# Killing of Intracellular *Leishmania donovani* by Human Mononuclear Phagocytes

## EVIDENCE FOR OXYGEN-DEPENDENT AND -INDEPENDENT LEISHMANICIDAL ACTIVITY

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ABSTRACT Human peripheral blood monocytes were cultivated for 1–30 d before assay for $H_2O_2$ release or challenge with *Leishmania donovani* promastigotes (LDP) or amastigotes (LDA). 1-d cells readily generated $H_2O_2$ in response to both phorbol myristate acetate triggering (1,013±58 nmol/mg protein · 90 min) and LDP ingestion, and killed 50% of LDP within 6 h, and 90% by 24 h. In contrast, the same cells released little $H_2O_2$ during LDA ingestion, killed no LDA at 6 h and <30% by 24 h, and supported intracellular LDA replication. Monocyte-derived macrophages (cells first cultivated for ≥7 d) generated <125 nmol $H_2O_2$/mg · 90 min after phorbol myristate acetate triggering, killed neither LDP nor LDA, and permitted both forms to replicate. The addition of mitogen- or antigen-stimulated lymphokines, however, prevented the decline in monocyte oxidative capacity, enhanced macrophage $H_2O_2$ release by more than sixfold, and, in parallel, induced 1-d monocytes to kill LDA and cultivated macrophages to display both promastigocidal and amastigocidal activity.

In comparison to 1-d monocytes and lymphokine-activated macrophages from normal donors, the same cells from patients with chronic granulomatous disease (CGD) or normal cells whose oxidative activity had been impaired by catalase pretreatment or glucose deprivation exerted considerably less or no antileishmanial activity during the early (6–24 h) postphagocytic period. By 48 h after infection, however, 1-d CGD monocytes and oxidatively impaired normal cells killed 40 and >80% of LDP, respectively. Although a longer period of lymphokine stimulation was required and the resulting antileishmanial effects were not as rapid as with normal cells, activated CGD monocytes and macrophages also eventually achieved promastigocidal and amastigostatic activity.

These results indicate that human mononuclear phagocytes utilize both oxygen-dependent and -independent mechanisms to achieve activity against ingested Leishmania, and also demonstrate (*a*) the differential susceptibilities of the two forms of *L. donovani* to intracellular killing, (*b*) the key role of oxygen intermediates in effective mononuclear phagocyte antimicrobial activity, (*c*) the capacity of lymphocyte products to enhance oxygen-dependent as well as -independent pathways, and (*d*) the vulnerability of the monocyte-derived macrophage to Leishmania infection in the absence of lymphokine stimulation.

## INTRODUCTION

The ability to survive and replicate within host mononuclear phagocytes is a pathogenetic characteristic shared by the intracellular protozoa, Leishmania, *Toxoplasma gondii*, and *Trypanosoma cruzi*. In models utilizing both mouse peritoneal macrophages and human peripheral blood monocytes, considerable effort has recently been directed at identifying how these pathogens resist the mononuclear phagocyte's killing mechanisms (1–11). For example, from the standpoint of oxygen-dependent microbicidal activity, multiple factors appear to underlie the capacity of *T. gondii* to parasitize normal resident mouse peritoneal macrophages. These include the protozoan's abundant endogenous stores of oxygen intermediate scavengers

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(superoxide dismutase [SOD],[1] catalase, glutathione peroxidase) (1, 12), resistance to superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) (13), and the ability to avoid effective triggering of the macrophage oxidative burst during ingestion (7, 8). Host cell factors include the low levels of $O_2^-$ and $H_2O_2$ which nonactivated resident macrophages can generate (2, 10), and perhaps the absence of granular myeloperoxidase (MPO), an enzyme which can augment the toxicity of even minute amounts of $H_2O_2$ (9). Recent studies with human monocytes appear to support these findings (8, 9).

In contrast to *T. gondii*, the flagellate (promastigote) form of *L. donovani* is virtually devoid of catalase and glutathione peroxidase and is exquisitely susceptible to $H_2O_2$ (1). Promastigotes also readily trigger the macrophage oxidative burst, and >85% are killed by normal resident cells at least in part by a $H_2O_2$-dependent mechanism (1, 2). The intracellular fate of the amastigote form of *L. donovani* (to which surviving promastigotes transform within phagolysosomes and which is responsible for persistent tissue infection), however, is quite different. Amastigotes are appreciably more resistant to $H_2O_2$, evade substantial stimulation of the macrophage respiratory burst during ingestion, and readily parasitize normal mouse cells (3, 4, 14). Lymphokine treatment, however, induces resident macrophages to display both an enhanced oxidative response (4, 7, 10) and striking amastigocidal activity, and the latter also appears to be largely mediated by $H_2O_2$ (4, 15).

The present report extends this analysis to human mononuclear phagocytes, and examines the interaction of *L. donovani*, the etiologic agent of visceral leishmaniasis, with monocytes obtained from normal individuals and patients with chronic granulomatous disease (CGD). Our results indicate that *L. donovani* amastigotes (LDA) are more resistant than promastigotes (LDP) to killing by human mononuclear phagocytes, and that monocytes are considerably more active against both LDA and LDP than monocyte-derived macrophages. The latter finding appears to reflect the rapid and parallel decline in the monocyte's oxygen-dependent and independent antileishmanial mechanisms during in vitro cultivation. The activity of both mechanisms, however, can be effectively restored by lymphokine activation.

[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; Con A, concanavalin A; OH•, hydroxyl radical; KRP, Krebs-Ringer phosphate buffer; KRPG, KRP plus 5.5 mM glucose; LDA, *L. donovani* amastigotes; LDP, *L. donovani* promastigotes; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; PMA, phorbol myristate acetate; $O_2^-$, superoxide anion; SOD, superoxide dismutase.

## METHODS

*Parasites.* As previously described, LDA (1 Sudan strain) were obtained from homogenates of infected hamster spleens (3). LDP (the same strain), which were harvested from the log phase of growth (3), were maintained at 25°C in tissue culture flasks containing medium 199 (Gibco Laboratories, Grand Island, NY), 20% heat-inactivated fetal bovine serum (Gibco Laboratories), and penicillin (100 U/ml) and streptomycin (100 μg/ml) (3).

*Cells.* Heparinized peripheral venous blood was obtained from healthy volunteers, three male patients with well-documented CGD and the mother of one CGD patient. Cells from these latter four individuals were generously provided by Drs. M. Hilgartner, E. Smithwick, and D. Miller. In addition, leukocyte concentrates (buffy coats) were purchased from The Greater New York Blood Center, New York City. After dilution and separation by standard Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation (16), the mononuclear cell fraction that contained 16–33% monocytes as judged by morphology and peroxidase staining (16), was washed twice, and adjusted to 10–13 × $10^6$ cells/ml in RPMI 1640 medium (Flow Laboratories, Rockville, MD) supplemented with 15–20% heat-inactivated heterologous human serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). 100 μl of the cell suspension was added to 12-mm round glass coverslips placed in 35-mm plastic tissue culture dishes. After 2 h at 37°C in 5% $CO_2$-95% air, nonadherent cells were removed by washing and fresh medium was added. Monocyte cultures were then incubated overnight (1-d cells) or for up to 30 d before infection or assay. The culture medium described above was replenished every 3 d. Similar to other studies (11, 17), between 15 and 30% of initially adherent cells detached during the first 24 h of cultivation. Thereafter, and during the next 4–7 d, total cell loss seldom exceeded 5–10%. Cells that had been cultivated for >7 d were arbitrarily designated as monocyte-derived macrophages, and demonstrated typical changes including marked increases in size, cytoplasmic spreading, and protein content, and the appearance of bi- and multinucleated cells (16–18).

*Preparation of soluble lymphocyte products (lymphokines).* Nonadherent cells or the unseparated mononuclear cell fraction were cultivated at 3 × $10^6$ cells/ml with either mitogen (concanavalin A [Con A], 15 μg/ml) or antigen in 120-mm round plastic tissue culture dishes in 6 ml of RPMI 1640 medium containing 10–20% fresh heterologous human serum, penicillin, and streptomycin (11, 19). For antigen-induced lymphokines, cells from a *T. gondii*-immune donor (Sabin-Feldman dye test titer of 1:256) were incubated with 50 μg/ml of toxoplasma lysate antigen (7). After 48 h at 37°C in 5% $CO_2$-95% air, supernates were collected, centrifuged at 500 *g*, sterilized by filtration, and stored at 4°C. Control (sham) lymphokines consisted of (*a*) cells cultivated alone for 48 h with Con A added at the end of this period and (*b*) cells from dye test-negative donors incubated with toxoplasma antigen (7). For both mitogen- and antigen-stimulated lymphokines, there was no difference in activity if the supernate was prepared from nonadherent cells or from the unseparated mononuclear cell fraction. Various dilutions of lymphokine were added to monocytes preparations either at the outset of cultivation or after the cells had been in culture for a week or longer.

*Infection of monocytes and macrophages.* After 1–30 d of cultivation, cells were challenged for 1 h with either LDP (5 × $10^6$/ml) or LDA (10 × $10^6$/ml) (1, 3). More than 90% of LDP were motile at the time of challenge (1). Uningested parasites were removed by washing, the medium was replaced, and at the indicated intervals the percentage of cells infected and the number of LDP or LDA/100 cells were enumerated in Giemsa-stained preparations (1, 3). In 2 of 11 experiments with infected 1-d monocytes, >20% of adherent cells detached during the 72-h observation period, and these results were discarded. In the remaining experiments, cytocentrifuge preparations of the cell culture medium rarely showed free parasites or detached cells containing intracellular organisms.

To assess the effects of $O_2^-$ and $H_2O_2$ in intracellular killing, monocytes and macrophages were incubated 3 h before and during the 1-h infection period with medium containing SOD (1 mg/ml), catalase (1 mg/ml), or hydroxyl radical scavengers (50 mM mannitol, 10 mM benzoate) (1, 4, 6, 20), or with buffer free of glucose (Krebs-Ringer phosphate [KRP], pH 7.4) (6, 21). Standard medium was then replaced after the cells were infected. To inhibit monocyte MPO activity, $10^{-4}$ M azide was added 15 min before and during LDP or LDA ingestion (22). Exposure to $10^{-4}$ M azide for up to 12 h was not toxic to either form of the parasite as judged by microscopic appearance, motility, and the capacity of LDA to transform to LDP at 25°C (1, 4).

*$H_2O_2$ release and qualitative nitroblue tetrazolium (NBT) reduction.* The fluorometric scopoletin assay was used to measure monocyte and macrophage $H_2O_2$ release after triggering with phorbol myristate acetate (PMA) (Consolidated Midland Co., Brewster, NY) or parasite ingestion (4). Coverslips were washed, transferred to 16-mm wells of Costar tissue culture trays (Costar Data Packaging, Cambridge, MA), and incubated for 90 min at 37°C in 1.5 ml of KRP with 5.5 mM glucose (KRPG) and either PMA (100 ng/ml) or LDP or LDA at 10–30 × $10^6$/ml (1, 4). As previously reported (4), there was no detectable catalase or glutathione peroxidase activity in the LDA suspensions. Each 1.5 ml of reaction mixture also contained scopoletin, 5–20 nmol/ml, horseradish peroxidase, 0.44 purpurogallin U/ml, and in some assays $10^{-4}$ M azide was included to inhibit MPO (16). Controls consisted of wells containing (*a*) cells incubated without a triggering agent (no detectable $H_2O_2$ release), (*b*) parasites alone, and (*c*) blank wells with neither cells nor parasites (4). Adherent cell protein was determined using uninfected duplicated coverslips after digestion with 0.5 N NaOH (23), and the extent of parasite ingestion by infected cells was determined by counting the number of intracellular organisms after fixation and staining (1, 4).

Monocytes and macrophages were also stimulated for 1 h at 37°C with PMA, opsonized zymosan, LDP, or LDA suspended in medium containing NBT, 0.5 mg/ml. Cells were scored as positive if ingested zymosan or parasites were stained blue-black by precipitated formazan or if they showed clumped formazan after PMA triggering (1, 2, 4).

*Special reagents.* Scopoletin, horseradish peroxidase, SOD (bovine blood, type 1, 3,000 U/mg), catalase (bovine liver, 30,000 U/mg), NBT (grade III), mannitol, benzoate, and Con A (type III) were from the Sigma Chemical Co., St. Louis, MO.

## RESULTS

*Intracellular fate of LDP and LDA within unstimulated monocytes and monocyte-derived macrophages.* After 1 d in culture, monocytes from healthy donors displayed effective promastigocidal activity, and 24 h after infection eradicated >90% of ingested LDP (Fig. 1 *A*). Although considerable killing of intracellular LDP was achieved by 6 h as judged by dissolution of the organisms, within the first hour after ingestion obvious microscopic changes indicating dead LDP were also consistently present (Fig. 2 *A* and *B*). This striking leishmanicidal activity was not, how-

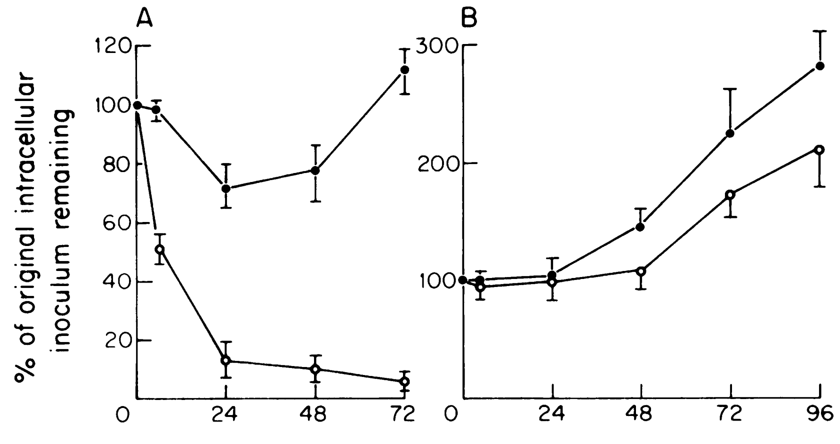


FIGURE 1 Intracellular fate of ingested LDP (O) and LDA (●) within (*A*) 1-d normal monocytes and (*B*) macrophages cultivated for 9–12 d before infection. For 1-d cells after the 1-h challenge period, 28–40% were infected with LDP with 41–92 LDP/100 cells and 31–42% were infected with LDA with 52–108 LDA/100 cells. For macrophages 1 h after challenge, 39–52% were infected with 52–108 LDP/100 cells and 42–64% had ingested LDA with 118–215 LDA/100 cells. Results are the means±SEM of five to nine experiments, and indicate the proportion (percent) of the original number of ingested parasites per 100 cells present at the indicated times (1–3).

ever, evident towards LDA (Fig. 1 *A*). Virtually none were killed by 1-d monocytes at 6 h, and <30% of LDA were digested by 24 h, after which time intracellular replication commenced (Fig. 2 *C*).

As illustrated in Figs. 1 *B* and 2 *D* and *E*, monocytes first cultivated for >7 d before challenge had undergone marked morphologic changes, and behaved quite differently towards both LDP and LDA. These monocyte-derived macrophages displayed little or no activity towards LDP and none towards LDA, and readily supported the replication of both parasite forms. Fig. 3 summarizes the decline in monocyte leishmanicidal capacity during in vitro maturation to the macrophage stage.

*Killing of LDP and LDA by lymphokine-activated monocytes and macrophages.* Since soluble products secreted by sensitized T lymphocytes induce human macrophages to exert activity against *T. gondii* and *T. cruzi* (11, 24, 25), we next examined the effects of lymphokine pretreatment on antileishmanial activity. The data in Fig. 4 demonstrate that peripheral blood lymphocytes readily generate both mitogen- and antigen-induced products capable of appreciably enhancing the activities of 1-d monocytes towards LDA and of cultivated macrophages towards both LDP and LDA. Although some degree of killing could be induced by pretreating macrophages for 24–48 h with as little as 1% lymphokine (data not shown), optimal results (Fig. 4 *B*) were achieved by 48–72 h of exposure to a concentration of 10–15% with fresh supernate material added each day before infection. These latter conditions were used in subsequent experiments, and were sufficient to activate cells that had remain in culture for as long as 30 d. The addition of lymphokine after infection only, however, did not enhance the activity of either monocytes or macrophages. Super-

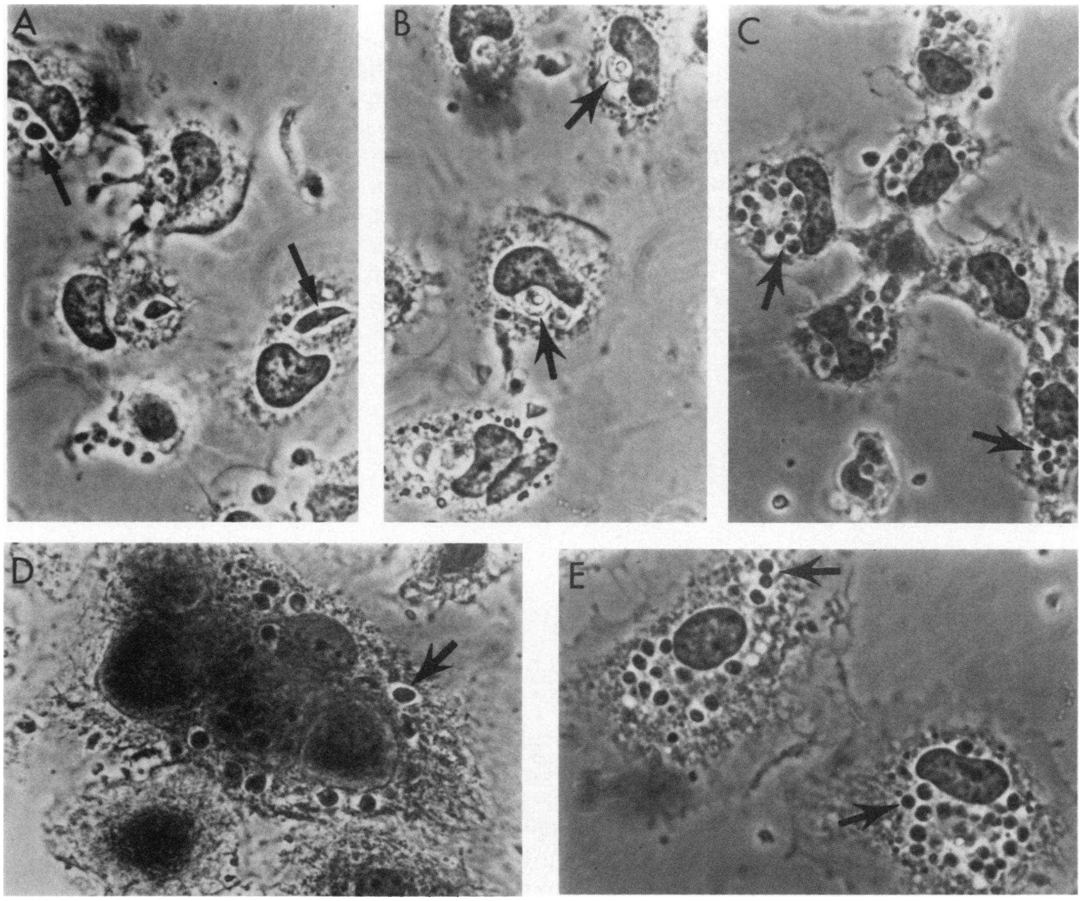


FIGURE 2 Phase-contrast micrographs showing intracellular *L. donovani*. (*A*) 1-d monocytes containing phase-dense and intact LDP. (*B*) Within 1 h, monocytes have degraded many intravacuolar LDP. (*C*) LDA replicating within 1-d monocytes 72 h after infection. (*D*) and (*E*) Monocyte-derived macrophages support the replication of both LDP (*D*) and LDA (*E*). Glutaraldehyde fixation, Giemsa stain. ×600–900.

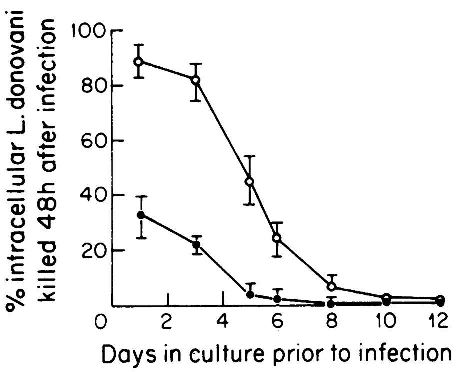


FIGURE 3 Decline in monocyte killing of LDP (O) and LDA (●) during in vitro cultivation. Results are the means±SEM of three to eight experiments at each time point.

nates prepared from mitogen- or antigen-stimulated cultures were equally effective in inducing antileishmanial activity, and increasing the pretreatment lymphokine concentration to 40% did not yield further activity.

*Monocyte oxidative activity during cultivation and effect of lymphokines.* Since oxygen-dependent mechanisms appear to contribute importantly to mononuclear phagocyte activity against intracellular protozoa (1, 2, 4, 6–10, 15), we next explored whether the capacity of monocytes and macrophages to generate oxygen intermediates correlated with their respective antileishmanial activities. The extracellular release of $H_2O_2$ was selected for study because this toxic intermediate (and not $O_2^-$) appears by itself to mediate oxygen-dependent leishmanicidal activity in the mouse peritoneal macrophage model (1, 2, 4, 15). As shown in Fig. 5, as monocytes differentiated into macrophages, the capacity to generate $H_2O_2$ after PMA triggering rapidly declined, and after 7 d in culture, macrophages released <10% of the $H_2O_2$ secreted by 1-d cells. The effect of lymphokine stimulation on monocyte and macrophage oxidative activity is also shown in Fig. 5. The presence of 10% mitogen- or antigen-induced lymphokines from the initiation of culture not only prevented the loss of the monocyte's $H_2O_2$-releasing capacity, but after 72 h of treatment, also enhanced the mature macrophage's oxidative activity by more than sixfold. In individual experiments, moreover, lymphokine-stimulated macrophages secreted up to 20 times more $H_2O_2$ than untreated or sham lymphokine-treated cells. These results agree closely with the recent studies of Nakagawara et al. (16, 26). Thus, for monocytes and macrophages, both in the unstimulated and activated state, there was a close correlation between oxidative capacity and the ability to display intracellular activity against LDP and LDA. In addition, the time course of the decline in $H_2O_2$-releasing capacity (Fig. 5) appeared to parallel the loss of antileishmanial activity (Fig. 3). Similar to the induction of leishmanicidal activity, optimal conditions for restoring macrophage $H_2O_2$ secretion were achieved by daily exposure to 10–15% fresh lymphokine for 72 h. Exposure to as little as 1% lymphokine for 3 d, however, resulted in an appreciable

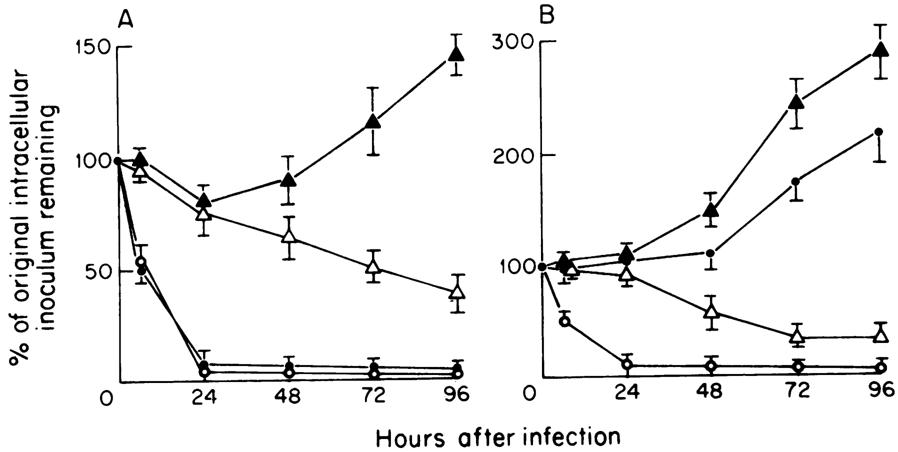


FIGURE 4 Enhancement of antileishmanial activity by lymphokine. (*A*) Fresh monocytes were cultivated with (open symbols) or without (closed symbols) 10% Con A lymphokine for the 24 h before infection with either LDP (circles) or LDA (triangles). In (*B*), cells were first cultivated in standard medium for 7–12 d and then fresh 10% Con A lymphokine was added for 3 d (open symbols) before challenge with LDP or LDA. Results are the means of three to four experiments. Parasite ingestion by lymphokine-treated cells was comparable to control cells. In two parallel experiments, antigen-induced lymphokine was as effective as Con A supernates, and sham lymphokines did not enhance either monocyte or macrophage antileishmanial activity.

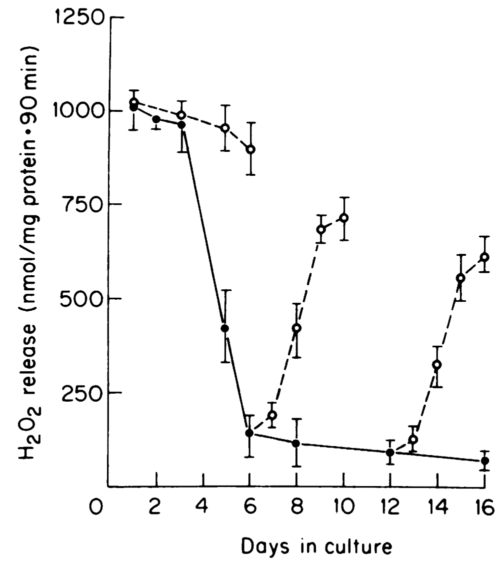


FIGURE 5 Capacity of monocytes to release $H_2O_2$ during cultivation. Monocytes were cultivated for 1–16 d in medium alone (●) or medium containing 10% Con A lymphokine (O) before triggering with PMA, 100 ng/ml. Fresh lymphokine was added daily either from the initiation of culture or after 6 or 12 d of cultivation in standard medium. Antigen-stimulated lymphokines produced simlar results, and $H_2O_2$ generation by sham lymphokine-treated cells was within 15–24% of that released by control cells. Results shown are the means±SEM of 4–18 experiments each in triplicate. Selected adherent cell protein values per coverslip for cells cultivated in medium alone were: 1 d (15.4±0.6 μg); 3 d (16.6±0.8 μg); 6 d (20.9±0.8 μg); and 12 d (36.2±2.1 μg). Protein values for monocytes treated for 72–96 h with lymphokines were 16–28% lower than control cells.

(3.6-fold) increase in $H_2O_2$ release, and concentrations >20% yielded no additional effects. To maintain enhanced oxidative activity, application of fresh lymphokine every 24–36 h was required; in its absence, $H_2O_2$ release for both activated monocytes and macrophages rapidly declined to control levels within 3 d (data not shown).

*Triggering of the oxidative burst by LDP and LDA.* To deliver toxic oxygen intermediates to parasite-containing phagocytic vacuoles, an obvious prerequisite is effective triggering of cell's $O_2^-$ and $H_2O_2$-generating mechanism. This aspect of monocyte and macrophage interaction with *L. donovani* was examined utilizing both qualitative and quantitative assays. In three to five experiments, 83–92% of 1-d monocytes promptly reduced NBT (primarily a $O_2^-$-dependent reaction (27)) upon ingestion of LDP (89±3%) and LDA (85±6%), and responded equally well to zymosan (83±5%) and stimulation with PMA (92±4%). Cells first cultivated for 7–18 d before challenge also responded to the same degree in this qualitative assay to LDP, zymosan, and PMA, but were appreciably less active towards LDA (48±8% of cells NBT positive). Data from the quantitative scopoletin assay, however, yielded additional information (not apparent from the NBT assay), which indicated that monocytes and macrophages display clear differences in their oxidative responses to LDP and LDA. These results (Fig. 6) demonstrated that LDA ingestion provoked remarkably little $H_2O_2$ generation by monocytes or macrophages at levels less than six times that released after phagocytizing comparable numbers of LDP, and, that ma-

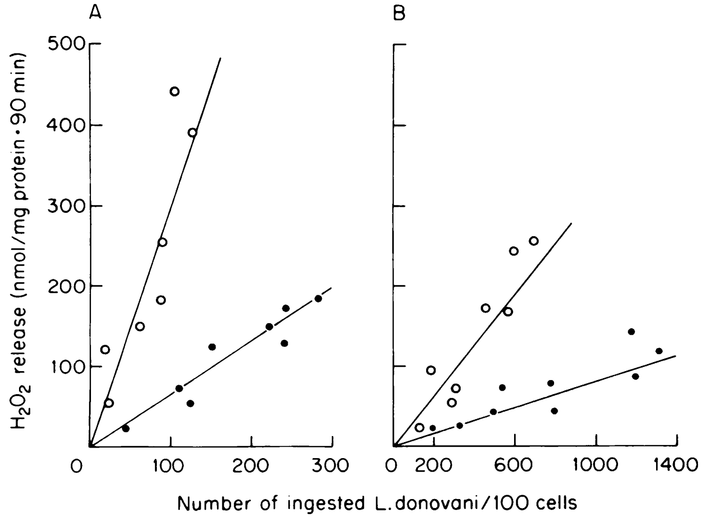


FIGURE 6 $H_2O_2$ release by (*A*) 1-d monocytes and (*B*) 10–16-d macrophages during ingestion of various loads of LDP (O) or LDA (●). Each symbol represents the mean of triplicate determinations for both $H_2O_2$ release and the number of organisms ingested.

ture macrophages secreted ~10-fold less $H_2O_2$ than 1-d monocytes in response to the ingestion of either form of *L. donovani.* The latter difference is consistent with the results obtained by using PMA as the respiratory burst stimulus (Fig. 5). Monocytes triggered by simultaneous exposure to both PMA and LDA released $H_2O_2$ in amounts similar to cells treated with PMA alone, indicating that neither the parasites for contaminating hamster spleen components were acting as $H_2O_2$ scavengers (4, 5). In addition, while 3 d of lymphokine treatment clearly enhanced macrophage $H_2O_2$ release in response to PMA (Fig. 5) or zymosan uptake (26), its effect on $H_2O_2$ generation triggered by LDP or LDA ingestion was somewhat less pronounced (a 2.6-fold increase, mean of three experiments, data not shown).

*Role of $O_2^-$ and $H_2O_2$ in intracellular antileishmanial activity.* Further studies were carried out to explore whether the mononuclear phagocyte's oxygen-dependent mechanism, which generates $O_2^-$ and $H_2O_2$ and more distal radicals such as $OH^\bullet$(20), actually mediates the observed intracellular killing of LDP and LDA. First, monocytes and monocyte-derived macrophages from three patients with CGD, cells that produce little or no oxygen intermediates (28), were challenged with the parasites after either 1 d or >10 d in culture. The oxidative and antileishmanial activities of cells from these patients and from a CGD carrier are shown in Table I and Fig. 7. 1-d CGD monocytes failed to kill any LDP or LDA at 6 h, and at 24 h, killed only 15% of LDP and no LDA. In a single experiment, 1-d cells from the CGD carrier generated 50% less $H_2O_2$ than normal monocytes, and killed 30–35% fewer LDP at both 6 and 24 h after infection. When compared with the activity of normal monocytes (Fig. 1), the findings with CGD cells not only indicated the key role of oxygen-dependent mechanisms, but also suggested that oxidative intermediates exert their leishmanicidal effects early in the post-phagocytic period. In addition, glucose deprivation (6, 21), (which as shown in Table II can reversibly inhibit NBT reduction and $H_2O_2$ generation), and catalase administration also effectively abrogated the early killing of intracellular LDP by both 1-d normal monocytes and lymphokine-activated macrophages (Table III). Heated catalase and scavengers of $O_2^-$ (SOD) and $OH^\bullet$ (50 mM mannitol and 10 mM benzoate) (20), however, had no effect in these experiments suggesting that $H_2O_2$ alone was the key leishmanicidal oxygen intermediate (1, 4).

*Role of MPO.* Since monocytes contain abundant levels of MPO and the $H_2O_2$-MPO-halide reaction is highly toxic to a number of microorganisms including LDP and LDA (1, 4), we also examined parasite intracellular fate after MPO inhibition by azide (9). Treatment of 1-d cells for 15 min before and during ingestion with 0.1 mM azide, a procedure that impairs monocyte candidacidal activity (22), inhibited LDA killing in three experiments by 38±6%, but had no effect on LDP killing. The latter observation may re-

TABLE I
*Oxidative Activity of CGD Monocytes**

| Monocyte population | Cells NBT positive | | | | $H_2O_2$ released‡ | |
|---|---|---|---|---|---|---|
| | Zymosan | PMA | LDP | LDA | −LK | +LK§ |
| | | | % | | | |
| 1-d cells | | | | | | |
| Normal controls (5) | 87±3 | 90±4 | 89±3 | 85±4 | 1,015±58 | 998±92 |
| CGD patients (5) | <1 | <1 | 1±0.5 | 1±0.3 | 24±9 | 27±12 |
| CGD carrier (1) | 44 | 51 | 45 | 49 | 478±39 | 496±21 |
| 10–16-d cells | | | | | | |
| Normal controls (3) | 83±4 | 81±6 | 84±4 | 54±7 | 124±29 | 654±66 |
| CGD patients (3) | 0 | <1 | 0 | <1 | 36±11 | 49±10 |

* Monocytes from three normal donors, three patients with CGD, and one CGD carrier were cultivated overnight (1-d cells) or for up to 16 d before either (*a*) a 1-h challenge with zymosan particles ($5 \times 10^6$/ml), PMA (100 ng/ml), or $5 \times 10^6$/ml LDP or LDA suspended in medium containing NBT, 0.5 mg/ml (1, 4), or (*b*) triggering with PMA (100 ng/ml) for $H_2O_2$ release. Results are the means±SEM of (*n*) experiments performed in duplicate (NBT assay) or triplicate ($H_2O_2$ release).

‡ Nanomoles of $H_2O_2$ released per milligram cell protein per 90 min.

§ 10% Con A lymphokine (LK) was added to fresh monocytes for 24 h before assay or for the preceeding 72 h to 10–16-d cells. In three experiments, 0–1% of lymphokine-activated 10–16-d CGD cells reduced NBT in response to the agents listed.

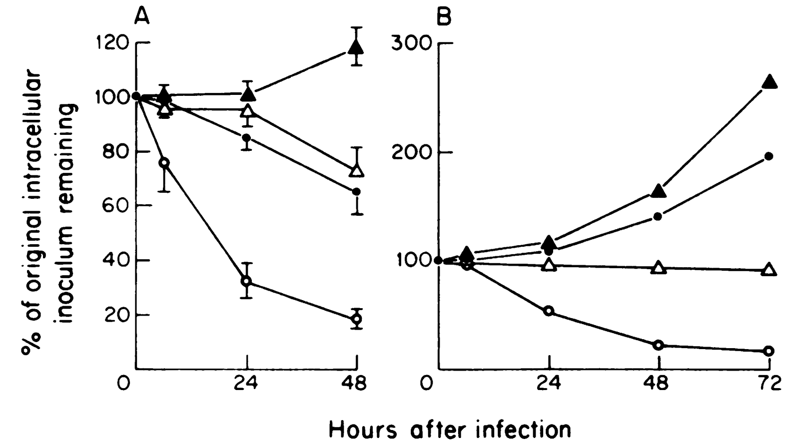


FIGURE 7 Survival of LDP (circles) and LDA (triangles) within (*A*) 1-d CGD monocytes and (*B*) 10–16-d CGD macrophages. Closed symbols indicate cells cultivated in medium alone; open symbols indicate cells treated for 24 h (*A*) or for 72 h (*B*) before infection with 10% Con A lymphokine as in Fig. 4. Parasite ingestion by CGD monocytes and macrophages was comparable to normal cells. Results are the means±SEM of four experiments in (*A*) and of two experiments in (*B*).

flect the exquisite susceptibility of LDP to $H_2O_2$ alone (1). Following azide treatment, there was a greater than twofold increase in monocyte PMA-triggered $H_2O_2$ release, presumably indicating azide's inhibition of MPO-mediated $H_2O_2$ catabolism (16). Mature human macrophages have little or no MPO activity (17), and azide administration did not alter the intracellular fate of either parasite within these cells nor enhance $H_2O_2$ generation irrespective of prior lymphokine stimulation (data not shown).

*Evidence for oxygen-independent antileishmanial activity.* Although the results depicted in Fig. 7 and Table III suggested that the early postphagocytic antileishmanial activity of 1-d monocytes and lymphokine-activated macrophages was primarily oxygen dependent, the same experiments also provided evidence for oxygen-independent activity as well. Thus, by 48 h after infection unstimulated 1-d CGD monocytes killed nearly 40% of LDP, and both the magnitude and rate of this activity could be appreciably enhanced by pretreatment with lymphokine (Fig. 7 *A*). In ad-

TABLE II
*Effect of Glucose Deprivation on Monocyte Oxidative Activity**

| Preincubation medium (3 h) | Assay buffer | Cells NBT positive: Zymosan | Cells NBT positive: LDP | $H_2O_2$ release: PMA |
|---|---|---|---|---|
| | | % | | |
| RPMI | KRPG | 86±4 | 82±5 | 764±47 |
| KRPG | KRPG | 84±3 | 88±2 | 715±66 |
| KRP | KRP | 16±6 | 18±3 | 51±22 |
| KRP | KRPG | 72±8 | 68±7 | 552±92 |

* Cultures of 1-d normal monocytes were preincubated for 3 h in standard medium (RPMI), buffer containing 5.5 mM glucose (KRPG), or glucose-free buffer (KRP), and then assayed in either KRPG or KRP for NBT reduction (1 h) or $H_2O_2$ release (nanomoles per milligram per 90 min) as in the legend to Table I. Results are the mean±SEM of three experiments. In two separate experiments with lymphokine-activated macrophages, glucose deprivation inhibited NBT reduction and $H_2O_2$ release by 78±4 and 86±6%, respectively.

TABLE III
*Effect of Oxygen Intermediate Scavengers and Glucose Deprivation on Intracellular Leishmanicidal Activity**

| Monocyte population/Treatment | Percent LDP killed at 6 h | 24 h | 48 h |
|---|---|---|---|
| 1-d monocytes | | | |
| No treatment | 68±7 | 90±3 | 91±2 |
| Glucose-deprived | 9±4 | 46±6 | 88±4 |
| Catalase | 12±6 | 64±4 | 74±6 |
| SOD | 55±6 | 89±5 | 90±4 |
| 10–15-d activated macrophages | | | |
| No treatment | 62±9 | 76±8 | 84±4 |
| Glucose-deprived | 13±4 | 51±13 | 80±8 |
| Catalase | 16±7 | 58±9 | 72±8 |
| SOD | 50±6 | 70±7 | 82±5 |

* Unstimulated 1-d monocytes and macrophages activated by 3-d of treatment with 10% Con A lymphokine were incubated for 3 h before and during a 1 h challenge with LDP ($5 \times 10^6$/ml) in buffer free of glucose (KRP) or medium containing catalase (1 mg/ml) or SOD (1 mg/ml). Results indicate the mean±SEM of three to four experiments.

dition, despite an impaired capacity to generate $H_2O_2$, glucose-deprived and catalase-treated 1-d normal monocytes and lymphokine-activated macrophages also achieved virtually normal levels of LDP killing by 24–48 h (Table III). Although unstimulated 1-d CGD monocytes displayed no activity towards LDA, lymphokine exposure also resulted in some LDA killing by 48 h as well as in inhibition of replication (Fig. 7 *A*).

Monocyte-derived CGD macrophages were also challenged with *L. donovani,* and as illustrated in Fig. 7 *B*, these cells behaved similar to normal macrophages and killed neither LDP nor LDA and supported intracellular replication. However, lymphokine pretreatment was also effective in inducing CGD macrophages to display antileishmanial activity that was microbicidal towards LDP and microbistatic against LDA (Fig. 7 *B*). Additional experiments (Fig. 8) compared the time course of activation of normal and CGD macrophages. In addition to indicating that normal cells require considerably less stimulation to display effective promastigocidal activity, these results also reemphasized that activated CGD macrophages, which lack the ability to generate oxygen intermediates (Table I), could kill LDP but not LDA. 72 h of lymphokine exposure did, however, achieve the inhibition of LDA replication depicted in Fig. 7 *B*.

*Activity of binucleated and multinucleated macrophages.* In monocyte cultures maintained >7 d, the appearance of bi- or multinucleated cells was typical, and their number and size (e.g., giant cells) were considerably enhanced by 3–4 d of lymphokine exposure (29). Monocyte from CGD patients formed these cells as readily as did monocytes from normal individuals. During the course of these studies we also had the opportunity to observe both the oxidative and antiprotozoal activities of this subpopulation of culture-derived human macrophages. Multinucleated cells from normal donors behaved similar to uninucleated macrophages in terms of qualitative NBT reduction (>80% of cells NBT-positive after triggering with PMA, LDP, or zymosan), ingestion of LDP and LDA, and in supporting LDP and LDA replication. In addition, the oxygen-dependent and -independent antileishmanial activities of multinucleated cells appeared to be comparably enhanced by lymphokine since in no experiment did these cells from either normal or CGD donors contain a disproportionate number of surviving *L. donovani.*

## DISCUSSION

The results of this study indicate that human mononuclear phagocytes utilize both oxygen-dependent and -independent mechanisms to achieve activity against the intracellular protozoal target, *L. donovani*. Although the activity of these two antimicrobial pathways appears to be well developed and vigorous in the peripheral blood monocyte, both mechanisms rapidly involute by the time the monocyte differentiates in culture to the macrophage stage. Thus, it is not surprising that it is this latter cell population that can be regularly parasitized in vitro by Leishmania (5, 30, 31), *T. gondii* (8, 24, 25), and *T. cruzi* (11). In addition, if these observations accurately reflect events in the intact host, they may also explain the basis of the tissue macrophage's susceptibility to infection by these three intracellular pathogens (32).

Evidence indicating the presence of both oxygen-dependent and -independent antileishmanial mecha-

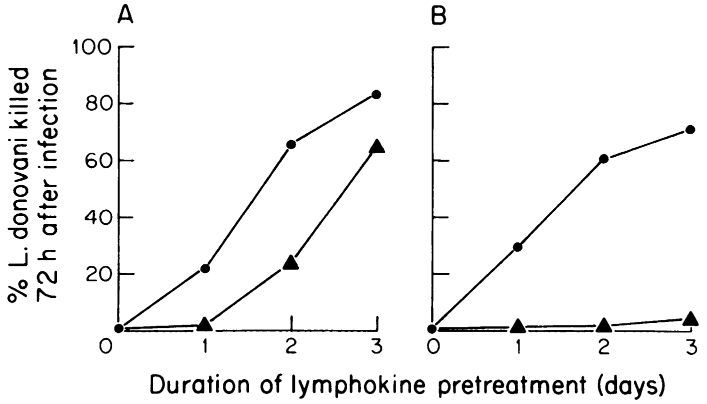


FIGURE 8 Comparison of the duration of pretreatment with 10% Con A lymphokine required to induce 10–16 d normal (●) or CGD (▲) macrophages to display activity against LDP (*A*) or LDA (*B*). Results are the means of two duplicate experiments in which the range of the values from the indicated means was 6–10% for normal cells and 2–8% for CGD cells.

nisms was derived from the same experiments that examined the fate of LDP and LDA within mononuclear phagocytes from normal individuals, patients with CGD, and normal cells treated with oxygen intermediate scavengers or deprived of exogenous glucose. Similar to its effect on mouse peritoneal cells (6, 21), the latter technique induces nearly complete (>90%) but promptly reversible inhibition of the oxidative activity of both human monocytes and macrophages. The results of these experiments provided firm evidence for the presence of oxygen-dependent antileishmanial activity during the initial 6–24 h postphagocytic period by demonstrating that 1-d monocytes and lymphokine-activated macrophages treated with catalase or deprived of glucose or obtained from CGD patients displayed considerably less activity than normal control cells towards both LDP and LDA. Utilizing human polymorphonuclear leukocytes (PMN) and a short-term killing assay, Pearson and Steigbigel (33) also arrived at much the same conclusion since CGD cells failed to kill LDP while normal PMN eradicated >80%. Since catalase, but not SOD, achieved an inhibitory effect in our studies, it appeared that $H_2O_2$ alone mediated the early events in intracellular *L. donovani* killing, and that neither $O_2^-$ nor the more distal intermediates such as $OH^\bullet$ were required for leishmanicidal activity. These results are consistent with previous findings derived from the mouse peritoneal macrophage model in which a key role for $H_2O_2$ was demonstrated in the killing of LDP and LDA by resident cells and lymphokine-activated macrophages, respectively (1, 2, 4, 15).

At the same time, our studies with CGD and oxidatively impaired normal cells also indicated the presence of an oxygen-independent antiprotozoal mechanism, and provided evidence for its responsiveness to lymphokine. Thus, unstimulated 1-d CGD monocytes eventually accomplished appreciable LDP killing by 48 h, and this capacity could be strikingly enhanced by lymphokine resulting in near-normal levels of promastigocidal activity by 24 h after infection. Lymphokine treatment also induced CGD macrophages to eradicate ingested LDP, and activated both CGD monocytes and macrophages to inhibit LDA replication. Similarly, despite displaying little early antileishmanial activity (e.g., at 6 h after infection), catalase-treated and glucose-deprived normal monocytes and lymphokine-stimulated macrophages also eventually achieved effective promastigocidal behavior as well. Since there presumably is no further plasma membrane perturbation following parasite ingestion, which might trigger the oxidative burst mechanism, it seems reasonable to suggest that subsequent antileishmanial activity also reflects oxygen-independent effects.

Comparison of the data in Figs. 4, 7, and 8 also provides further insight into the relative effectiveness and the requirements for activation of the monocyte and macrophage's antiprotozoal mechanisms. For example, although the oxygen-independent mechanisms of both unstimulated and lymphokine-activated CGD cells achieved promastigocidal effects, the same mechanisms appeared to be primarily microbistatic towards LDA. In addition, while clearly capable of killing LDP, CGD cells exerted this activity considerably more slowly than monocytes or lymphokine-activated macrophages from normal donors, and typically required 48 h or more to achieve the antileishmanial effects displayed within 6–24 h by normal cells. In addition, it also appeared that the macrophage's oxygen-independent mechanism required appreciably longer stimulation by lymphokine to become fully developed and effective (Fig. 8). Taken together, these observations serve to reemphasize both the importance of an intact oxidative burst mechanism and its capacity to deliver prompt intracellular antileishmanial activity. It is also worth pointing out that despite being able to generate $H_2O_2$ in amounts comparable to lymphokine-activated 1-d monocytes and 50% more $H_2O_2$ than lymphokine-activated macrophages (Fig. 5), unstimulated 1-d normal monocytes did not display the sustained amastigocidal activity exhibited by the other two cell populations. This finding also suggests that lymphokine can enhance the effects of multiple antileishmanial mechanisms.

In the absence of lymphokine stimulation, both the oxygen-dependent and -independent mechanisms of the cultivated monocyte declined rapidly and in parallel as indicated by the failure of either normal or CGD monocyte-derived macrophages to kill any LDP or LDA. Although normal cells cultivated for 7-d or longer lost relatively little of their oxidative responsiveness as judged by qualitative NBT reduction, in the quantitative scopoletin assay, macrophages released 90% less $H_2O_2$ after either PMA triggering or the ingestion of LDP and LDA. This spontaneous decline in monocyte oxidative capacity during in vitro differentiation has also been well-documented by two other recent studies (16, 34). At all stages of monocyte maturation, however, the addition of soluble lymphocyte products strikingly altered cellular behavior and readily restored both oxidative and antileishmanial activity. Nakagawara et al. (26) have also recently reported that lymphokines can enhance monocyte and macrophage $H_2O_2$ release, and it is pertinent to note that both mitogen- and antigen-stimulated lymphokines can induce human cells to inhibit or kill the other intracellular protozoa, *T. gondii* (24, 25) and *T. cruzi* (11). However, as judged by the eventual outcome of infection of activated normal cells whose $H_2O_2$ generation had been impaired by catalase and glucose deprivation and of stimulated CGD monocytes and macrophages, only the early phase of the lymphokine-enhanced antileishmanial response could be directly attributed to oxygen-dependent mechanisms. Nevertheless, it can be concluded from these results that both the oxygen-dependent and -independent mechanisms of the human mononuclear phagocyte are responsive to modulation by lymphokine. Since we did not use media and reagents known to be free of bacterial lipopolysaccharide (34), it is also worth noting the possibility that trace amounts of lipopolysaccharide, acting alone (34) or synergistically with lymphokine (35), may have contributed to the results of our experiments.

In comparison to the macrophage, the monocyte's oxygen-dependent and -independent mechanisms appeared to be more sensitive to lymphokine in terms of the duration of stimulation required to enhance antileishmanial activity. While 24 h of lymphokine treatment was sufficient to induce normal 1-d monocytes to display appreciable amastigocidal effects (Fig. 4 *A*), an additional 24–48 h of stimulation was required for normal macrophages. Similarly, although 24 h of lymphokine exposure induced 1-d CGD monocytes to kill 80% of ingested LDP, 3 d of lymphokine was necessary before CGD macrophages displayed comparable promastigocidal activity.

Our observation that the monocyte's oxygen-independent antileishmanial effects are not evident until ≥24 h after parasite ingestion contrasts with those recently reported by Locksley et al. (9). These investigators found that although significantly less active than normal cells, freshly obtained CGD monocytes could still kill 45% of phagocytized *T. gondii* within the first 6 h (9). Although we have examined CGD monocytes only after 1 d in culture, we have observed no toxoplasma killing or inhibition of replication by these cells in the absence of pretreatment with lymphokine. In contrast, 1-d monocytes from normal individuals readily display antitoxoplasma activity without lymphokine stimulation.[2] However, similar to previous studies with *T. gondii* and activated mouse peritoneal macrophages, which indicated the importance of effective oxidative burst activity (6–9), Locksley et al. (9) also noted that by 20 h after infection, normal monocytes effectively inhibit toxoplasma replication whereas CGD cells permit surviving organisms to multiply. In related experiments examining the effects of tumor cell-derived factors that markedly suppress mouse macrophage $O_2^-$ and $H_2O_2$ release (36), we have also observed virtually complete inhibition of the resident macrophage's capacity to kill LDP at 6 h (1), but by 18 h, killing occurs to a near-normal extent.[3] These results also suggest that the mononuclear phagocyte's oxygen-dependent mechanism is primarily responsible for antileishmanial activity during the early postphagocytic period.

This study also demonstrates a number of potentially key differences between the flagellate (promastigote) and the intracellular (amastigote) forms of *L. donovani* and their interaction with both human monocytes and macrophages. Previous work has indicated that LDP are not only highly susceptible to enzymatically generated $H_2O_2$ but also readily trigger its secretion by normal mouse peritoneal macrophages, cells which proceed to kill >85% of ingested LDP (1). In contrast, LDA are appreciably more resistant to $H_2O_2$, evade substantial triggering of the mouse macrophage's oxidative burst, and persist unharmed within the phagolysosomes of normal cells (4, 15). By extending this analysis to another mononuclear effector phagocyte, the human monocyte, our current studies serve to reemphasize the striking resistance of the amastigote form of *L. donovani* to intracellular killing. Thus, despite being capable of destroying 90% of LDP, 1-d monocytes killed few LDA, and failed to prevent this organism's subsequent replication. In addition to differential susceptibility to $H_2O_2$ (4), several other explanations for the contrasting intracellular fates of LDP and LDA within human monocytes may be pertinent. First, ingested LDA provoked substantially less $H_2O_2$ release than did LDP from all cells examined, which in conjunction with an intrinsically higher level of resistance to $H_2O_2$ (4), may allow the majority of LDA to evade oxidant-mediated injury. Using a chemiluminescence assay, Pearson et al. (5) have also recently found that LDA ingestion by 5-d human monocytes triggered <15% of the oxidative activity provoked by either LDP or zymosan. LDA also contain threefold more SOD than LDP, and appear to be resistant to enzymatically generated $O_2^-$ as well (4). Second, since LDA emerge to infect other cells from the vacuolar apparatus of a parasitized macrophage, it is possible that host cell-derived membranes or cytoplasmic constituents impart some degree of protection against killing, which cultivated LDP cannot acquire. Alternatively, replicating LDA themselves may secrete defensive factors or possess a less vulnerable outer membrane, either of which might enhance resistance and promote intracellular survival. Third, our experiments with unstimulated and lymphokine-activated CGD cells also indicate that in contrast to LDP, LDA

[2] Murray, H. W. Unpublished observations.

[3] Murray, H. W., A. Szuro-Sudol, and C. F. Nathan. Unpublished observations.

are not killed by oxygen-independent mechanisms. LDA resistance to an acid environment and potentially toxic lysosomal contents, for example, appears certain since Leishmania do not inhibit phagolysosomal fusion and readily replicate within fused vacuoles (30, 31).

Given sufficient exposure to active lymphokine, however, it is clear that macrophages from normal individuals can acquire the capacity to kill and/or effectively inhibit LDA replication in vitro. This activity is presumably achieved by focusing both stimulated oxygen-dependent and -independent pathways in a coordinated fashion. Based upon these results, those of our recent study in which lymphocytes from *L. donovani*-infected mice were actively suppressed and failed to generate effective macrophage-activating lymphokines (3), and the well-documented capacity of Leishmania infection to suppress both human and animal lymphocyte proliferation (37–40), it would seem reasonable to suggest and next explore the possibility that patients with uncontrolled leishmaniasis have defects in the production of macrophage-activating lymphokines. In the absence of such a stimulus shown here to be important in amplifying both oxygen-dependent and -independent microbicidal mechanisms, host macrophages presumably remain vulnerable to and perpetuate Leishmania infection.

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