INFECTION AND IMMUNITY, Mar. 1987, p. 587–593
0019-9567/87/030587-07$02.00/0

Vol. 55, No. 3

# Impairment of the Oxidative Metabolism of Mouse Peritoneal Macrophages by Intracellular *Leishmania* spp.

YOLANDE BUCHMÜLLER-ROUILLER* AND JACQUES MAUËL
*Institute of Biochemistry, CH-1066 Epalinges, Switzerland*

Received 4 August 1986/Accepted 18 November 1986

**When stimulated in vitro with macrophage-activating factor or lipopolysaccharide, mouse peritoneal macrophages acquire the capacity to develop a strong respiratory burst when they are triggered by membrane-active agents. The presence of intracellular parasites of the genus *Leishmania* (*L. enriettii*, *L. major*) significantly inhibited such activity, as measured by chemiluminescence, reduction of cytochrome *c* and Nitro Blue Tetrazolium, and hexose monophosphate shunt levels. On the contrary, inert intracellular particles such as latex beads strongly increased the macrophage respiratory burst, suggesting that the *Leishmania*-linked inhibition resulted from a specific parasite effect. Impairment of macrophage oxidative metabolism by intracellular *Leishmania* spp. was a function of the number of infecting microorganisms and was more pronounced in macrophages infected with living than with dead parasites. Moreover, the metabolic inhibition was less apparent in *L. enriettii*-infected macrophages that were exposed to both macrophage-activating factor and lipopolysaccharide, i.e., conditions leading to complete parasite destruction. The mechanisms of respiratory burst inhibition by intracellular *Leishmania* spp. are unclear, but these observations suggest that such effects may contribute significantly to intracellular survival of the microorganism.**

When triggered by a phagocytic stimulus or certain membrane-active agents, macrophages activated in vivo or in vitro undergo a respiratory burst that is characterized by the production of high levels of oxygen metabolites. Such a respiratory burst appears to result from the activation of a membrane-bound NADPH oxidase, the $K_m$ of which for NADPH is reduced. The oxygen metabolites are thought to play an important role in the destruction of various microorganisms and tumor cells (5, 10, 14, 22, 23–26, 30). Additional factors, however, appear to be necessary for the killing of certain targets. Indeed, cells stimulated with lipopolysaccharide (LPS) do secrete large amounts of $O_2$ metabolites, but they fail to destroy intracellular *Leishmania* parasites (6). The manner in which microorganisms evade intracellular killing is still not completely understood and may involve the impairment of both oxygen-dependent and -independent mechanisms.

This study was designed to examine whether intracellular *Leishmania* spp. might interfere with the oxidative metabolism of their host macrophages. The effect of the presence of these intracellular parasites on the respiratory burst of lymphokine-activated and LPS-stimulated mouse macrophages was determined by several assays, i.e., chemiluminescence, Nitro Blue Tetrazolium (NBT) and cytochrome *c* reduction, and hexose monophosphate shunt activity. Results obtained by all four procedures show an impairment of the oxidative metabolism of infected versus noninfected cells.

## MATERIALS AND METHODS

**Mice.** CBA/T6 mice of either sex (age, 2 to 5 months) were obtained from the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland.

* Corresponding author.

**Cell cultures.** Mice were inoculated intraperitoneally with sterile hydrolyzed starch (2% in pyrogen-free saline) (16). Peritoneal exudate cells were harvested 3 days later, washed, and suspended in Dulbecco medium (Seromed, Munich, Federal Republic of Germany) supplemented with 10% fetal calf serum (Seromed) and distributed as follows. For chemiluminescence studies cells were distributed into flat-bottom glass tubes (diameter, 10 mm; $2 \times 10^5$ to $2.5 \times 10^5$ peritoneal cells; 0.5 ml per tube). For hexose monophosphate shunt (HMPS) determination cells were distributed into 24-well tissue culture clusters (3524; Costar, Cambridge, Mass.) ($5 \times 10^5$ cells; 0.5 ml per well). For cytochrome *c* and NBT reduction tests and for protein determination, cells were distributed into flat-bottom, 96-well microtiter plates (3596; Costar) ($1.5 \times 10^5$ to $2.0 \times 10^5$ cells; 150 μl per well).

**Infection and activation of macrophage cultures.** *Leishmania enriettii* prepared from infected guinea pig tissue (15) and *Leishmania major* (Liverpool reference strain LV39) isolated from infected mouse footpads were propagated as promastigotes in Hosmem II liquid medium (3). Parasites were washed by centrifugation (1,200 × *g* for 10 min) before they were added to the macrophages at various concentrations, as indicated throughout the text. Polystyrene latex particles (diameter, 1.1 μm; Sigma Chemie GmbH, Munich, Federal Republic of Germany) were washed 3 times with Hanks balanced salt solution (HBSS; Seromed) and suspended in fetal calf serum-supplemented Dulbecco medium. At 2 h after plating peritoneal exudate cells (PECs), parasites, or latex beads were added to the cultures as follows: for *L. enriettii*, at a parasite/PEC ratio of 20:1 (unless indicated otherwise), resulting in a final infection ratio of 100 to 200 parasites per 100 macrophages on the day of the test; for *L. major*, at a parasite/PEC ratio of 10:1 (unless indicated otherwise), resulting in a final infection ratio of 800 to 900 parasites per 100 macrophages; for latex beads, at concentrations of 25 or 50 μg per well. Cultures were then incubated

overnight at 37°C. Cultures without added particles were run in parallel. Cells were then thoroughly washed with warm HBSS and reincubated for 24 h (unless otherwise indicated) either with a macrophage-activating factor (MAF)-containing supernatant (cf. below) or with control medium in the presence or absence of 10 ng of endotoxin (LPS from *Escherichia coli* O55:B5; Difco Laboratories, Detroit, Mich.) per ml. The final volumes were 500 μl for flat-bottom glass tubes, 1,000 μl per well for 24-well tissue culture clusters, and 200 μl per well for 96-well microtiter plate.

MAF-containing supernatants were prepared by stimulating CBA/T6 or C57BL/6 spleen cells with concanavalin A (Pharmacia, Uppsala, Sweden) for 72 h, as described previously (4).

**Metabolic studies.** Macrophage cultures treated as described above were washed twice with HBSS without phenol red before they were tested for oxidative metabolism.

**Chemiluminescence.** Macrophages were incubated with 0.5 ml of $10^{-4}$ M lucigenin (Sigma) in HBSS without phenol red. One μg of phorbol myristate acetate (PMA; Sigma) per ml was added as a stimulus. Luminescence was recorded every 5 to 6 min in a luminometer (LKB Instruments, Inc., Rockville, Md.), as described previously (6). Data are expressed either as integrals of the total number of millivolts recorded over a 60-min period or as the percentage of inhibition of luminescence in infected versus noninfected macrophages over a 60-min period.

**NBT reduction test.** NBT reduction by macrophages was determined by the semiautomated densitometric method described by Pick et al. (31). Briefly, washed cells in 96-well microtiter plates were preincubated either with 100 μl of HBSS or with 10 mM iodoacetamide (IAA) in HBSS for 30 min at 37°C. Wells containing IAA, an inhibitor of the respiratory burst, were used as blanks in later photometric determinations. Plates were then emptied, and the wells were supplemented with either 100 μl of an NBT solution (1 mg/ml in HBSS) containing 500 ng of PMA per ml or 100 μl of a NBT solution containing 10 mM IAA (blanks). The cells were reincubated at 37°C. At desired times (0, 15, 30, 45, 60, and 90 min) plates were placed in a micro-enzyme-linked immunosorbent assay (ELISA) reader (Easy Reader EAR 400; Kontron Analytik, Zurich, Switzerland) fitted with a 550-nm filter, and the absorbance of the formazan deposits in the cells was measured. Results were expressed as the difference in absorbance per milligram of cell protein ($\Delta E$/mg of protein) between cultures incubated in the absence (−IAA) and presence (+IAA) of IAA at each time point (*ti*), according to the following formula:

$$\Delta E/\text{mg of protein} = \frac{\Delta E_{ti\text{–}to}\,[-\text{IAA}] - \Delta E_{ti\text{–}to}\,[+\text{IAA}]}{\text{mg of protein}}$$

**Cytochrome *c* reduction test.** The method used for the cytochrome *c* reduction test was the semiautomated microassay described by Pick and Mizel (32). Washed cells were incubated with 100 μl of a solution of ferricytochrome *c* (from horse heart, type VI; Sigma) at a concentration of 2 mg/ml in HBSS containing 500 ng of PMA per ml. Parallel wells used as blanks included 30 μg of superoxide dismutase (SOD) (Sigma) per ml in the reaction medium. After various times (0, 30, 60, 90, and 120 min) of incubation at 37°C, plates were placed in a micro-ELISA reader (Easy Reader EAR 400; Kontron Analytik), and reduction of cytochrome *c* was measured at 550 nm with a reference filter at 492 nm. Results were expressed as the difference in absorbance per milligram of cell protein ($\Delta E$/mg of protein) between cultures incubated in the absence (−SOD) and presence (+SOD) of SOD at each time point (*ti*), according to the following formula:

$$\Delta E/\text{mg of protein} = \frac{\Delta E_{ti\text{–}to}\,[-\text{SOD}] - \Delta E_{ti\text{–}to}\,[+\text{SOD}]}{\text{mg of protein}}$$

**HMPS activity.** HMPS activity was determined as described previously (17). Briefly, washed cells in wells (diameter, 16 mm) were incubated with 300 μl of medium (25% medium 199–75% F12 medium without glucose) containing 0.1 μCi of [1-$^{14}$C]glucose (3.94 Ci/mmol; Radiochemical Centre, Amersham, England) and 1 μg of PMA per ml. The wells were immediately sealed with a silicone rubber disk fitted with a piece of filter paper moistened with 1.0 M NaOH to trap the radioactive carbon dioxide ($^{14}CO_2$) evolved by the cells. After 120 min at 37°C, 50 μl of 1.0 M $H_2SO_4$ was injected into each well. After an additional 15 min of incubation period at room temperature, the filters were removed, dried, and counted.

**Determination of cellular protein.** The method used to determine cellular protein was that described by Baumgarten (1). Briefly, cells were thoroughly washed in serum-free HBSS and then air dried for 60 min at 37°C. Coomassie blue dye (30 μl; 500-0006; Bio-Rad, Munich, Federal Republic of

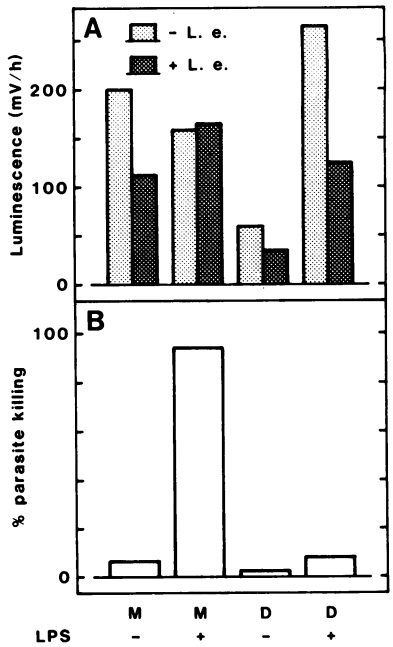


FIG. 1. Effect of intracellular *L. enriettii* on macrophage luminescence. CBA/T6 macrophages, infected or noninfected with *L. enriettii* (L. e.), were incubated with a 1:8 dilution of MAF-rich (M) or fetal bovine serum (FBS)-supplemented control Dulbecco (D) medium in the presence (+) or absence (−) of added LPS (10 ng/ml). After 24 h luminescence was recorded over a 60-min period following triggering with PMA. Results are reported as millivolts integrated over the 60-min test period. (A) A representative experiment of 13 experiments. The differences in luminescence between infected and noninfected cells were highly significant in MAF-stimulated, LPS-stimulated, and control cultures by both the paired-sample *t* test ($P < 0.00001$) and the Wilcoxon rank signed test ($P = 0$). Much less difference was observed between infected and noninfected cultures stimulated with MAF-LPS ($P = 4\%$ by the paired-sample *t* test and $P = 2\%$ by the Wilcoxon rank signed test). Cultures treated similarly were used to determine intracellular parasite killing (B).

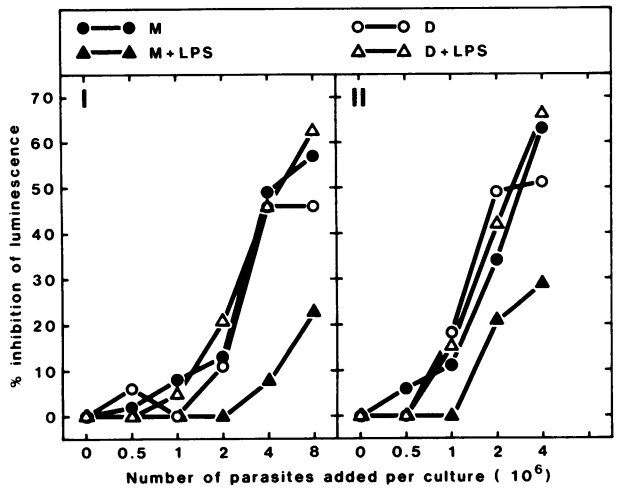


FIG. 2. Dose response of luminescence inhibition by intracellular *L. enriettii*. Cultures of CBA/T6 macrophages infected with increasing numbers of *L. enriettii* were incubated with a 1:8 dilution of MAF-rich (M) or FBS-supplemented control Dulbecco (D) medium in the presence (+) or absence (−) of added LPS (10 ng/ml). After 24 h luminescence was recorded over a 60-min period following triggering with PMA. Results are reported as the percent inhibition of luminescence of infected versus noninfected cultures. Results of two representative experiments (I and II) are shown.

Germany) was added into each well, and the plates were shaken for 5 min on a rotary shaker. The wells were then supplemented with 120 μl of water, and the plates were shaken again for 5 min. The optical density was then read in a micro-ELISA reader (Easy Reader EAR 400; Kontron Analytic) at 630 nm with a reference filter at 492 nm. Standards were prepared by adding 50 μl of increasing doses of ovalbumin (0 to 50 μg) into microtiter wells, which were then air dried at 37°C overnight. Standard protein determination was done in parallel with cellular protein determination.

**Statistical analysis.** The significance of the data was evaluated by the paired-sample *t* test and the Wilcoxon rank signed test.

## RESULTS

**Impairment of chemiluminescence of macrophages by intracellular *L. enriettii*.** Peritoneal cells of CBA/T6 mice were parasitized with *L. enriettii* and then incubated with MAF or control medium in the presence or absence of LPS. Luminescence of infected macrophages was determined after 24 h and compared with that of similarly treated uninfected cells. The results of a representative experiment are shown in Fig. 1A. Treatment of macrophages with MAF- or LPS-containing fluids, or with a combination of both, considerably increased their luminescence over that exhibited by controls. The presence of the intracellular parasites, however, impaired the development of such luminescence, particularly in macrophages incubated in LPS-supplemented medium or in MAF-rich fluids without added LPS. Inhibition of

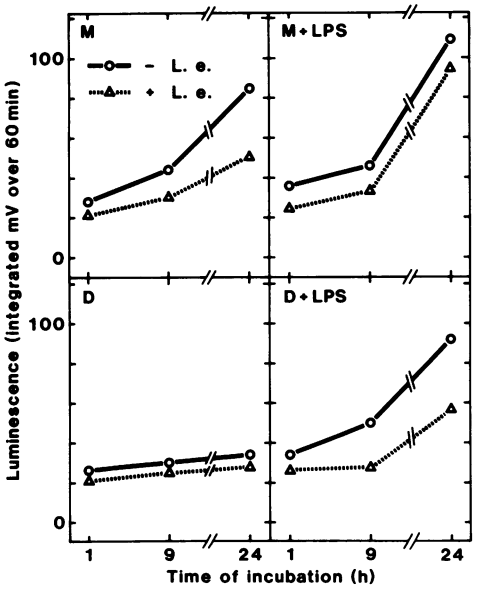


FIG. 3. Kinetics of luminescence inhibition by intracellular *L. enriettii*. *L. enriettii* (L. e.)-infected and noninfected CBA/T6 macrophages were incubated with a 1:8 dilution of MAF-rich (M) or FBS-supplemented control Dulbecco (D) medium in the presence (+) or absence (−) of added LPS (10 ng/ml). After 1, 9, and 24 h, luminescence was recorded over a 60-min period following triggering with PMA. Results are reported as millivolts integrated over the 60-min test period.

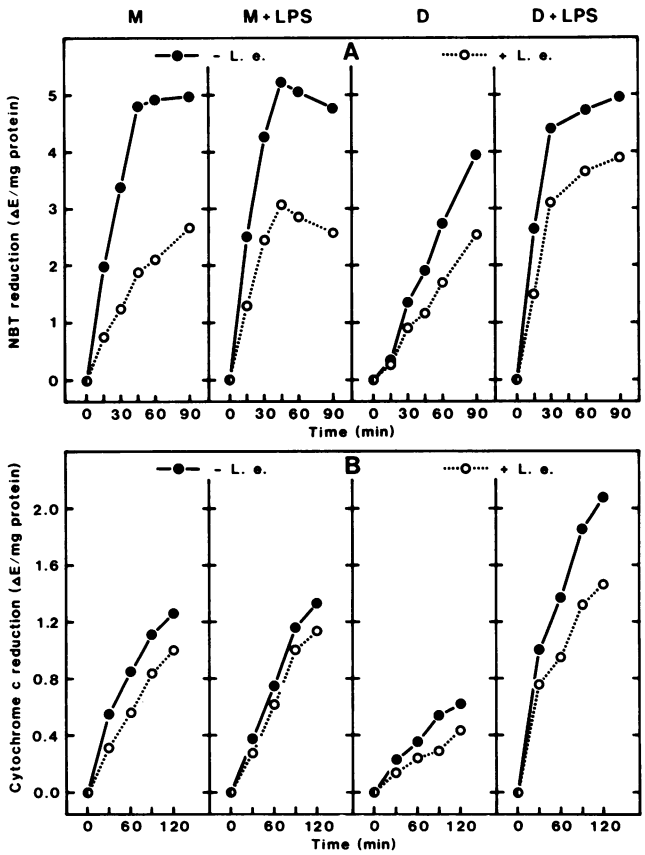


FIG. 4. Impairment of NBT and cytochrome *c* reduction by intracellular *L. enriettii*. *L. enriettii* (L. e.)-infected and noninfected macrophages were incubated with a 1:8 dilution of MAF-rich (M) or FBS-supplemented control Dulbecco (D) medium in the presence (+) or absence (−) of added LPS (10 ng/ml). After 24 h $O_2^-$ production was determined by the NBT reduction test (A) and the cytochrome *c* reduction test (B). Results are expressed as $\Delta E$/mg of protein (for more details, see the text).

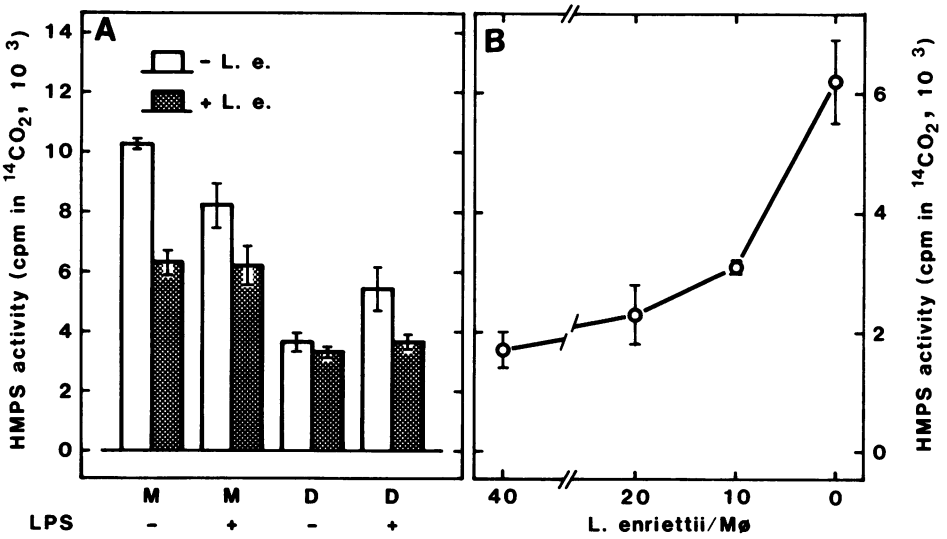


FIG. 5. Impairment of HMPS activity by intracellular *L. enriettii*. *L. enriettii* (L. e.)-infected and noninfected PECs (Mϕ) were incubated with a 1:8 dilution of MAF-rich (M) or FBS-supplemented control Dulbecco (D) medium in the presence (+) or absence (−) of added LPS (10 ng/ml). (A) HMPS activity was determined after 24 h. (B) Cultures were infected with increasing numbers of *L. enriettii* promastigotes and then activated with a 1:8 dilution of MAF-rich medium. HMPS activity was determined 24 h later.

luminescence was a function of the number of infecting parasites. Thus, little inhibition was observed in cultures infected with less than $2 \times 10^6$ parasites corresponding to a parasite/PEC ratio of 10:1 and resulting in the presence of approximately 50 intracellular parasites per 100 macrophages on the day of the test (Fig. 2). Chemiluminescence, however, was reduced by 50 to 70% in macrophages that were exposed to a greater number of parasites. Less inhibition was observed in infected macrophages that were incubated with MAF in the presence of LPS, correlating with the killing and disappearance of the parasites in such cells (Fig. 1B) (6).

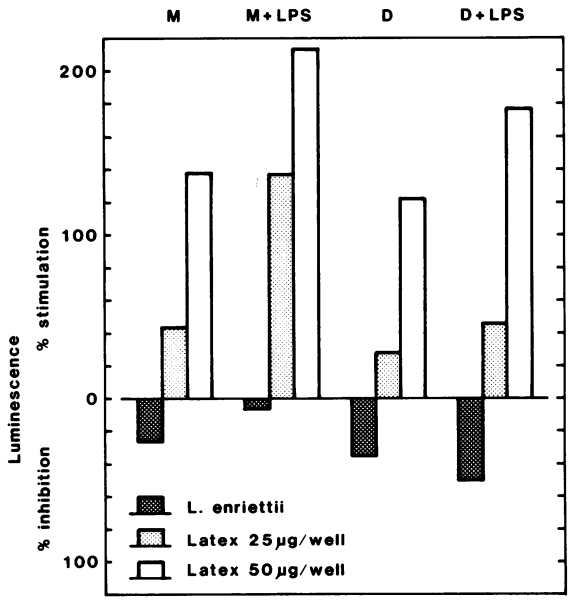


FIG. 6. Effect of intracellular latex on macrophage luminescence. CBA/T6 macrophages were allowed to ingest *L. enriettii* at a parasite/PEC ratio of 40:1 (final infection ratio of 200 parasites per 100 macrophages) or latex beads and were then incubated with a 1:8 dilution of MAF-rich (M) or FBS-supplemented control Dulbecco (D) medium in the presence (+) or absence (−) of added LPS (10 ng/ml). After 24 h luminescence was recorded over a 60-min period following triggering with PMA. Results are given as percent inhibition or percent stimulation of luminescence of infected cultures versus noninfected cultures using the following formula: % inhibition = 100 − [(integrated mV/60 min in infected cultures)/(integrated mV/60 min in noninfected cultures) × 100].

Luminescence in macrophages exposed to MAF, LPS, or both was followed as a function of time (Fig. 3). The respiratory burst activity was found to increase up to 24 h in both infected and noninfected cells. Inhibition by the intracellular parasites was evident at each time point. The increase in luminescence between 9 and 24 h was similar in infected and control macrophages when the cells were exposed to both MAF and LPS, suggesting that intracellular parasite killing, which occurred within 12 to 15 h in this model system (data not shown), released the inhibition induced by the microorganism.

**Impairment of NBT reduction, cytochrome *c* reduction, and HMPS activity by intracellular *L. enriettii*.** The impairment of respiratory burst activity in *L. enriettii*-infected macrophages, as determined by luminescence inhibition, was confirmed by using three other assay procedures, i.e., NBT and cytochrome *c* reduction and measurements of HMPS levels. NBT and cytochrome *c* reduction was inhibited in *L. enriettii*-infected compared with noninfected macrophages after 24 h of incubation with MAF, LPS, or both (Fig. 4). HMPS activity was also impaired by the presence of intracellular *L. enriettii* (Fig. 5); this inhibition was proportional to the number of intracellular parasites (Fig. 5B).

**Effect of inert particles or dead parasites on macrophage oxidative metabolism.** To determine whether inhibition of the respiratory burst observed in infected macrophages was specific for intracellular parasites rather than a consequence of the presence of any intracellular particle, macrophages were allowed to ingest latex beads. They were then exposed to MAF, LPS, or both. Luminescence was determined 24 h later. The presence of intracellular latex beads did not inhibit luminescence (Fig. 6). On the contrary, such cells developed a much stronger respiratory burst than control cells that

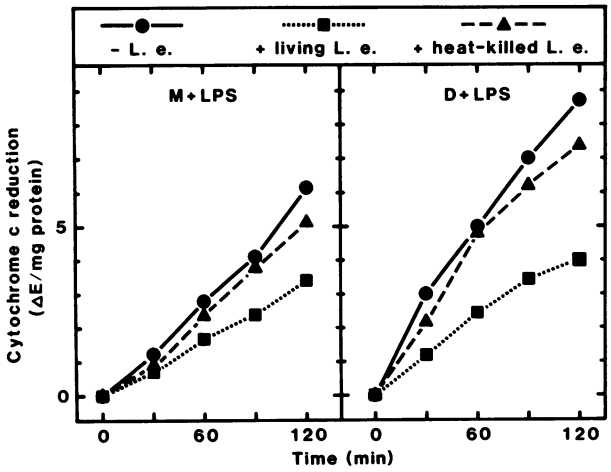


FIG. 7. Inhibition of the macrophage respiratory burst by *Leishmania* spp.; comparison of the effect of living versus dead parasites. Macrophages were infected overnight with living or heat-killed (15 min at 56°C) *L. enriettii* amastigotes (at a ratio of 7.5 parasites per PEC) and then washed and reincubated with a 1:16 dilution of MAF-rich (M) or FBS-supplemented control Dulbecco (D) medium in the presence of added LPS (10 ng/ml). After 24 h $O_2^-$ production was determined by the cytochrome *c* reduction test. Results are expressed as $\Delta E$/mg of protein.

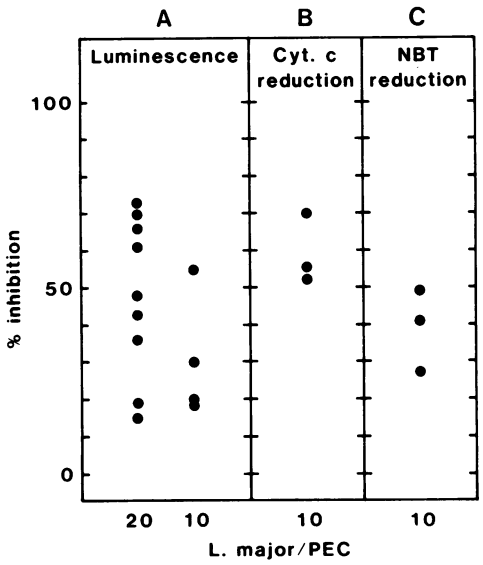


FIG. 8. Inhibition of the oxidative metabolism of MAF-activated macrophages by intracellular *L. major*. Infected and noninfected CBA/T6 macrophages were incubated with a 1:8 dilution of MAF-rich medium. After 24 h luminescence (A) was recorded over a 60-min period following triggering with PMA (13 experiments); $O_2$ release was determined by the SOD-inhibitable reduction of cytochrome *c* (Cyt c) (B) (3 experiments) and by the NBT reduction test (C) (3 experiments) after triggering with PMA. Results are expressed as percent inhibition of the oxidative metabolism of infected versus noninfected cells.

were not treated with latex. Parallel cultures of *L. enriettii*-infected macrophages, however, underwent an inhibition of their metabolic burst (Fig. 6).

To answer the question whether live, metabolizing parasites are required for inhibition of the respiratory burst, macrophages were allowed to ingest either living or heat-killed *L. enriettii* amastigotes, prior to exposure to the activating agents. Superoxide production was determined 24 h later. Inhibition of the respiratory burst was more pronounced in macrophages that were infected with live than with dead microorganisms (Fig. 7). Impairment of the respiratory burst by *L. enriettii* was also evident when uninfected macrophages were activated with MAF-LPS for 24 h and then tested for PMA-triggered cytochrome *c* reduction during parasite ingestion (Table 1). Again, a stronger inhibition was observed in macrophages that were challenged with living compared with dead parasites, especially when the promastigote form of the parasite was used.

TABLE 1. Respiratory burst inhibition on phagocytosis of living versus dead *L. enriettii* in PMA-triggered macrophages[a]

| Parasites | No. added per well | % Inhibition of respiratory burst in[b]: | |
|---|---|---|---|
| | | Expt 1 | Expt 2 |
| Living promastigotes | $3 \times 10^6$ | 71 ± 3 | – |
| Fixed promastigotes | $3 \times 10^6$ | −19 ± 9 | – |
| Living amastigotes | $7.5 \times 10^5$ | – | 57 ± 4 |
| Heat-killed amastigotes | $7.5 \times 10^5$ | – | 37 ± 5 |
| Living amastigotes | $10^6$ | 77 ± 2 | – |
| Fixed amastigotes | $10^6$ | 44 ± 7 | – |
| Living amastigotes | $1.5 \times 10^6$ | – | 71 ± 5 |
| Heat-killed amastigotes | $1.5 \times 10^6$ | – | 51 ± 4 |

[a] Macrophages ($1.5 \times 10^5$ PEC per well) were incubated with a 1:16 dilution of MAF-rich medium in the presence of added LPS (10 ng/ml). After 24 h cultures were washed and tested for PMA-triggered cytochrome *c* reduction during ingestion of various doses of living, heat-killed (15 min at 56°C), and glutaraldehyde-fixed (0.5% in HBSS for 45 min) parasites. Results for two experiments are shown.

[b] Percent inhibition of the respiratory burst of phagocytosing versus resting cells (± standard deviation).

**Inhibition of the oxidative metabolism of MAF-activated macrophages by *L. major*.** Another parasite of the genus *Leishmania*, *L. major*, was also tested for its capacity to inhibit oxidative metabolism in macrophages. To examine this effect, infected and noninfected macrophages were incubated for 24 h with MAF and then washed and assayed for luminescence and cytochrome *c* and NBT reduction. In several experiments the presence of intracellular *L. major* inhibited macrophage respiratory burst activity, as determined by the three assay procedures described above (Fig. 8).

## DISCUSSION

It is known that on exposure to MAF, LPS, or both, macrophages develop the capacity to respond to certain triggering agents by producing an intense respiratory burst. As shown in this study, however, the presence of intracellular *Leishmania* parasites impairs such metabolic activity; thus, macrophages infected in vitro with *L. enriettii* and then exposed to MAF or LPS displayed lower stimulation compared with uninfected cells in assays for chemiluminescence, cytochrome *c* and NBT reduction, and HMPS activity. Such inhibition was evident when either PMA (see above) or zymosan (data not shown) was used as a triggering agent and increased with the number of intracellular parasites. It lasted for at least 72 h after the initiation of infection (data not shown). Infection by another parasite of the *Leishmania* genus, i.e., *L. major*, led to similar inhibition of the respiratory burst of MAF-activated cells.

The question of the possible influence of intracellular *Leishmania* spp. on macrophage oxidative metabolism has

been addressed by other investigators. Passwell et al. (28) did not observe any inhibitory effect of intracellular *L. major* on cytochrome *c* reduction by unstimulated human monocytes triggered with PMA. As shown by the results of the experiments reported here, however, the *Leishmania*-induced metabolic inhibition was readily demonstrable in murine macrophages that were primed for high respiratory burst activity by treatment with MAF or LPS for 24 h. Infection by *L. donovani* was also shown by Pearson et al. (29) to impair markedly the chemiluminescent response of human macrophages that were treated with zymosan. An important issue is whether such inhibition represents a unique parasite effect or whether it results from the presence of any intracellular particle. The latter alternative was favored by the observation that macrophages that are allowed to ingest zymosan also display a reduced oxidative response after 24 h (29). It is noteworthy, however, that the respiratory burst triggered by membrane-active agents or phagocytic stimuli is short-lived (2, 9), and phagocytes may then remain refractory to restimulation for up to 48 h (21). On the contrary, as shown in this study, phagocytosis of latex beads results in the strong metabolic stimulation of macrophages on triggering with PMA after 48 h, concomitant with the respiratory burst inhibition in *Leishmania*-infected cells. This suggests that the latter phenomenon is specific to the intracellular parasite.

The experiments described here were designed to determine whether respiratory burst inhibition is dependent on the presence of living (as opposed to dead) intracellular parasites. Little impairment of the oxidative metabolism was detectable in *L. enriettii*-infected macrophages exposed to both MAF and LPS, conditions that allow full intracellular parasite killing within 24 h. This suggests that dead parasites were no longer able to block the macrophage oxidative machinery. Moreover, macrophages suffered a higher inhibition of their respiratory burst when exposed to live than to heat-killed or glutaraldehyde-fixed parasites. It should be noted, however, that when macrophages were challenged with high numbers of parasites, respiratory burst inhibition was significant, even when dead microorganisms were used.

The mechanisms whereby *Leishmania* spp. interfere with the oxidative metabolism of their host cells are unknown. Although macrophages were infected with promastigotes in most of the experiments described here, transformation to the amastigote stage in the intracellular environment appeared to be a rapid event. Within 48 h of the initiation of phagocytosis (i.e., at the time when macrophages were tested for respiratory burst activity), no morphological difference could be detected between intracellular parasites in cultures infected with amastigotes or with promastigotes of *L. enriettii* (data not shown). *Leishmania* amastigotes contain scavenging enzymes such as catalase, SOD, and glutathione peroxidase (7, 18, 20) which might detoxify hydrogen peroxide and superoxide generated by the phagocytes. In this connection, it is significant that phagocytosis of amastigotes leads to a reduced respiratory burst (as measured by chemiluminescence and by NBT and cytochrome *c* reduction) compared with that caused by promastigote ingestion (8, 13). It is conceivable that enzymes of the parasite surface membrane might also play a role in the survival of the parasites within host cells. Indeed, *L. donovani* displays on its outer surface an acid phosphatase (12, 33) which is able to block superoxide anion production in neutrophils stimulated by the chemoattractant peptide fMet-Leu-Phe (34). One effect of this enzyme might be the dephosphorylation of the surface proteins that are involved in the generation of oxygen metabolites by the phagocytes. Furthermore, it has been suggested that parasite lipids might detoxify hydrogen peroxide by serving as substrates for harmless peroxidation reactions (7).

Whatever the mechanism(s), inhibition of respiratory burst activity by *Leishmania* spp. may contribute significantly to the intracellular survival of the parasite. Thus, treatments that interfere with the capacity of macrophages to generate oxygen metabolites inhibit parasite killing (for a review, see reference 22). Promastigotes, which are poorly endowed in enzymes that are capable of decomposing $H_2O_2$ (18, 19), are destroyed within hours of ingestion by macrophages. In this connection, it is interesting that the infectivity of amastigotes is superior to that of promastigotes (27), presumably correlating with the increased resistance of the former developmental stage to oxidant damage.

Although oxygen metabolites appear to play an important role in the destruction of microorganisms, other oxygen-independent mechanisms may be involved in the microbicidal activity of phagocytes (23, 35). With regard to the latter, Eilam et al. (11) have suggested that the *Leishmania* surface membrane may be protected from degradation by lysosomal enzymes through an excreted factor that is composed of negatively charged glycopeptides. Thus, parasite survival in the intracellular environment may involve the impairment of both oxygen-dependent and -independent mechanisms.

## ACKNOWLEDGMENTS

We thank Adriana Ransijn for expert technical assistance, Eric Rouiller for help with statistical analysis of the data, and Michèle Markert for the use of a luminometer and advice during the course of these studies.

This study was supported by grants 3.249-082 and 3.172-085 from the Swiss National Fund.

## LITERATURE CITED

1. **Baumgarten, H.** 1985. A simple microplate assay for the determination of cellular protein. J. Immunol. Methods **82**:25–37.
2. **Baxter, M. A., R. G. Q. Leslie, and W. G. Reeves.** 1983. The stimulation of superoxide anion production in guinea pig peritoneal macrophages and neutrophils by phorbol myristate acetate, opsonized zymosan, and $IgG_2$-containing soluble immune complexes. Immunology **48**:657–665.
3. **Berens, R. L., and J. J. Marr.** 1978. An easily prepared defined medium for cultivation of *Leishmania donovani* promastigotes. J. Parasitol. **64**:160.
4. **Buchmüller, Y., and J. Mauël.** 1979. Studies on the mechanisms of macrophage activation. II. Parasite destruction by macrophages activated by supernates from concanavalin A-stimulated lymphocytes. J. Exp. Med. **150**:359–370.
5. **Buchmüller, Y., and J. Mauël.** 1981. Studies on the mechanisms of macrophage activation: possible involvement of oxygen metabolites in killing of *Leishmania enriettii* by activated mouse macrophages. J. Reticuloendothel. Soc. **29**:181–192.
6. **Buchmüller-Rouiller, Y., and J. Mauël.** 1986. Correlation between enhanced oxidative metabolism and leishmanicidal activity in activated macrophages from healer and non healer mouse strains. J. Immunol. **136**:3884–3890.
7. **Channon, J. Y., and J. M. Blackwell.** 1985. A study of the sensitivity of *Leishmania donovani* promastigotes and amastigotes to hydrogen peroxide. II. Possible mechanisms involved in protective $H_2O_2$ scavenging. Parasitology **91**:207–217.
8. **Channon, J. Y., M. B. Roberts, and J. M. Blackwell.** 1984. A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine resident peritoneal macrophages. Immunology **53**: 345–355.
9. **DeChatelet, L. R., G. D. Long, P. S. Shirley, D. A. Bass, M. J. Thomas, F. W. Henderson, and M. S. Cohen.** 1982. Mechanism

of the luminol-dependent chemiluminescence of human neutrophils. J. Immunol. **129**:1589–1593.
10. **Di Stefano, J. F., G. Beck, and S. Zucker.** 1983. Mechanism of BCG-activated macrophage induced tumor cell cytotoxicity: evidence for both oxygen-dependent and independent mechanisms. Int. Arch. Allergy Appl. Immunol. **70**:252–260.
11. **Eilam, Y., J. El-On, and D. T. Spira.** 1985. *Leishmania major*: excreted factor, calcium ions and the survival of amastigotes. Exp. Parasitol. **59**:161–168.
12. **Gottlieb, M., and D. M. Dwyer.** 1981. *Leishmania donovani*: surface membrane acid phosphatase activity of promastigotes. Exp. Parasitol. **52**:117–128.
13. **Haidaris, C. G., and P. F. Bonventre.** 1982. A role for oxygen-dependent mechanisms in killing of *Leishmania donovani* tissue forms by activated macrophages. J. Immunol. **129**:850–855.
14. **Locksley, R. M., and S. J. Klebanoff.** 1983. Oxygen-dependent microbicidal systems of phagocytes and host defense against intracellular protozoa. J. Cell. Biochem. **22**:173–185.
15. **Mauël, J., R. Behin, B. Noerjasin, and D. S. Rowe.** 1975. Mechanisms of protective immunity in experimental cutaneous leishmaniasis of the guinea pig. I. Lack of effect of immune lymphocytes and of activated macrophages. Clin. Exp. Immunol. **20**:339–350.
16. **Mauël, J., Y. Buchmüller, and R. Behin.** 1978. Studies on the mechanisms of macrophage activation. I. Destruction of intracellular *Leishmania enriettii* in macrophages activated by cocultivation with stimulated lymphocytes. J. Exp. Med. **148**:393–407.
17. **Mauël, J., J. Schnyder, and M. Baggiolini.** 1984. Intracellular parasite killing induced by electron carriers. II. Correlation between parasite killing and the induction of oxidative events in macrophages. Mol. Biochem. Parasitol. **13**:97–110.
18. **Meshnick, S. R., and J. W. Eaton.** 1981. Leishmanial superoxide dismutase: a possible target for chemotherapy. Biochem. Biophys. Res. Commun. **102**:970–976.
19. **Murray, H. W.** 1981. Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. J. Exp. Med. **153**:1302–1315.
20. **Murray, H. W.** 1982. Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. J. Immunol. **129**:351–357.
21. **Murray, H. W.** 1982. Pretreatment with phorbol myristate acetate inhibits macrophage activity against intracellular protozoa. J. Reticuloendothel. Soc. **31**:479–487.
22. **Murray, H. W.** 1984. Macrophage activation: enhanced oxidative and antiprotozoal activity. Curr. Top. Immunobiol. **13**:97–115.
23. **Murray, H. W., and D. M. Cartelli.** 1983. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and independent leishmanicidal activity. J. Clin. Invest. **72**:32–44.
24. **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**: 938–949.
25. **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**:950–964.
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**:100–113.
27. **Pan, A. A., and B. M. Honigberg.** 1985. *Leishmania mexicana pifanzi*: *in vivo* and *in vitro* interactions between amastigotes and macrophages. Parasitenkd. **71**:3–13.
28. **Passwell, J., R. Shor, G. Keren, G. Messer, and J. El-On.** 1984. Comparison of the effect of various stimuli on the leishmaniacidal capacity of human monocytes in vitro. Clin. Exp. Immunol. **56**:553–558.
29. **Pearson, R. D., J. L. Harcus, P. H. Symes, R. Romito, and G. R. Donowitz.** 1982. Failure of the phagocytic oxidative response to protect human monocyte-derived macrophages from infection by *Leishmania donovani*. J. Immunol. **129**:1282–1286.
30. **Philippeaux, M. M., and J. Mauël.** 1984. Extracellular cytolysis by activated macrophages: studies with macrophages on permeable membranes. Immunobiology **167**:301–317.
31. **Pick, E., J. Charon, and D. Mizel.** 1981. A rapid densitometric microassay for nitroblue tetrazolium reduction and application of the microassay to macrophages. J. Reticuloendothel. Soc. **30**:581–593.
32. **Pick, E., and D. Mizel.** 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J. Immunol. Methods **46**:211–216.
33. **Remaley, A. T., S. Das, P. I. Campbell, G. M. LaRocca, M. T. Pope, and R. H. Glew.** 1985. Characterization of *Leishmania donovani* acid phosphatase. J. Biol. Chem. **260**:880–886.
34. **Remaley, A. T., R. H. Glew, D. B. Kuhns, R. E. Basford, A. S. Waggoner, L. A. Ernst, and M. Pope.** 1985. *Leishmania donovani*: surface membrane acid phosphatase blocks neutrophil oxidative metabolite production. Exp. Parasitol. **60**:331–341.
35. **Scott, P., S. James, and A. Sher.** 1985. The respiratory burst is not required for killing of intracellular and extracellular parasites by a lymphokine-activated macrophage cell line. Eur. J. Immunol. **15**:553–558.