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Mechanisms Involved in Mycobacterial Growth Inhibition by Gamma Interferon-Activated Bone Marrow Macrophages: Role of Reactive Nitrogen Intermediates

INGE E. A. FLESCH AND STEFAN H. E. KAUFMANN*

Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, D-7900 Ulm, Federal Republic of Germany

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Murine bone marrow-derived macrophages are able to inhibit the growth of *Mycobacterium bovis* after stimulation with recombinant gamma interferon. This antimycobacterial activity was inhibited by N^G-monomethyl-L-arginine, a specific inhibitor of nitrite and nitrate synthesis from L-arginine. Furthermore, there was a complete lack of mycobacterial growth inhibition in a medium deficient in L-arginine. Nitrite is generated by gamma interferon-activated bone marrow-derived macrophages after infection with *M. bovis*, and a correlation between mycobacterial growth inhibition and nitrite production was observed. These results indicate that reactive nitrogen intermediates derived from L-arginine are crucially involved in macrophage antimycobacterial activity.

Evidence that the reactive nitrogen intermediates (RNI) nitric oxide ($NO^{\cdot}$), nitrite (NO_2^-), and nitrate (NO_3^-) are potent effector molecules of macrophage-mediated extracellular and intracellular cytotoxicity (5, 12, 18, 21, 24) is accumulating. For example, it has been shown recently that killing by macrophages of the protozoa *Toxoplasma gondii* (1) and *Leishmania major* (11, 22, 23), the opportunistic fungus *Cryptococcus neoformans* (9), the metazoan pathogen *Schistosoma mansonii* (17, 18), and tumor cells (12, 13, 19, 33) involves RNI.

Mycobacterium bovis and *M. tuberculosis* are facultative intracellular bacteria capable of persisting and replicating within resting mononuclear phagocytes. After stimulation with interleukins, macrophages acquire the capacity to reduce their load of intracellular mycobacteria, and there is strong evidence that gamma interferon (IFN-γ) is particularly effective (26). By using murine bone marrow-derived macrophages (BMMφ) preactivated with recombinant IFN-γ (rIFN-γ) or lymphokines from antigen-specific T-cell clones, we have observed powerful tuberculostatic effects in vitro (7).

Although the precise mechanism(s) of this antimycobacterial activity is unclear, it has been claimed that the reactive oxygen intermediates (ROI), O_2^-, H_2O_2, $^1O_2$, and $OH^{\cdot}$, are important for macrophage antimycobacterial activities (15, 16, 35). In contrast, we obtained evidence arguing against an involvement of ROI in the growth inhibition of *M. bovis* by rIFN-γ-activated BMMφ (i) because phagocytosis of *M. bovis* by rIFN-γ-activated BMMφ failed to trigger the oxidative burst (7) and (ii) because scavengers of reactive oxygen metabolites did not reverse mycobacterial growth inhibition (8).

In this report, we provide evidence for an involvement of RNI, rather than ROI, in macrophage tuberculostasis. The data presented show that RNI are generated from L-arginine by rIFN-γ-activated BMMφ after infection with *M. bovis* and that growth inhibition of *M. bovis* is markedly reversed by means which specifically interfere with RNI production in macrophages.

MATERIALS AND METHODS

Microorganisms. *M. bovis* BCG Phipps was grown in Dubos broth (Difco Laboratories, Detroit, Mich.) supplemented with bovine serum albumin and Tween 80 with shaking. Cultures were centrifuged, washed twice in phosphate-buffered saline, and stored frozen at −70°C. Numbers of viable organisms were determined by plating 1:10 dilutions on Middlebrook Dubos agar plates (Difco). Plates were incubated at 37°C, and the number of CFU was determined.

Cytokines and culture reagents. Murine rIFN-γ was kindly provided by G. Adolf, Ernst Boehringer-Institut für Arzneimittel-Forschung, Vienna, Austria. N^G-monomethyl-L-arginine (MLA) was purchased from Calbiochem (La Jolla, Calif.). Nω-nitro-L-arginine (NLA) and L-arginine were obtained from Sigma Chemical Co. (St. Louis, Mo.). L-arginine-depleted RPMI 1640 medium was prepared by using an RPMI 1640 select amine kit (GIBCO).

Macrophage cultures. Bone marrow cells were prepared from the femora of 6- to 8-week-old C57BL/6 male or female mice from our own breeding colony. BMMφ were grown in Teflon film bags (Heraeus, Hanau, Federal Republic of Germany) in a serum-free culture medium as described previously (6, 7). The serum-free medium consisted of Iscove's modified Dulbecco's medium (IMDM) without antibiotics and was supplemented with 15 μg of transferrin per ml, 5×10^{-5} M 2-mercaptoethanol, and 30% (vol/vol) serum-free supernatant from L-929 cells as a source of macrophage colony-stimulating factor. BMMφ were harvested after 9 days in culture.

Inhibition of mycobacterial growth by BMMφ. Mycobacterial growth inhibition was assessed as described previously (7). BMMφ (10^5 cells per well) were seeded in flat-bottom microdilution plates (Nunc, Roskilde, Denmark) in IMDM without additives and were stimulated with rIFN-γ. After 24 h, the medium was removed and BMMφ were infected with 10^6 live *M. bovis* organisms per well. After incubation for 4

* Corresponding author.

days at 37°C in 10% CO_2 in air, macrophages were washed to remove extracellular bacteria. Cells were lysed by the addition of IMDM containing 0.1% saponin, and the viability of mycobacteria was assessed by the addition of 0.5 μCi of [^{3}H]uracil per well. After 7 to 8 h, cultures were harvested onto glass fiber filters. The incorporation of radioactivity was counted in a β-counter (Beta-plate; LKB).

To assess the effects of MLA and NLA on the viability and growth of *M. bovis*, mycobacteria were seeded into microdilution plates (10^6 bacteria per well) in IMDM containing MLA or NLA, respectively. After 4 days of incubation, the growth of *M. bovis* was determined by the addition of [^{3}H]uracil (0.5 μCi per well); the solution was allowed to stand for 7 to 8 h.

Nitrite determination. BMMϕ supernatants were collected at different time points, as indicated in Results. The nitrite concentration was measured by Griess reagent (4, 10). Briefly, 50-μl aliquots were mixed with equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4) and incubated at room temperature for 10 min. A_{570} was measured in an Immunoreader NJ 2000 (Intermed). NO_2^- was determined by using $NaNO_2$ dissolved in IMDM as a standard, with IMDM alone as a blank. NO_2^- production is reported as nanomoles per 10^5 BMMϕ. Griess reagent detects nitrite in the range of 1 to 300 μM (10).

Incubation of *M. bovis* with RNI at reduced pH. *M. bovis* organisms were seeded into flat-bottom microdilution plates with 5×10^5 bacteria per well in bicarbonate-free IMDM with increasing concentrations of $NaNO_2$. After 3 days of incubation, [^{3}H]uracil (0.5 μCi per well) was added and the solution was allowed to stand for 7 h. The incorporation of radioactivity was counted in a Beta-plate β-counter.

RESULTS

In the following experiments, BMMϕ obtained by cultivation in serum-free medium were used. As shown previously, these cells represent a quiescent macrophage population. After activation with rIFN-γ, growth of *M. bovis* as well as *M. tuberculosis* H37Rv is inhibited (7, 8). The viability of *M. bovis* was determined by the uptake of [^{3}H]uracil, which is incorporated into the RNA of mycobacteria. An 80 to 95% reduction in [^{3}H]uracil uptake reflects a 10- to 100-fold decrease in the number of intracellular bacteria as determined by CFU (7). Since BMMϕ were lysed with saponin before the addition of [^{3}H]uracil, the incorporation of radioactivity into cellular RNA could be ruled out.

RNI production by BMMϕ. Among RNI, NO· has been identified as the intermediate effector molecule. NO· is short-lived (half-life, 4 to 50 s) and reacts with itself, oxygen, and water to generate NO_2^- and NO_3^-, which accumulate in macrophage culture supernatants at a ratio of 3:2 (4, 10, 33). With the Griess reaction, the amount of accumulated NO_2^- is measured (10). Since RNI are small molecules which diffuse through cell membranes, an active secretion process is not required. We assume a steady state between intracellular and extracellular nitrite on the day of measurement (day 4).

RNI production, as measured by NO_2^-, was investigated in BMMϕ. Cells were stimulated with rIFN-γ for 24 h and subsequently treated with either viable *M. bovis* organisms or zymosan particles. After 24 h, supernatants were collected for NO_2^- detection, macrophages were washed, and fresh medium was added. After 48 and 72 h, supernatants were collected in the same way. As shown in Table 1, unstimulated BMMϕ or BMMϕ stimulated with rIFN-γ failed to release detectable amounts of NO_2^-. *M. bovis* infection of rIFN-γ-stimulated BMMϕ induced marked NO_2^- production for 48 h; afterwards, NO_2^- release declined. Also, phagocytosis of unopsonized zymosan particles induced NO_2^- release in BMMϕ which were activated with rIFN-γ. However, the amount of NO_2^- detected was significantly lower than that produced by cells costimulated by IFN-γ and viable *M. bovis*. Obviously, RNI synthesis by murine BMMϕ requires two signals which are provided by costimulation with rIFN-γ and *M. bovis*.

TABLE 1. NO_2^- production by BMMϕ[a]

Phagocytic stimulus and amt of rIFN-γ (U/ml)	NO_2^- production, nmol/10^5 BMMϕ (mean ± SD)		
	24 h	48 h	72 h
M. bovis			
—[b]	0	0	0
100	0.9 ± 0.1	0.7 ± 0.1	
500	2.9 ± 0.2	4.8 ± 0.1	
2,500	3.2 ± 0.1	5.6 ± 0.2	0.5 ± 0.2
Zymosan			
—	0	0	0
100	0	0	0
500	0.3 ± 0.2	0.3 ± 0.06	0
2,500	0.9 ± 0.1	1.2 ± 0.2	0

[a] BMMϕ (10^5 per well) were stimulated with rIFN-γ for 24 h, washed, and treated with either viable *M. bovis* organisms or zymosan. Every 24 h, supernatants were collected for NO_2^- detection, cells were washed, and fresh medium was added. Either *M. bovis* (10^6 per well) or unopsonized zymosan (10 μg per well) were used as phagocytic stimuli. In the absence of any phagocytic stimulus, results showed no NO_2^- production. Data are presented as means from fourfold determinations ± standard deviations.

[b] —, none.

Antimycobacterial activity of and RNI production by BMMϕ. Previously, we demonstrated that rIFN-γ is capable of inducing antimycobacterial capacities in BMMϕ (7). To determine whether mycobacterial growth inhibition is paralleled by NO_2^- production, BMMϕ were stimulated with increasing concentrations of rIFN-γ for 24 h and subsequently infected with *M. bovis* organisms. After 4 days of incubation, supernatants were collected for NO_2^- determination; afterwards BMMϕ were lysed and mycobacterial growth was assessed by [^{3}H]uracil incorporation. As shown in Fig. 1, increasing concentrations of rIFN-γ induced the release of NO_2^- by BMMϕ in a dose-dependent fashion, which was paralleled by intracellular growth inhibition of *M. bovis*.

Effect of L-arginine depletion on BMMϕ antimycobacterial activity. RNI produced by macrophages are derived from L-arginine (14, 24, 25, 34), and hence a lack of L-arginine should prevent RNI production. BMMϕ were stimulated with rIFN-γ in medium containing L-arginine to guarantee normal protein synthesis during the activation period. Then medium was removed and infection with *M. bovis* was performed in L-arginine-deficient medium. As shown in Fig. 2, the depletion of L-arginine from the medium completely abolished the antimycobacterial activity of BMMϕ. The depletion of L-arginine not only affects RNI production by macrophages; it also enhances superoxide production, the phagocytosis of latex beads, and protein synthesis (2).

Reversion of NO_2^- production and antimycobacterial activity by L-arginine analogs. MLA and NLA are two *N*-guanidino-substituted specific analogs of L-arginine which inhibit

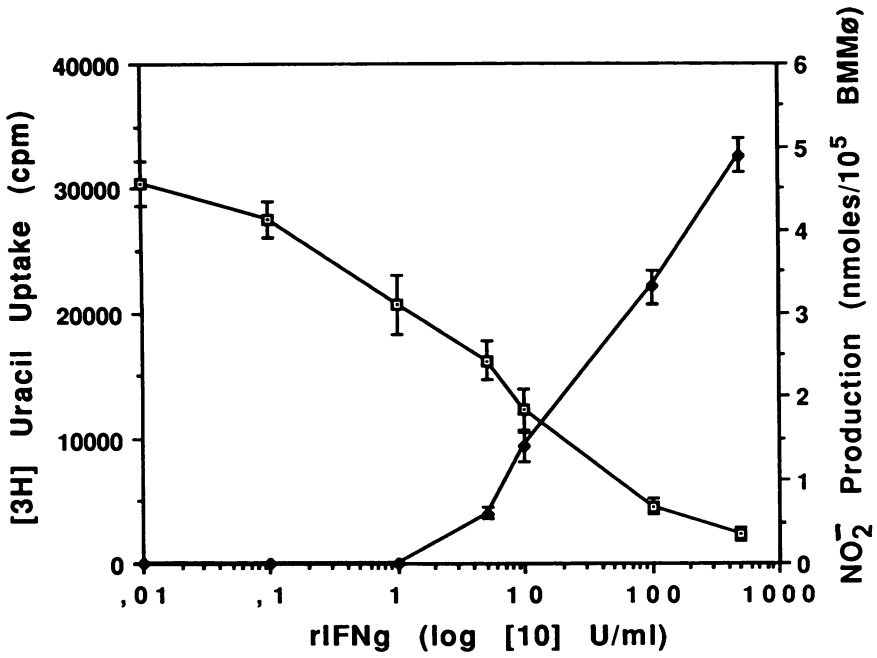

FIG. 1. Antimycobacterial activity and NO_2^- production by rIFN-γ-activated BMMϕ. BMMϕ were stimulated with increasing concentrations of rIFN-γ for 24 h, washed, and infected with *M. bovis* organisms. Four days later, supernatants were collected for NO_2^- determination (◆). Intracellular growth of mycobacteria was measured by [^{3}H]uracil incorporation (⊡). Data are presented as means from triplicate cultures ± standard deviations. Take note that nitrite concentrations below 1 μM (0.2 nmoles per 10^5 BMMϕ) cannot be detected with Griess reagent (10).

RNI synthesis by macrophages (12, 14). BMMϕ were stimulated with rIFN-γ for 24 h, washed, and infected with *M. bovis* in the presence of increasing concentrations of MLA or NLA, respectively. After 4 days of incubation, supernatants were collected to measure NO_2^- production. As shown in Table 2, NO_2^- production was inhibited in a concentration-dependent manner by MLA but not by NLA. In parallel, mycobacterial growth inhibition was determined. The addition of MLA or NLA to the cultures during activation with rIFN-γ and the subsequent infection with *M. bovis* in medium without analogs had no effect on intracellular growth inhibition of mycobacteria (Fig. 3). In contrast, when the analogs were added during infection with *M. bovis*, MLA markedly reversed the growth inhibition induced by rIFN-γ, whereas NLA had little effect (Fig. 4). MLA and NLA had no direct effect on the viability of *M. bovis*, and the addition of excess L-arginine partially counteracted the effect of MLA (data not shown). We do not have an explanation for the different effects of MLA and NLA in our assay system. Both analogs have been shown to reverse the RNI-mediated inhibition of malaria parasite development induced by tumor necrosis factor and interleukin 6 in cocultures of hepatocytes and nonparenchymal cells (27).

Direct effect of RNI on the growth of *M. bovis*. The direct effect of RNI on the growth of *M. bovis* was assessed by incubating bacteria with increasing concentrations of $NaNO_2$ in bicarbonate-free IMDM at pH 6.5 and 6.0 for 3 days. Upon mild acidification, HNO_2 is formed from $NaNO_2$, the dismutation of which generates NO· (33). Accordingly, $NaNO_2$ at pH 6.0 and less so at pH 6.5 inhibited [^{3}H]uracil incorporation by *M. bovis* in a concentration-dependent way (Table 3).

DISCUSSION

Although it is generally accepted that interleukin-activated macrophages represent the crucial effector cells of host resistance against tubercle bacilli and other intracellular bacteria, little is known about the underlying mechanisms (26). Low-molecular-weight components including ROI (O_2^-, H_2O_2, $^1O_2$, and OH·) and RNI (NO, NO_2^-, and NO_3^-) could be important. For a long time, ROI have been considered crucial for antimycobacterial resistance (15, 16, 35). Our attempts to show a role for ROI in mycobacterial growth inhibition by rIFN-γ-activated BMMϕ have thus far failed (8). We used murine BMMϕ obtained in a serum-free culture medium, which represent a quiescent macrophage population. After activation with rIFN-γ, these macrophages express marked antimycobacterial activities; however, scavengers of ROI had no effect on the growth inhibition of *M. bovis* (8).

By using the very same cell system, we now provide evidence for a major role of RNI in mycobacterial growth inhibition. Our claim is based on the following findings: (i) mycobacterial infection of rIFN-γ-stimulated BMMϕ caused marked RNI production, as measured by NO_2^-; (ii) mycobacterial growth was inhibited by RNI generated by mild acidification of IMDM containing $NaNO_2$; and (iii) inhibition of RNI production by BMMϕ either by the addition of the L-arginine analog MLA or by L-arginine depletion-reversed growth inhibition of *M. bovis*.

Synthesis of RNI appears to be a general property of macrophages (21, 24). After activation, NO_2^-, NO_3^-, and citrulline are synthesized from L-arginine with NO· as an intermediate (25). Evidence that NO· rather than NO_2^- and NO_3^- is the bacteriostatic effector molecule has been previ-

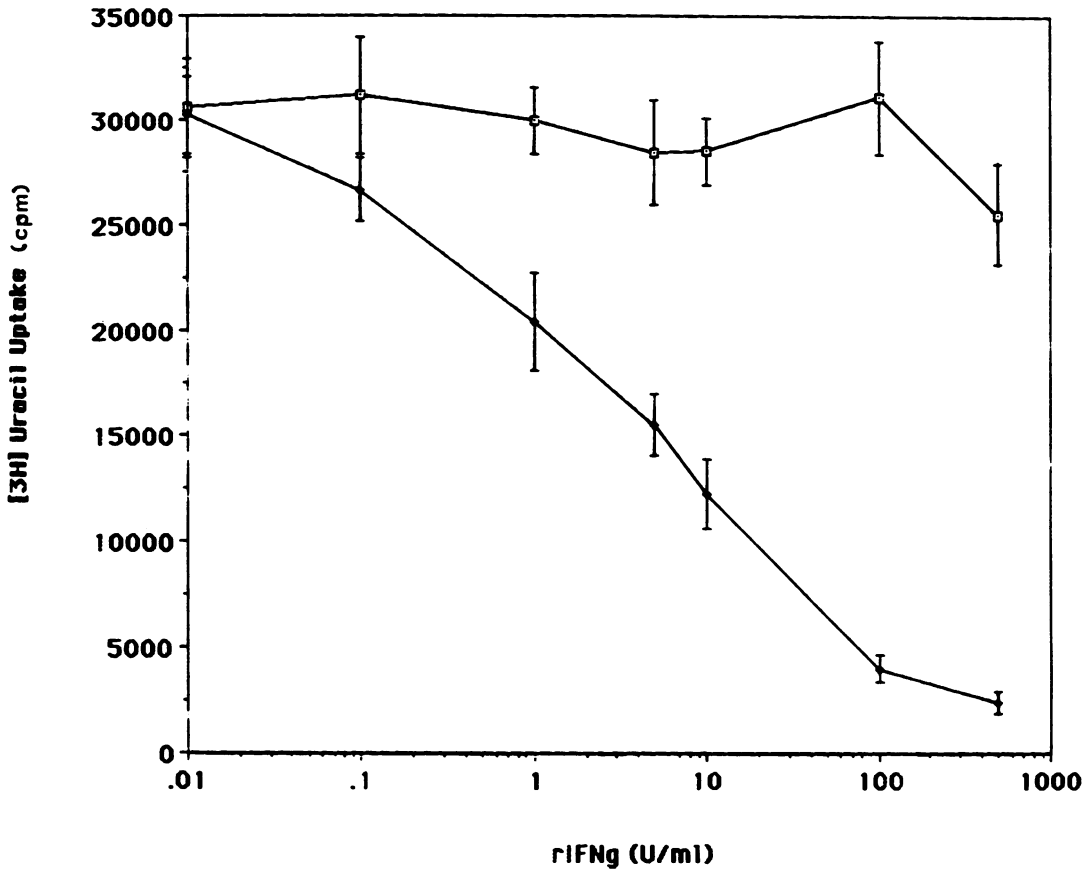

FIG. 2. Effect of L-arginine depletion on growth inhibition of *M. bovis*. BMMϕ were stimulated with rIFN-γ for 24 h, washed, and infected with *M. bovis* organisms in medium without (⊡) or with (◆) 0.5 mM of L-arginine. Intracellular growth of *M. bovis* was measured after 4 days by [^{3}H]uracil incorporation. Data are presented as means from triplicate cultures ± standard deviations.

ously presented (33). The enzyme nitric oxide synthase has been found in the cytosol of macrophages costimulated with IFN-γ plus lipopolysaccharide but not in the cytosol of unstimulated cells (34).

By using either resident peritoneal macrophages or peritoneal macrophages elicited with periodate, casein, or thioglycolate, Ding et al. (4) showed that, of 12 cytokines, IFN-γ is the only one which induces substantial NO_2^- secretion. IFN-γ plus lipopolysaccharide as well as IFN-γ plus tumor necrosis factor interacted synergistically. *M. bovis* infection of mice results in an increased excretion of NO_3^- (31). Peritoneal macrophages from *M. bovis*-infected mice also produce high levels of NO_2^- and NO_3^- (32). Murine BMMϕ obtained in a serum-containing medium could also be induced to secrete NO_2^- after stimulation with macrophage-activating lymphokines (19). In our system, NO_2^- production was not induced by rIFN-γ alone; rather, mycobacterial infection was necessary. In some but not all macrophage preparations, viable *M. bovis* alone was sufficient to induce NO_2^- secretion. Since serum-free BMMϕ represent a completely quiescent macrophage population (in contrast to peritoneal macrophages and BMMϕ cultivated with serum), these findings suggest that a triggering signal provided by bacteria is necessary for RNI production by macrophages.

TABLE 2. Effect of MLA and NLA on NO_2^- production[a]

RNI inhibitor (μg/ml)	NO_2^- production, nmoles/10^5 BMMϕ (mean ± SD)
MLA	
—[b]	10.3 ± 0.4
1	9.8 ± 0.8
10	8.0 ± 0.3
50	3.9 ± 0.08
100	1.8 ± 0.3
NLA	
1	9.5 ± 0.6
10	9.7 ± 0.6
50	8.9 ± 0.2
100	9.0 ± 0.2

[a] BMMϕ were stimulated with rIFN-γ (500 U/ml) for 24 h, washed, and infected with *M. bovis* in the presence of MLA or NLA. Accumulation of NO_2^- in the culture supernatant was measured after 4 days of incubation. Data are presented as the means from triplicate cultures ± the standard deviations.

[b] —, no inhibitor.

Previous studies have already revealed that RNI are involved in the elimination of the intracellular protozoa *L. major* and *T. gondii* by interleukin-activated macrophages (1, 11, 22, 23). Also, macrophages activated with IFN-γ and lipopolysaccharide are cytostatic for the opportunistic fungus *C. neoformans* and for some tumor cell lines (9, 12, 13, 19, 33). Furthermore, cytotoxic activity of lymphokine-

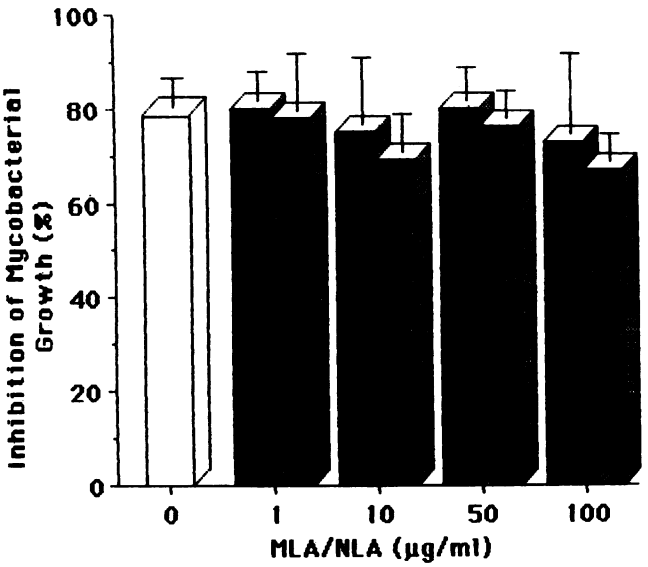

FIG. 3. Effect of L-arginine analogs MLA and NLA on growth inhibition of *M. bovis*. Analogs were present during activation of BMMϕ with rIFN-γ. BMMϕ were stimulated with rIFN-γ (500 U/ml) in the presence of MLA (closed columns) or NLA (hatched columns) or in the absence of either compound (open column). After 24 h, cells were washed and infected with *M. bovis* organisms. Growth of mycobacteria was measured after 4 days by [^{3}H]uracil incorporation. Percent inhibition = (1 − [^{3}H]uracil uptake after culture with rIFN-γ-activated BMMϕ/[^{3}H]uracil uptake after culture with unstimulated BMMϕ) × 100. Data are presented as means from triplicate cultures ± standard deviations.

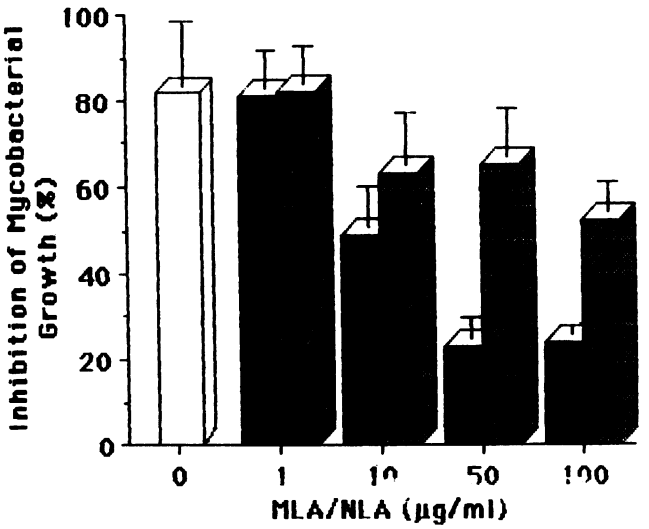

FIG. 4. Effect of the L-arginine analogs MLA and NLA on the growth inhibition of *M. bovis*. Analogs were present during the infection of BMMϕ with *M. bovis*. BMMϕ were stimulated with rIFN-γ (500 U/ml) for 24 h, washed, and infected with *M. bovis* organisms in the presence of MLA (closed columns) or NLA (hatched columns) or in the absence of either compound (open columns). Growth of mycobacteria was measured after 4 days by [^{3}H]uracil uptake. Percent inhibition was calculated as described in the legend to Fig. 3. Data are presented as means from triplicate determinations ± standard deviations.

activated macrophages for the extracellular helminth *S. mansoni* seems to depend on RNI (17, 18). These studies as well as ours employed inhibition of the L-arginine-dependent production of RNI by the L-arginine analog MLA or by the use of L-arginine-depleted medium. In our experiments, the L-arginine analog NLA showed negligible effects for unknown reasons.

When generated in a cell-free system, both ROI and RNI expressed marked antimycobacterial activity. On the other hand, the infection of rIFN-γ-activated BMMϕ with *M. bovis* did not result in the secretion of ROI (7) but induced production of RNI only. It has been shown previously that mycobacteria are preferentially taken up via complement receptors and that this type of receptor-mediated phagocytosis bypasses ROI production (29). In addition, low-molecular-weight fractions of mycobacteria, including sulfolipids (28, 36), lipoarabinomannan (30), and phenolic glycolipid-1 (3), are able to inhibit ROI production by macrophages. Taken together, these data suggest that mycobacteria are well equipped to interfere with the oxidative burst, but not with RNI production. Obviously, RNI play a more important role in antituberculous resistance than ROI.

TABLE 3. Effect of $NaNO_2$ on the [^{3}H]uracil incorporation of *M. bovis* at pHs 6.0 and 6.5[a]

$NaNO_2$, µg/ml (µM)	Effect at pH 6.0		Effect at pH 6.5	
	[^{3}H]uracil uptake, cpm (mean ± SD)	% Inhibition[b]	[^{3}H]uracil uptake, cpm (mean ± SD)	% Inhibition
—	3,164 ± 98		2,893 ± 258	
0.05 (0.72)	2,919 ± 159	8	2,835 ± 193	2
0.5 (7.2)	2,849 ± 114	10	2,634 ± 61	9
5 (72)	2,370 ± 183	25	2,407 ± 170	17
50 (720)	1,015 ± 184	68	2,373 ± 234	18
125 (1,800)	151 ± 65	95	1,431 ± 158	51
250 (3,600)	64 ± 20	98	307 ± 89	89

[a] *M. bovis* organisms (5×10^5 per well) were seeded into microdilution plates in IMDM with $NaNO_2$ at pHs 6.0 and 6.5. After 3 days of incubation, [^{3}H]uracil was added for 7 h to assess the viability of mycobacteria. —, no $NaNO_2$.

[b] Percent inhibition = (1 − [^{3}H]uracil uptake of bacteria treated with $NaNO_2$/[^{3}H]uracil uptake of untreated bacteria) × 100. [^{3}H]uracil uptake data are presented as means from fourfold determinations (± standard deviations).

Several groups including ourselves have provided evidence for an additional role of phagosome-lysosome fusion in mycobacterial growth inhibition (8, 26). With what we know now, it appears that RNI release and the introduction of lysosomal enzymes into the infected phagosome are of major importance for the host defense against tubercle bacilli. It is unclear at the moment whether the two mechanisms are independent or synergistic in action.

Tuberculosis is typically a disease of the lung, and this organ represents the preferential port of entry for tubercle bacilli. Recent studies have shown that nitric oxide synthase is induced by endotoxin in rat lungs (20). We, therefore, assume that the mechanism delineated here in an in vitro system may also contribute to the defense against tuberculosis in vivo. Further studies employing the inhibition of RNI production in vivo by MLA are planned to decide whether RNI are indeed involved in in vivo resistance against tuberculosis.

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ADDENDUM IN PROOF

After this paper was submitted, Denis published similar results (M. Denis, Cell. Immunol. **132**:150–157, 1991).

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