIFBS) for infection of macrophage monolayers (1). *T. gondii* trophozoites were obtained from infected mouse peritoneal exudates (4), and were administered to macrophages as previously reported (1, 2).

Cells. Resident peritoneal cells from inbred male BALB/c, DBA/2, and CBA/ca mice (The Jackson Laboratory, Bar Harbor, Maine) were harvested and cultivated on 12-mm round glass cover slips placed in 35-mm plastic tissue culture dishes (1). As classified by others (8, 9) and in this laboratory (10), BALB/c mice are considered susceptible to LD and LT infection, whereas DBA/2 are resistant to LD and CBA/ca are resistant to LT. Macrophages were also obtained from DBA/2 and BALB/c mice 10–14 d after an initial intraperitoneal injection of 0.2 ml (1.4 mg) of formalin-killed *Corynebacterium parvum* (Coparvax; Welcome Research Laboratories, Beckenham, Kent, England) followed by an intraperitoneal boosting dose (0.2 ml) 3 d before cell harvest (2). For all experiments, macrophages were cultivated overnight before assay or infection in either D_{10}HIFBS alone or D_{10}HIFBS plus 25% active or control concanavalin A (Con-A)-stimulated spleen cell supernates which were prepared as described elsewhere (2).

Cell-free Microbicidal Assays. Washed promastigotes were suspended in either KRPG (for the glucose oxidase [GO] reaction [4]) or PBS (for the xanthine oxidase [XO] reaction [4]). 5×10^6 promastigotes were added to the components indicated in the legends to the Tables and Figures in a final vol of 1 ml, and were incubated in 12- × 75-mm plastic tubes in a shaking 37°C water bath. After 60 min, 0.05-ml aliquots were placed on glass slides, overlayed with cover slips, and the motility and morphology of promastigotes were assessed using phase-contrast microscopy. Obvious swelling and distortion of the promastigote body readily identified lysed organisms. In addition, promastigote viability was also examined by pelleting control and treated organisms, and resuspending them in 2 ml of culture medium. After 2–3 d at 25°C, samples of these cultures were examined microscopically for persistence and/or multiplication of promastigotes.

Leishmania Enzyme Activities. 1×10^8–3×10^8 washed promastigotes in 1 ml of PBS (representing 0.8–2.4 mg of parasite protein/ml) were twice sonicated for 60 s (Cell Disruptor; 50% duty cycle; output control 4; Heat Systems-Ultrasonics, Inc., Planview, N. Y.). Sonicates were cleared by centrifugation at 4°C for 15 min at 8000 *g*, and the supernates were assayed spectrophotometrically for SOD (11), catalase (12), and GPO (13) as described (5). Examination of the sonicate pellet revealed only a rare intact promastigote. Supernate protein was determined by the method of Lowry et al. (14), and enzyme activities are expressed per milligram of parasite protein. Boiling the sonicate supernates for 20 min ablated the activity of all three enzymes.

Qualitative Nitroblue Tetrazolium (NBT) Reduction. Cultivated macrophages were exposed for 60 min at 37°C to either 5×10^6 promastigotes, toxoplasmas, or opsonized zymosan particles suspended in 1 ml of D_{10}HIFBS containing 0.5 mg/ml of NBT (grade III; Sigma Chemical Co., St. Louis, Mo.). Cover slips were washed and fixed with 1.25% glutaraldehyde. Attached or ingested parasites or particles were identified using phase-contrast optics, and then were viewed by bright-field microscopy. Macrophages were scored as positive if ingested organisms or zymosan were deeply stained blue-black by precipitated formazan (2).

Assays for O_2^- and H_2O_2 Generation. The production of O_2^- by the xanthine-XO reaction was measured by the ferricytochrome *c* reduction method (11) using the extinction coefficient $\Delta E_{550\,nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ (15). The H_2O_2 generated by glucose-GO interaction and that released by macrophages during phagocytosis of promastigotes was assayed by the fluorometric scopoletin technique (16). For the latter experiments, dishes containing three cover slips were

thoroughly washed and overlayed with 1.5 ml of KRPG containing promastigotes, 5×10^6/ml; scopoletin, 10 nmol/ml; and horseradish peroxidase, 0.44 purpurogallin units/ml (1, 2). After up to 2 h at 37°C (water bath), the reaction medium was aspirated, centrifuged at 750 *g* for 15 min to pellet the organisms, and the supernate was assayed for scopoletin oxidation. Adherent cell protein was determined using uninfected duplicate cover slips (2). Infected cover slips were washed once, fixed, and the percent of macrophages which had ingested promastigotes was enumerated after Giemsa staining. Control preparations for H_2O_2-release experiments consisted of identically processed dishes containing (*a*) cells alone, (*b*) parasites alone, and (*c*) blank dishes with neither.

Infection of Untreated, Scavenger-treated, and Glucose-deprived Macrophages. Overnight macrophage cultures were challenged for 2 h at 37°C in 5% CO_2 with 5×10^6 promastigotes suspended in 1 ml of D_{10}HIFBS. More than 85% of the infecting promastigotes were motile. For experiments examining the effects of oxygen intermediate scavengers or glucose deprivation, cultures were first incubated for 3 h before infection with medium containing no glucose (2) or containing SOD (2 mg/ml), catalase (2 mg/ml), mannitol (50 mM), benzoate (10 mM), histidine (10 mM), or diazabicyclooctane (DABCO, 1 mM), which were obtained and prepared as described elsewhere (2). For these experiments, the challenge promastigote inoculum was also suspended in medium free of glucose or containing the same concentration of scavenger. After the 2-h infection period, uningested parasites were removed by washing, and cultures were reincubated for up to 18 h in standard medium alone. At the indicated times, duplicate cover slips were removed and fixed in either glutaraldehyde or methanol, and the percent of cells infected and number of intracellular *Leishmania* per 100 macrophages was recorded after Giemsa staining. For the early time points, cover slips were fixed in 1.25% glutaraldehyde which better preserved macrophage cytoplasmic morphology and allowed accurate counting of intracellular parasites using phase contrast optics. For the later examinations, absolute methanol was used because this fixative rendered the amastigote form (to which the promastigote transforms) more easily identifiable using bright-field microscopy.

Special Reagents. GO (type V), XO (milk type, 65 mg/ml), xanthine, scopoletin, horseradish peroxidase, and ferricytochrome *c* (type VI) were obtained from the Sigma Chemical Co. Xanthine was prepared in 0.05 M potassium phosphate buffer (pH 7.8) with EDTA (10^{-4} M). Lactoperoxidase (lyophilized, B grade) was from Calbiochem-Behring Corp., American-Hoechst Corp., San Diego, Calif., and was assayed by the orthodianisidine method before use (17).

Statistical Analysis. Results were analyzed by the paired-sample *t* test.

Results

Susceptibility of Leishmania to H_2O_2 and H_2O_2-peroxidase-halide. We first examined the susceptibility of promastigotes to fluxes of H_2O_2 by exposing them to glucose and GO, a reaction that generates no oxygen intermediate other than H_2O_2 (18). As shown in Fig. 1 A, all LT and most LD promastigotes were readily immobilized or lysed after exposure for 1 h to as little as 5 nmol H_2O_2/min. Assuming no catabolism, the concentration of H_2O_2 in the medium after this period would approximate 3×10^{-4} M. LT promastigotes were considerably more susceptible than LD to H_2O_2; appreciable effects were seen with 0.5 nmol/min and almost all LT (90%) were immobilized by 1 nmol/min. For both strains, killing was abolished upon the addition of 100 μg/ml of catalase. Lack of motility proved to be a reliable index of killing because all promastigotes exposed to 100% lethal (LD_{100}) fluxes of H_2O_2 (5 nmol/min for LT; 10 nmol/min for LD, Fig. 1 A) subsequently lysed and failed to persist in culture when examined 48–72 h later. Parasites incubated in KRPG alone or with the respective LD_{100} of H_2O_2 in the presence of catalase (100 μg/ml), however, remained motile, survived, and readily multiplied after 48 h (data not shown). Heated catalase had no protective effect.

Although macrophages lack granular peroxidase (17), monocytes do contain mye-

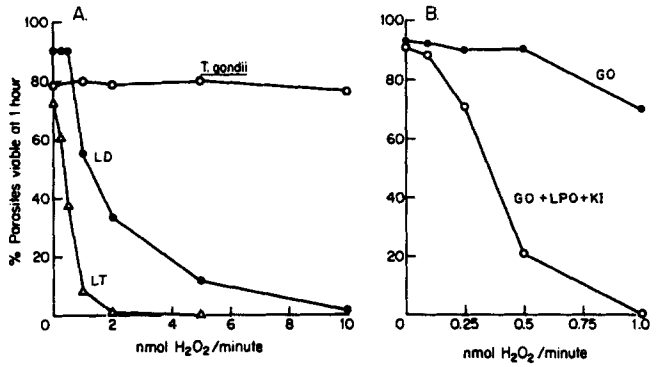

FIG. 1. Susceptibility of *Leishmania* to H_2O_2. (A) 5 × 10^6 promastigotes in a final vol of 1 ml of KRPG were incubated for 1 h at 37°C with various dilutions of GO which generated 0.25–10 nmol H_2O_2/min. Data for *T. gondii* are from a previous report (5), and are included for comparison. At 1 h, the 50% lethal dose (LD_{50}) of H_2O_2 was 1.5 nmol/min for LD and 0.5 nmol/min for LT. (B) 5 × 10^6 LD promastigotes were incubated as above with GO alone or GO plus LPO (10 mU) and KI (0.05 μmol). Enhanced killing was abolished by the omission of either GO, LPO, or KI, or if catalase (100 μg/ml) was included. Results for (A) and (B) are the means of three experiments.

loperoxidase and phagocytize *Leishmania* (19); thus we also investigated whether promastigote susceptibility to H_2O_2 could be further augmented by the addition of a peroxidase and an oxidizable halide cofactor. This potent oxidizing system has been shown to be highly microbicidal against virtually all classes of microorganisms (20) including protozoans such as *T. gondii* (4). As illustrated in Fig. 1 B, killing was markedly enhanced in the presence of lactoperoxidase (LPO) and KI, and a comparable leishmanicidal effect could be achieved with 5- to 10-fold less H_2O_2. In addition, 100% lysis of LD by 1 nmol of H_2O_2/min was observed within 15 min in the presence of LPO and KI, whereas 60 min and 10-fold more H_2O_2 were required for similar killing by GO alone. The susceptibility of LT to H_2O_2 was also enhanced, and with LPO-KI, all LT were killed by 0.1 nmol H_2O_2/min, the lowest flux tested (data not shown).

Leishmania Susceptibility to Other Oxygen Intermediates. The effect of other oxygen intermediates was examined by exposing promastigotes to xanthine and XO, a reaction which generates O_2^-, H_2O_2, OH·, and probably $^1O_2$ (21). The dose-related killing of LD by XO is shown in Fig. 2. No appreciable effect was noted if either xanthine or XO was omitted, and LT were killed in a similar fashion (data not shown). Studies with soluble scavengers of oxygen intermediates (Table I) provided data indicating that H_2O_2 alone was sufficient for the leishmanicidal activity of the XO system. Thus, only catalase effectively inhibited killing, whereas SOD (which promotes the dismutation of O_2^- to H_2O_2) and scavengers and quenchers of OH· and $^1O_2$ (mannitol, benzoate, DABCO, and histidine) (21) all failed to do so. These findings also indicated that both LD and LT are resistant to up to 4.8 nmol of O_2^-/min because catalase, which does not diminish O_2^- formation, consistently reversed leishmanicidal activity. In addition, there was a reasonably close correlation between the extent of promastigote killing in the GO and XO models and the fluxes of H_2O_2 generated by each of these two enzymatic systems. Under the conditions employed in Table I, 4.8 nmol of O_2^-/min was formed which theoretically should yield 2.4 nmol of H_2O_2/min (21).

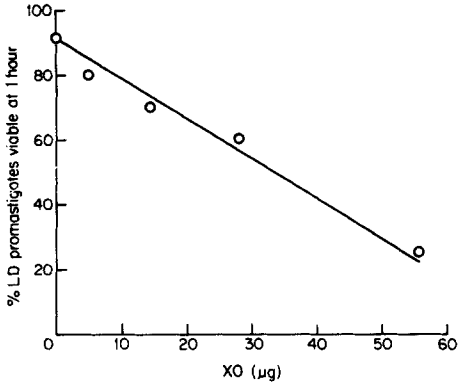

FIG. 2. 5×10^6 promastigotes were incubated with xanthine (1.5×10^{-4} M) and increasing amounts of XO in a final vol of 1 ml of PBS, pH 7.4. Killing was abolished if either xanthine or XO was omitted (see legend to Table I).

TABLE I

*Effect of Oxygen Intermediate Scavengers on Killing by XO**

Scavenger added	Percentage viable at 1 h	
	LD	LT
None	18 ± 7	21 ± 5
SOD, 100 μg/ml	25 ± 10	25 ± 5
Catalase, 100 μg/ml	62 ± 9	72 ± 5
Catalase, heated	26 ± 10	28 ± 7
Mannitol, 50 mM	18 ± 6	23 ± 5
Benzoate, 10 mM	15 ± 4	24 ± 6
DABCO, 1 mM	17 ± 6	—
Histidine, 10 mM	19 ± 8	—

* 5×10^6 promastigotes were incubated for 60 min at 37°C in a final vol of 1 ml of PBS, pH 7.4, with xanthine (1.5×10^{-4} M) and XO (50 μg/ml) with or without scavengers added in the indicated concentrations. As determined by the reduction of ferricytochrome *c* (11, 15), this xanthine-XO reaction generated 4.8 ± 0.3 nmol of O_2^-/min, which could be abolished by the addition of 25 μg/ml of SOD. Results are the means ± SEM of three to four experiments. The percent of viable promastigotes in the presence of PBS alone, PBS plus xanthine alone, or PBS plus XO alone was: 80 ± 7, 76 ± 4, and 74 ± 9 for LD, respectively; and 87 ± 4, 84 ± 3, and 82 ± 2 for LT, respectively.

Promastigote SOD, Catalase, and GPO Activities. The marked susceptibility of *Leishmania* to H_2O_2 suggested that these parasites may contain low levels of the protective endogenous scavengers catalase and GPO (22, 23). We have previously reported that *T. gondii* is richly endowed with these enzymes and is resistant to H_2O_2 (5). As shown in Table II, both LD and LT possess scant amounts of catalase and GPO at levels over 100-fold less than *T. gondii*. The SOD levels of *Leishmania* and *T. gondii* were, however, comparable and, as discussed, both protozoans appear to be resistant to O_2^- alone (5).

Promastigote Triggering of the Macrophage Oxidative Burst. Because mononuclear phagocytes generate and release O_2^- and H_2O_2 as well as other oxygen intermediates in response to plasma membrane perturbation (1, 15, 16, 24), we next explored whether

TABLE II
*Endogenous Parasite Scavengers of O_2^- and H_2O_2**

Parasite	SOD	Catalase	GPO
	U/mg	*BU/mg × 10^{-2}*	*nmol/min per mg*
T. gondii	6.1 ± 2.2 (4)	4.8 ± 0.4 (5)	117 ± 8 (3)
LD	4.1 ± 0.9 (3)	0.05 ± 0.02 (3)	0.5 ± 0.5 (3)
LT	6.4 ± 0.5 (3)	0.03 ± 0.02 (3)	0.2 ± 0.2 (3)

* Up to 3×10^8 parasites were sonicated in 1 ml of PBS, and assayed for SOD (11), catalase (Baudhuin units [BU] [12]), and GPO (15) activities as described (5). Results are the means ± SEM (number of experiments is in parentheses), and GPO activity (nanomoles of NADPH oxidized to NADP) has been corrected for the spontaneous oxidation of NADPH by H_2O_2 alone (15). Data for *T. gondii* is from a previous report (5).

promastigote ingestion effectively triggered the macrophage oxidative burst, including the production of the leishmanicidal agent, H_2O_2. During the course of these experiments, we also investigated whether macrophages from genetically susceptible and resistant mice could be distinguished by their oxidative response to *Leishmania*. As judged by the qualitative NBT reduction technique (2, 3), a reaction which is mostly O_2^--dependent (25), resident macrophages from both susceptible and resistant mice readily responded to phagocytosis of LD and LT with dense formazan precipitation around ingested promastigotes (Fig. 3, Table III). In some instances, it appeared that flagellar contact alone was sufficient to trigger NBT reduction. In contrast (Table III), as reported by others (3) and confirmed by us (2), *T. gondii* avoids triggering the oxidative burst of normal resident cells, which also permits its intracellular replication. Depriving macrophages of exogenous glucose inhibits their capacity to generate oxygen intermediates (2, 26), and this treatment resulted in a >80% decrease in the number of cells able to reduce NBT after ingestion of zymosan particles or *Leishmania* promastigotes (Fig. 3).

Because the results of the cell-free experiments indicated that H_2O_2 alone was sufficient for *Leishmania* killing, it was important to determine if promastigote ingestion stimulated macrophage H_2O_2 generation. As shown in Fig. 4, resident cells from both susceptible and resistant mice promptly released H_2O_2 to a similar extent in response to promastigote challenge. LT and LD were equally effective as triggers for macrophage H_2O_2 production which was dependent upon both the size and the duration of exposure to the challenge inoculum.

Fate of Intracellular Promastigotes and Effect of Exogenous Oxygen Intermediate Scavengers and Glucose Deprivation. The prior evidence indicating that promastigotes are both susceptible to H_2O_2 and trigger its generation by resident macrophages suggested that these cells should be able to exert leishmanicidal activity assuming effective delivery of sufficient H_2O_2. Fig. 5 demonstrates the prompt killing and digestion of intracellular LT and LD by all cells examined. There were no appreciable differences between the activities of BALB/c and DBA/2 macrophages against LD nor of BALB/c and CBA/ca cells toward LT, and therefore, data were pooled. As illustrated, LT were considerably more susceptible than LD to macrophage killing. *T. gondii*, however, readily persisted and multiplied in these same resident cells (1), and 18 h after infection there were 4–5 toxoplasmas/vacuole (data not shown). Macrophages from *C. parvum*-immunized mice and normal cells activated before infection by Con-A

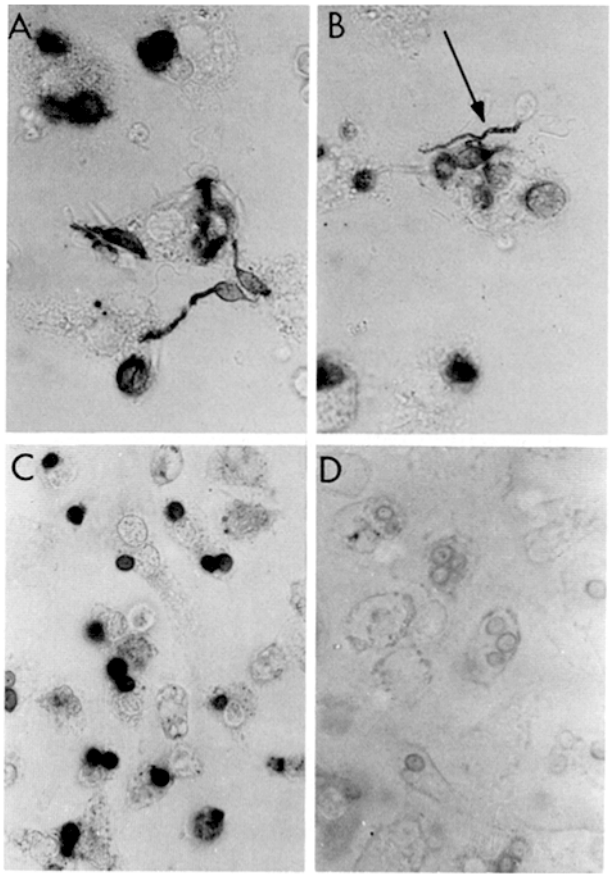

FIG. 3. Bright-field micrographs showing resident macrophage NBT reduction 1 h after challenge with 5×10^6 promastigotes or zymosan particles suspended in 1 ml of D_{10}HIFBS containing NBT (0.5 mg/ml). (A) Ingested promastigotes are deeply stained blue-black with precipitated formazan. (B) Flagellar contact alone (arrow) stimulates macrophage NBT reduction. Extracellular promastigote bodies are not stained. (C) Most intracellular zymosan particles are deeply stained with formazan. (D) Absence of precipitated formazan around intracellular zymosan indicates inhibition of macrophage NBT reduction. Cells are same as in (C), but were deprived of exogenous glucose 3 h before and during ingestion of zymosan. × 600.

lymphokine both eradicated more LD than unstimulated resident macrophages (Fig. 5).

To provide evidence indicating a role for oxidative metabolites in the leishmanicidal activity of normal resident cells, glucose-free medium and soluble scavengers of oxygen intermediates were administered to macrophages 3 h before and during the promastigote challenge. The latter agents included SOD (2 mg/ml), catalase (2 mg/ml), mannitol (50 mM), benzoate (10 mM), DABCO (1 mM), and histidine (10 mM). We have previously reported that treatment with these agents, as well as the absence of glucose, reverses the anti-toxoplasma activity of both in vivo and in vitro activated macrophages (1, 2). As shown in Table IV, both exogenous catalase and glucose deprivation significantly inhibited the destruction of intracellular promastigotes, and resulted in a twofold (for LD) to fivefold (for LT) increase in the proportion of the

TABLE III

*Qualitative NBT Reduction by Resident Macrophages**

Macrophage source	Percentage of cells with precipitated formazan 1 h after ingestion of			
	LD	LT	Zymosan	*T. gondii*
BALB/c	81 ± 4 (3)	82 ± 4 (3)	78 ± 4 (3)	21 ± 4 (3)
DBA/2	78 ± 3 (4)	81 ± 3 (3)	85 ± 2 (3)	15 ± 3 (3)
CBA/ca	—	85 ± 2 (3)	80 ± 5 (2)	16 ± 5 (3)

* Macrophages from BALB/c, DBA/2, and CBA/ca mice were challenged as in legend to Fig. 3 with 5 × 10^6 promastigotes, toxoplasmas, or zymosan particles for 1 h. Results are the mean ± SEM for experiments performed in duplicate (number of experiments is in parentheses). The addition of SOD (1 mg/ml) to the promastigote-NBT reaction mixture decreased the percent of cells reducing NBT by only 26 ± 3% (two experiments); however, glucose deprivation (see Fig. 3) inhibited NBT reduction by macrophages ingesting promastigotes and zymosan by 82 ± 6% and 86 ± 3%, respectively (three to four experiments). Data for *T. gondii* is similar to our previous results using cells from outbred mice (2). Neither extracellular parasites nor particles reduced NBT.

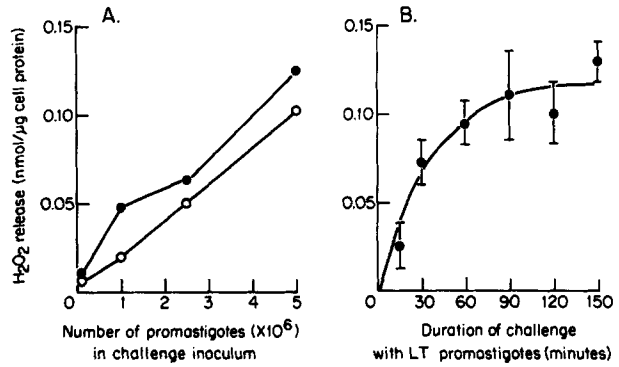

FIG. 4. (A) Resident macrophage H_2O_2 release after challenge with 0.1 × 10^6–5 × 10^6 LD promastigotes/ml for 2 h at 37°C. Cells were from BALB/c (●) or DBA/2 (○) mice, and results are the means of two experiments for each cell type performed in triplicate. After 2 h of exposure to 5 × 10^6 LD/ml, 46 ± 5% of BALB/c and 39 ± 4% of DBA/2 cells had ingested promastigotes. In four experiments, after a similar 2-h challenge with 5 × 10^6 LT/ml, BALB/c macrophages released 0.09 ± 0.02 and CBA/ca cells released 0.11 ± 0.03 nmol H_2O_2/μg protein with 50 ± 5% and 45 ± 6% of macrophages infected, respectively. (B) Resident CBA/ca macrophages were challenged for 15–150 min with 5 × 10^6 LT promastigotes/ml. Results are the means (± SEM) of three experiments in triplicate. Data in (A) and (B) have been corrected for the spontaneous oxidation of scopoletin in control dishes containing cells or promastigotes alone (Materials and Methods).

original intracellular inoculum remaining intact and transforming to the amastigote stage at 18 h. Heated catalase and the other five scavengers had no effect. Fig. 6 shows the morphologic counterpart of these observations for catalase-treated macrophages.

Discussion

The results of this study indicate that macrophages exert microbicidal activity against the infective promastigote form of LD and LT by an oxygen-dependent mechanism which generates H_2O_2. These two hemoflagellates are world-wide human

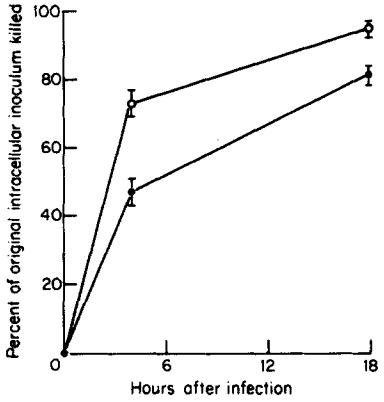

FIG. 5. Leishmanicidal activity of normal macrophages. Resident macrophages were challenged for 2 h with 5×10^6 LD (●) or LT (○) promastigotes, which resulted in infection of 51 ± 4% of cells with 85 ± 9 *Leishmania* per 100 macrophages (mean ± SEM). Immediately after the 2-h infection period (time 0) and at the indicated times, the number of *Leishmania* per 100 macrophages was determined. Data are expressed as the proportion (percentage) of the original intracellular inoculum present at time 0 which was subsequently digested 4 h and 18 h after infection. Results are the means ± SEM of 10 experiments with LD-infected macrophages (BALB/c: 3; DBA/2: 7) and 7 experiments for LT (BALB/c: 4; CBA/ca: 3). In three parallel experiments, the percent of the original LD inoculum killed at 4 h and 18 h was 67 ± 5 and 91 ± 3, respectively, for *C. parvum*-immunized DBA/2 cells and 65 ± 5 and 90 ± 1, respectively for normal DBA/2 cells activated by Con-A lymphokine ($P < 0.05$ for both cell types compared to unstimulated normal DBA/2 macrophages).

TABLE IV

*Effect of Exogenous Catalase and Glucose Deprivation on the Leishmanicidal Activity of Normal Macrophages**

Treatment	Percentage of original intracellular inoculum killed‡			
	LD		LT	
	4 h	18 h	4 h	18 h
None (control)	47 ± 4 (10)	81 ± 3 (10)	73 ± 4 (7)	95 ± 2 (7)
Catalase	28 ± 9 (3)	42 ± 10 (3)	38 ± 2 (3)	71 ± 3 (3)
Glucose deprivation	33 ± 4 (3)§	49 ± 8 (3)	—	—

* Normal resident macrophages were exposed to catalase (2 mg/ml) or deprived of exogenous glucose (1, 2) 3 h before and during the 2-h challenge with 5×10^6 promastigotes. Results are the means ± SEM (number of experiments is in parentheses), and leishmanicidal activity is expressed as in the legend to Fig. 5. Heated catalase (2 mg/ml), SOD, mannitol, benzoate, DABCO, and histidine all had no effect on promastigote killing.

‡ Results for all 4 h and 18 h catalase-treated cells and 18 h glucose-deprived cells are significantly different from untreated control macrophages ($P < 0.05$).

§ Not different from control cells ($P > 0.05$).

pathogens, and are the etiologic agents of visceral and cutaneous leishmaniasis, respectively. The present observations with *Leishmania* are similar to our previous findings with *T. gondii* (1, 2, 4, 5) in that they provide further evidence suggesting that the generation of toxic oxygen intermediates is a principal mechanism underlying the antiprotozoal activity of mononuclear phagocytes. At the same time, however, these studies also demonstrate clear differences between these intracellular parasites in

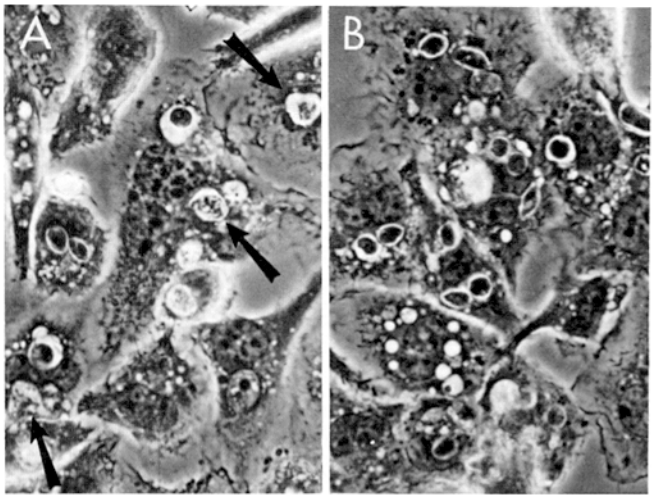

FIG. 6. Phase-contrast micrographs showing resident macrophages from BALB/c mice 4 h after challenge with LD promastigotes. (A) Approximately one-half of parasites ingested by untreated control cells have been degraded to intravacuolar debris (arrows). (B) In contrast, most LD ingested by catalase-treated macrophages appear phase-dense and intact. Giemsa stain, × 600.

terms of susceptibility to oxidative products, triggering of the macrophage oxidative burst, and fate within the cytoplasm of normal, unstimulated macrophages.

In a cell-free oxidative environment, 90–100% of LD and LT promastigotes were promptly killed by low fluxes (5 nmol/min) of enzymatically generated H_2O_2. The 50% lethal dose (LD_{50}) of H_2O_2 was 0.5 nmol/min for LT and 1.5 nmol/min for LD. In similar parasite models, the LD_{50} of H_2O_2 for *Trypanosoma cruzi* trypomastigotes was 9.4 nmol/min (27), whereas *T. gondii* was not appreciably affected by either 20 nmol of H_2O_2/min or 10^{-3} M reagent H_2O_2 (4). A plausible explanation for the enhanced sensitivity of *Leishmania* promastigotes to H_2O_2 and the contrasting resistance of toxoplasmas is the virtual absence of catalase and GPO in *Leishmania* and their abundance within *T. gondii* (5). Together, these enzymatic scavengers act as effective endogenous defense mechanisms against the toxicity of H_2O_2 (22, 23). We have previously suggested that catalase plays such a protective role for toxoplasmas because pretreatment with aminotriazole, a catalase inhibitor, enhances their susceptibility to H_2O_2 (5).

Data derived from exposing promastigotes to the xanthine-XO system, indicated that H_2O_2 alone was sufficient for leishmanicidal activity. Because killing by XO was inhibited by catalase and was unaffected by the O_2^- scavenger, SOD, it appeared that O_2^- by itself was not toxic (21). The presence of SOD within promastigotes may explain resistance to O_2^- alone. In addition, OH· and $^1O_2$, potent intermediates more distal to H_2O_2 in the reduction pathways of molecular oxygen (21), did not appear necessary for leishmanicidal activity. Thus, SOD, which in most systems inhibits OH· formation (5, 21, 24), failed to reverse killing, and proposed scavengers and quenchers of OH· and $^1O_2$ (mannitol, benzoate, DABCO, histidine [21]) also afforded no protection. These observations contrast with the toxoplasmacidal activity of the same XO system which appears to be mediated by neither O_2^- or H_2O_2 alone, but by products of their interaction, presumably OH· and $^1O_2$ (4).

Because the intracellular survival and replication of *T. gondii* within normal macrophages may be related in part to their capacity to avoid triggering the oxidative burst of these cells (2, 3), it was important to ascertain if *Leishmania* promastigotes also shared this ability. As indicated by NBT reduction (25) and H_2O_2 release, however, normal resident macrophages readily responded to promastigote ingestion with the generation of oxygen intermediates. Macrophage H_2O_2 release after phagocytosis of both LD and LT was comparable to that observed after stimulation with phorbol myristate acetate (2). Using assumptions and calculations determined by Nathan et al. (27), if the H_2O_2 (8 nmol/10^6 cells per h) released after promastigote ingestion were accumulated within the macrophage cytoplasm, its concentration would theoretically approach 2×10^{-2} M, far exceeding the LD_{100} of both LD and LT. The concentration of H_2O_2 within individual phagolysosomes would presumably be even higher. Thus, *Leishmania* promastigotes are not only susceptible to H_2O_2 in cell-free systems, but readily trigger its formation by macrophages in amounts which theoretically are more than sufficient for leishmanicidal activity. It was not surprising, therefore, to observe as have most investigators (9, 10, 28–32), that unstimulated resident macrophages killed and digested the bulk of phagocytized *Leishmania* promastigotes. These same cells, however, exert no activity against the intracellular protozoans *T. gondii* (1) and *T. cruzi* (27), both of which are considerably more resistant to H_2O_2 (4, 27). Macrophages first activated in vivo by *C. parvum* immunization or in vitro by lymphokine destroyed LD more efficiently than normal cells, consistent with the enhanced anti-microbial and oxidative capacity of immunologically activated macrophages (2, 16, 24, 27, 33).

Evidence suggesting that an oxygen-dependent mechanism participates in the killing of *Leishmania* promastigotes by normal macrophages was derived from examining the effects of soluble scavengers of oxidative metabolites and glucose deprivation. The latter technique markedly reduces macrophage H_2O_2 release (1, 26), and as demonstrated here (Fig. 3), also inhibits NBT reduction presumably by limiting macrophage O_2^- generation (25, 26). Of the six exogenous scavengers tested using our technique of preinfection administration (1, 2), only catalase influenced the destruction of intracellular *Leishmania*. Depriving macrophages of glucose achieved comparable effects; however, neither SOD nor scavengers and quenchers of $OH\cdot$ and $^1O_2$ prevented promastigote killing. Thus, these findings, which correlated with the results of the cell-free XO system, suggested that H_2O_2 was a key leishmanicidal oxygen intermediate. It should be pointed out, however, that although catalase and glucose deprivation inhibited the killing of both LD and LT, neither treatment prevented macrophages from eradicating a considerable proportion of intracellular *Leishmania* by 18 h (Table IV). Although glucose deprivation markedly diminishes the oxidative response of macrophages (1, 2, 26), this technique does not totally abolish H_2O_2 release (1) or NBT reduction. Similarly, pretreatment with exogenous catalase decreases but does not ablate macrophage H_2O_2, release (5). Thus, the residual capacity of glucose-deprived or catalase-treated macrophages to generate some H_2O_2 may still permit the killing of a portion of susceptible intracellular *Leishmania*. Alternatively, these observations might also reflect the presence of synergistic but oxygen-independent antiprotozoal mechanisms that appear to participate in the killing of *T. gondii* by human monocytes (3).

During the course of these studies, we also investigated whether macrophages from

mice classified on a genetic basis as susceptible or resistant to *Leishmania* infection (8–10) differed in their response to in vitro challenge with LD or LT. As judged by NBT reduction, H_2O_2 release, and the capacity to digest intracellular promastigotes, it appears that these susceptibility patterns are not reflected at the level of the unstimulated resident macrophage (9, 10). Using similar techniques and the amastigote form (10), we are currently investigating whether this genetic variability is expressed in other macrophage populations, including those activated by exposure to products generated by *Leishmania* antigen-stimulated lymphocytes.

Finally, it was important to initiate our studies of *Leishmania*-macrophage interaction using the promastigote because the hemoflagellate is the form first encountered by phagocytic cells. The amastigote, however, to which the promastigote rapidly transforms within phagolysosomes, is responsible for persistent tissue infection (6). Thus, although most LT and LD promastigotes can be readily eradicated in our model by unstimulated macrophages, there is a small proportion that resists killing and survive as amastigotes. These are presumably the organisms that proceed to replicate within and infect other mononuclear cells at local or distant sites culminating in cutaneous (LT) or visceral (LD) leishmaniasis, respectively (6). The mechanisms by which amastigotes resist the oxidative microbicidal activity of mononuclear phagocytes is the subject of our current investigations.

Summary

This study demonstrates that the promastigote form of virulent *Leishmania donovani* and *Leishmania tropica* are both deficient in endogenous enzymatic scavengers of H_2O_2 (catalase, glutathione peroxidase) and susceptible to low fluxes of H_2O_2 in a cell-free model. In addition, the killing of promastigotes by H_2O_2 is markedly enhanced in the presence of a peroxidase and halide. Promastigotes also readily trigger the macrophage oxidative burst including the generation of H_2O_2, and most intracellular promastigotes are killed within 18 h by unstimulated normal resident cells. Catalase, but not scavengers or quenchers of O_2^-, $OH\cdot$, or $^1O_2$, protected promastigotes in a cell-free xanthine oxidase microbicidal system, and catalase also partially inhibited the leishmanicidal activity of resident macrophages. Thus, amongst various oxygen intermediates, H_2O_2 alone appeared to be both necessary and sufficient for promastigote killing. Depriving macrophages of exogenous glucose, which inhibits the generation of oxygen intermediates, achieved effects similar to catalase treatment. These observations directly contrast with the intracellular parasite, *T. gondii* which is richly endowed with catalase and glutathione peroxidase, highly resistant to H_2O_2, and requires products of O_2^--H_2O_2 interaction for effective oxidative killing. Toxoplasmas also fail to trigger the respiratory burst of normal macrophages, and readily multiply within these cells (1–5). Macrophages first activated by in vivo or in vitro immunologic stimuli, however, display an enhanced capacity to generate oxygen intermediates beyond O_2^- and H_2O_2, and are able to kill toxoplasmas or inhibit their intracellular replication (1, 2).

These studies illustrate the wide spectrum of susceptibility to oxidative products which appears to exist for virulent intracellular protozoans, and indicate that such differences may be reflected in contrasting fates of parasites within cell-free oxidative environments and the cytoplasm of normal resident macrophages. In addition, these observations also demonstrate that nonactivated phagocytes may display effective

microbicidal activity against certain intracellular pathogens utilizing an oxygen-dependent mechanism.

The author thanks Ms. Judy Adams for photographic assistance.

Received for publication 11 December 1980 and in revised form 22 January 1981.

References

1. Murray, H. W., C. W. Juangbhanich, C. F. Nathan, and Z. A. Cohn. 1979. Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. *J. Exp. Med.* **150:**950.
2. Murray, H. W., and Z. A. Cohn. 1980. Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation. *J. Exp. Med.* **152:**1596
3. Wilson, C. B., V. Tsai, and J. S. Remington. 1980. Failure to trigger the oxidative burst by normal macrophages. Possible mechanism for survival of intracellular pathogens. *J. Exp. Med.* **151:**328.
4. Murray, H. W., and Z. A. Cohn. 1979. Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. *J. Exp. Med.* **150:**938.
5. Murray, H. W., C. F. Nathan, and Z. A. Cohn. 1980. Macrophage oxygen-dependent antimicrobial activity. IV. The role of endogenous scavengers of oxygen intermediates. *J. Exp. Med.* **152:**1601
6. Zuckerman, A. 1975. Parasitological review. Current status of the immunology of blood and tissue protozoa. I. *Leishmania. Exp. Parasitol.* **38:**370.
7. Keithly, J. S. 1976. Infectivity of *Leishmania donovani* amastigotes and promastigotes for golden hamsters. *J. Protozool.* **23:**244.
8. Bradley, D. J. 1977. Regulation of *Leishmania* populations within the host. II. Genetic control of acute susceptibility of mice to *Leishmania donovani* infection. *Clin. Exp. Immunol.* **30:** 130.
9. Behin, R., J. Mavel, and B. Sordat. 1979. *Leishmania tropica*: pathogenicity and in vitro macrophage function in strains of inbred mice. *Exp. Parasitol.* **48:**81.
10. Masur, H., J. Keithly, and T. C. Jones. 1980. The interaction in vitro between *Leishmania* and macrophages from susceptible and resistant inbred mice. *In* 20th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans. American Society for Microbiology, Washington, D. C. Abstract 728.
11. McCord, J. M., and I. Fridovich. 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244:**6049.
12. Baudhuin, P., H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques, and C. deDuve. 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase, and catalase in rat liver tissue. *Biochem. J.* **193:**265.
13. Paglia, D. E., and W. N. Valentine. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70:**158.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265.
15. Johnston, R. B., C. A. Godzik, and Z. A. Cohn. 1978. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. Exp. Med.* **148:**115.
16. Nathan, C. F., and R. K. Root. 1977. Hydrogen peroxide release from mouse peritoneal macrophages. *J. Exp. Med.* **146:**1648.
17. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J. Cell Biol.* **55:**186.

18. Boveris, A., E. Martino, and A. O. M. Stoppani. 1977. Evaluation of the horseradish peroxidase-scopoletin method for the measurement of hydrogen peroxide formation in biological systems. *Anal. Biochem.* **80:**145.
19. Berman, J. D., D. M. Dwyer, and D. J. Wyler. 1979. Multiplication of *Leishmania* in human macrophages in vitro. *Infect. Immun.* **26:**375.
20. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Sem. Hematol.* **12:**117.
21. Rosen, H., and S. J. Klebanoff. 1979. Bactericidal activity of a superoxide anion-generating system. A model for the polymorphonuclear leukocyte. *J. Exp. Med.* **149:**27.
22. Nichols, P. 1965. Activity of catalase in the red cell. *Biochim. Biophys. Acta.* **99:**286.
23. Cohen, G., and P. Hochstein. 1963. Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxidase in erythrocytes. *Biochemistry.* **2:**1420.
24. Badwey, J. A., and M. L. Karnovsky. 1980. Active oxygen species and the functions of phagocytic leukocytes. *Annu. Rev. Biochem.* **49:**695.
25. Baehner, R. L., L. A. Boxer, and J. Davis. 1976. The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. *Blood.* **48:**309.
26. Nathan, C. F., S. C. Silverstein, L. H. Brukner, and Z. A. Cohn. 1979. Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. *J. Exp. Med.* **149:**100.
27. Nathan, C. F., N. Nogueira, C. Juangbhanich, J. Ellis, and Z. A. Cohn. 1979. Activation of macrophages in vivo and in vitro. Correlation between hydrogen peroxide release and killing of *Trypanosoma cruzi*. *J. Exp. Med.* **149:**1056.
28. Pulvertaft, R. J. V., and G. F. Hoyle. 1960. Stages in the life-cycle of *Leishmania donovani*. *Trans. Roy. Soc. Trop. Med. Hyg.* **54:**191.
29. Akiyama, H. J., and R. D. Haight. 1971. Interaction of *Leishmania donovani* and hamster peritoneal macrophages. *Am. J. Trop. Med. Hyg.* **20:**539.
30. Lewis, D. H., and W. Peters. 1977. The resistance of intracellular *Leishmania* parasites to digestion by lysosomal enzymes. *Ann. Trop. Med. Parasitol.* **71:**295.
31. Farah, F. S., S. A. Samra, and N. Nuwayri-Salti. 1975. The role of the macrophage in cutaneous leishmaniasis. *Immunology.* **29:**755.
32. Miller, H. C., and D. W. Twohy. 1967. Infection of macrophages in culture by leptomonads in *Leishmania donovani*. *J. Protozool.* **14:**781.
33. North, R. J. 1978. The concept of the activated macrophage. *J. Immunol.* **121:**806.