

INTERACTION OF *LEISHMANIA* WITH A MACROPHAGE CELL
LINE

Correlation between Intracellular Killing and the Generation of Oxygen
Intermediates*

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In a recent report (1), we demonstrated that the ingestion of *Leishmania donovani* (LD) and *Leishmania tropica* (LT) promastigotes, hemoflagellates that are susceptible to hydrogen peroxide (H_2O_2), readily triggers the macrophage oxidative burst, resulting in the generation of toxic oxygen intermediates, including superoxide anion (O_2^-) and H_2O_2 . In addition, this oxygen-dependent mechanism appears to contribute importantly to the prompt killing of most intracellular promastigotes by normal macrophages (1). The present study extends this analysis by demonstrating the strikingly different fate of these protozoa within phagocytes derived from a macrophage cell line, which in the absence of lymphokine stimulation display remarkably little oxidative activity.

Materials and Methods

Cells. The macrophage-like cell line, J774, derived from a BALB/c murine reticulum cell sarcoma (2), was provided by Dr. Jay Unkeless (The Rockefeller University) who designated this clone J774G8 (3). Cells were cultivated at 37°C in 5% CO_2 in 75-cm² tissue culture flasks (Corning, Corning Glass Works, Corning, N. Y.) and passed weekly in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 20% heat-inactivated fetal bovine serum (HIFBS; Grand Island Biological Co.), 100 U/ml penicillin, and 100 μ g/ml streptomycin. 5×10^4 cells suspended in Eagle's modified minimum essential medium (MEM; Flow Laboratories, McLean, Va.) with 10% HIFBS, penicillin, and streptomycin were added to 12-mm round glass coverslips (1), and after 1 h at 37°C in 5% CO_2 , the cultures were washed and reincubated for 18 h in MEM alone or MEM plus 25% active or control concanavalin A (Con A)-stimulated supernates (lymphokines) prepared using spleen cells from normal BALB/c mice (Charles River Breeding, Co., Wilmington, Mass.; 4). Resident peritoneal macrophages were also obtained BALB/c mice, and were cultivated in MEM alone (1).

Parasites and Infection of Cell Monolayers. The promastigote forms of LD (1 Sudan strain) and LT (strain 252, Iran) were maintained and harvested as described (1). Overnight cell cultures were challenged with 5×10^6 – 10×10^6 promastigotes for 2 h, which resulted in the infection of 53–91% of the cells. Quantitative observations (e.g., the number of *Leishmania* per 100 cells; 1) were not carried out beyond 48 h, because J774G8 cells occasionally replicated after this time.

Assays for O_2^- and H_2O_2 Release and Qualitative Nitroblue Tetrazolium (NBT) Reduction. The reduction of ferricytochrome *c* (5) and the fluorometric scopoletin techniques were used to measure the release of O_2^- and H_2O_2 , respectively (1, 4, 6). Respiratory burst triggering agents

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TABLE I
Oxidative Activity of BALB/c Macrophages and J774G8 Cells

Stimulus	Percent cells NBT-positive*			O ₂ ⁻ release‡			H ₂ O ₂ release‡		
	BALB/c	J774G8	J774G8 + Con A LK§	BALB/c	J774G8	J774G8 + Con A LK§	BALB/c	J774G8	J774G8 + Con A LK§
PMA	97 ± 1	3 ± 1	34 ± 5	178 ± 12	46 ± 7	114 ± 6	112 ± 19	13 ± 4	28 ± 2
Zymosan	87 ± 3	8 ± 2	60 ± 4	211 ± 14	18 ± 13	66 ± 16	89 ± 16	14 ± 6	35 ± 11
LT	83 ± 4	12 ± 2	70 ± 4	188 ± 16	18 ± 11	78 ± 11	82 ± 17	16 ± 7	—
LD	82 ± 3	10 ± 1	65 ± 6	157 ± 11	16 ± 10	61 ± 4	76 ± 8	13 ± 3	—

* Overnight cell cultures were exposed for 1 h to medium containing NBT (0.5 mg/ml) and either PMA (100 ng/ml), zymosan particles, 5×10^6 /ml, or *Leishmania* promastigotes, 5×10^6 /ml. Cells were scored as positive if they showed precipitated formazan as described (1). See also Fig. 1. Results are the means \pm SEM of three to eight experiments. Increasing the concentration of PMA to up to 6 μ g/ml (9) had no effect on J774G8 cells. The addition of SOD (1 mg/ml) to Con A lymphokine (LK)-activated cells inhibited the NBT reaction for all stimuli by 63–73%, indicating dependence on O₂⁻. Heated SOD (1 mg/ml) had no effect.

‡ Results are expressed as nmol of O₂⁻ or H₂O₂ released per mg of cell protein per 90 min (1, 4, 6), and represent the mean \pm SEM of three to four experiments in triplicate. PMA was used at 100 ng/ml. To achieve comparable ingestion, control J774G8 cells were exposed to twofold more zymosan (1 mg/ml) and promastigotes (10×10^6 /ml) than BALB/c macrophages or Con A LK-treated J774G8 cells. These adjustments resulted in 84–98% of the cells with ingested zymosan and infection of 63–77% of cells with promastigotes. The addition of SOD, 30 μ g/ml (5), abolished O₂⁻ release.

§ J774G8 cells were cultivated for 18 h before assay in medium containing 25% Con A LK. Control Con A supernate had no appreciable effect on J774G8 cell NBT reduction or O₂⁻ or H₂O₂ release.

(1; and legend to Table I) were suspended in Krebs-Ringer phosphate buffer with 5.5 mM glucose. These agents were also administered in medium containing 0.5 mg/ml NBT (1, 4). The latter's reduction to formazan is mostly O₂⁻-dependent (7), and cells were scored as positive if ingested zymosan particles or *Leishmania* were stained blue-black by precipitated formazan (1, 4), or if they showed clumped formazan after phorbol myristate acetate (PMA) stimulation.

Results

Oxidative Activity of J774G8 Cells. Depending upon the clone examined and the assay employed, J774 cells have been reported to generate varying amounts of O₂⁻ and H₂O₂ ranging from none (8, 9) to equal to (5, 9) or exceeding (5, 9, 10) that produced by resident peritoneal macrophages from normal mice. The J774G8 cells were first screened using the qualitative reduction of NBT, and as shown in Table I and Fig. 1, most adherent J774G8 cells failed to respond to soluble (PMA) and particulate phagocytic stimuli, including zymosan and LD and LT promastigotes.

In contrast, these same agents effectively triggered the oxidative burst of normal BALB/c macrophages. The quantitative data in Table I also demonstrate that, depending upon the triggering stimulus, J774G8 cells released up to 10-fold less O₂⁻ and H₂O₂ than BALB/c macrophages.

Fate of Leishmania within J774G8 Cells and Effect of Lymphokine Activation. J774G8 cells readily ingested LD and LT promastigotes (Fig. 1G). However, in striking contrast to BALB/c macrophages, which killed 80–95% of phagocytized LD and LT (1), J774G8 cells cultivated in standard medium failed to exert any appreciable leishmanicidal activity (Fig. 2A and B), and permitted virtually all promastigotes to survive, transform, and persist as amastigotes (Fig. 1H and I). 18 h of exposure to Con A lymphokine, however, clearly enhanced the oxidative response of J774G8 cells to all triggering agents including promastigotes (Table I), which was paralleled by the capacity to display intracellular anti-leishmanial activity (Fig. 2).

Role of H₂O₂. To define the role of oxygen intermediates in this *Leishmania* killing, activated J774G8 cells were deprived of exogenous glucose (11) and exposed to soluble scavengers of O₂⁻, H₂O₂, and OH \cdot (1, 6; Table II). As was observed with BALB/c

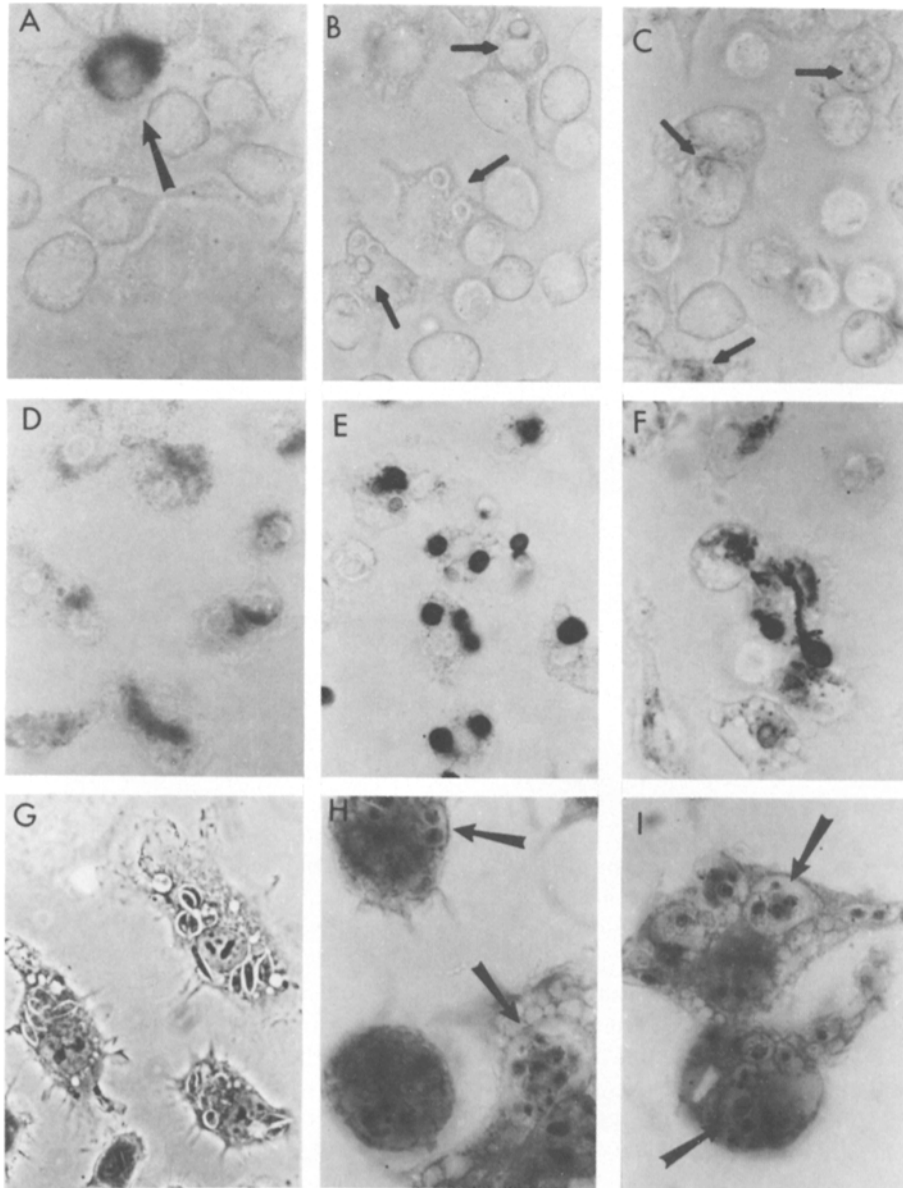


FIG. 1. Bright-field micrographs of J774G8 cells (top row) and resident BALB/c macrophages (middle row) 1 h after exposure to NBT and either PMA (A, D), opsonized zymosan (B, E), or LD promastigotes (C, F), as described in the legend to Table I ($\times 600$). In (A), only one J774G8 cell show formazan precipitation (arrow) after PMA; in (B), none respond to ingested zymosan (arrows); and in (C), LD-infected cells (arrows) show little formazan deposition. In contrast, most BALB/c macrophages demonstrate prominent formazan staining after exposure to PMA (D), zymosan (E), and LD promastigotes (F). Bottom row: Giemsa-stained J774G8 cells 2 h (G) (phase-contrast, $\times 600$) and 48 h (H, I) (bright-field, $\times 900$) after infection with LT promastigotes. In (H) and (I), cells contain up to 15 LT amastigotes, and arrows indicate vacuoles housing two to eight replicating parasites.

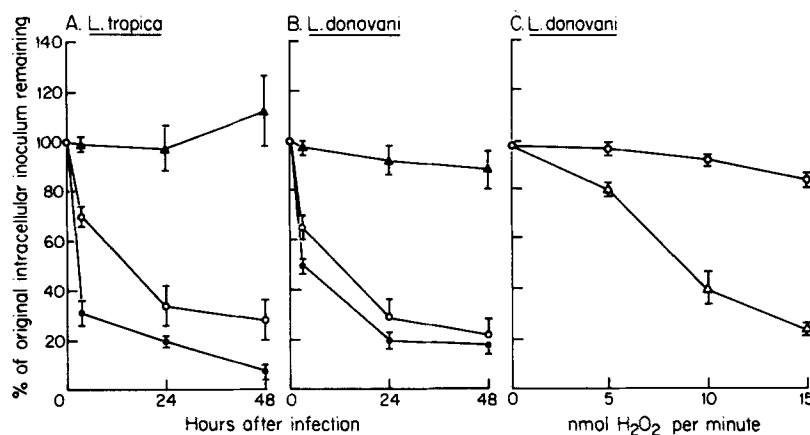


FIG. 2. In (A) and (B), overnight cultures of BALB/c macrophages (●), unstimulated J774G8 cells (▲), and Con A-lymphokine-activated J774G8 cells (○) were challenged with promastigotes for 2 h, which resulted in ingestion of 92–181 *Leishmania* per 100 cells. Results represent the mean \pm SEM of three to five experiments, and indicate the percentage of the original (time zero) intracellular inoculum that persisted up to 48 h later. Control Con A-lymphokine did not induce anti-leishmanial activity, and adding active lymphokine after infection had no effect. In (C), unstimulated J774G8 cells were infected with LD promastigotes, washed, and reincubated in MEM. After 2 h the culture medium was replaced for 1 h by 1 ml of Krebs-Ringer phosphate buffer, to which glucose oxidase (generating 5–15 nmol of H_2O_2 /min [1, 12]) was added with (○) or without (Δ) 100 μ g/ml of catalase. Cultures were then washed and incubated for 18 h in MEM alone. Results are the means \pm SEM of three experiments.

TABLE II
Inhibition of Leishmanicidal Activity of Lymphokine-activated J774G8 Cells
by Glucose Deprivation and Catalase

Treatment	Percent of original intracellular inoculum killed 24 h after infection	
	LT	LD
None	65 \pm 9	72 \pm 7
Glucose deprivation	11 \pm 9	16 \pm 2
Catalase, 2 mg/ml	19 \pm 10	20 \pm 5
SOD, 2 mg/ml	61 \pm 7	63 \pm 2

After 18 h of cultivation with 25% Con A lymphokine, J774G8 cells were incubated in glucose-free medium (11) or in medium containing catalase or SOD for 3 h before and during the 2-h challenge with $5\text{--}10 \times 10^6$ promastigotes (1, 4, 6). Results are the means \pm SEM of three to five experiments. In two parallel experiments, heated catalase (2 mg/ml) and the proposed $OH\cdot$ scavengers, mannitol (50 mM) and benzoate (10 mM; 16), had no effect on promastigote killing.

macrophages (1), both glucose deprivation and the administration of catalase, techniques that decrease macrophage H_2O_2 generation (4, 6, 11, 12), consistently reversed the leishmanicidal activity of lymphokine-treated J774G8 cells. Scavengers of O_2^- (SOD) and $OH\cdot$ had no effect. In addition (Fig. 2C), unstimulated J774G8 cells were infected and then provided with an exogenous source of enzymatically generated H_2O_2 (6, 12). This extracellular treatment readily resulted in the killing of intracellular *Leishmania*.

Discussion

Homogeneous macrophage-like cell lines, including the J774 line, have proven useful as tools for studying a variety of macrophage intracellular and plasma mem-

brane-associated activities (13). The finding that certain of the J774 clones are deficient in the post-phagocytic production of oxygen intermediates (9) provided a convenient model by which to extend our analysis of the role O_2^- and H_2O_2 in macrophage antimicrobial activity (1, 4, 6, 12). Thus, the demonstration that *Leishmania* promastigotes can effectively parasitize J774G8 cells, but not macrophages that actively generate oxygen intermediates, appears to reflect the disparate abilities of these cells to mount an effective oxidative response. Although the intrinsic activity of the O_2^- - H_2O_2 generating system of J774G8 cells was quite low, it could readily be stimulated by lymphocyte products resulting in appreciable increases in O_2^- and H_2O_2 production and the simultaneous induction of leishmanicidal activity. Moreover, the enhanced generation of H_2O_2 by lymphokine-treated J774G8 cells appeared to be an important intracellular event as judged by the inhibitory effects of glucose deprivation and exogenous catalase (1, 4, 6, 11, 12). On the basis of these and other studies (4, 14), it now seems clear that enhanced oxidative metabolism and antimicrobial activity are two interrelated expressions of the complex process of macrophage activation.

The inability of unstimulated J774G8 cells to generate an effective oxidative burst may explain why these cells are a favorable intracellular environment for the long-term propagation of amastigotes of other *Leishmania* strains (e.g., *L. mexicana amazonensis*; 15). We have also found that LT and LD amastigotes persist for weeks in flask cultures of J774G8 cells. In other unpublished studies, it was observed that like normal macrophages (4), J774G8 cells fail to reduce NBT upon ingestion of *Toxoplasma gondii*, a parasite that replicates freely within both types of phagocytes. *Trypanosoma cruzi* epimastigotes, however, behave in a manner similar to *Leishmania* promastigotes, and trigger the oxidative burst of >80% of BALB/c macrophages, but only 25% of J774G8 cells. Moreover, virtually no epimastigotes survive within BALB/c macrophages, whereas >50% persist intracellularly after infection of J774G8 cells (H. Murray, unpublished observations). It should also be pointed out that there is a broad spectrum of susceptibility to H_2O_2 among *T. gondii*, *Leishmania*, and *T. cruzi*; the first pathogen is resistant and the latter two are susceptible (1, 14). Thus, as judged by the interaction of these three protozoa with J774G8 cells and BALB/c macrophages, cells that display varying capacities to generate toxic oxygen intermediates, it appears that the ability of parasites to differentially trigger the phagocyte respiratory burst, the magnitude of the oxidative response, and the organism's susceptibility to H_2O_2 may all be important determinants of the intracellular fate of ingested protozoa.

Summary

The promastigote form of *Leishmania donovani* and *Leishmania tropica*, the etiologic agents of visceral and cutaneous leishmaniasis, respectively, readily parasitize unstimulated J774G8 macrophage-like cells, whereas 80–95% of the same promastigotes are killed within resident macrophages from normal BALB/c mice. This striking difference in intracellular anti-leishmanial activity correlated closely with the capacity to generate toxic oxygen intermediates. Thus, after triggering with phorbol myristate acetate or phagocytosis of zymosan or promastigotes, 90% of the J774G8 cells failed to reduce nitroblue tetrazolium, and released 5–10-fold less O_2^- and H_2O_2 than BALB/c macrophages. Exposure to concanavalin A-stimulated lymphokine, however, effectively enhanced the oxidative response of J774G8 cells, and, similarly, induced intracellular anti-leishmanial activity. Inhibiting macrophage H_2O_2 production consistently decreased the killing of *Leishmania* by lymphokine-treated J774G8 cells. These

observations illustrate the usefulness of examining homogeneous macrophage cell lines that are deficient in a particular effector function, and also serve to reemphasize the important role of oxygen intermediates in the microbicidal response of mononuclear phagocytes to intracellular parasites.

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