The Journal of Immunology

Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide.

F Y Liew, Y Li and S Millott

J Immunol 1990; 145:4306-4310; ;
http://www.jimmunol.org/content/145/12/4306

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

0022-1767/90/14512-4306$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 145, 4306–4310, No. 12, December 15, 1990
Printed in U.S.A.

TUMOR NECROSIS FACTOR-α SYNERGIZES WITH IFN-γ IN MEDIATING KILLING OF *Leishmania major* THROUGH THE INDUCTION OF NITRIC OXIDE

F. Y. LIEW,[1] YUN LI,[2] AND STEPHANIE MILLOTT

From the Department of Experimental Immunobiology, Wellcome Biotech, Beckenham, Kent BR3 3BS, U. K.

CBA mice develop cutaneous lesions when infected with *Leishmania major*. The disease development was significantly reduced by injecting into the lesion a combination of rIFN-γ and rTNF-α. The doses of IFN-γ and TNF-α used were suboptimal in that either cytokine alone did not have any effect. The therapeutic effect of IFN-γ and TNF-α in vivo is reflected in their ability to activate macrophages to kill the intracellular parasites in vitro. The macrophage leishmanicidal activity induced by TNF-α and IFN-γ can be completely inhibited by a specific inhibitor (L-N^G monomethyl arginine) of nitric oxide synthesis. There was a direct correlation between the intracellular killing of the parasites and the production of nitric oxide by the macrophages. In contrast, there was no correlation between leishmanicidal activity and superoxide production by macrophages.

Cell-mediated immunity is deemed to be the major host defense mechanism against the protozoa parasite *Leishmania* spp. (1). For cutaneous leishmaniasis, IFN-γ secreted by the Th-1 subset of CD4 T cells (2–4) appears to be principally involved in activating macrophages for killing the intracellular parasites (5–8). Recently, it has been demonstrated that TNF-α can also mediate host-protection against cutaneous leishmaniasis (9, 10). Furthermore, the protective effect of TNF-α is associated with its ability to induce macrophage leishmanicidal activity in vitro (10) in a manner similar to that of IFN-γ.

It has recently been reported that IFN-γ-activated macrophages kill intracellular leishmania parasites by means of NO (11, 12). Currently, there has been much interest in the biologic role of NO[3] (13). It is now generally accepted that NO is the endothelium-derived relaxing factor (14) and that it also participates in the regulation of the nervous and immune systems (13). Activated macrophages form NO_2^- and NO_3^- from the terminal guanidino nitrogen atoms of L-arginine (15, 16) by a process now known to proceed via the formation of NO (17–19). This pathway is inhibited by the L-arginine analog (L-NMMA) (15, 17–20), forms L-citrulline as a co-product (15, 17–18, 21) and is responsible for the cytotoxic action of macrophages (22). In the leishmania system, the leishmanicidal activity of IFN-γ-treated macrophages can be completely inhibited by the addition of L-NMMA, an antagonist specific for the L-arginine → NO pathway (11, 12). Furthermore, *Leishmania major* promastigotes are killed when incubated at room temperature in PBS containing NO (12). The importance of NO in vivo is demonstrated by the finding that CBA mice infected with *L. major* developed exacerbated disease when L-NMMA was injected into the lesions (12).

We report here that TNF-α synergizes with IFN-γ in mediating host resistance against *L. major* infection in vivo and in inducing macrophage leishmanicidal activity in vitro. The leishmanicidal effect induced by the combination of TNF-α and IFN-γ can also be completely inhibited by L-NMMA, leading to a dose-dependent inhibition of NO production but enhanced respiratory burst. These data demonstrate that TNF-α synergizes with IFN-γ in mediating host resistance against *L. major* infection through NO, which is necessary for the macrophage killing of the intracellular parasite.

MATERIALS AND METHODS

Mice. CBA/T6T6 mice aged 8 to 10 wk were obtained from the colonies at Wellcome Biotech, Beckenham, Kent.

Parasites. The isolation, cultivation, and maintenance of the promastigote stage of the parasite *L. major* (LV39) have been described in detail elsewhere (23). For animal infection, groups of mice were injected s.c. (in 0.05 ml PBS) in the left hind footpad with 1×10^7 stationary-stage promastigotes. The lesion that developed in the footpads was measured with a dial calliper and expressed as footpad thickness increase ($\times 10^{-1}$ mm). In some experiments, 10^3 U of TNF-α, IFN-γ, a combination of the two cytokines in 20 μl of PBS, or PBS alone was injected daily for 6 days into the lesion, starting from day 6 after infection. At the end of the experiment, the mice were sacrificed and the footpads removed. The parasite loads in the footpads were estimated by a method described by Heinzel et al. (4). Briefly, the footpad tissue was homogenized and supernatants containing the released parasites were serially diluted in Schneider's medium containing 30% FCS. Quadruplicate cultures were incubated at 28°C for 3 days and then pulsed with 1 μCi of [^{3}H]TdR. Cultures were harvested 18 h later. The incorporation of radioactivity was counted in a β-counter (β-plate, LKB, Bromma, Sweden). The results were expressed as cpm at 10^{-1} dilution or the highest dilution (log 10) at which viable parasites are detectable (2 SD over background cpm of cultures without parasites).

Materials. L-NMMA and its enantiomer, D-NMMA, were generously provided by Dr. H. Hodson of the Department of Medicinal Chemistry, Wellcome Research Laboratories, Beckenham, U. K.; Murine rIFN-γ and murine rTNF-α were kindly provided by Dr. G. Adolf, Ernst Boehringer-Institute für Arzneimittel-Forschung, Vienna, Austria; LPS was obtained from Sigma, St. Louis, MO; and [^{3}H]TdR was obtained from the Radiochemical Centre, Amersham U. K. (26 Ci/nmol).

Leishmanicidal assay. This has been described in detail elsewhere (24). Briefly, PEC were collected in culture medium (RPMI 1640 plus 10% FCS, L-glutamine, penicillin, and streptomycin) from CBA mice injected i.p. 6 days previously with 3 ml of a 2% sterile, hydrolyzed starch solution (BDH Chemicals, Poole, Dorset, UK). The

Received for publication June 19, 1990.
Accepted for publication September 21, 1990.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[2] Address correspondence and reprint requests to: Dr. F. Y. Liew, Department of Experimental Immunobiology, Wellcome Biotech, Langley Court, Beckenham, Kent BR3 3BS, U. K.

[2] Y. Li is supported by a scholarship from the British Council, U.K.

[3] Abbreviations used in this paper: NO, nitric oxide; L-NMMA, L-N^G monomethyl arginine; PEC, peritoneal exudate cells.

cells were plated at 1×10^5 cells/0.1 ml culture medium/well in 96-well flat bottom microtiter plates (Nunc, Roskilde, Denmark) and were incubated at 37°C in an atmosphere of 5% CO_2 in air for 24 h. Nonadherent cells were removed and the adherent cells washed three times with prewarmed medium. To each well was added 100 μl of medium containing 10 ng/ml of LPS with or without TNF-α and IFN-γ. In some cultures, graded doses of L-NMMA or D-NMMA were also added. Cultures were then incubated as above for a further 2 to 4 h before addition into each well of 1×10^5 *L. major* promastigotes in 100 μl of culture medium containing 10 ng/ml of LPS. The mixture was cultured for an additional 72 h. At the end of 72 h, 25 μl of supernatant were removed from each well and kept at −20°C for the analysis of NO_2^- content and the cultures were washed extensively with prewarmed medium. To each well were added 100 μl of 0.01% SDS solution in serum-free medium at 37°C for 20 to 30 min. Schneider's medium supplemented with 30% FCS and L-glutamine was added (100 μl/well) and the cultures were incubated at 28°C for an additional 72 h. The cultures in three to six replicates were then pulsed with 1 μCi/well of [^{3}H]TdR and the incorporation of radioactivity by viable parasites after 18 h of further culturing was determined as above.

In some experiments, the macrophage leishmanicidal activity was determined by visual counting of intracellular parasites as described in detail previously (24). Briefly, the CBA peritoneal cells obtained as above were dispensed into eight-well slides (Lab-Tek tissue culture chamber slides; Miles Laboratories, Naperville, IL) at 2×10^6 cells/0.2 ml culture medium/well. The slides were incubated for 6 h at 37°C and nonadherent cells removed by washing the wells three times with prewarmed medium. To each well were then added 200 μl of medium containing 10 ng/ml of LPS and 40 U/ml of IFN-γ, 400 U/ml of TNF-α or a combinations of IFN-γ and TNF-α. Cultures were incubated for 4 h at 37°C and 5% CO_2 followed by addition into each well of 4×10^7 promastigotes/100 μl of medium containing 10 ng/ml of LPS. At intervals of 2, 24, 48, and 72 h the slides were removed, washed in PBS (pH 7.2), and fixed for 10 min with 1% glutaraldehyde (BDH Chemicals, Poole, UK). After fixing, the cells were washed three times in distilled water and stained with 0.25% Giemsa (Gurr) for 90 min. They were then destained for 10 min in PBS (pH 7.2), washed twice in distilled water, dipped in acetone, in a 1/1 mixture of acetone and xylol, and finally dipped in xylol before being mounted in mountant (Gurr). Parasite killing was estimated by determining the number of intact parasites in 200 macrophages.

Measurement of NO_2^-. NO_2^- in culture supernatants was determined by chemiluminescence as described previously (14).

Measurement of H_2O_2. H_2O_2 in the culture supernatant was assayed by a colorimetric method as described by Pick and Keisari (25). PEC from mice stimulated i.p. 3 to 5 days previously with 3 ml of 2% hydrolyzed starch were dispensed into a 24-well Costar plate at 2.5×10^6 cells/1 ml in a culture medium (phenol red-free RPMI 1640 containing 10% FCS). The plate was incubated at 37°C and 5% CO_2 for 24 h. Nonadherent cells were removed by washing with prewarmed phenol red-free balanced salt solution. The macrophages were then treated with various concentrations of TNF-α and IFN-γ with or without L-NMMA/D-NMMA in the culture medium for 48 to 72 h at 37°C and 5% CO_2. At the end of the culture the cells were again washed with prewarmed balanced salt solution and to each well was added 1 ml of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer (pH 7.0), 5.5 mM dextrose, 0.56 mM (0.2 g/liter) phenol red, and 19 U/ml (100 μg/ml) of horseradish peroxidase and 10 μg/ml of PMA. The plate was returned to the incubator for an additional hour. The cellfree supernatants were transferred to conical glass tubes and clarified by centrifugation for 5 min at 2000 × *g* at 4°C. The supernatants were then brought up to pH 12 by addition of 10 μl 1 N NaOH and read in a photometer at 600 nm. The results were referred to a standard curve and expressed as μmoles of H_2O_2 per 2.5×10^6 cells per 60 min.

RESULTS

Synergistic effect of TNF-α and IFN-γ on the disease development in CBA mice infected with L. major. CBA mice were infected in the footpad with 10^7 *L. major* promastigotes and injected in the same footpad with 10^3 U of TNF-α or 10^3 U of IFN-γ or a combination of the two cytokines. These doses of cytokines on their own were suboptimal in inducing disease-regression as determined in preliminary experiments. Thus, the lesions developed in mice receiving TNF-α alone or IFN-γ alone were not significantly different from those of the control mice similarly injected with PBS. In contrast, mice injected with a combination of TNF-α and IFN-γ produced significantly smaller lesions and parasite loads compared with the controls (Table I).

Synergistic effect of TNF-α and IFN-γ in vitro. The synergistic effect of TNF-α and IFN-γ was also apparent in the induction of macrophage leishmanicidal activity in vitro. TNF-α at 400 U/ml did not activate macrophages to kill intracellular *L. major*. On the other hand, IFN-γ at 40 U/ml induced a significant but modest level of macrophage leishmanicidal activity. In contrast, macrophages treated with a combination of TNF-α and IFN-γ were highly effective in killing the intracellular parasite (Fig. 1 and Table II). This was demonstrated by the [^{3}H]TdR incorporation method and the visual counting of surviving intracellular organisms. The treatment did not affect the parasite uptake by the macrophages because there was no apparent difference in the number of attached parasites examined 2 h after the infection. Some of the killed parasites were clearly visible with disintegrated membrane and cytoplasm.

Production of superoxide and NO by activated macrophages. The mechanism of the leishmanicidal activity of TNF-α- and IFN-γ treated macrophages was investigated. The supernatants of the macrophage cultures were analyzed for NO_2^- concentration, and the macrophages were stimulated with PMA to test for their ability to produce respiratory burst. The level of NO_2^- measured was indicative of NO production (14), whereas the contents of H_2O_2 examined reflected the O_2^- released (25). Both IFN-γ and TNF-α alone were able to activate macrophages to produce NO and O_2^-. However, whereas TNF-α and IFN-γ induced the production of NO synergistically, their effect on O_2^- production appeared to be only additive (Fig. 2).

Inhibition of macrophage leishmanicidal activity by L-NMMA. Mouse PEC were activated in vitro with TNF-α plus IFN-γ in the presence of L-NMMA or D-NMMA (0.5 to 500 μm) and infected with *L. major* promastigotes. PEC treated with TNF-α + IFN-γ acquired a strong leishmanicidal activity compared with cells treated with the medium alone. However, the ability of the activated macrophages to kill *Leishmania* was progressively decreased and was completely abrogated at 50 μM of L-NMMA (Fig. 3). In contrast, the leishmanicidal activity was not affected by the enantiomer, D-NMMA. Thus, the TNF-α + IFN-γ-induced macrophage leishmanicidal activity is completely inhibitable by a specific inhibitor of the L-arginine/NO pathway, indicating that the macrophage leishmanicidal activity in the present system is mediated by NO because NO_2^- has no effect on *L. major* promastigotes (12). In an earlier reports, L-NMMA was shown not to affect the uptake of *L. major* by macrophage (11, 12).

The inhibition of NO production by L-NMMA. The ability of TNF-α + IFN-γ-activated macrophages to kill intracellular leishmania was paralleled by an increase in NO_2^- in the culture supernatant (Figs. 3 and 4). The level of NO_2^- was progressively decreased in the culture supernatant of macrophages treated with increasing doses of L-NMMA but not D-NMMA (Fig. 4). Complete inhibition was achieved with 50 μM of L-NMMA.

The enhancement of O_2^- production by L-NMMA. The ability of activated macrophages to produce O_2^- in the presence of L-NMMA and D-NMMA was also determined in parallel with the NO measurement. The level of H_2O_2

TABLE I

Synergistic effect of TNF-α and IFN-γ on the disease development in CBA mice infected with L. major

Treatment[a]	Lesion Size (× 10^{-1} mm)			Parasite Load (d49)[b]	
	d21	d35	d49	cpm (× 10^{-3})	Dilution
TNF-α	7.9 ± 0.4	7.6 ± 0.5	6.1 ± 0.6	7.34 ± 0.73	10^3
IFN-γ	8.4 ± 0.4	7.5 ± 0.4	5.8 ± 0.4	7.44 ± 0.73	10^3
TNF + IFN-γ	*6.1 ± 0.2*[c]	*4.5 ± 0.8*	*2.5 ± 0.5*	*4.64 ± 0.35*	*10^1*
Control	8.6 ± 0.4	8.2 ± 0.6	5.4 ± 0.5	7.32 ± 0.61	10^3

[a] CBA mice were infected in the footpad with 10^7 *L. major* promastigotes, and from day 6 to day 13 after infection they were injected daily into the lesion with 10^3 U of IFN-γ, 10^3 U of TNF-α, or a combination of the two cytokines in 20 μl of PBS. Control mice were injected with 20 μl of PBS alone.

[b] Parasite loads in the infected footpads were estimated as described in *Materials and Methods* and expressed as [^{3}H] TdR incorporation and at the highest dilution in which viable parasites are detectable.

[c] Figures italicized are significantly different ($p < 0.05$) from controls (last line). Mean ± SEM, $n = 5$. The results are representative of two experiments.

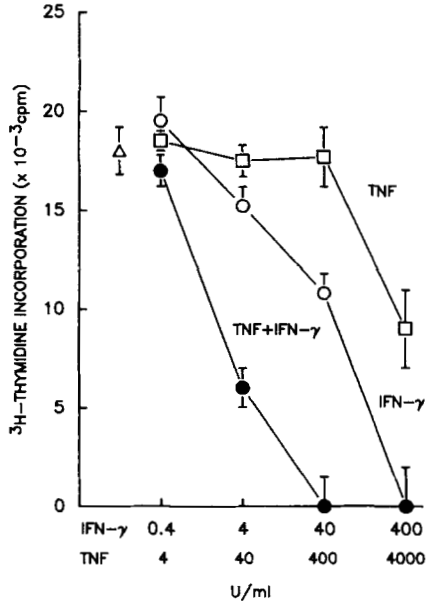

Figure 1. The activation of macrophage leishmanicidal activity by TNF-α, IFN-γ, or a combination of the two cytokines. For details, see *Materials and Methods*. Vertical bars = 1 SEM; $n = 3$. The data is representative of three similar experiments.

was progressively enhanced by L-NMMA but not by D-NMMA (Fig. 5). This is in agreement with an earlier report by Ding et al. (26). These results indicate that the leishmanicidal activity of activated macrophages in this system is not correlated with the production of superoxide anions.

DISCUSSION

Earlier reports demonstrate that TNF-α can mediate host resistance against *L. major* infection (9, 10). Mice developed exacerbated lesions when they were injected with neutralizing doses of anti-TNF antibodies. Conversely, the lesions were significantly reduced when TNF-α was injected into the lesions. Furthermore, TNF-α was found to activate mouse peritoneal macrophages to kill intracellular *L. major* (10). In the present report, we extend these observations by showing that TNF-α can synergize with IFN-γ for the host resistance against cutaneous leishmaniasis.

The mechanism for the synergistic effects of TNF-α and IFN-γ in the present system is unclear. TNF alone, in the presence of LPS, is able to activate murine PEC to produce NO (27) (F. Y. Liew, Y. Li, and S. Millott, unpublished observations). TNF has been shown to synergize with IFN-γ in activating macrophage cytotoxicity against TNF-insensitive tumor target cells (27–31). Furthermore, IFN-γ was able to enhance the TNF secretion by LPS-treated monocytes (32). Whatever the mechanism, the combination of IFN-γ and TNF-α appears to be highly effective in activating macrophages for NO synthesis (26). The presence of LPS was found to be essential for the consistent activation of macrophages by IFN-γ and TNF. The precise role of LPS in this system is unclear. It may provide a priming signal for the activation pathway. The kinetics of the relative requirement of LPS, TNF, and IFN-γ in macrophage NO synthesis and leishmanicidal activity is currently under investigation.

The precise mechanism of NO-mediated killing of intracellular pathogen is at present unknown, but is likely to be similar to that described for macrophage-mediated lysis of tumor cells (33). In the cytolytic system, NO has been shown to react with the Fe-S groups resulting in the formation of iron-nitrosyl complex (18) causing the inactivation and degradation of Fe-S prosthetic groups of aconitase and complex I and complex II of the mitochondria electron transport chain. Although NO can assert direct cytostatic effects on tumor cells (18) and leish-

TABLE II

Synergistic effect of TNF-α and IFN-γ in the induction of macrophage leishmanicidal activity in vitro

Macrophages[a] Cultured with	[^{3}H]TdR[b] Incorporation (× 10^3 cpm)	No. of *L. major*/100 Macrophages[c]		
		2 h	48 h	72 h
TNF-α (400 U/ml)	14.6 ± 0.8	259 ± 20	240 ± 18	221 ± 15
IFN-γ (40 U/ml)	*8.2 ± 0.7*	301 ± 25	*175 ± 10*	*15 ± 2*
TNF-α + IFN-γ	*0.7 ± 0.1*	281 ± 15		
Medium alone	15.1 ± 1.2	275 ± 15	245 ± 21	289 ± 25

[a] Peritoneal macrophages were treated with TNF, IFN-γ, or a combination of the two cytokines at 400 U/ml and 40 U/ml, respectively. For details, see *Materials and Methods*.

[b] [^{3}H]TdR incorporation as a measurement of surviving parasite at the end of 72-h culture period. Mean ± 1 SEM; $n = 6$. Data are pooled from two experiments.

[c] Mean of three to four cultures ± 1 SEM. Figures italicized are statistically different from controls (medium alone). Readings at 24 h were similar to those at 2 h.

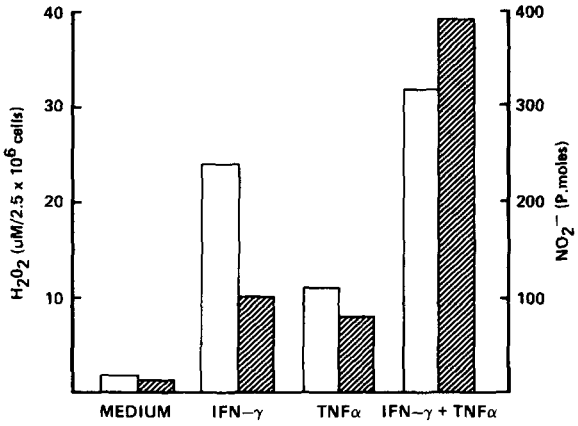

Figure 2. Production of H_2O_2 and NO_2^- by PEC activated with TNF-α and IFN-γ. For details see *Materials and Methods*. H_2O_2 (*open columns*) and NO_2^- (*hatched columns*) were determined in the pooled supernatants of three to four cultures.

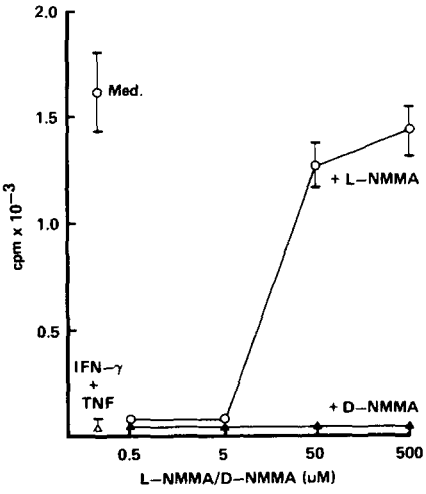

Figure 3. Effect of L-NMMA on the macrophage leishmanicidal activity induced by a combination of IFN-γ (40 U/ml) and TNF-α (400 U/ml). The surviving parasites at the end of 72-h culture were quantitated by the uptake of [^{3}H]TdR. Vertical bars = 1 SEM; $n = 6$.

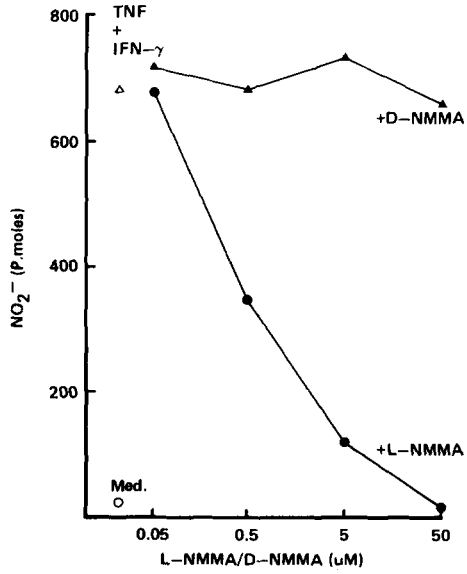

Figure 4. Inhibition of NO_2^- production by L-NMMA. Supernatants from the 72-h cultures described in Figure 2 were assayed for NO_2^- (14). Each point represents a pool of six cultures.

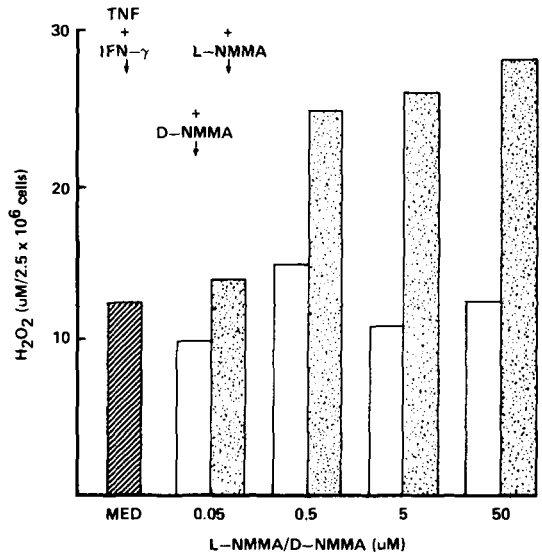

Figure 5. Effect of L-NMMA on H_2O_2 production by activated macrophages. PEC were treated with TNF-α (400 U/ml) + IFN-γ (40 U/ml) in the absence (*hatched column*) or presence of various concentrations of L-NMMA (*spotted columns*) or D-NMMA (*open columns*). The culture supernatants were assayed for H_2O_2 (25). Each reading represents a pool of three cultures.

mania promastigotes (12) in vitro in the absence of macrophages, the hydroxyl radical ($HO^\cdot$) derived from O_2^- and NO may also be involved as suggested by Beckman et al. (34). In this model, NO reacts efficiently with O_2^- to form peroxynitrite anion ($ONOO^-$), which decays rapidly once protonated to form $HO^\cdot$ and NO_2 (35). Hydroxyl radical is highly reactive combining with almost all molecules found in living cells with rate constants of 10^9 to 10^{10} M^{-1} s^{-1} (36).

Earlier reports indicated that H_2O_2 is the principal agent for killing *Leishmania donovani* in lymphokine-activated macrophages (37, 38). Catalase inhibited amastigote killing in a cellfree, acetaldehyde-xanthine oxidase system, indicating that H_2O_2 alone was sufficient to achieve leishmanicidal activity. This is in contrast to the *L. major* system in which respiratory burst is consistently shown not to be related with the killing of the parasites. Scott et al. (39) showed that *L. major* can be eliminated by a murine macrophage cell line, IC-21, which is deficient in the production of oxygen metabolites. The leishmanicidal activity of IFN-γ-activated mouse PEC infected with *L. major* amastigotes can be completely inhibited by L-NMMA (11, 12) (J. Mauel, A. Ransijn, and Y. Buchmüller-Rouiller, personal communication). Here, we show that L-NMMA not only inhibits macrophage leishmanicidal activity and NO production but can also enhance H_2O_2 output by the macrophages (Fig. 5) (26). These results strongly suggest that NO rather than H_2O_2 is principally involved in the killing of *L. major*. However, the data reported here do not exclude the possibility that killing of early logarithmic growth phase promastigotes may well involve respiratory oxygen intermediates produced by normal macrophages (unactivated by cytokines), whereas those parasites that survive this initial defense (amastigotes) are only destroyed by cytokine activated-macrophages involving NO. The relative role of NO in the *L. donovani* system is at present unclear.

Granger et al. (40) first demonstrated that macrophages

activated with IFN-γ and LPS have a cystostatic effect in vitro for the fungal pathogen *Cryptococcus neoformans* through the production of NO. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* also involves arginine-dependent production of reactive nitrogen intermediates (41). The same mechanism also appears to be involved in the macrophage microbiostatic capacity for *Toxoplasma gondii* (42). The role of NO in other infectious diseases, including bacterial and virus infections, will no doubt be revealed in the near future.

REFERENCES

1. **Howard, J. G.** 1985. Host immunity to leishmaniasis. In *Leishmaniasis.* K. P. Chang, and R. S. Bray, eds. Elsevier Science Publishers BV, Amsterdam, p. 140.
2. **Scott, P., P. Natovitz, R. L. Coffman, E. Pearce, and A. Sher.** 1988. Immunoregulation of cutaneous leishmaniasis: T-cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. Exp. Med. 168:1675.*
3. **Liew, F. Y.** 1989. Functional heterogeneity of $CD4^+$ T-cells in leishmaniasis. *Immunol. Today 10:40.*
4. **Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley.** 1989. Reciprocal expression of interferon-gamma or interleukin-4 during the resolution or progression of murine leishmaniasis: evidence for expansion of distinct helper T-cell subsets. *J. Exp. Med. 169:59.*
5. **Mauel, J., and R. Behin.** 1982. Leishmaniasis. In *Immunology of Parasitic Infections.* S. Cohen, and K. S. Warren, eds. Blackwell Scientific Publications, Cambridge, MA, p. 299.
6. **Murray, H. W., B. Y. Rubin, and C. D. Rothermel.** 1983. Killing of intracellular *Leishmania donovani* by lymphokine-stimulated human mononuclear phagocytes: evidence that interferon-gamma is the activating lymphokine. *J. Clin. Invest. 72:1506.*
7. **Hoover, D. L., C. A. Nacy, and M. S. Meltzer.** 1985. Human monocyte activation for cytotoxicity against intracellular *Leishmania donovani* amastigotes: induction of microbicidal activity by interferon-gamma. *Cell. Immunol. 94:5005.*
8. **Titus, R. G., A. Kelso, and J. A. Louis.** 1984. Intracellular destruction of *L. tropica* by macrophages activated with macrophage activating factor/interferon. *Clin. Exp. Immunol. 55:157.*
9. **Titus, R. G., B. Sherry, and A. Cerami.** 1989. Tumor necrosis factor plays a protective role in experimental cutaneous leishmaniasis. *J. Exp. Med. 170:2097.*
10. **Liew, F. Y., C. Parkinson, C., S. Millott, A. Severn, and M. Carrier.** 1990. Tumour necrosis factor (TNFα) in leishmaniasis. I. TNFα mediated host protection against cutaneous leishmaniasis. *Immunology 69:570.*
11. **Green, S. J., M. S. Meltzer, J. B. Hibbs, Jr., and C. A. Nacy.** 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. *J. Immunol. 144:278.*
12. **Liew, F. Y., S. Millott, C. Parkinson, R. M. J. Palmer, and S. Moncada.** 1990. Macrophage killing of leishmania parasite in vivo is mediated by nitric oxide from L-arginine. *J. Immunol. 144:4793.*
13. **Moncada, S., R. M. J. Palmer, and E. A. Higgs.** 1989. Biosynthesis of nitric oxide from L-arginine: a pathway for the regulation of cell function and communication. *Biochem. Pharmacol. 38:1709.*
14. **Palmer, R. M. J., A. G. Ferrige, and S. Moncada.** 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature 327:524.*
15. **Hibbs, J. B., Jr., R. R. Taintor, and Z. Vavrin.** 1987. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science 235:473.*
16. **Iyengar, R., D. J. Stuehr, and M. A. Marletta.** 1987. Macrophage synthesis of nitrite, nitrate and *N*-nitrosamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. USA 84:6369.*
17. **Marletta, M. A., P. S. Yoon, R. Iyengar, C. D. Leaf, and J. S. Wishnok.** 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry 27:8706.*
18. **Hibbs, J. B., Jr., R. R. Taintor, Z. Vavrin, and E. M. Rachlin.** 1988. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun. 157:87.*
19. **Stuehr, D., S. Gross, I. Sakuma, R. Levi, and C. Nathan.** 1989. Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. *J. Exp. Med. 169:1011.*
20. **Palmer, R. M. J., D. S. Ashton, and S. Moncada.** 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature 333:664.*
21. **Palmer, R. M. J., and S. Moncada.** A novel citrulline-forming enzyme inplicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun. 158:348.*
22. **Hibbs, J. B., Jr., Z. Vavrin, and R. R. Taintor.** 1987. L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol. 138:550.*
23. **Liew, F. Y., J. G. Howard, and C. Hale.** 1984. Prophylactic immunisation against experimental leishmaniasis. III. Protection against fatal *Leishmania tropica* infection induced by irradiated promastigotes involves Lyt-1^+2^- T-cells that do not mediate cutaneous DTH. *J. Immunol. 132:456.*
24. **Liew, F. Y., and J. S. Dhaliwal.** 1987. Distinctive cellular immunity in genetically susceptible BALB/c mice recovered from *Leishmania major* infection or after subcutaneous immunization with killed parasites. *J. Immunol. 138:4456.*
25. **Pick, E., and Y. Keisari.** 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J. Immunol. Methods 38:161.*
26. **Ding, A. H., C. F. Nathan, and D. J. Stuehr.** 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. *J. Immunol. 141:2407.*
27. **Drapier, J. C., J. Wietzerbin, and J. B. Hibbs, Jr.** 1988. Interferon-gamma and tumour necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. *Eur. J. Immunol. 18:1587.*
28. **Esparza, I., D. Mannel, A. Ruppel, W. Falk, and P. Krammer.** 1987. Interferon-gamma and lymphotoxin or tumor necrosis factor act synergistically to induce macrophage killing of tumor cells and schistosomula of *Schistosoma mansoni. J. Exp. Med. 166:589.*
29. **Hori, K., M. J. Ehrke, K. Mace, and E. Mihich.** 1987. Effect of 9 recombinant tumor necrosis factor in tumoricidal activation of murine macrophages: synergism between tumor necrosis factor and gamma-interferon. *Cancer Res. 47:5868.*
30. **Chen, L., Y. Susuki, and E. F. Wheelock.** 1987. Interferon-γ synergizes with tumor necrosis factor and with interleukin-1 and requires the presence of both monokines to induce anti-tumor cytotoxic activity in macrophages. *J. Immunol. 139:4096.*
31. **Heidenreich, S., M. Weyers, J. H. Gong, H. Sprenger, M. Nain, and D. Gemsa.** 1988. Potentiation of lymphokine-induced macrophage activation by tumor necrosis factor-α. *J. Immunol. 140:1511.*
32. **Burchett, S. K., W. M. Weaver, J. A. Westall, A. Larsen, S. Kronheim, and C. B. Wilson.** 1988. Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. *J. Immunol. 140:3473.*
33. **Hibbs, J. B., Jr., R. R. Taintor, Z. Vavrin, D. L. Granger, J.-C. Drapier, I. J. Amber, and J. R. Lancaster, Jr.** 1990. Synthesis of nitric oxide from a terminal guanidino atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In *Argine:NO Pathway.* S. Moncada, ed. Elsevier Press, New York. p. 189.
34. **Beckman, J. S., T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman.** 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA 87:1620.*
35. **Blough, N. V., and O. C. Zafiriou.** 1985. Reaction of superoxide with nitric oxide to form peroxonitrite in alkaline aqueous solution. *Inorg. Chem. 24:3502.*
36. **Anbar, M., and P. Neta.** 1967. A compilation of specific biomolecular rate constants for the reactions of hydrated electrons hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in aqeous solution. *Int. J. Appl. Rad. Isotopes 18:495.*
37. **Murray, H. W.** 1982. Cell-mediated immune response in experimental leishmaniasis. *J. Immunol. 129:351.*
38. **Channon, J. Y., and J. M. Blackwell.** 1985. A study of the sensitivity of *Leishmania donovani* promastigotes and amastigotes to hydrogen peroxide. I. Differences in sensitivity correlate with parasite-mediated removal of hydrogen peroxide. *Parasitology 91:197.*
39. **Scott, P., S. James, and A. Sher.** 1985. The respiratory burst is not required for killing of intracellular and extracellular targets by a lymphokine-activated macrophage cell line. *Eur. J. Immunol. 15:553.*
40. **Granger, D. L., J. B. Hibbs, Jr., J. R. Perfect, and D. T. Durack.** 1990. Metabolic fate of L-arginine in relation to microbiostatic capacity of macrophages. *J. Clin. Invest. 85:264.*
41. **James, S. L., and J. Glaven.** 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J. Immunol. 143:4208.*
42. **Adams, L. B., J. B. Hibbs, Jr., R. R. Taintor, and J. L. Krahenbuhl.** 1990. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*: role for synthesis of inorganic nitrogen oxides from L-arginine. *J. Immunol. 144:2725.*