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Resistance to Nitric Oxide in *Mycobacterium avium* Complex and Its Implication in Pathogenesis

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Susceptibility of three different strains of *Mycobacterium avium* complex (MAC), i.e., one strain of *M. avium* (Mino) and two strains of *M. intracellulare* (31F093T and KUMS 9007), to nitric oxide (NO) generated by rat alveolar macrophages (Mϕ) or NO generated chemically by acidification of NO_2^- was examined in vitro. We also investigated the effects of NO on phagocytosis and superoxide anion (O_2^-) generation by Mϕ. The intracellular growth of *M. avium* Mino was significantly suppressed by NO generated by gamma interferon (IFN-γ)-stimulated Mϕ, whereas that of two strains of *M. intracellulare* (31F093T and KUMS 9007) was not. *M. avium* Mino was also more susceptible to NO generated chemically by acidification of NO_2^- than the two *M. intracellulare* strains. In L-arginine (1 mM)-containing medium, NO release from the Mϕ assessed by measuring NO_2^- increased as the concentration of IFN-γ increased. The enhancing potential of IFN-γ for NO release became more pronounced when Mϕ were infected with 31F093T, an NO-resistant strain. A large amount of NO generated by IFN-γ-stimulated Mϕ suppressed both phagocytosis and O_2^- generation by the Mϕ, especially after infection of the Mϕ with strain 31F093T. These results indicate that the intracellular growth of MAC is not always inhibited by NO generated by immunologically activated Mϕ; rather, NO generation induced by infection with an NO-resistant MAC strain suppresses phagocytosis of the Mϕ, which may allow extracellular spreading of such NO-resistant mycobacteria. Therefore, the pathogenic potential of MAC may be partly attributed to its resistance to NO.

Physiological roles of nitric oxide (NO) are presently a focus of considerable interest in the signal transduction mechanism of a variety of cells, e.g., neural and endothelial cells (13, 30, 38). Activated macrophages (Mϕ) generate NO from the terminal guanidino-nitrogen atoms of L-arginine, and the generated NO is subsequently converted to NO_2^- and NO_3^- by a rapid oxidizing process (27, 40, 42). This pathway is inhibited by the L-arginine analog N^G-monomethyl L-arginine (L-NMMA) (27). Some of these nitrogen oxides appear to play an important role in the antitumor effects (27, 28, 40, 42) and in some antimicrobial actions (24, 26, 32) of activated Mϕ.

Gamma interferon (IFN-γ), which is secreted by activated T lymphocytes, activates Mϕ to exhibit antimicrobial activity in vivo (15, 21, 29, 36) and in vitro (12, 19). Ding et al. (18) demonstrated that IFN-γ was the most potent inducer of reactive nitrogen oxides in mouse peritoneal Mϕ. Activated Mϕ stimulated with IFN-γ seem to exert antitumor effects and some antimicrobial activities via induction of NO or other reactive nitrogen oxides. It is also reported that in the murine system, IFN-γ-stimulated Mϕ show not only mycobacteristatic but also mycobactericidal activity via generation of reactive nitrogen oxides (14, 16, 23). In contrast, in the human system, several studies have shown that IFN-γ has no growth-suppressing effect on the intracellular growth of mycobacteria such as *Mycobacterium avium* complex (MAC) (11, 12) and *M. tuberculosis* (19) in vitro. Until now, it has been reported that human Mϕ cannot produce reactive nitrogen oxides in vitro (34, 37). Therefore, one possibility for the conflicting results concerning growth inhibition of mycobacteria in vitro might be the capacity of Mϕ from different sources to produce reactive nitrogen oxides or not. However, even in the murine system, it is also reported that paradoxical enhancement of intracellular growth of *M. lepraemurium* (33) in IFN-γ-stimulated mouse peritoneal Mϕ is observed. Furthermore, Flesch and Kaufmann (22) also showed by using bone marrow-derived Mϕ from mice that the intracellular growth of both *M. bovis* BCG and *M. tuberculosis* H37Rv was inhibited by IFN-γ-stimulated Mϕ, whereas that of *M. tuberculosis* Middleburg was not. Thus, there seems to be a difference in susceptibility to NO or other reactive nitrogen oxides produced by immunologically activated Mϕ among the different strains of mycobacteria. That is, NO or other reactive nitrogen oxides may exhibit antimycobacterial actions against some strains of mycobacteria, but not against others.

Drapier and Hibbs (20) showed that reactive nitrogen oxides derived from L-arginine in activated Mϕ inhibited mitochondrial respiration in the Mϕ. Albina et al. (5, 6) also demonstrated the down-regulation of phagocytosis and superoxide anion (O_2^-) generation by Mϕ-derived reactive nitrogen oxides for rat peritoneal Mϕ. Thus, it is expected that inhibition of Mϕ functions by NO or other reactive nitrogen oxides might occur after infection with NO-inducible microbial pathogens.

MAC is a facultative intracellular pathogen that is able to survive and multiply within cultured Mϕ (12), but the detailed mechanisms of its growth or death within Mϕ remain to be clarified. The present studies were done with IFN-γ-stimulated rat alveolar Mϕ to examine the effect of NO on the intracellular fate of the three strains composing the MAC (i.e., one strain of *M. avium* and two strains of *M. intracellulare*) and its effect on phagocytosis and O_2^- generation of MAC-infected or noninfected Mϕ. Our results showed a significant difference in susceptibility among the

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three strains of MAC toward NO generated by immunologically activated Mϕ or that generated chemically by acidification of NO_2^-. In addition, it was found that some functions of MAC-infected alveolar Mϕ such as phagocytosis and O_2^- generation were suppressed by an enhanced generation of NO by the Mϕ. Consequently, NO generated by Mϕ may facilitate extracellular spreading of NO-resistant mycobacteria.

MATERIALS AND METHODS

Animals. Male Wistar rats, weighing 250 to 300 g, were used to obtain alveolar Mϕ. Male Fischer rats, weighing 200 to 250 g, were used for immunization against MAC.

Bacteria and culture. Three MAC strains, i.e., two strains of *M. intracellulare* and one strain of *M. avium*, were used in this study. One of the two *M. intracellulare* strains is a murine pathogenic strain of 31F093T (31), which was kindly provided by F. Kuze, Kyoto University, Kyoto, Japan, and the other strain, KUMS 9007, is a clinically isolated strain from a patient with bronchiectasis in our university. An *M. avium* Mino strain was kindly provided by R. Nakamura, National Institute of Health, Tokyo, Japan. Each strain of MAC was cultured for 7 to 10 days at 37°C in Dubos broth (Eiken Chemical Co., Tokyo, Japan) supplemented with 0.5% bovine serum albumin and stored frozen at −70°C until use. In each experiment, a frozen stock medium was thawed, and each strain of MAC was collected by centrifugation (20,000 × *g* for 15 min) and washed three times in 0.01 M phosphate-buffered 0.15 M saline (PBS) (pH 7.3) and then suspended in Krebs-Ringer phosphate buffer (KRP) (pH 7.4). The suspension of each strain of MAC was vigorously agitated with a Pasteur pipette and a vortex for 1 min, and the number of MAC cells in the suspension was determined by measuring the optical density at 570 nm after dilution with KRP and by correlating it with CFU.

Immunization. Immunization of rats with MAC was performed by using *M. intracellulare* 31F093T with 10^8 cells of autoclaved MAC suspended in 0.2 to 0.3 ml of PBS. The rats were immunized by intravenous injection every 7 to 10 days for a total of 10 times. Seven days after the last injection, blood samples were collected by cardiac puncture, and serum was obtained by centrifugation after the blood clotted at room temperature.

Preparation of anti-MAC IgG antibody and opsonized MAC. Anti-MAC immunoglobulin G (IgG) antibody was purified from the antiserum prepared as just described. IgG was isolated by precipitation with ammonium sulfate and column chromatography as described previously (8). The titration of anti-MAC antibody was determined by the indirect fluorescent antibody method (43). Fluorescein-labeled anti-rat IgG was obtained from Sigma Chemical Co., St. Louis, Mo. To opsonize MAC, it was incubated with anti-MAC IgG antibody in KRP at 37°C for 1 h.

Harvest and culture of alveolar Mϕ. Rats were anesthetized with pentobarbital sodium and were exsanguinated by severing the abdominal aorta with a sharp blade. Alveolar Mϕ were obtained by repeated lavage of the airways with a total of 100 ml (10 ml ten times) of warmed PBS containing 2.0 mM EDTA as reported previously (3). The fluid samples from the serial lavage were pooled on ice and centrifuged at 400 × *g* for 10 min at 4°C. The resulting pellets were washed three times with KRP. In all experiments, more than 95% of the cells in these suspensions were Mϕ as judged by Giemsa staining and by nonspecific esterase staining, and the proportion of polymorphonuclear leukocytes was less than 1%. More than 97% of the alveolar Mϕ harvested were viable (by the trypan blue dye exclusion method). Adherent alveolar Mϕ monolayers were obtained by seeding the cells on 24-well tissue culture plates (Falcon 3047; Becton Dickinson Labware, Lincoln Park, N.J.) at 2.5 × 10^5 cells per well and by incubating the plates at 37°C for 2 h in 5% CO_2–95% air. Nonadherent cells were removed by repeated washing with warmed KRP, and fresh medium was added to each well with or without various reagents. The number of adherent Mϕ was counted by the method of Nakagawara and Nathan (35).

Medium for cell culture. L-Arginine-depleted RPMI 1640 medium (Select Amine Kit; GIBCO Laboratories, Grand Island, N.Y.) without antibiotics was prepared with 10% fetal calf serum; fetal calf serum was heated at 56°C for 30 min and dialyzed against PBS for 2 days. Various concentrations of L-arginine were added before use. Two buffer systems were used depending on the pH of the medium desired to study the direct antimycobacterial effect of NO: 25 mM succinic acid buffer for an acidic medium (pH 5.0), and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer for a neutral medium (pH 7.0).

Cytokine and reagents. Recombinant murine IFN-γ was purchased from Genzyme Co., Cambridge, Mass. The original solution of IFN-γ was diluted to 10^5 U/ml in L-arginine-depleted RPMI 1640 medium and stored at −70°C until use. Before each experiment, the cytokine was diluted again in the medium to the desired concentration. Cu,Zn-superoxide dismutase (from bovine erythrocytes), ferricytochrome *c* (type III, from horse heart), phorbol myristate acetate (PMA), and latex beads (diameter, 1.06 μm) were purchased from Sigma Chemical Co. Naphthylethylenediamine dihydrochloride, sulfanilamide, saponin, and phosphoric acid were from Nacalai Tesque, Inc., Kyoto, Japan. L-NMMA was from Calbiochem Co., La Jolla, Calif.

Infection of alveolar Mϕ with MAC. After preincubation of alveolar Mϕ without cytokine for 2 days, MAC suspensions were added to monolayers of the Mϕ, and incubation proceeded for 4 h at 37°C. The ratio of bacteria to Mϕ ranged from 1:1 to 50:1; a ratio of 10:1 was usually used throughout the experiments unless otherwise indicated. After incubation, the wells were washed four times with warmed KRP to remove extracellular uningested bacteria. There were no appreciable extracellular bacteria left after four washings as judged by a colony-forming assay of the supernatant and by electron microscopy (data not shown). The intracellular number of *M. avium* Mino, *M. intracellulare* 31F093T, or *M. intracellulare* KUMS 9007 ingested by the Mϕ during the 4-h incubation was 5.2 ± 0.9, 5.9 ± 0.7, or 4.5 ± 0.6, respectively. Then 1.0 ml of the fresh medium (RPMI 1640) with 10% dialyzed fetal calf serum containing L-arginine or IFN-γ at the desired concentration was added to each well, and incubation was continued. Phagocytosis of sterile latex beads by the Mϕ was performed similarly to phagocytosis of MAC; the ratio of latex beads to Mϕ was 10:1, and ingested latex beads could be detected and quantitated under a microscope.

Measurement of phagocytosis of MAC by alveolar Mϕ. The suspension of MAC opsonized with anti-MAC IgG antibody was added to the monolayer of alveolar Mϕ; the ratio of opsonized MAC to Mϕ was 20:1, and the Mϕ were incubated at 37°C for 2 h. Then the wells were washed vigorously four times with KRP at 37°C to remove unphagocytized bacteria. By using electron microscopy, we confirmed that all Mϕ-associated MAC cells remaining after four washings were actually inside the macrophages. Phagocytosis of MAC by

Mϕ was determined by the colony-forming assay or by the visual inspection of Mϕ.

In the colony-forming assay, the cells were lysed with saponin (0.1% [wt/vol], final concentration) and then vigorously scraped with a rubber policeman and flushed with Pasteur pipettes. The suspension was then agitated with a vortex for 1 min to lyse Mϕ completely. The suspension was diluted serially, and 0.1 ml of the final suspension was seeded on plastic dishes (diameter, 88 mm; Eiken Chemical Co.) for counting viable bacteria after colony formation in Mycobacteria 7H11 agar (Difco Laboratories, Detroit, Mich.). The dishes were incubated at 37°C for 2 weeks, and colonies of MAC in the agar were counted. No effect of saponin on the viability of MAC was observed.

In the microscopical visual inspection, the percentage of MAC-associated Mϕ and the number of phagocytized MAC cells per macrophage were determined after incubation for 2 h by counting a total of 400 adherent cells in multiple fields of triplicate cultures under oil immersion (magnification, ×1000) after Ziehl-Neelsen staining in a Lab-Tek chamber slide (Nunc Inc., Naperville, Ill.).

Assay for intracellular growth of MAC in alveolar Mϕ. Immediately or 3 days after infection with each MAC strain, alveolar Mϕ were lysed with saponin as described above, and the number of viable MAC cells in the Mϕ was quantitated by the colony-forming assay and was expressed as CFU.

Antimycobacterial effect of NO generated by acidification of NO_2^-. Approximately 2×10^5 cells of each MAC strain were incubated at 37°C in 1 ml of RPMI 1640 medium containing different concentrations of sodium nitrite (0.01 to 20 mM) at different pHs (5.0 and 7.0). The amount of NO generated by acidification of NO_2^- in this system was quantitated by using electron spin resonance spectroscopy with imidazolineoxyl *N*-oxides (4). At pH 5.0, 191.04 and 465.60 nmol of NO per ml per 24 h were generated from 5.0 and 10.0 mM of NO_2^-, respectively, whereas at these concentrations of NO_2^-, no appreciable NO generation could be observed at pH 7.0. The amount of NO generated by this NO-generating system is sufficiently larger than that generated by the Mϕ used in this study or other investigators. That is, the maximal amount of NO_2^- released from IFN-γ-stimulated murine Mϕ infected with mycobacteria was 27.92 nmol per 2.5×10^5 cells per 72 h in this study and 10.3 nmol per 1.0×10^5 cells per 96 h in the study of Flesch and Kaufmann (23). Furthermore, the IFN-γ-stimulated mouse peritoneal Mϕ used by Chan et al. (14), which is reported to kill *M. tuberculosis* Erdman within the Mϕ in vitro, release very large amount of NO_2^- (maximally 202.4 nmol per 2×10^5 cells per 48 h). Thus, the Mϕ used in this study or by previous investigators generated less NO than the NO-generating system used in this study. Therefore, it is sufficient and reasonable to choose pH 5.0 to determine the difference in susceptibility to NO among MAC strains used. Sodium nitrite was not added to the control. After 24 h of incubation, the number of viable MAC cells was quantitated by the colony-forming assay and was expressed as CFU.

Measurement of O_2^- generation. Generation of O_2^- was assayed spectrophotometrically by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (2). Briefly, monolayers of alveolar Mϕ were washed twice with KRP and were incubated with assay mixture (1 ml) containing 80 μM ferricytochrome *c* and PMA (200 ng/ml) in KRP in the presence or absence of superoxide dismutase (100 μg/ml) at 37°C for 60 min. The reduction of cytochrome *c* was measured as an increase in the optical density at 550 to 540 nm, and the optical density reading was converted to nanomoles of cytochrome *c* reduced by using an ϵ_{mol} value of 19.1 $mM^{-1}\ cm^{-1}$.

Measurement of NO_2^-. NO_2^- released from rat alveolar Mϕ was quantitated as described by Green et al. (25). The supernatant (500 μl) of the culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2% H_3PO_4) and was incubated at room temperature for 10 min. Supernatants from mycobacteria-infected cultures were filter sterilized before measurement. The optical density at 550 nm was measured, and the amount of NO_2^- was determined by using $NaNO_2$ as the standard.

Measurement of lactate dehydrogenase activity. Lactate dehydrogenase activity in the supernatant was determined spectrophotometrically by using a lactate dehydrogenase assay kit (Wako Pure Chemical Industries, Osaka, Japan) with lactate as the substrate (9).

Statistical analysis. All values are expressed as the mean ± standard error of the mean (SEM) of duplicate or triplicate determinations from three or four separate experiments. Statistical analyses were performed by the two-tailed *t* test for unpaired data.

RESULTS

Different susceptibility among three MAC strains toward NO generated by IFN-γ-stimulated Mϕ or by acidification of NO_2^-. The potential of NO generated by IFN-γ-stimulated alveolar Mϕ to kill MAC intracellularly was examined. Immediately after infection of the Mϕ with MAC, IFN-γ was added at a final concentration of 100 U/ml. Intracellular growth of MAC within the Mϕ during further incubation with IFN-γ for 3 days in the presence or absence of 1 mM of L-arginine or 0.5 mM of L-NMMA was measured. Although NO_2^- release from the IFN-γ-stimulated Mϕ was not completely inhibited, the higher concentration of L-NMMA could not be used because direct cytotoxic effects of L-NMMA on both the Mϕ and the mycobacteria were observed (data not shown). The amount of released NO was assessed by measuring the amount of NO_2^- in the culture supernatant. The amount of NO_2^-, which was converted from NO after generation by Mϕ, accumulated in the culture supernatant during the 3-day incubation was measured as described in Materials and Methods.

Viability of the Mϕ was not affected by the presence of L-arginine or by treatment with IFN-γ under our experimental condition, as judged by the trypan blue dye exclusion method after the 3-day incubation. Furthermore, very few mycobacteria were found outside the Mϕ on day 3. In Mϕ-depleted medium, none of MAC strains used grew (data not shown). Therefore, the growth of each MAC strain during the 3-day incubation can mostly be attributed to intracellular growth.

As shown in Table 1, intracellular killing of MAC by the Mϕ was not observed under any conditions in this study. The growth of *M. avium* Mino was significantly ($P < 0.01$) inhibited by IFN-γ-stimulated Mϕ in L-arginine-containing medium, in which a large amount of released NO_2^- was detected, but not in L-arginine-depleted or L-NMMA-containing medium (Table 1). However, we did not find any significant inhibitory effects of NO on intracellular growth of two *M. intracellulare* strains (31F093T and KUMS 9007) (Table 1).

NO is spontaneously generated by the disproportionation of NO_2^- in acidic aqueous solutions (7). Therefore, the

TABLE 1. Difference in susceptibility among three different MAC strains toward NO produced by IFN-γ-stimulated Mϕ[a]

MAC strain	IFN-γ (100 U/ml)	L-Arginine (1 mM)	L-NMMA (0.5 mM)	10^5 CFU[b]		NO_2^- (nmol/well/3 days)[b]
				Day 0	Day 3	
Mino	−	−	−	2.48 ± 0.12	6.51 ± 0.56	1.99 ± 0.58
	−	+	−		4.85 ± 1.08	13.51 ± 0.47[c]
	+	−	−		7.00 ± 0.71	2.26 ± 0.81
	+	+	−		3.75 ± 0.38[c]	25.71 ± 1.46[c]
	+	+	+		5.47 ± 0.10	8.23 ± 0.38[c]
31F093T	−	−	−	2.73 ± 0.17	5.27 ± 0.59	2.03 ± 0.61
	−	+	−		6.31 ± 0.63	14.99 ± 0.38[c]
	+	−	−		5.51 ± 0.83	1.86 ± 0.36
	+	+	−		5.99 ± 0.28	25.07 ± 1.81[c]
	+	+	+		6.19 ± 0.67	6.92 ± 0.49[c]
KUMS 9007	−	−	−	2.01 ± 0.18	9.65 ± 1.40	1.93 ± 0.35
	−	+	−		11.17 ± 2.45	8.90 ± 0.44[c]
	+	−	−		11.81 ± 1.93	1.49 ± 0.46
	+	+	−		10.10 ± 1.01	24.89 ± 1.33[c]
	+	+	+		10.95 ± 0.49	8.56 ± 0.89[c]

[a] Intracellular growth of each MAC strain (*M. avium* Mino, *M. intracellulare* 31F093T, or *M. intracellulare* KUMS 9007) during the 3-day incubation was measured and is indicated as CFU.
[b] Results are mean ± SEM of duplicate determinations from four separate experiments.
[c] $P < 0.01$ compared with control (no IFN-γ and L-arginine) cultures.

direct antimycobacterial effect of NO on each MAC strain could also be assessed by incubating mycobacteria with increasing concentrations of $NaNO_2$ in RPMI 1640 medium at pH 5.0 and 7.0. As indicated in Fig. 1, nitrite itself did not exhibit any antimycobacterial activity against all MAC strains tested at pH 7.0. At pH 5.0, the viability of all the MAC strains decreased as the concentration of nitrite increased. However, a more significant decrease in viability was observed with *M. avium* Mino than with the two *M. intracellulare* strains. The viability of *M. avium* Mino was only about 4% of that of control (no nitrite added) by treatment with 10 mM of nitrite at pH 5, while that of *M. intracellulare* 31F093T and KUMS 9007 was 32 and 25%, respectively, after the 24-h treatment. These results suggest that *M. avium* Mino is more susceptible to NO than *M. intracellulare* 31F093T or KUMS 9007.

NO_2^- release from MAC-infected or noninfected alveolar Mϕ stimulated with IFN-γ. Alveolar Mϕ were incubated for 2 days before the addition of IFN-γ, and then both phagocytosis and O_2^- generation stimulated by PMA were tested on day 3 after addition of IFN-γ throughout these experiments.

The effect of infection of alveolar Mϕ with MAC on NO_2^- release, which is known to be an oxidized metabolite of NO, was examined (Fig. 2) by using *M. intracellulare* 31F093T, a strain resistant to NO produced by the Mϕ (Table 1). Alveolar Mϕ infected with MAC or ingesting latex beads were incubated for 3 days in the presence or absence of IFN-γ at the desired concentration, and the amount of NO_2^- accumulated in the culture supernatant was measured on day 3 after the addition of IFN-γ. In the L-arginine-containing (1 mM) medium, NO_2^- release from the Mϕ increased as the concentration of IFN-γ increased. This enhancing potential of IFN-γ for NO_2^- release became more significant when Mϕ were infected with MAC. For instance, MAC-infected Mϕ stimulated with 1 U of IFN-γ per ml released a larger amount of NO_2^- than MAC-noninfected Mϕ stimulated with 100 U of IFN-γ per ml (Fig. 2). Furthermore, the amount of NO_2^- released from IFN-γ-stimulated Mϕ infected with either *M. avium* Mino or *M. intracellulare* KUMS 9007 was almost equal to that from Mϕ infected with *M. intracellulare* 31F093T (Table 1). NO_2^- release from Mϕ infected with autoclaved MAC was the same as that obtained with live MAC (data not shown). Ingestion of latex beads had no effect on NO_2^- release from the Mϕ in culture (data not shown). Thus, NO_2^- release augmented by MAC infection seems to have resulted from immunological activation of alveolar Mϕ by MAC itself.

Effect of NO on phagocytosis of MAC by MAC-infected or noninfected alveolar Mϕ stimulated with IFN-γ. The effect of NO on the phagocytic function of alveolar Mϕ against MAC opsonized with anti-MAC IgG antibody was determined. Alveolar Mϕ which were infected with NO-resistant MAC strain *M. intracellulare* 31F093T or noninfected, were incubated with IFN-γ in the presence or absence of L-arginine. The amount of NO_2^- released from the MAC-infected or noninfected Mϕ incubated in L-arginine-containing medium was similar to that indicated in Fig. 2 in this experimental setting (data not shown). Three days after addition of IFN-γ, i.e., 3 days after the first infection, the MAC-infected or noninfected Mϕ were superinfected with MAC. The number of MAC cells within the Mϕ was measured by the colony-forming assay and expressed as colony formation by total viable phagocytized MAC cells per 2.5×10^5 Mϕ. The number of MAC cells phagocytized during (2 h) the second infection was calculated by subtracting the number of MAC cells within the Mϕ not given the second infection from the number of MAC cells within the Mϕ treated with the second infection. As shown in Fig. 3A, the phagocytic activity of noninfected alveolar Mϕ was significantly suppressed in the presence of L-arginine at 10 U of IFN-γ per ml or more compared with activity in the absence of L-arginine. Furthermore, the phagocytic activity of MAC-infected alveolar Mϕ was also significantly suppressed in the presence of L-arginine by incubation with just 1 U of IFN-γ per ml (Fig. 3B). At that concentration of IFN-γ, no suppressive effect of L-arginine on phagocytosis by noninfected Mϕ was observed (Fig. 3A).

The effect of NO on the phagocytic function of noninfected alveolar Mϕ stimulated with IFN-γ was also deter-

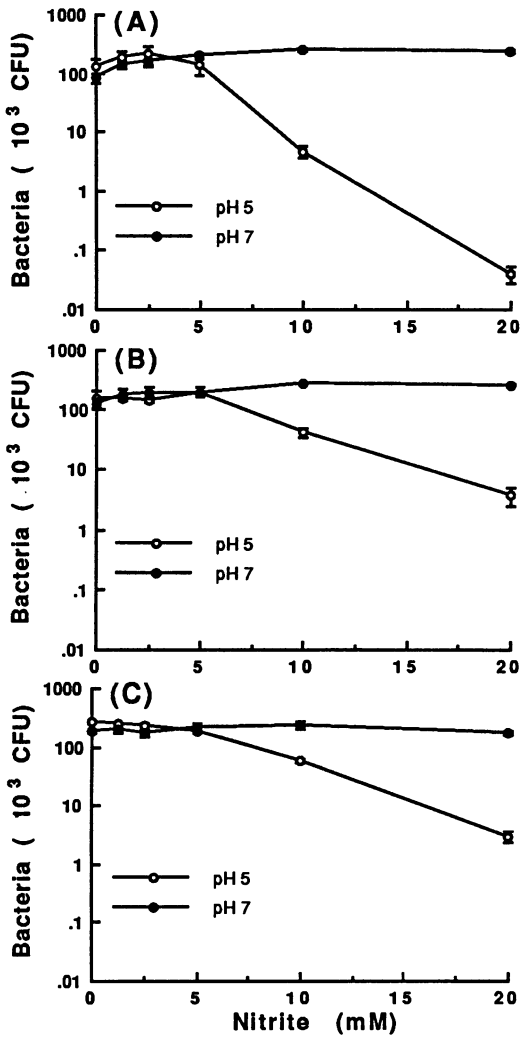

FIG. 1. Difference in susceptibility among three MAC strains toward NO generated by acidification of NO_2^-. Approximately 2 × 10^5 cells of *M. avium* Mino (A), *M. intracellulare* 31F093T (B), or *M. intracellulare* KUMS 9007 (C) were incubated at 37°C for 24 h in 1 ml of RPMI 1640 medium containing different concentrations of sodium nitrite at different pHs (5.0 or 7.0). The number of viable MAC cells after the incubation period was quantitated by a colony-forming assay and expressed as CFU (see text for details).

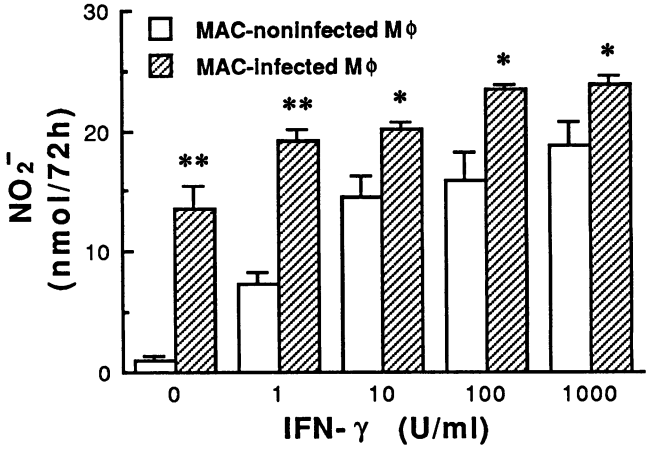

FIG. 2. Induction of NO_2^- release from MAC-infected or noninfected alveolar Mϕ stimulated with IFN-γ. Immediately after infection of Mϕ with *M. intracellulare* 31F093T, various concentrations (units per milliliter) of IFN-γ were added. The amount of NO_2^-, which was derived from NO, accumulated in the supernatant was measured 72 h after the addition of IFN-γ. L-Arginine was added at a final concentration of 1.0 mM. Results are mean ± SEM (bars) of triplicate determinations from three separate experiments. Symbols: * and **, significant difference from the value for MAC-noninfected Mϕ at $P < 0.01$ and $P < 0.001$, respectively.

mined by visual inspection analysis. The test was performed on day 3 after the addition of IFN-γ as described above. There was a significant decrease in the percentage of Mϕ phagocytizing MAC at 10 U of IFN-γ per ml or more in the presence of 1 mM of L-arginine compared with that in the absence of L-arginine (Fig. 4A). Furthermore, at 10 U of IFN-γ per ml or more, there was a significant decrease in the number of phagocytized MAC cells per macrophage in the presence of 1 mM L-arginine compared with that in the absence of L-arginine (Fig. 4B).

The suppressive effect of NO on phagocytosis was confirmed by examination with the L-arginine analog L-NMMA, which indeed inhibited NO generation by Mϕ (Table 2). Suppression of phagocytosis, which was determined by visual inspection analysis, was nullified by the addition of 0.5 mM of L-NMMA to the medium. Although NO_2^- release from the Mϕ was not completely inhibited at 100 U of IFN-γ per ml or more, the higher concentration of L-NMMA could not be used because of the direct cytotoxic effects of L-NMMA on the Mϕ as described above.

These results indicate that phagocytosis of MAC is suppressed by NO generated by IFN-γ-stimulated Mϕ and that the suppression is augmented after infection with MAC, which potentiates NO generation.

Effect of NO on PMA-stimulated O_2^- generation by noninfected alveolar Mϕ cultured with IFN-γ. After incubation of the Mϕ with the desired concentration of IFN-γ for 3 days, released NO_2^-, an oxidized form of NO, and PMA-stimulated O_2^- generation by the Mϕ were measured. The amount of NO_2^- accumulated in the culture supernatant during the 3-day incubation was measured. PMA-stimulated O_2^- generation was measured in the absence of L-arginine following sufficient washing of each well with KRP to eliminate the effect of NO derived from residual L-arginine on O_2^-, which is known to be captured by NO (10, 39).

A dose-dependent increase in NO_2^- release was induced by IFN-γ in L-arginine-containing (1.0 mM) medium. In L-arginine-depleted medium, however, no dose-dependent increase in NO_2^- release was observed with IFN-γ (Fig. 5A).

In the L-arginine-depleted medium, an increase in O_2^- generation with IFN-γ was observed maximally at 10 U of IFN-γ per ml (Fig. 5B). On the contrary, the presence of L-arginine significantly suppressed O_2^- generation by alveolar Mϕ stimulated with IFN-γ (Fig. 5B).

As shown in Fig. 6, an L-arginine-dependent increase in NO_2^- release and decrease in O_2^- generation were observed with noninfected alveolar Mϕ incubated with 100 U of IFN-γ per ml. The dose-dependent changes in the amount of NO_2^- released with L-arginine are inversely proportional to those in PMA-stimulated O_2^- generation (Fig. 5 and 6). An L-arginine analog, L-NMMA, which strongly inhibited NO_2^- release from the Mϕ, enhanced PMA-stimulated O_2^- generation by the Mϕ (Table 3).

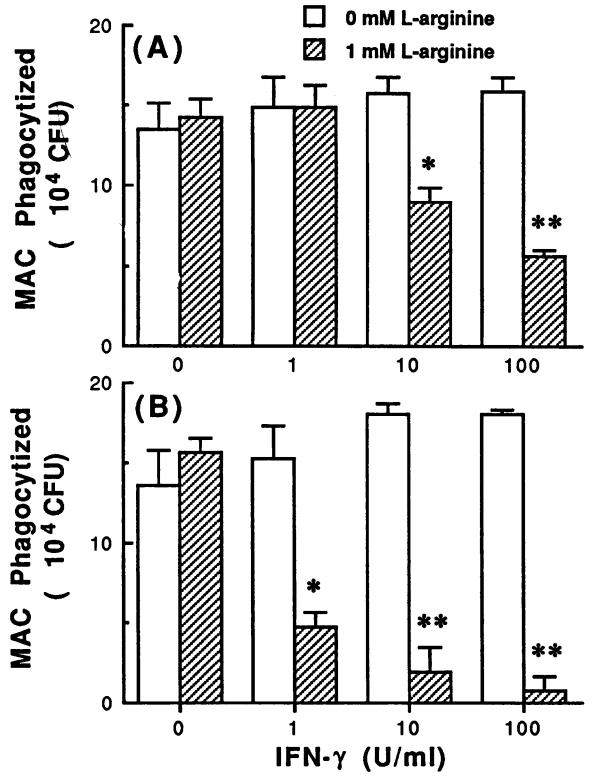

FIG. 3. Effect of NO on phagocytosis of MAC by noninfected (A) or MAC-infected (B) alveolar Mϕ stimulated with IFN-γ. Alveolar Mϕ, which were noninfected or infected with an NO-resistant MAC strain, *M. intracellulare* 31F093T, were incubated with various concentrations (units per milliliter) of IFN-γ in L-arginine-depleted or -containing (1 mM) medium. Three days after the addition of IFN-γ, i.e., 3 days after the first infection, the noninfected or MAC-infected Mϕ were superinfected with opsonized MAC. The number of MAC cells phagocytized by 2.5×10^5 Mϕ during (2 h) the second infection was expressed as CFU (see text for details). Results are mean ± SEM (bars) of triplicate determinations from three separate experiments. Symbols: * and **, significant difference from the value for L-arginine-depleted medium at $P < 0.01$ and $P < 0.001$, respectively.

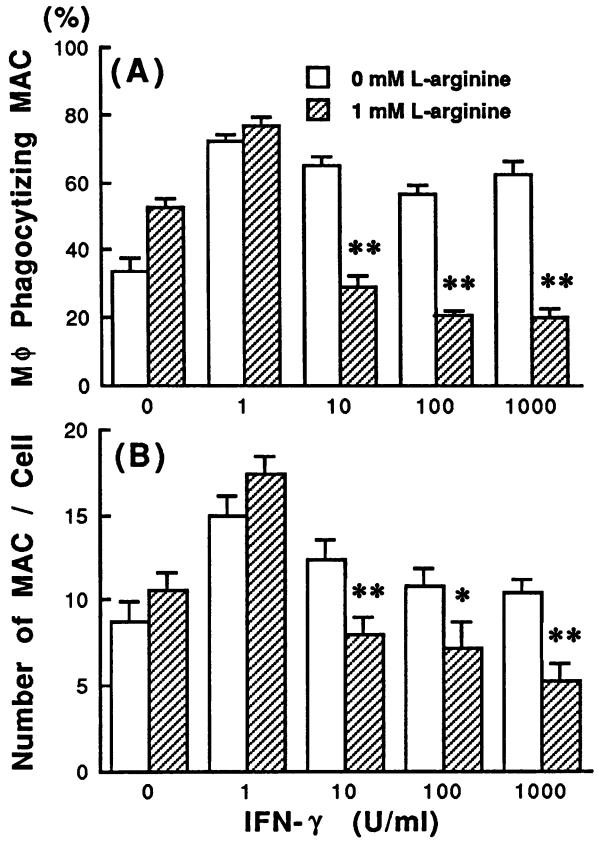

FIG. 4. Effect of NO on phagocytosis of MAC by noninfected Mϕ stimulated with IFN-γ. The percentage of alveolar Mϕ (400 adherent cells) phagocytizing MAC (A) and the number of phagocytized MAC cells per macrophage (B) were determined by microscopic observation of multiple fields of the culture slide. Phagocytosis was performed for 2 h as described in the text after 72 h of incubation with various concentrations (units per milliliter) of IFN-γ in the presence or absence of 1.0 mM of L-arginine. Results are mean ± SEM (bars) of triplicate determinations from three separate experiments. Symbols: * and **, significant difference from the value for L-arginine-depleted medium at $P < 0.05$ and $P < 0.01$, respectively.

Viability of alveolar Mϕ was not affected by treatment with IFN-γ, as judged by the release of lactate dehydrogenase and by the trypan blue dye exclusion method, despite the marked generation of NO_2^-, which was effectively suppressed by depletion of L-arginine (data not shown). These results indicate that suppression by L-arginine of the respiratory burst is not caused by the cytocidal effect of NO or other reactive nitrogen oxides. We could not find detectable amounts (>0.1 nmol) of NO_2^- released from the Mϕ in the culture supernatant during the 60-min incubation following stimulation with PMA even in the presence of a sufficient amount of superoxide dismutase (100 μg/ml). Suppression of PMA-stimulated O_2^- generation was not affected by treatment of the Mϕ with L-NMMA during the 60-min incubation following stimulation of the Mϕ with PMA. Thus, in this assay system, the amount of NO generated should be very small (<0.1 nmol/60 min) even if NO was produced from intracellular residual L-arginine by the stimulation of the Mϕ with PMA. Therefore, it is suggested that the decrease in PMA-stimulated O_2^- generation is really due to actual suppression of ability of the Mϕ to generate O_2^- and not due to O_2^- and NO interaction.

DISCUSSION

In the present experiments, we demonstrated a difference in susceptibility among three different MAC strains toward NO generated by IFN-γ-stimulated Mϕ (Table 1) or NO generated chemically by acidification of NO_2^- (Fig. 1). We also showed a suppressive effect of NO on phagocytosis and O_2^- generation in IFN-γ-stimulated rat alveolar Mϕ in vitro. The down-regulation of these functions (phagocytosis and O_2^- generation) by Mϕ-derived NO has already been demonstrated by Albina et al. (5, 6) for rat peritoneal Mϕ. In this study, we demonstrated further by using rat alveolar Mϕ that the suppressive effect of NO on the antimicrobial functions of Mϕ was significantly augmented by infection of the Mϕ with MAC (Fig. 3 and 4), perhaps due to enhancement of NO generation (Fig. 2). Therefore, it is reasonable to suggest that generation of NO by Mϕ is not always beneficial for hosts, particularly when they are infected with NO-resistant microbial pathogens.

In recent years, increasing attention has been focused on the role of NO as a mediator of a number of physiological

TABLE 2. Effect of L-NMMA on NO_2^- release and phagocytosis of MAC by noninfected alveolar Mϕ[a]

IFN-γ (U/ml)	− L-NMMA		+ L-NMMA[b]	
	Mϕ Phagocytizing MAC (%)[c]	NO_2^- (nmol/well)	Mϕ Phagocytizing MAC (%)[c]	NO_2^- (nmol/well)
0	48.6 ± 1.5	1.30 ± 0.40	49.9 ± 2.5	1.80 ± 0.10
1	62.5 ± 3.2	7.23 ± 1.21	63.9 ± 3.9	2.47 ± 0.11
10	40.6 ± 2.2	14.40 ± 1.42	61.6 ± 1.5	2.47 ± 0.04
100	34.1 ± 4.9	18.32 ± 1.63	65.9 ± 3.0	5.72 ± 0.05
1,000	19.1 ± 1.1	22.21 ± 1.92	67.8 ± 2.0	6.47 ± 0.47

[a] Effects of L-NMMA on NO_2^- release and phagocytosis of MAC by noninfected Mϕ stimulated with IFN-γ were measured after 72 h of incubation with different concentration of IFN-γ as shown in the table in the presence of 1.0 mM of L-arginine. Both released NO_2^- and phagocytosis were measured with the same culture of the Mϕ (see text for details).

[b] L-NMMA was added at a final concentration of 0.5 mM.

[c] Phagocytosis was performed for 2 h as described in the text. The percentage of alveolar Mϕ (400 adherent cells) phagocytizing MAC was determined by microscopic observation of multiple fields of the culture slide. Results are mean ± SEM of triplicate determinations from three separate experiments.

reactions such as relaxation of smooth muscle and signal transmission in neural systems (13, 30, 38, 40). Activated Mϕ appear to exhibit some antimicrobial actions as well as antitumor effects via generation of NO, which depends on the L-arginine oxidation pathway (1, 24, 26–28, 32, 40). Ding et al. (18) demonstrated that IFN-γ was the most potent inducer of reactive nitrogen oxides in mouse peritoneal Mϕ. In the present study with rat alveolar Mϕ, we also demonstrated the strong capacity of IFN-γ to induce NO_2^- (Fig. 2, 5, and 6). In addition, we showed that MAC-infected alveolar Mϕ released larger amounts of NO_2^- without incubation with cytokine than noninfected Mϕ did (Fig. 2). NO_2^- release was significantly augmented in the presence of IFN-γ (Fig. 2). The evidence of augmentation of NO_2^- release from alveolar Mϕ after infection with MAC agrees with previous reports on mouse peritoneal Mϕ infected with other intra-

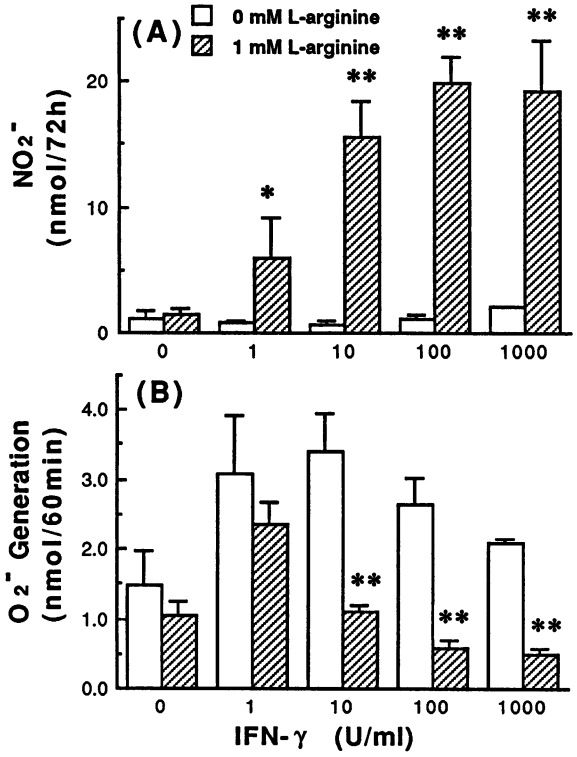

FIG. 5. NO_2^- release (A) and PMA-stimulated O_2^- generation (B) from noninfected alveolar Mϕ were measured after 72 h of incubation with different concentrations of IFN-γ as shown in the figure in the presence or absence of 1 mM of L-arginine. The supernatants were harvested and then subjected to measurement of released NO_2^-. O_2^- generated upon stimulation with PMA (200 ng/ml) was quantitated by the cytochrome *c* reduction assay. Both released NO_2^- and PMA-stimulated O_2^- generation were measured with the same culture of the Mϕ (see text for details). Results are mean ± SEM (bars) of triplicate determinations from two different experiments. Symbols: * and **, significant difference from the value for L-arginine-depleted medium at $P < 0.05$ and $P < 0.01$, respectively.

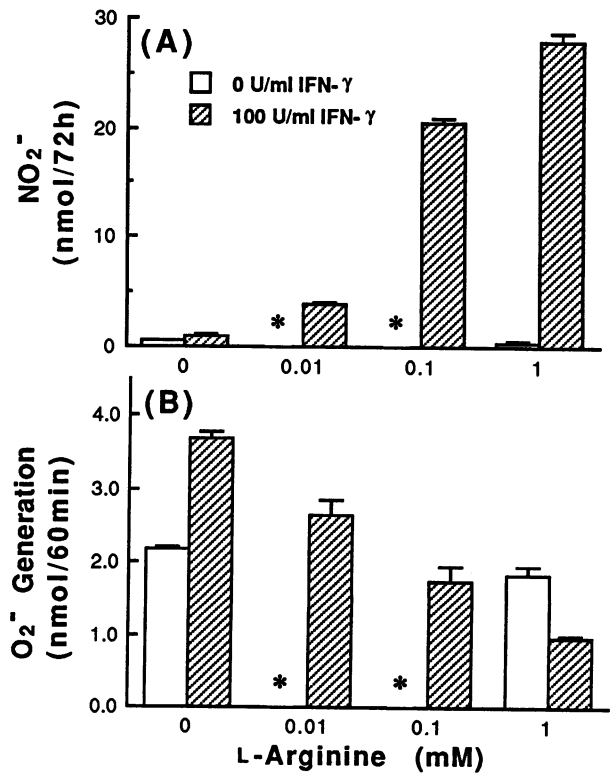

FIG. 6. Effect of L-arginine on NO_2^- release (A) and on PMA (200 ng/ml)-stimulated O_2^- generation (B) by noninfected alveolar Mϕ after 72 h of incubation with 100 U of IFN-γ per ml. Both NO_2^- release and O_2^- generation were measured by the same method as described in the legend to Fig. 5 except that the concentration of L-arginine varied from 0 to 1.0 mM. Results are mean ± SEM of triplicate determinations from three separate experiments. *, Not done.

TABLE 3. Effect of L-NMMA on NO_2^- release and PMA-stimulated O_2^- generation by noninfected alveolar Mϕ stimulated with IFN-γ[a]

L-NMMA (mM)	− IFN-γ		+ IFN-γ	
	O_2^- generation (nmol/60 min)	NO_2^- (nmol/well/72 h)	O_2^- generation (nmol/60 min)	NO_2^- (nmol/well/72 h)
0	0.98 ± 0.07	2.02 ± 0.10	1.87 ± 0.07	22.17 ± 0.07
0.01	1.05 ± 0.13	1.82 ± 0.02	2.16 ± 0.09	18.34 ± 1.02
0.10	1.31 ± 0.06	1.67 ± 0.12	4.38 ± 0.53	14.38 ± 1.44
0.50	1.31 ± 0.39	2.07 ± 0.63	4.03 ± 0.07	6.47 ± 0.47
1.00	0.88 ± 0.07	1.84 ± 0.10	3.50 ± 0.46	4.45 ± 0.30

[a] The amount of released NO_2^- acumulated in the supernatant and PMA (200 ng/ml)-stimulated O_2^- generation by noninfected alveolar Mϕ were measured after 72 h of incubation with 100 U of IFN-γ per ml in L-arginine (1.0 mM)-containing medium in the presence of various concentrations of L-NMMA. Both released NO_2^- and PMA-stimulated O_2^- generation were measured with the same culture of Mϕ (see text for details). Results are mean ± SEM of triplicate determinations from three separate experiments.

cellular pathogens such as BCG (41) or *Leishmania major* (26) and on human monocyte-derived Mϕ infected with *M. avium* (17). In this study, we demonstrated that the capacities of three MAC strains to induce NO release from the IFN-γ-stimulated Mϕ were similar (Table 1).

Recently, Denis (16) demonstrated that NO or other reactive nitrogen oxides are important effector molecules in restricting growth of *M. tuberculosis* in IFN-γ-pulsed murine Mϕ. Flesch and Kaufmann (23) also demonstrated the inhibitory effect of reactive nitrogen oxides released from mouse bone marrow-derived Mϕ stimulated by IFN-γ on the intracellular growth of *M. bovis* BCG. Furthermore, Chan et al. (14) have shown that reactive nitrogen oxides are used by Mϕ to kill *M. tuberculosis* Erdman in vitro.

If NO effectively inhibits the growth of or kills all the mycobacteria within Mϕ, similar to the cases of *Cryptococcus neoformans* (24) or *Leishmania* species (26, 32), intracellular growth of all the MAC strains used in this study should be at least inhibited by IFN-γ-stimulated Mϕ. On the contrary, although the intracellular growth of *M. avium* Mino was inhibited by NO generated by IFN-γ-stimulated Mϕ, we did not find any significant inhibitory effect of the reactive nitrogen oxide on intracellular growth of two *M. intracellulare* strains (31F093T and KUMS 9007) (Table 1).

Mor et al. (33) showed the paradoxical enhancement of intracellular growth of *M. lepraemurium* in mouse peritoneal Mϕ stimulated with IFN-γ. Furthermore, it was reported by Flesch and Kaufmann (22) using mouse bone marrow-derived Mϕ that the intracellular growth of both *M. bovis* BCG and *M. tuberculosis* H37Rv was inhibited by IFN-γ-stimulated Mϕ, whereas that of *M. tuberculosis* Middleburg was not. These reports disagree with the reports of an inhibitory effect of IFN-γ on intracellular growth of some mycobacterial species via NO generation by murine Mϕ (14, 16, 23). These conflicting results may be attributed to a difference in susceptibility to NO among different strains of mycobacteria as revealed in our experiment. It should be noted that the two *M. intracellulare* strains (31F093T and KUMS 9007) used in the present experiments are highly resistant to NO generated by Mϕ (Table 1) or NO generated chemically by acidification of NO_2^- (Fig. 1). *M. tuberculosis* Erdman used by Chan et al. (14) is killed by reactive nitrogen oxides generated by IFN-γ-activated murine Mϕ and is also very susceptible to NO generated by acidification of NO_2^-; this mycobacterium is completely killed at 5 mM of nitrite concentration at pH 4.5. In the present study, however, even with 10 mM of nitrite, none of MAC strains tested was completely killed at pH 5.0 (Fig. 1). The amount of NO generated by acidification of nitrite at 5 mM of nitrite at pH 4.5 was almost equal to that at 10 mM of nitrite at pH 5.0 when quantitated by electron spin resonance spectroscopy (unpublished observation). It has been reported that some species of mycobacteria have the capacity to reduce NO_2^- and/or NO_3^- (44). Reduction of NO_2^- may result in generation of NO as a metabolic intermediate in mycobacteria. Thus, one possibility for the difference in susceptibility to NO might be the different capacity of mycobacteria to reduce nitrogen oxides, although further study is necessary to identify NO generation from mycobacteria. To our knowledge, this is the first report to show clearly the difference in susceptibility among different strains of MAC toward NO in an Mϕ cell-free system, which is correlated very well with intracellular fate of the mycobacteria.

In addition, we demonstrated that IFN-γ at 10 U/ml or more suppressed both phagocytosis and O_2^- generation by the MAC-infected or noninfected Mϕ via the L-arginine-dependent generation of NO (Tables 2 and 3 and Fig. 3 to 6). Furthermore, even at the lower concentration of IFN-γ, inhibition of phagocytosis by L-arginine was observed with MAC-infected Mϕ (Fig. 3B), which induced a larger amount of NO_2^- than did noninfected Mϕ (Fig. 2). Therefore, it seems that NO derived from L-arginine suppresses the functions of Mϕ, especially after infection with MAC, through its inhibitory effects on phagocytosis and O_2^- generation by Mϕ.

Mϕ harboring NO-resistant mycobacteria such as 31F093T and KUMS 9007 appear to become impaired in their own functions by the constant generation of the large amount of NO induced by these NO-resistant intracellular organisms. This result is in clear contrast to the fungistatic, leishmanicidal, and tumoricidal actions of reactive nitrogen oxides (24, 27, 32, 42). It seems that *C. neoformans*, *L. major*, and tumor cells are very susceptible to reactive nitrogen oxides, as evidenced by intracellular and extracellular growth inhibition and killing by Mϕ in vitro, even though the function of Mϕ is impaired by NO or other reactive nitrogen oxides.

On the basis of these results, we suggest that enhanced NO generation induced by infection with NO-resistant mycobacteria under L-arginine-rich conditions results in facilitating extracellular spreading of the mycobacteria via impairment of some functions of the Mϕ by NO. Therefore, the pathogenic potential of MAC may be, at least in part, attributed to its resistance to NO.

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