Oxidant-Mediated Damage of *Leishmania donovani* Promastigotes

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Received 9 November 1981/Accepted 23 February 1982

Dissemination of *Leishmania* within the host is related to parasites undergoing unchecked proliferation. We therefore studied the effects of oxidant generating systems on promastigote multiplication by (i) direct determinations of organism proliferation and (ii) the incorporation of [³H]uracil into promastigote nucleoprotein. These two parameters correlated closely as measures of organism replication as demonstrated by parallel suppression of them by the protein synthesis inhibitors puromycin and cycloheximide and the nucleic acid synthesis inhibitors actinomycin D and mitomycin C. Promastigotes showed dose-related susceptibility to reagent and generated hydrogen peroxide (H₂O₂) as reflected in quantitatively similar decreases in multiplication and [³H]uracil incorporation. These effects were specific for H₂O₂ as catalase abrogated the diminution in multiplication. The generation of superoxide anion by acetaldehyde-xanthine oxidase (10 mU/ml) did not alter promastigote replication or nucleoprotein synthesis. These results indicate that *Leishmania donovani* promastigotes are damaged by H₂O₂ and that the incorporation of [³H]uracil into promastigote nucleoprotein may be useful for studying the interaction of this parasite with host effector cells.

Infections with species of *Leishmania* pathogenic for humans and other mammals are characterized by the presence of parasite packages in phagolysosomal vacuoles within host mononuclear phagocytes. Since dissemination of parasites within the host and morbidity due to leishmaniasis occur when these organisms undergo unchecked proliferation, the crucial balance between infection and disease may in large part be determined by the ability of the host to restrict parasite multiplication. Numerous determinants of host defense may be operative in leishmanial killing; however, the localization of *Leishmania* within mononuclear phagocytes which generate reduced oxygen products dictates that the ability of these cells to kill the parasites be carefully examined. Previous studies directed at evaluating oxidative effector mechanisms have examined the susceptibility of *Leishmania* to reduced oxygen products by using parameters such as motility, ultrastructural changes, and parasite lysis. In contrast, the effects of these possible cytotoxic mechanisms on parasite multiplication were not critically evaluated (4, 9, 11). We therefore studied the effects of hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻) on parasite replication. Two measures of parasite viability were used for this purpose: direct enumeration of organism replication and incorporation of [³H]uracil into promastigote nucleoproteins (13).

MATERIALS AND METHODS

Parasites. Sudan strain 2S of *Leishmania donovani* was kindly provided by K.-P. Chang, Rockefeller University, New York, N.Y. Promastigotes were maintained by weekly subculture at 27°C in medium 199 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (300 µg/ml), and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (5.9 mg/ml). Parasites used for experiments were in the log phase of growth.

Measurements of promastigote multiplication and [³H]uracil or [³H]thymidine incorporation. Samples of 4×10^5 parasites in 0.1 ml of medium 199 without serum were placed in wells of flat-bottom microtiter trays (Falcon Plastics, Oxnard, Calif.). Inhibitors of protein or nucleic acid synthesis and reagents required for the oxidant generating systems were added as outlined below, and the plates were incubated for 1 h at 37°C. Heat-inactivated fetal bovine serum (10%) was then added to each well, and the organisms were incubated for 72 h at 27°C. Parasite multiplication was assessed by enumeration of Formalin-fixed organisms in a Nebauer chamber at the initiation of the experiment and after 72 h. Then 0.5 µCi of either [³H]uracil ([5,6-³H]uracil; specific activity, 40 to 60 µCi/mmol; Amersham Corp., Arlington Heights, Ill.) or [³H]thymidine (specific activity, 6 µCi/mmol; Schwarz/Mann, Orangeburg, N.Y.) was added to each well for the final 18 to 24 h of incubation, and nucleoprotein synthesis was estimated by incorporation of isotope into trichloroacetic acid-precipitable material held at 4°C.

TABLE 1. Effects of inhibition of protein synthesis in *L. donovani* promastigotes upon multiplication and correlation with uptake of [³H]uracil and [³H]thymidine

Reaction mixture ^a	Promastigotes/ml (10 ⁶)	cpm incorporated (10 ³)	
		[³ H]uracil	[³ H]thymidine
Control	26.6 ± 0.8 ^b	5,415 ± 264	14,553 ± 846
Puromycin (10 ⁻³ M)	0.0 ^c (100) ^d	994 ± 304 ^c (82)	1,160 ± 140 ^c (92)
Cycloheximide (10 ⁻⁵ M)	1.8 ± 0.1 ^c (93)	394 ± 65 ^c (93)	878 ± 36 ^c (94)

^a The reaction mixtures contained medium 199 (pH 7.44) and 5.5 mM glucose. Puromycin or cycloheximide was added in the final concentration indicated.

^b Data are expressed as mean ± standard error of the mean.

^c The difference between parasite multiplications or isotope incorporations by promastigotes in control wells and treated wells is significant at the ≤0.1% level.

^d Numbers within parentheses indicate percent inhibition.

TABLE 2. Effects of inhibition of nucleoprotein synthesis in *L. donovani* promastigotes upon multiplication and correlation with uptake of [³H]uracil

Reaction mixture (μg/ml) ^a	Promastigotes/ml (10 ⁶)	cpm of [³ H]uracil incorporated (10 ³)
Control	8.50 ± 0.33 ^b	12.69 ± 0.85
Actinomycin D (10.0)	0.55 ± 0.07 (93) ^c	0.51 ± 0.13 (96)
Actinomycin D (1.0)	0.69 ± 0.23	0.81 ± 0.00
Actinomycin D (0.1)	5.80 ± 0.36 ^d (32)	7.83 ± 0.79 ^e (38)
Mitomycin C (25)	0.77 ± 0.23 (91)	0.24 ± 0.05 (94)
Mitomycin C (12.5)	0.67 ± 0.16 (92)	0.68 ± 0.06 (98)

^a The reaction mixtures contained medium 199 (pH 7.44) and 5.5 mM glucose. Actinomycin D or mitomycin C was added in the final concentrations indicated.

^b Data are expressed as mean ± standard error of the mean.

^c Numbers within parentheses indicate percent inhibition.

^d The difference in multiplication of promastigotes in control wells versus wells with actinomycin D (0.1 μg/ml) is significant at the 0.5% level.

^e The difference between [³H]uracil incorporations by promastigotes in control wells and wells with actinomycin D (0.1 μg/ml) is significant at the 2.0% level.

Parasites were harvested with a Mash II automated microharvester (model M24; Brandel, Rockville, Md.) onto glass fiber filter paper (Brandel). Counts were determined in a liquid scintillation counter (Nuclear Chicago, Chicago, Ill.). All experiments were done in triplicate.

Inhibition of protein or DNA synthesis. To inhibit protein and nucleic acid synthesis by parasites, puromycin dihydrochloride (Sigma Chemical Co., St. Louis, Mo.; 6255, lot 107C-0335) or cycloheximide (Sigma; 6255, lot 320-2840), and actinomycin D (Merck Sharp & Dohme, West Point, Pa.; 3298, lot 3678D) (0.1, 1, and 10 μg/ml, final concentration) or mitomycin C (Sigma; M-0503, lot 11F-0199) (12.5 and 25.0 μg/ml),

respectively, were dissolved in sterile distilled water and added to promastigote cultures.

Cell-free systems for generation of reactive oxygen intermediates. Reagent-grade H₂O₂ (Fisher Scientific Co., Fairlawn, N.J.) was adjusted to the desired concentration after spectrophotometric determination (DU-8 spectrophotometer; Beckman Instruments Inc., Fullerton, Calif.) of its absorbance at 230 nm (extinction coefficient of 81 M⁻¹ cm⁻¹) (16). Continuous generation of H₂O₂ in the absence of other oxygen intermediates was provided by glucose (5.5 mM)-glucose oxidase (0.033 to 3.33 mU/ml, final concentration; type II; Sigma) (2). The effect of O₂⁻ on promastigote viability was assessed by addition of acetaldehyde (0.2 mM; Eastman Kodak Co., Rochester, N.Y.) and xanthine oxidase (10 mU/ml, type II from buttermilk; Sigma) (15). Catalase (Sigma) was added in some experiments. The rate of H₂O₂ generation in the glucose-glucose oxidase system was measured by the scopoletin method (14) with an Aminco-Bowman spectrofluorometer (American Instrument Corp., Silver Spring, Md.). Superoxide production by acetaldehyde-xanthine oxidase was assessed by superoxide dismutase-inhibitable reduction of ferricytochrome c (1).

RESULTS

Effects of inhibition of protein or nucleic acid synthesis upon promastigote multiplication and incorporation of [³H]uracil or [³H]thymidine. To establish that measurement of [³H]uracil or [³H]thymidine incorporation into promastigote nucleoproteins accurately reflected parasite proliferation, protein synthesis was inhibited with puromycin or cycloheximide. Treatment with puromycin (10⁻³ M) resulted in complete arrest of promastigote multiplication and reduced incorporation of [³H]uracil and [³H]thymidine into parasite nucleoproteins by 82 and 92%, respectively (Table 1). Similar results were obtained with cycloheximide (10⁻⁵ M) (greater than 90% reductions in promastigote multiplication and incorporation of isotopes) (Table 1).

The nucleic acid synthesis inhibitor actinomycin D had dose-related effects upon promastigote multiplication and [³H]uracil incorporation (Table 2). At 10 μg/ml, multiplication and iso-

TABLE 3. Effects of reagent H_2O_2 upon *L. donovani* promastigote multiplication and incorporation of $[^3H]$ uracil or $[^3H]$ thymidine^a

H_2O_2 concn (M)	% Decrease		
	Promastigote multiplication	$[^3H]$ uracil incorporation	$[^3H]$ thymidine incorporation
10^{-5}	10 ± 6^b	2 ± 3	1.0 ± 7
10^{-4}	94 ± 5	97 ± 7	95 ± 5
10^{-3}	99 ± 3	98 ± 10	97 ± 15

^a The reaction mixtures contained medium 199 (pH 7.44) and 5.5 mM glucose. H_2O_2 was added to the final concentrations indicated.

^b Data are expressed as mean $\pm$ standard error of the mean.

tope incorporation were inhibited by 93 and 96%, respectively. A lower concentration of actinomycin D (0.1 μ g/ml) had a reduced effect (32 and 38% inhibition of proliferation and $[^3H]$ uracil uptake, respectively). Mitomycin C at concentrations of 25 and 12.5 μ g/ml also reduced promastigote multiplication (91 to 92% decrease) and isotope incorporation (94 to 98% decrease).

H_2O_2 -mediated parasite cytotoxicity. H_2O_2 at 10^{-5} M had no significant effects on promastigote multiplication or $[^3H]$ uracil and $[^3H]$ thymidine incorporation. In contrast, 10^{-4} and 10^{-3} M H_2O_2 reduced these activities by greater than 90% (Table 3). There was a progressive reduction of parasite multiplication and $[^3H]$ uracil incorporation by H_2O_2 at concentrations between 10^{-5} and 10^{-4} M (Fig. 1).

FIG. 1. Dose-dependent effects of H_2O_2 upon replication and $[^3H]$ uracil incorporation by promastigotes of *L. donovani*. Points represent the mean $\pm$ standard error of the mean for triplicate determinations and illustrate data from one of three representative experiments.

Continuous generation of H_2O_2 by glucose-glucose oxidase also had a profound suppressive effect on promastigote proliferation. Generation of this molecule at a rate of 0.24 nmol/min (5.5 mM glucose plus 3.33 mU of glucose oxidase per ml) reduced the number of organisms after 72 h of incubation by 92% (from $16.3 \times 10^6 \pm 1.6 \times 10^6$ in controls to $1.3 \times 10^6 \pm 0.2 \times 10^6$ promastigotes per ml, $P < 0.001$) and the incorporation of $[^3H]$ uracil by 91% (from $16,230 \pm 4,021$ cpm to $1,477 \pm 120$ cpm, $P < 0.025$) (Table 4). The inhibitory effect was less marked at lower concentrations of glucose oxidase and completely lost at 0.03 mU/ml (Table 4). Heat inactivation (100°C for 30 min) of glucose oxidase resulted in complete loss of toxicity. Inhibition of parasite multiplication was specifically due to generated H_2O_2 , as destruction of this molecule by catalase (3,400 U/ml) completely abrogated the inhibitory activity of glucose-glucose oxidase (16.3 ± 1.6 promastigotes per ml in the control versus 12.9 ± 3.2 promastigotes per ml with glucose-glucose oxidase plus catalase, $P > 0.1$) (Table 4).

Acetaldehyde-xanthine oxidase-mediated parasite cytotoxicity. Incubation of promastigotes with 0.2 mM acetaldehyde and 10 mU of xanthine oxidase per ml had no effect on parasite division or uracil incorporation ($8.3 \times 10^6 \pm 0.7 \times 10^6$ promastigotes per ml in treated cultures versus $11.0 \times 10^6 \pm 1.1 \times 10^6$ in controls, $P > 0.1$; $14,423 \pm 3,860$ cpm in treated cultures versus $15,864 \pm 1,698$ cpm in controls; $P > 0.1$). At these concentrations of enzyme and substrate, superoxide anion was generated at a rate of 0.9 nmol/min. The addition of superoxide dismutase (10 μ g/ml) did not alter these results. The effects of greater fluxes of O_2^- could not be evaluated, as higher concentrations of acetaldehyde (1 mM) or xanthine oxidase (20 mU/ml) were independently toxic for the organisms.

DISCUSSION

Intracellular localization within host mononuclear phagocytes is a prerequisite for successful parasitism by protozoa such as *Toxoplasma*

TABLE 4. Toxic effects of glucose-glucose oxidase-generated H_2O_2 and their reversal by catalase on *L. donovani* promastigotes as assessed by promastigote multiplication and incorporation of [3H]uracil

Group	Glucose oxidase concn (mU/ml) ^a	Promastigotes/ml (10 ⁶)	cpm of [3H]uracil incorporated (10 ³)
I	Control	16.3 ± 1.6 ^b	16.2 ± 4.02
	3.33	1.3 ± 0.2 ^c	1.5 ± 1.20 ^d
	0.33	10.0 ± 0.2	12.4 ± 2.6
	0.03	13.9 ± 2.2	16.5 ± 1.8
	3.3 (+ catalase)	12.9 ± 3.2	ND ^e
II	Control	25.9 ± 0.5	ND
	3.33	0.9 ± 0.1	ND
	3.33 (boiled)	24.7 ± 3.4	ND

^a The reaction mixture contained medium 199 (pH 7.44) and 5.5 mM glucose. In a final volume of 0.2 ml, glucose oxidase was added at the concentrations indicated and catalase was added at 3,400 U/ml.

^b Data are expressed as mean ± standard error of the mean.

^c The difference in multiplication of promastigotes in control wells versus wells with glucose oxidase (3.33 mU/ml) is significant at the 0.1% level.

^d The difference between [3H]uracil incorporations by promastigotes in control wells and wells with glucose-oxidase (3.33 mU/ml) is significant at the 2.5% level.

^e ND, Not determined.

gondii, *Trypanosoma cruzi*, and *Leishmania* spp. (6, 8, 10, 17). Although the ingestion of many types of microorganisms by polymorphonuclear and mononuclear phagocytes is followed by death due to a variety of lysosomal enzymes and toxic oxygen products, unicellular protozoa seem to successfully parasitize and proliferate within the hostile environment of the host cells. *Toxoplasma* spp. have been shown to avoid damage in a variety of ways, including failure to trigger the metabolic burst, inhibition of phagolysosomal fusion, and possession of large amounts of enzymes capable of degrading toxic oxygen products such as O_2^- and H_2O_2 (6, 7, 18). *Leishmania* spp. are also avidly ingested by host mononuclear phagocytes and packaged in phagolysosomal vacuoles and trigger macrophages to reduce oxygen to H_2O_2 and O_2^- (3, 5, 9, 11). Previous studies designed to examine the susceptibility of *Leishmania* spp. to these molecules have assessed damage in terms of lysis, motility, or morphology (4, 9, 11). No detailed examinations of the effect of these products on leishmanial replication, which is perhaps the most biologically relevant determinant of disease, were attempted. In the present study we examined the effects of reagents and generated H_2O_2 and superoxide anion on in vitro parasite multiplication and incorporation of [3H]uracil and [3H]thymidine into nucleoproteins. All of these parameters provided an objective and reproducible index of parasite damage; furthermore, parallel changes in uracil and thymidine handling were observed.

Multiplication of *L. donovani* and incorporation of uracil into parasite nucleoproteins was reduced drastically by H_2O_2 , but not affected by O_2^- . Since 10^{-4} M H_2O_2 was required for a

~100% lethal effect on promastigotes in our system (Fig. 1), the sensitivity of the Sudan 2S strain of *L. donovani* utilized in the present study appears to be similar to that of the 1S strain reported by Murray (9). In contrast, the capacity of promastigotes to divide and incorporate uracil was not significantly reduced by acetaldehyde-xanthine oxidase. Since this combination of substrate and enzyme generates O_2^- (15), these results suggest that *L. donovani* organisms are relatively resistant to superoxide anion and are consistent with previous observations (9). *L. donovani* organisms may not be damaged by O_2^- and be susceptible to H_2O_2 because they possess the scavenger enzyme superoxide dismutase and only small amounts of catalase (9). In the present study, the enzymatic conversion of superoxide to H_2O_2 by superoxide dismutase presumably resulted in insufficient H_2O_2 generation to induce parasite damage.

The fate of *Leishmania* parasites within host cells is a subject of considerable importance regardless of whether oxidative or nonoxidative effector mechanisms are operative. Past studies designed to quantify parasite viability have primarily utilized morphological criteria and have not systemically evaluated effects upon parasite multiplication. Our results show that *L. donovani* promastigotes incorporate [3H]uracil and [3H]thymidine into trichloroacetic acid-precipitable nucleoprotein in a parallel fashion. These parameters of metabolic activity are decreased by H_2O_2 concomitant with reductions in parasite division. As a measure of parasite metabolism, [3H]uracil incorporation not only may be a useful tool for studies of the cytotoxic effects of host products in cell-free systems but may also facilitate studies with intact cells, since the

utilization of preformed uracil by mammalian cells is negligible in comparison to that of protozoa (12).

ACKNOWLEDGMENTS

We thank Adel A. F. Mahmoud for his advice and support and Barbara Joyce Jackson and Pat Amato for their invaluable assistance in the preparation of the manuscript.

This work was supported by Public Health Service grant AI-15351 from the National Institutes of Health and by the Rockefeller Foundation.

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