Rapid Induction of mRNA for Nitric Oxide Synthase II in Rat Alveolar Macrophages by Intratracheal Administration of *Mycobacterium Tuberculosis* and *Mycobacterium Avium* (43876)

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Abstract. **_Mycobacterium avium_ complex (MAC) organisms are among the most common bacterial cause of disseminated infection in patients with acquired immune deficiency syndrome (AIDS). An increase in the incidence of virulent _Mycobacterium tuberculosis_ (MTB) is also occurring throughout the world. _In vitro_ data suggest that nitric oxide (NO) may be important in restricting the growth of MAC. However, the ability of MTB to stimulate NO production and the susceptibility of MTB to the bactericidal activity of NO produced by murine alveolar macrophages (AM) is controversial. This study tested the hypothesis that _in vivo_ administration of heat-killed MAC (strain 100 and 101) and human virulent MTB (strain F1) to rats stimulated NO production by rat AM, _ex vivo_. We show that heat-killed MTB instilled into rat lungs rapidly induced mRNA for NO synthase (iNOS) II in AM obtained by bronchoalveolar lavage (BAL). In contrast, expression of AM iNOS mRNA was only found in 40% of the rats given MAC. Moreover, the change in iNOS mRNA in the AM obtained from rats given MTB and MAC correlated with the production of the reactive nitrogen intermediates (RNI) NO_2^- and NO_3^- in BAL fluid, lung homogenate, and the spontaneous generation of RNI by isolated AM _ex vivo_ and occurred without measurable increases in BAL fluid tumor necrosis factor-α (TNF-α). L-N^G-monomethylarginine (50 mg/kg, ip) given 30 min before MAC or MTB attenuated the increase in RNI in lung homogenates and BAL fluid. This is the first demonstration that _in vivo_ exposure to MTB results in rapid upregulation of gene expression for iNOS which is associated with functional RNI production by rat AM. These results show that MTB human virulent strain 1 has the ability to rapidly upregulate iNOS mRNA in AM. If human AM generate NO from L-arginine by either iNOS or other NADPH oxidases then NO may play a role in the overall host-defense response of the lung to MAC and MTB.** [P.S.E.B.M. 1995, Vol 209]

Nitric oxide synthase (NOS) converts L-arginine into L-citrulline and nitric oxide (NO). NOS exists as several isozymes. These include Ca^{2+}-calmodulin activated, constitutive bNOS and cNOS in neurons and endothelial cells, and inducible Ca^{2+}-independent NOS II (iNOS) in neutrophils, macrophages, and other cells (1–4). NO is believed to play a role in pulmonary host-defense mechanisms in response to infectious stimuli and in phagocyte-

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mediated killing of intra- and extracellular pathogenetic microorganisms in rodents and higher order mammals (3–6). At this time, it is unclear what role, if any, NO plays in human pulmonary host defense mechanisms. *Mycobacterium avium* complex (MAC) organisms are the most common cause of disseminated infection in patients with acquired immune deficiency syndrome (AIDS). An increase in the incidence of virulent *Mycobacterium tuberculosis* (MTB) is also occurring throughout the world (6–9). *In vitro* data suggest that NO may be important in restricting the growth of MAC. However, it is uncertain if the human virulent strain F1 *Mycobacterium tuberculosis* (MTB) either induces NO in alveolar macrophages or is susceptible to the bacteriostatic or bactericidal activity of NO (10–16).

Bermudez (11) evaluated the role of NO generated by murine peritoneal and human monocyte-derived macrophages infected with *Mycobacterium avium* intracellulare in the killing of mycobacteria *in vitro*. Neither competitive inhibition of iNOS II with 1-N^G-nitromethylarginine (LNMA) nor depletion of arginine by arginase had any effect on the inhibition of growth and/or intracellular killing of mycobacteria by activated human and murine macrophages. It was concluded that *M. avium* did not induce NO production because of their resistance to the cytotoxic effects of NO (11). Yet, activated macrophages obtained from mice infected with *Mycobacterium bovis* (strain bacillus Calmette-Guerin, *M. bovis* BCG) and that were cultured in medium with *Escherichia coli* endotoxin (LPS) and L-arginine produced the reactive nitrate intermediates, nitrate and nitrate anions (RNI) (16) and iron-nitrosyl complexes formed from the destruction of iron-sulfur centers by NO (16). Since *M. bovis* BCG (12) and MAC (13) appear resistant to NO-mediated arrest of cellular growth and/or killing, it was proposed that the resistance of mycobacteria to the bacteriostatic and bactericidal activity of the generated NO contributes to the virulence and dissemination of these organisms throughout the host (12, 13). It has also been suggested that the function of mycobacteria generated NO is to suppress the growth of other intracellular opportunistic microorganisms, which would compete with the tubercle bacilli for nutrients and cellular survival (12), or to suppress phagocytosis of the tubercle bacilli by the alveolar macrophages within the lung, thereby allowing the extracellular dissemination of the tubercle bacilli (13).

The concept that MTB is resistant to the bactericidal effects of NO was challenged by Denis (14), who demonstrated *in vitro* that γ-interferon-treated murine peritoneal macrophages inhibited the growth of the human MTB bacilli via the generation of RNI. Moreover, Moreno *et al.* (15) demonstrated that lipoarabinomannan, an immunostimulatory component of the MTB cell wall analogous to endotoxin lipopolysaccharide (LPS) obtained from *E. coli,* also induced the production of tumor necrosis factor-α (TNF-α). TNF-α has been implicated in the induction of iNOS II activity and NO *in vitro* and *in vivo* (17–20). This suggests that MTB may upregulate the NO system *in vivo* by way of the intermediary cytokine TNF-α. However, *in vitro* events do not always predict *in vivo* actions. We recently demonstrated that intratracheal administration of endotoxin to rats and mice upregulated iNOS gene expression and RNI production independent of the presence of endogenous TNF-α (21). Thus, the ability of MTB to upregulate cytokine and NO production *in vitro* does not insure the capability of the bacilli to do so *in vivo*. This study evaluated the effect of *in vivo* intratracheal injections of heat-killed human virulent F1 MTB and MAC (strains 101 and 102) on rat alveolar macrophage production of TNF-α, RNI, and mRNA for iNOS II.

Methods and Materials

Animal Experimentation. All experiments were performed with approved LSU and Delta Regional Primate Center Institutional Animal Care and Use Committee Protocols. Male, Sprague-Dawley rats (190–250 g; Hilltop Farms, Scottsdale, PA) were anesthetized with ether and the trachea isolated using aseptic techniques. Rats were pretreated with L-N^G-monomethylarginine (LNMMA; 50 mg/kg, ip) or sterile phosphate buffered saline (PBS; 1 ml/kg, ip) 30 min before experimentation. At the time of experimentation, the rats were given PBS (1 ml/kg), heat-killed MAC strains 100 and 101 (2 mg protein/ml of PBS/kg) or heat-killed human virulent strain F1 MTB (0.5 or 2 mg protein/ml/kg) (Department of Microbiology, Tulane University Medical Center), into the lung via an intratracheal injection under ether anesthesia. Heat-killed mycobacteria were used since access to a P-3 facility, an absolute requirement for the study of live MTB, was unavailable to the investigators at the time of the study. The neck wound was closed, and the rats were allowed to recover for the next 2.5 hr, at which time they were again anesthetized with ether. Blood was obtained by cardiac puncture and assayed for TNF-α with the L929 bioassay and RNI with ozone chemiluminescence as described previously in detail (21–24). The heart and lung were removed *en bloc* and the lungs of the challenged rats were lavaged three times with a total of 30 ml of sterile HEPES-EDTA buffer. An initial aliquot of lavage fluid was assayed for TNF-α activity and for RNI by ozone chemiluminescence (21–24). The pool of lavage fluid was assayed for total alveolar macrophages and recruited neutrophil content and their relative percentage of distribution (25) and for isolation of alveolar macrophages and measurement of mRNA for iNOS II by a competitive, equalized reverse transcription poly-

merase chain reaction (cERT-PCR) (21–26). The lung was then removed from the BAL apparatus, weighed, and homogenized at 4°C in 3.5 times the volume of PBS using four cycles of a polytron homogenizer (Buchler Instruments, New Brunswick, NJ) with a setting of 7 for 15 sec on and 10 sec off. The homogenate was centrifuged at 1000*g* for 15 min, and an aliquot of the supernatant was assayed for RNI, corrected for the volume of supernatant and lung weight. The MTB contained 56 ng of *E. coli* endotoxin activity per 10 mg of protein as determined by the Limulus assay. The *E. coli* endotoxin activity of the MAC was 524 ng/10 mg of stock suspension, which yielded less than 25 ng of LPS-equivalents/rat.

Alveolar Macrophage Preparation. The combined lavage fluids were centrifuged (200*g* for 10 min) and the cells resuspended in 4 ml of HEPES-EDTA buffer. The mixture was then layered on 5 ml of Ficol-Hypaque (1.077 g/ml; Sigma Chemical Co., St. Louis, MO) and centrifuged (1500*g* for 15 min). The less dense alveolar macrophages were at the buffer-Ficol interface, and any neutrophils present sedimented to the bottom of the gradient. Each group of cells was recovered and washed twice in PBS. Direct cell counts were performed with a hemocytometer. Morphologic differentiation of the cell population was performed on the original BAL fluid and on each fraction using cell monolayers prepared by cytocentrifugation and staining with Wright-Giensa solution. Separation of lavaged cells by this method produced a greater than 99% pure population of alveolar macrophages since (i) the neutrophils settle to the bottom of the gradient; and (ii) 2.5 hr after administration of MAC or MTB the major cell population of the BAL fluid is the alveolar macrophage (Table I), although it is possible that some airway macrophages and lymphocytes were also present. The percentage of viable cells was >98% as determined by exclusion of Trypan blue dye. One million alveolar macrophages, contained in 0.5 ml of solution, from each rat lung of the experimental groups were either flash frozen and assayed for mRNA for iNOS II or incubated at 37°C in HEPES buffer for 60 min at 37°C to evaluate their spontaneous production of RNI (21–26). The HEPES buffered salt solution contained (m*M*): NaCl (128), KCl (4.9), $MgCl_2$ (1.2), $CaCl_2$ (1.6), dextrose (10), NaHEPES/HEPES buffer (18.7), and NaH_2PO_4 (1.18).

Measurement of RNI. Plasma, BAL fluid, lung supernatant, or incubates of alveolar macrophages (10–50 μl) were added to 200 ml of a reducing solution (2.3% vanadium chloride in 2 *N* HCl at 100°C) under a stream of nitrogen gas. Quantification of the NO formed from RNI was determined from the specific chemiluminescence resulting from the reaction of NO with ozone using a Dasibi Model 821 Nitric Oxide-NO_X Analyzer (Dasibi Environmental Inc., Glendale, CA). Conversion of standard mixtures of nitrite and nitrate solutions to NO was 94%–96% when compared with calibrated standards of NO gas (21–26).

Assay of mRNA for iNOS II. Transcripts for NOS II were measured by cDNA cERT-PCR in 10^6 lavaged alveolar macrophages as previously described (21–26). Briefly, total RNA was isolated by homogenizing the cells in RNAzol (Biotecx, Friendsville, TX) following the manufacturer's protocol. Reverse transcription was carried out in 40 μl reactions with a final concentration of 500 pm random hexamer as primer, 1 m*M* dATP-dGTP-dTTP and 0.03 m*M* dCTP (all from Pharmacia, Gaithersburg, MD), 1 × PCR Buffer (50 m*M* KCl, 25 m*M* $MgCl_2$, and 10 m*M* Tris, pH 8.3), 40 units RNasin (Promega), 1 m*M* dithiothreitol, and 400 units Moloney Murine Leukemia Virus-Reverse Transcriptase (Gibco/BRL, Bethesda, MD). The resulting cDNA was labeled by the addition of 1 μCi of α-^{32}P-dCTP (Dupont/NEN) and quantitated by electrophoresing an aliquot of the reaction on a denaturing polyacrylamide gel and scanning the gel on a PhosporImager (Molecular Dynamics, Mountain View, CA). After this quantitation, equal amounts of cDNA from each sample were analyzed and quantitated using competitive PCR as previously described (21–26). Primer sequence for iNOS II were as follows: iNOS-A—5′-AATGGCAACATCAGGTCGGCCATCACT-3′; and INOS-B—5′-GCTGTGTGTCACAGAAGTC-TCGAACTC-3′. The cDNAs were co-amplified using PCR with denaturation at 94°C, annealing at 55°C and extension at 72°C for 30 cycles. Both competitor and signal were labeled by adding α-^{32}P-dCTP into the PCR. Restriction enzyme digestion of the PCR products confirmed the specificity of these primers for the desired gene (28). An aliquot of the PCR reaction was separated on a 6% polyacrylamide gel and exposed to a storage phosphor screen and scanned on a PhosphorImager. The amount of iNOS II transcript was calculated from the ratio of the signal of iNOS to the competitor. We have previously demonstrated that this method generates a standard curve with a correlation coefficient ≥0.94 and can be utilized with as little as 100,000 cells (22, 25). Results are expressed as picogram iNOS II mRNA per nanogram cDNA. The cDNA linearly competes over a four logarithmic range of iNOS II mRNA concentrations (21–26).

Analyses of Data. Data were analyzed with an analysis of variance (ANOVA) (29). A *P* value of 0.05 or less was accepted as a significant difference between and among means.

Results

Initial Parameters of Rats. Table I summarizes the hematologic and humoral parameters of the rats in the four treatment groups. With the exception of a

slight increase in BAL fluid TNF-α and a decrease in the number of cells recoverable in the BAL fluid of the MTB-treated rats of recovered cells, the initial parameters of tested did not differ among the four groups of rats. The body and lung weights, BAL fluid volume, percentage of distribution of alveolar macrophages within the BAL fluid and plasma TNF-α levels did not differ among the four experimental groups ($P > 0.36$). BAL fluid TNF-α was undetectable in the rats treated with PBS. Although some TNF-α was detectable within the BAL fluid of the rats given intratracheal MAC or MTB these values were within experimental error of the assay. Under equivalent conditions intratracheal administration of LPS (0.2 mg/rat) would product TNF-α levels of 2,000 to 10,000 U/ml within 2.5 hr (21–26). The number of cells recovered following BAL was reduced in the rats treated with the low and high dose of MTB but no dose response relationship was evident (Table I). However, the recovered cells were exclusively alveolar macrophages and the percentage of alveolar macrophages did not differ among the four groups of rats.

RNI in Plasma, BAL Fluid, and Lung Homogenates. Plasma obtained from rats treated with PBS, MAC, or MTB contained low concentrations of RNI, which did not differ ($P > 0.3$; Fig. 1). In contrast, RNI in BAL fluid and lung homogenates obtained from rats treated with PBS or MAC was barely detectable. However, MTB significantly increased both BAL fluid and lung homogenate RNI ($P < 0.05$) in a dose-dependent manner when obtained 2.5 hr after intratracheal administration of MTB (Fig. 1). RNI was undetectable in incubates of PBS containing 0.1 and 0.5 mg of MTB or 0.5 mg of MAC, amounts of heat-killed mycobacteria equal to that injected into the trachea of the rats (data not shown). Moreover, the picogram amounts of LPS which would have been associated with the mycobacterial protein were devoid of any measurable effect on BAL fluid or lung homogenate RNI (data not shown).

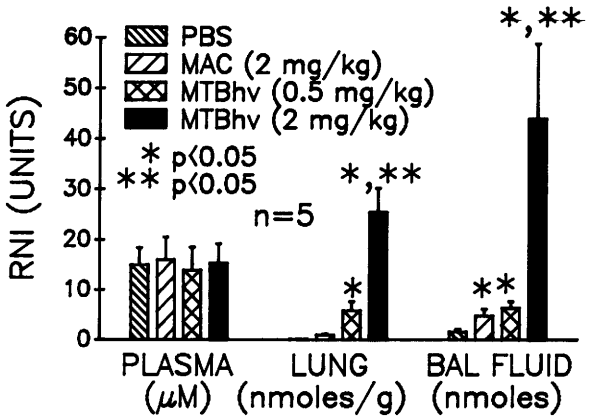

Figure 1. Effect of *in vivo* treatment of rats with intratracheal PBS (1 ml/kg), heat-killed *M. avium* complex (2 mg protein/ml or PBS/kg) or heat-killed human virulent F1 *M. tuberculosis* (MTB, 0.5 and 2 mg protein/ml of PBS/kg) on plasma, bronchoalveolar lavage (BAL) fluid, and lung homogenate nitrite and nitrate (RNI). Samples were obtained 2.5 hr after administration of PBS or mycobacteria. MTB is a more effective stimulator of RNI in the lung than an equal amount of MAC. Vertical lines are the SEM from five experiments. *Differs from PBS ($P < 0.05$); **differs from MAC ($P < 0.05$).

Assay of Alveolar Macrophages for mRNA for iNOS II and RNI. MAC and MTB are engulfed by the alveolar macrophages *in vivo* and remain immunocompetent until the alveolar macrophages are extracted with RNAzol. Since 1.5 hr was required for the isolation and purification of the alveolar macrophages, at which time the RNAzol was added, we consider the actual time of exposure of the macrophages to MAC or MTB as 4 hr, rather than the 2.5-hr time at which the rats were sacrificed. mRNA for iNOS II was undetectable in the polyacrylamide gels obtained from the alveolar macrophages of rats treated with PBS (Fig. 2). A small induction of iNOS II transcripts occurred in alveolar macrophages from two of the five rats given

Table I. Hematologic and Humoral Parameters of Rats

Parameter	PBS	MAC (2 mg/kg)	MTB (0.5 mg/kg)	MTB (2 mg/kg)
Body wt (g)	205 ± 7	220 ± 3	193 ± 9	199 ± 3
Lung wt (g)	1.6 ± 0.2	1.5 ± 0.3	1.6 ± 0.2	1.6 ± 0.2
BAL Fluid (ml)	7.6 ± 0.3	7.6 ± 0.3	7.7 ± 0.2	7.4 ± 0.2
TNF-α (U/ml)				
Plasma	0 ± 0	0 ± 0	0 ± 0	0 ± 0
BAL fluid	0 ± 0	15 ± 8	32 ± 9	36 ± 24
BALf cells (10^6)	7.4 ± 0.8	7.6 ± 0.6	5.3 ± 0.2*	6.6 ± 0.7
AM (% of total)	100 ± 0	99.6 ± 0.5	99.6 ± 0.5	99.6 ± 0.2
PMN (% of total)	0 ± 0	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
Number of rats	5	5	5	5

Note. Ether-anesthetized rats were administered PBS (1 ml/kg, intratracheal), heat-killed *M. avium* complex (MAC; 2 mg protein/ml PBS/kg, intratracheal), or human virulent strain F1-MTB (0.5 or 2 mg protein/ml PBS/kg, intratracheal). TNF-α was measured in plasma, and TNF-α, alveolar macrophages (AM), and recruited neutrophils (PMN) were measured in bronchoalveolar lavage (BAL) fluid 2.5 hr after intratracheal administration of PBS or pathogen.

[a] Differs from PBS ($P < 0.05$).

[b] Differs from MTB (0.5 mg/kg) ($P < 0.05$).

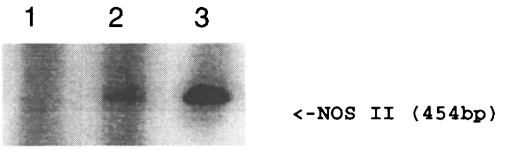

Figure 2. A representative polyacrylamide gel of RT-PCR of rat alveolar macrophage (AM) mRNA for iNOS II obtained from rats treated with intratracheal heat-killed MTB (0.5 and 2 mg of protein/ml of PBS/kg). AM were obtained 2.5 hr after administration of PBS or MTB and required 1.5 hr for isolation during which time the MTB may have been active within the AM. Therefore, 4 hr is indicated as the time of exposure of the AM to MTB prior to exposure of the AM to RNAzol (see Materials and Methods). (Lane 1) PBS treatment; (Lane 2) MTB 0.5 mg/kg; and (Lane 3) MTB 2 mg/kg.

M. avium complex, *in vivo* (data not shown). This was associated with the low levels of spontaneous release of RNI by alveolar macrophages isolated from MAC treated rats and incubated *ex vivo* (Fig. 3). Intratracheal administration of MTB to rats produced dose-dependent increases in mRNA for iNOS II, which were so great at the high dose of MTB that none of the competitor cDNA initially present remained on the gel (Fig. 2). Subsequent quantitation of MTB-mediated increases of mRNA for iNOS II showed that heat-killed MTB produced protein-dependent increases in both the mRNA content of the alveolar macrophage as well as in the spontaneous release of RNI by the alveolar macrophage *ex vivo* (Fig. 3).

Effect of LNMMA on RNI Production. Pretreatment of rats with LNMMA (50 mg/kg, ip) 30 min before administration of MAC or MTB did not affect plasma levels of RNI. However, pretreatment of rats with LNMMA inhibited MAC and MTB-induced RNI production in the BAL fluid and in the lung (Fig. 4).

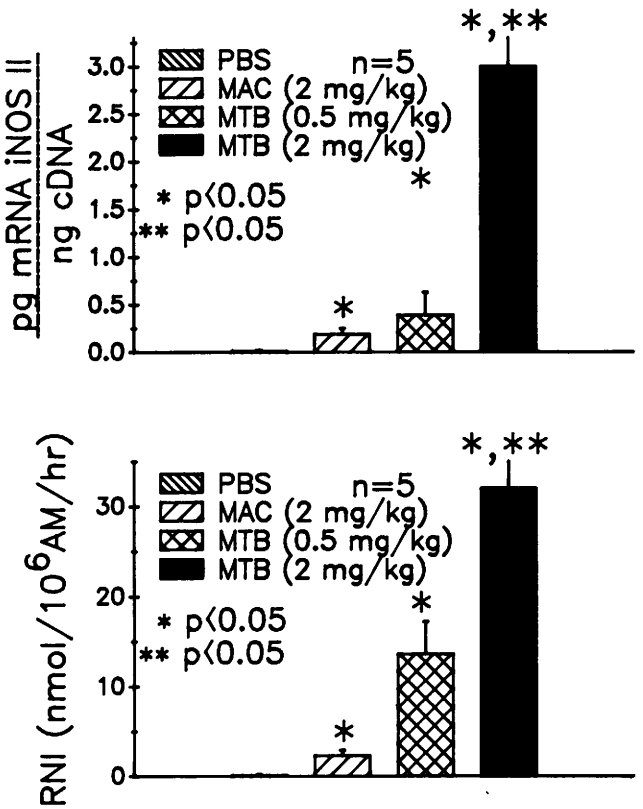

Figure 3. (Top Panel) Rat alveolar macrophage (AM) mRNA for iNOS II obtained from rats pretreated with intratracheal PBS (1.0 ml/kg), heat-killed MAC (2 mg protein/ml of PBS/kg), or heat-killed human virulent strain F1-MTB (0.5 and 2 mg protein/ml of PBS/kg). AM were obtained 2.5 hr after administration of PBS or mycobacteria and mRNA was extracted 1.5 hr after sacrifice. Each data point is the mean and SEM from five experiments. Data are expressed as picogram of mRNA for iNOS II per nanogram of cDNA. (Bottom Panel) Spontaneous RNI production by AM obtained from the rats described in the top panel incubated for 60 min in HEPES-buffer physiologic salt solution. Vertical lines are the SEM from five animals per group. *Differs from PBS ($P < 0.05$); **differs from MAC ($P < 0.05$).

Discussion

Within 2.5 hr after *in vivo* intratracheal administration of heat-killed MAC or MTB to rats the intrapulmonary NO system is upregulated. It is mildly upregulated by MAC, whereas the more virulent human strain of MTB exhibited pronounced induction of the NO system within the lung. The effect of the mycobacteria on induction of iNOS II activity is relatively rapid, since increased iNOS II gene expression and RNI production occurred within 2.5 hr after intratracheal administration of the heat-killed MTb to the rats. Moreover, the induction of the iNOS II system appears to be localized within the lung, since plasma levels of RNI were unchanged at a time when BAL fluid levels of RNI were elevated. In addition, the RNI appeared to originate from L-arginine-derived NO, because LNMMA attenuated MAC- and MTB-induced increases in BAL fluid and lung homogenate RNI without any effect on plasma RNI. MAC were less efficacious than MTB in their ability to upregulate iNOS II gene expression and RNI production in alveolar macrophages. Finally, both MAC- and MTB-induced upregulation of the iNOS system occurred without any significant increase in BAL fluid TNF-α. This suggests that, during exposure of the rat lung to heat-killed mycobacteria, (i) the MTB is sufficiently immunocompetent to upregulate the lung and alveolar macrophage iNOS II gene expression and (ii) MTB-induced upregulation of mRNA for iNOS II can occur independently of released TNF-α. This is the first demonstration that *in vivo* administration of MTB into the intact rat can selectively and rapidly upregulate mRNA for iNOS II in alveolar macrophages and that the increased gene expression is rapidly translated into RNI production *in vivo* and *ex vivo*, and is supported by additional studies demonstrating that *in vivo* (30) and *in vitro* (31, 32) administration of ethanol suppresses mycobacteria-induced upregulation of the iNOS system in macrophages.

We demonstrated that LNMMA inhibited mycobacteria-induced RNI production in lung homogenates

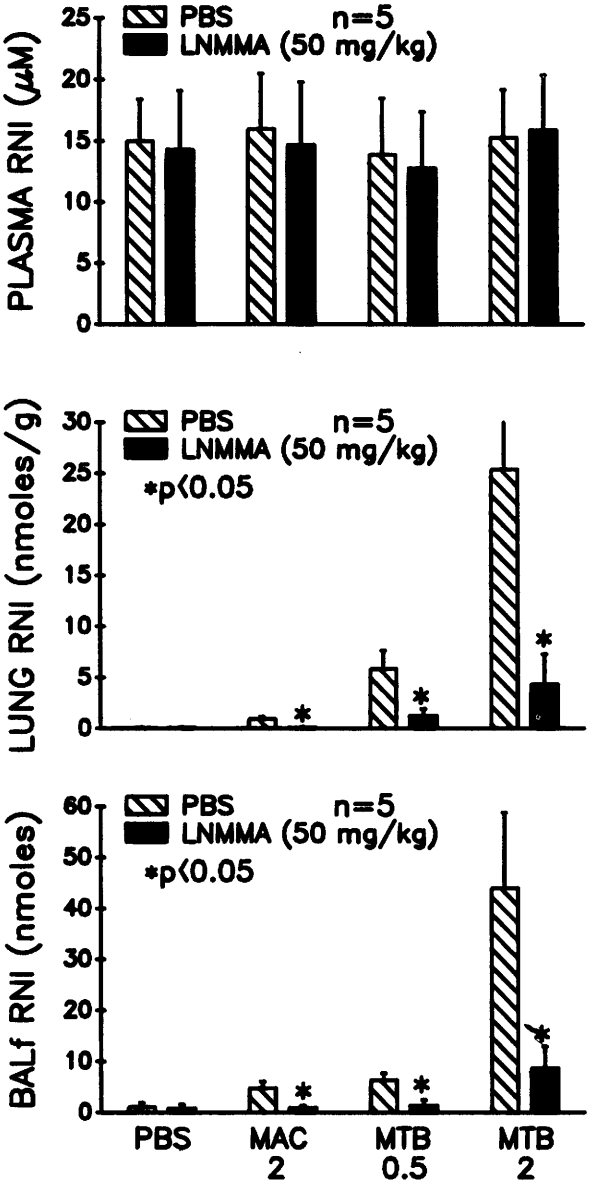

Figure 4. Effect of *in vivo* pretreatment of rats with LNMMA (50 mg/kg, ip) 30 min before experimentation on RNI levels in plasma, bronchoalveolar lavage (BAL) fluid, and lung homogenate 2.5 hr after administration of PBS (1 ml/kg), heat-killed *M. avium* complex (2 mg protein/ml of PBS/kg), or heat-killed human virulent strain F1-MTB (0.5 and 2 mg protein/ml of PBS/kg). Vertical lines are the SEM from five experiments. *Differs from PBS ($P < 0.05$).

and BAL fluid, and that MTB upregulated iNOS gene expression in alveolar macrophages. Nevertheless, one may challenge the conclusion that mycobacteria-induced RNI production by the alveolar macrophage reflects iNOS II activity, since we did not measure iNOS enzyme activity or levels in the macrophage. A temporally related increase in iNOS mRNA and RNI production does not necessarily imply that the former was causal to the latter. However, unlike vascular endothelium, alveolar macrophages do not contain constitutive NOS activity (2, 17) but only exhibit RNI production upon induction of gene expression for iNOS II. RNI production by alveolar macrophages can be inhibited by glucocorticoids and inhibitors of iNOS II activity (2–4, 6, 17). Also, we could only measure RNI production when iNOS II mRNA was detectable. The relative amounts of iNOS II mRNA correlated well with the relative levels of RNI produced by the alveolar macrophage (Figs. 1–3). Finally, the levels of RNI released from the alveolar macrophage incubated in HEPES buffer, *ex vivo*, were consistent with the nanogram per minute rate of NO production by iNOS II (22–24, 26).

One may also feel that our conclusions should be challenged because MAC- and MTB-induced upregulation of the NO system was not associated with increased release of TNF-α in the BAL fluid. Alveolar macrophages play a critical role in the prevention and control of pulmonary tuberculosis and MAC related diseases. Resident, activated alveolar macrophages engulf and digest more than 90% of inhaled mycobacteria (6–9). Those bacilli resistant to destruction multiply within the alveolar macrophage. Upon the death of the macrophage the bacilli are released into the alveolar lumen where they release chemotactic factors and recruit unactivated circulating monocytes and peripheral macrophages into the alveolar space. These monocyte/macrophages then engulf the MTB and provide a symbiotic environment suited for further replication of the MTB bacilli without the death of the macrophage (6–9). This results in the formation of the primary tubercle (8). Thus, highly activated alveolar macrophages will kill mycobacteria, whereas partially activated or unactivated alveolar macrophages or recruited monocyte/macrophages will be less effective in clearing the lung of the bacilli (6–9). The alveolar macrophage is also the major of the lung and BAL fluid TNF-α. Bacterial wall stimulation of the alveolar macrophage is believed to elicit TNF-α, which in turn acts in an autocrine and paracrine manner to upregulate other cytokines and iNOS II in the alveolar macrophage (3–5, 10, 12, 18, 19, 33, 34). Although cell wall fragments of lipoarabinomannan (LAM) from human nonvirulent strains of MTB and other mycobacteria stimulate the release of TNF-α from the alveolar macrophage (15), the human virulent strain of MTB does not appear to stimulate TNF-α release from the alveolar macrophage (15, 33, 34). This could suggest that the active site of the LAM in the intact and/or phagocytosed MTB is not accessible to the binding sites on the alveolar macrophage required for induction of TNF-α activity. Speculatively, the inability of MTB and other strains of mycobacteria, including MAC, to stimulate TNF-α may limit the activation of the alveolar macrophage. The production of NO by mycobacteria may augment the absence of TNF-α especially if the mycobacteria are resistant to the bactericidal activity of NO. The induction of NO, which can suppress

macrophage phagocytosis (13), combined with the absence of TNF-α secretion may contribute to retaining the alveolar macrophage in a subactivated state. This could contribute to the characteristic virulence of some strains of MAC and MTB (6–9, 15, 33, 34). Moreover, it could also suggest that, although MTB upregulates the iNOS system and NO may contribute in some manner to the killing of intracellular mycobacteria, its activity is insufficient to clear the lung of mycobacteria in the absence of alveolar macrophage activation by TNF-α.

The present data do not allow us to distinguish between a direct effect of MAC and MTB on induction of gene expression for iNOS II and an indirect effect resulting from mycobacteria-induced stimulation of other cytokines such as interleukins or γ-interferon, which also upregulate gene expression for iNOS II (4, 6–10, 14). Nevertheless, the data clearly show that intratracheal administration of heat-killed human virulent F1 strain MTB, whether directly or indirectly through induction of cytokines other than TNF-α, upregulates iNOS II mRNA with subsequent phenotypic expression of RNI activity by rat alveolar macrophages. Further studies are now required to determine (i) if human alveolar macrophages generate iNOS II mRNA and NO in response to MTB and (ii) whether human alveolar macrophage NO can affect the viability and virulence of the MTB bacilli. Studies are in progress to answer these questions.

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