

To investigate effect of MMLA, an inhibitor of nitric oxide (NO) production, on regulation of inflammatory responses to *Bordetella pertussis* infection, mice were infected intranasally, and treated with various concentrations of MMLA. Ten days after infection, mice treated with MMLA at dosage of 100 mg/kg, given intraperitoneally in a single dose or for 5 consecutive days, showed at histopathologic examination, a significant decrease of intensity of inflammation (scores, 0.6 ± 0.2 and 0.9 ± 0.5 respectively). A decrease of cellular accumulation of neutrophils and lymphocytes in the bronchoalveolar lavage (BAL) fluid was observed in infected mice treated with MMLA, especially at dosage of 10 mg/kg, given in a single dose intraperitoneally. In addition, BP-infected mice treated with MMLA (100 mg/kg, intraperitoneally) for 5 consecutive days showed higher mortality rate than untreated mice infected with *B. pertussis*, and the number of *B. pertussis* in lungs of mice treated with MMLA was significantly increased. However, MMLA treatment of infected mice had some effect on levels of IFN- γ and nitrite/nitrate (end-stable products of NO) in the BAL fluid. This study indicates that NO may play a role either as microbiocidal agent or as a modulator of immune regulation, inasmuch as it may upregulate tissue inflammatory response to *B. pertussis*.

Regulation of inflammatory responses to *Bordetella pertussis* by N^G-monomethyl-L-arginine in mice intranasally infected

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Introduction

In recent years, it has become apparent that nitric oxide (NO) and reactive nitrogen intermediates are effector molecules responsible for the antimicrobial functions of macrophages.¹ The release of NO and reactive nitrogen intermediates is an important part of macrophage effector function directed against a variety of intracellular pathogenic microorganisms, including fungi, bacteria and parasites.^{2–6} *Bordetella pertussis* is considered a facultative intracellular pathogen, which invades and survives within macrophages.⁷ The mechanisms by which macrophage-derived NO kills a microbial target have not been fully clarified, but are believed to involve toxic byproducts of reactions with oxygen, superoxide and transition metals. Although the role of macrophage activation in the killing of facultative intracellular pathogens, including *B. pertussis*, is still debated, we have previously shown that *in vitro* murine peritoneal macrophages induce NO production in response to *B. pertussis* and pertussis toxin.⁸ In addition, NO production was also observed in alveolar macrophages from mice infected intranasally with *B. pertussis*.⁸ The capacity to release NO is not a constitutive trait of macrophages, but is dependent on the

induction of a specific cytosolic enzyme, inducible NO synthase.⁹ Regarding the pulmonary pathogen *B. pertussis*, the role of NO and macrophage inducible NO synthase in host responses to infection are unknown.

Ding *et al.*¹ have shown that murine macrophages, when stimulated with gamma interferon (IFN- γ) catabolize L-arginine to produce NO, by releasing reactive nitrogen intermediates. Thus, activated macrophages stimulated with IFN- γ seem to exert some antimicrobial activities via induction of NO or other reactive nitrogen oxides.^{10,11} We have previously demonstrated that mice infected intranasally with *B. pertussis* show an early production of endogenous IFN- γ in the bronchoalveolar lavage (BAL) fluid.¹²

This study evaluates the effect of N^G-monomethyl-L-arginine (MMLA), an inhibitor of NO synthase, in mice infected intranasally with *B. pertussis*.

Materials and methods

Experimental animals and reagents

Eight-to-10 week old female NMRI mice (Morini, San Polo d'Enza, Italy), weighing 15–18 g were housed throughout the experiments. The mice were caged in

groups of eight mice and given food and water *ad libitum*.

B. pertussis 18323 (ATCC 9797) was grown at 35°C on Bordet-Gengou agar containing 20% defibrinated horse blood. The cells from freeze-dried cultures were transferred on Bordet-Gengou agar no more than twice before use. On the final passage, cells were allowed to grow 20 h, and then suspended in PBS containing 1% casamino acids (Difco Laboratories, Detroit, MD). Viable counts of *B. pertussis* were determined by diluting samples in sterile PBS, spreading 0.1 ml of appropriate dilutions on Bordet-Gengou plates, and incubating at 35°C for 3–4 days.

MMLA was purchased from Alexis Corp. (San Diego, CA, USA), nitrate reductase from *Aspergillus* sp. was purchased from Boehringer Biochemica Mannheim (Milan, Italy), and FAD and NADPH from Sigma Chemicals (St Louis, MO, USA).

Respiratory tract infection

An intranasal mouse model was used, as previously described.¹² Briefly, bacterial strains were dispensed in 1% casamino acids, and adjusted to concentration of 1×10^6 organisms/ml. Mice were lightly anaesthetized with diethyl ether. A 50- μ l bacterial suspension was placed in the tips of their noses for inhalation. Control mice were treated with saline solution. Lungs were prepared for quantitative bacteriologic examination by removal of the heart and lungs en bloc. Lungs and heart were then separated and placed in 10 ml of 1% casamino acids. Viable counts were made after the organs were homogenized for 10 s in a homogenizer and appropriately detected. At least five mice were used for each experimental group. Bronchoalveolar lavage (BAL) fluid was recovered from the lungs by lavage with three 1-ml aliquots of sterile saline via a polyethylene tubing attached to a syringe. All lavages yielded equivalent amounts, 90% of the volume of the BAL returned. Lavage fluids from each animal were pooled in sterile tubes and centrifuged at $400 \times g$ for 10 min. Total cells were counted with a haemocytometer. Differential counts were done on preparations stained with Giemsa stain.

Histopathology

After lavage, the lungs were immersed in formalin. Parasagittal sections of fixed lung tissue were embedded in paraffin, and processed for histologic examination, using haematoxylin-eosin stain. The intensity of inflammation caused by *B. pertussis* infection was graded blindly and validated by three investigators, using a semiquantitative scale ranging from 0 (no inflammatory cells in alveolar/interstitial spaces; absence of oedema; haemorrhage with < 5 erythrocytes in alveolar/interstitial spaces) to three (large

numbers of inflammatory cells, neutrophils and lymphocytes, in a generalized distribution; increased swelling of alveolar walls and fibrinous exudate; > 15 erythrocytes in alveolar/interstitial spaces). Our observations showed a close correlation between the infection and inflammation scores obtained by the three investigators.

Nitric oxide assay

NO was measured through the spectrophotometric determination of nitrate/nitrite. To reduce nitrate to nitrite, samples were incubated in the presence of 40 mU of nitrate reductase, 5 mM FAD, and 0.6 mM NaDPH. Nitrite was measured by a procedure based on the Griess reaction. Samples were incubated with the Griess reagent (1% sulphanilamide in 1 N HCl and 0.15% N-naphthylethylenediamine dihydrochloride) for 30 min in the dark at room temperature in 96 microtitre plates. The absorbance at 550 nm was determined in an automatic reader.

IFN assay

IFN was assayed as previously reported.¹² Briefly, L-929 cells were suspended and cultured on microculture plates. Plates were incubated in the presence of serial twofold dilution of samples (1:2 to 1:256). Cells were then challenged with vesicular stomatitis virus (Istituto Zooprofilattico, Turin, Italy) at a 1:1 MOI. When cytopathic effect reached 100% in controls, the plates were stained with crystal violet. The number of units of IFN per millilitre for each sample was estimated by comparing sample titres with the known number of units of IFN activity of the reference standard of IFN at a 50% end point.

Statistics

Data are expressed as mean and standard deviation values, and the results from various set of experiments were compared by the non-parametric Mann-Whitney *U*-test, Welch's *t*-test and Fischer's exact test when appropriate.

Results

Table 1 shows cellular accumulation in the BAL fluid of mice infected with *B. pertussis*, and treated with various concentrations of MMLA, given in a single dose intraperitoneally. As shown, decrease of cellular accumulation in the BAL fluid, particularly of neutrophils, was noted in mice treated with MMLA at various concentrations, and in particular in those treated at the dosage of 100 mg/kg ($13.6 \pm 20.9 \times 10^5$ cells/ml vs. control treated mice: $30.8 \pm 23.0 \times 10^5$ cells/ml).

Table 1. Effect of MMLA, given at various concentrations intraperitoneally in a single dose, on cells recovered from bronchoalveolar lavage fluid of mice intranasally infected with *B. pertussis* (six mice for each experimental group). Mice were sacrificed 10 days after infection.

Treatment	Total cell count ($\times 10^5$ cells/ml)	Differential cell count ($\times 10^5$ cells/ml)		
		Neutrophils	Lymphocytes	Macrophages
BP	30.8 \pm 23.0	24.6 \pm 20.1	5.0 \pm 4.8	1.2 \pm 1.1
BP + MMLA 50 mg/kg	15.7 \pm 18.2	14.2 \pm 15.8	1.0 \pm 1.8	0.5 \pm 0.7
BP \pm MMLA 100 mg/kg	13.6 \pm 20.9*	11.2 \pm 13.3	1.9 \pm 2.0	0.5 \pm 1.0

P = not significant.

Intensity of inflammation caused by *B. pertussis* infection was evaluated in lung tissue of mice treated with MMLA. As can be seen in Fig. 1, mice sacrificed 10 days after infection with *B. pertussis*, showed severe inflammation in lung tissue, with a mean inflammatory score of 2.5 ± 0.3 ($n = 6$). At the same time interval after infection, infected mice treated with MMLA at the dosage of 100 mg/kg in a single dose showed a uniform decrease of inflammation (inflammatory score, 0.6 ± 0.2 , $n = 4$; $P = 0.0286$). In addition, mice treated with MMLA at the dosage of 100 mg/kg for 5 consecutive days after infection also showed a significant decrease of inflammatory score (0.9 ± 0.5 , $n = 4$; $P = 0.0286$) (Fig. 2). The groups of mice treated with MMLA in a single dose, and for 5 consecutive days had a 10% and 20% mortality rate ($P = 0.0367$) at day 10 post-infection respectively, with respect to the control group (mice only treated with *B. pertussis*), which had a 10% mortality rate (Table 2). In addition, there was a marked and significant increase in the number of bacteria in the lungs of mice treated with MMLA for 5 consecutive days ($P = 0.0296$).

Furthermore, we evaluated levels of nitrite/nitrate in the BAL fluid of infected mice treated with various concentrations of MMLA, given in a single dose intraperitoneally. As shown in Fig. 3, treatment with MMLA did not significantly increase BAL levels of nitrite/nitrate, although a moderate increase of nitrite/nitrate was noted at a concentration of 100 mg/kg.

Finally, levels of IFN- γ were determined in the BAL fluid of infected mice treated with MMLA. As shown in Fig. 4, IFN- γ levels were not significantly decreased

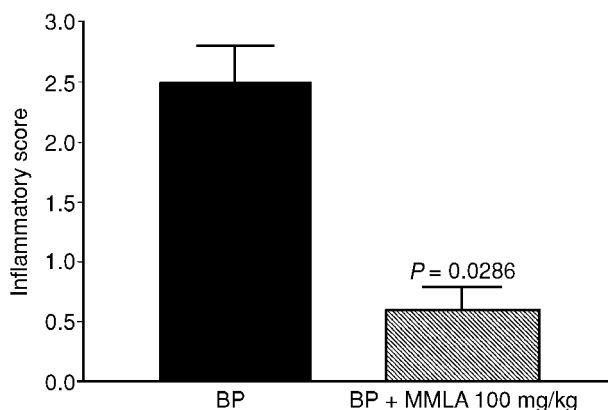


FIG. 1. Mean inflammatory score of mice infected intranasally with *B. pertussis*, and treated with MMLA (100 mg/kg i.p. in a single dose). Mice were sacrificed 10 days after infection.

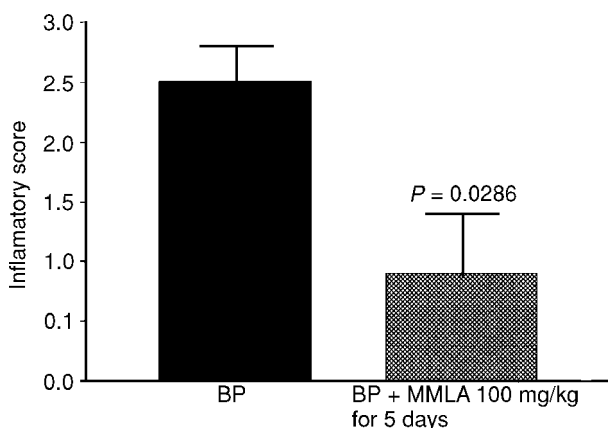


FIG. 2. Mean inflammatory score of mice infected intranasally with *B. pertussis*, and treated with MMLA (100 mg/kg i.p. for 5 consecutive days). Mice were sacrificed 10 days after infection.

Table 2. Number of colony-forming units of *B. pertussis* in lungs of mice treated or not with MMLA, and mortality rate 10 days after they were infected intranasally with *B. pertussis* (10^5 cfu)

Treatment group	<i>B. pertussis</i> (cfu $\times 10^4$)	Mortality rate (%)
BP	105.3 \pm 45.8	10
BP + MMLA (100 mg/kg in a single dose)	145.5 \pm 99.8	10
BP + MMLA (100 mg/kg for 5 days)	190.7 \pm 74.6*	20**

* $P = 0.0296$ respect to BP-infected mice group (Welch's *t*-test);

** $P = 0.0367$ respect to BP-infected mice group (Fischer's exact test).

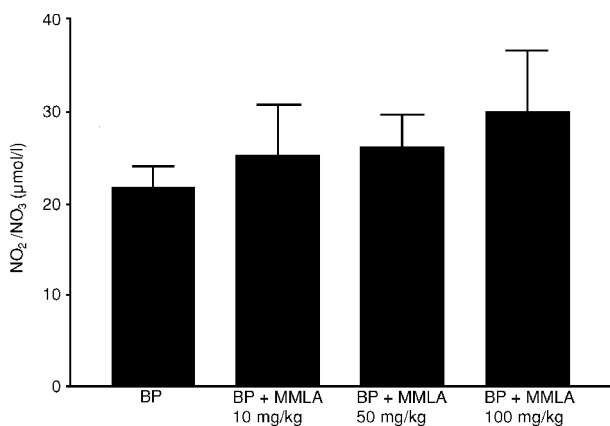


FIG. 3. Levels of NO₂/NO₃ in the BAL fluid of mice infected with *B. pertussis*, and treated with MMLA, at various concentrations in a single dose intraperitoneally. Mice were sacrificed 10 days after infection.

in mice treated with various concentrations of MMLA, though a diminution of IFN- γ levels in the BAL fluid was noted in mice treated with MMLA at dosage of 50 mg/kg.

Discussion

The results of this study indicate that MMLA, as inhibitor of NO synthase, may play a role in reducing inflammatory response in mice infected with *B. pertussis*, even though infected mice treated with MMLA for 5 consecutive days showed higher mortality rate with a concomitant increase of the number of *B. pertussis* in lungs of the infected mice. Our data are somewhat surprising, because release of NO has been proposed as a major cellular clearance pathway for intracellular pathogens.¹³ However, the *in vivo* role of macrophage killing of *B. pertussis* through NO remains unknown. Our study indicates that NO might amplify tissue inflammation through its effects on

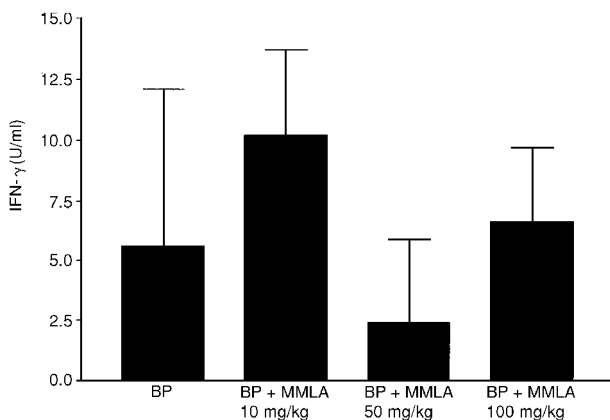


FIG. 4. Levels of IFN- γ in the BAL fluid of mice infected with *B. pertussis*, and treated with MMLA, at various concentrations in a single dose intraperitoneally. Mice were sacrificed 10 days after infection.

leukocyte adhesion to endothelium. Kubes *et al.*¹⁴ showed that endothelium-derived NO may be an important endogenous modulator of leukocyte adherence, inasmuch as inhibition of NO production causes vasoconstriction and a reduction in the shear forces that tend to push neutrophils along venular endothelium. In addition, the simultaneous generation of superoxide and NO by macrophages and activated neutrophils may cause a direct lung injury via peroxynitrite formation.¹⁵ Kuo *et al.*¹⁶ have shown that endogenous NO may contribute to neurogenic inflammation in the airways of guinea-pig, through effects on vascular smooth muscle and tissue oedema, inasmuch as an inhibitor of NO, N^G-nitro-L-arginine methyl ester, may inhibit neurogenic plasma exudation in the guinea-pig airways. However, the reduction of the inflammatory score in MMLA treated mice found in our study may be due to the severe vasoconstriction caused by the high doses of MMLA. Shellito *et al.*¹⁷ have shown that inducible NO synthase does not upregulate in murine lung macrophages as part of the normal host response to clear infectious challenge with *Pneumocystis carinii*.

We have previously demonstrated that alveolar macrophages from mice intranasally infected with *B. pertussis* show a significant production of NO *in vitro*.⁸ Thus, we suggest that macrophage release of NO in murine infection with *B. pertussis* may be important in amplifying the tissue inflammatory response to the pathogen, although we did not find a correlation between MMLA treatment and BAL nitrite/nitrate levels. However, endothelial cells and activated neutrophils are mainly responsible for NO production in the lungs after inflammatory or infectious stimuli, as shown by increased expression of NO synthase by these cells.¹⁴ Furthermore, NO production by alveolar macrophages, detected in the BAL as nitrite/nitrate, is not increased during lung inflammation, as these cells are not probably involved respect to parenchymal cells, including endothelial cells and activated neutrophils. It is interesting to note that release of NO by alveolar macrophages can be blocked by glucocorticoids *in vitro*.¹⁸ In a histopathologic study, we evaluated effects of betamethasone in mice treated with pertussis toxin, a major protein-toxin of *B. pertussis* responsible for establishing and maintaining the respiratory tract infection.¹⁹ In this study, we have demonstrated that mice treated with pertussis toxin and betamethasone showed a decreased congestion and oedema of the lung, and reduction of cellular accumulation in parenchyma. Inhibition of NO release by lung macrophages and consequent suppression of tissue inflammation may be one mechanism by which glucocorticoids improve outcome in human pertussis.²⁰ Finally, the increased mortality rate of infected mice treated with MMLA together with elevation of the number of *B. pertussis* in lungs of the infected mice support the role of NO

as a major clearance pathway for intracellular facultative pathogens.

IFN- γ is an important cytokine that modulates NO activity against intracellular infection.^{21,22} We have previously demonstrated, *in vitro*, that IFN- γ partially increased intracellular killing of human monocytes in response to *B. pertussis*.²³ In addition, no significant increase of superoxide production was noted in human monocytes in response to *B. pertussis*, when stimulated with IFN- γ .²³ In our study, levels of IFN- γ in the BAL fluid did not significantly change in mice treated with MMLA. In fact, NO levels in the BAL fluid were not significantly decreased in mice treated with various concentrations of MMLA.

In conclusion, we may suggest that NO may have a dual function both as microbiocidal agent and as a modulator of immune regulation, in particular NO may upregulate tissue inflammatory response to *B. pertussis*.

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