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Neutralization of Gamma Interferon and Tumor Necrosis Factor Alpha Blocks In Vivo Synthesis of Nitrogen Oxides from L-Arginine and Protection against *Francisella tularensis* Infection in *Mycobacterium bovis* BCG-Treated Mice

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Peritoneal cells from *Mycobacterium bovis* BCG-infected C3H/HeN mice produced nitrite (NO_2^-, an oxidative end product of nitric oxide [NO] synthesis) and inhibited the growth of *Francisella tularensis*, a facultative intracellular bacterium. Both NO_2^- production and inhibition of bacterial growth were suppressed by N^G-monomethyl-L-arginine, a substrate inhibitor of nitrogen oxidation of L-arginine, and monoclonal antibodies (MAbs) to gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α). Intraperitoneal injection of mice with BCG increased urinary nitrate (NO_3^-) excretion coincident with development of activated macrophages capable of secreting nitrogen oxides and inhibiting *F. tularensis* growth in vitro. Eight days after BCG inoculation, mice survived a normally lethal intraperitoneal challenge with *F. tularensis*. Treatment of these BCG-infected mice with MAbs to IFN-γ or TNF-α at the time of BCG inoculation reduced urinary NO_3^- levels to those found in normal uninfected mice for up to 14 days. The same anticytokine antibody treatment abolished BCG-mediated protection against *F. tularensis*: mice died within 4 to 6 days. Intraperitoneal administration of anti-IFN-γ or anti-TNF-α antibody 8 days after BCG infection also reduced urinary NO_3^- and abolished protection against *F. tularensis*. Isotype control (immunoglobulin G) or anti-interleukin 4 MAbs had little effect on these parameters at any time of treatment. IFN-γ and TNF-α were clearly involved in the regulation of macrophage activation by BCG in vivo. Protection against *F. tularensis* challenge by BCG depended upon the physiological generation of reactive nitrogen oxides induced by these cytokines.

The formation of endogenous nitrate (NO_3^-) is a ubiquitous mammalian process (28, 29, 39, 46, 58, 75). In humans, L-arginine is the precursor for NO_3^- synthesis, and recent reports document increases in NO_3^- concentrations in urine of patients receiving interleukin 2 (IL-2) therapy and in sera of acutely ill patients (39, 46, 58). These findings are consistent with earlier animal studies showing that endogenous NO_3^- production is augmented during inflammatory responses. For example, germfree rats treated with lipopolysaccharide show a ninefold increase in NO_3^- excreted in urine (75). Mice infected with *Mycobacterium bovis* BCG also excrete high levels of NO_3^- during the course of disease (72). Urinary nitrogen oxides in BCG-infected mice peak 8 to 10 days after intraperitoneal (i.p.) inoculation with BCG, a time coincident with the appearance of macrophages activated for tumoricidal and microbicidal activity in vitro (32, 72). These activated macrophages produce NO_3^- and NO_2^- in vitro (71, 72). Nitric oxide (NO) and NO_3^--NO_2^- production by activated macrophages in vitro depends upon extracellular L-arginine (37, 38). Activated macrophages do not synthesize NO or display certain tumoricidal or antimicrobial activity in the absence of L-arginine or in the presence of N^G-monomethyl-L-arginine (N^GMMLA), a substrate inhibitor of nitrogen oxidation of L-arginine (25, 27, 37, 38). L-Arginine also serves as the substrate for NO production in animals with infection: oral administration of N^GMMLA blocks BCG-induced urinary NO_3^- excretion by infected mice (25).

Macrophage activation during BCG infection occurs during the synthesis and release of cytokines by lymphocytes and other cellular participants in immune reactions. In vitro, cytotoxicity and NO_2^--NO_3^- production by activated macrophages is regulated by gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) is a cofactor for IFN-γ-primed macrophages to express certain cytotoxic effector functions (19, 31, 50, 68). Macrophages activated during BCG infection display potent nonspecific immunity (51); BCG infection protects animals during subsequent exposure to such diverse infections and neoplastic agents as *Listeria*, *Rickettsia*, and *Leishmania* spp. and sarcomas (22, 35, 51, 55, 76). A strong correlation between antimicrobial activity and production of nitrogen oxides by activated macrophages in vitro has been demonstrated (1, 2, 6, 15, 21, 23, 24, 26, 30–32, 41, 50, 52, 53, 57, 64, 74). However, the relationship between production of reactive nitrogen oxides in vivo and nonspecific protection against a lethal infection with any pathogen remains unexplored.

The live vaccine strain of *Francisella tularensis*, a facultative intracellular bacterium, causes a lethal infection in certain mouse strains, and i.p. inoculation of <10 organisms leads to fulminating infection and death (24). Independently, Anthony et al. (6) and our group showed that *F. tularensis* is

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susceptible to NO produced by activated macrophages (6, 23). In this report, we document the potent protective effect of BCG against in vivo infection with *F. tularensis* and demonstrate that in vivo neutralization of the cytokines required for induction of NO and related reactive nitrogen oxides blocks both elevated urinary NO_3^- excretion and nonspecific protection against this pathogen afforded by BCG.

MATERIALS AND METHODS

Animals. Specific-pathogen-free C3H/HeNHSD male mice were obtained from Harlan Sprague Dawley (Frederick, Md.) and were housed in a barrier facility. Mice were routinely screened and found to be negative for bacterial pathogens and subclinical viral infections through the serodiagnostic services of Microbiological Associates (Bethesda, Md.). All mice were used at 5 to 7 weeks of age.

Antibodies and reagents. Characterization of neutralizing monoclonal antibodies (MAbs) to IFN-γ (H22) (66) and TNF-α (TN3 19.2) (67) and the isotype (immunoglobulin G [IgG]) control antibody (Organon Teknika Corp., West Chester, Pa.) was described previously. Anti-IL-4 MAb (11B11) was generously provided by W. Paul, National Institutes of Health Bethesda, Md. (59). N^GMMLA was purchased from Calbiochem (San Diego, Calif.).

Bacteria and growth conditions. The live vaccine strain of *F. tularensis* (ATCC 29684, Rockville, Md.) was cultured on Mueller-Hinton plates supplemented with 10% glucose, ferric pyrophosphate, and IsoVitaleX (Becton Dickinson, Cockeysville, Md.) for 4 days at 37°C in 5% CO_2 and 95% humidity. Colonies were selected for growth in modified Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with ferric pyrophosphate and IsoVitaleX. Broth cultures were incubated for 2 days to a bacterial density of 10^8 to 10^9 CFU/ml, aliquoted, and frozen at −70°C. One-ml aliquots were periodically thawed, and viable bacteria were quantified by plating serial dilutions in phosphate-buffered saline (PBS) on modified Mueller-Hinton plates.

M. bovis BCG (Statens Seruminstitut, Dk-2300, Copenhagen, Denmark) was grown in 250-ml flasks containing 50 ml of 7H9 Middlebrook broth (Difco) for 12 days at 37°C, washed three times by centrifugation (3,200 × *g*, 4°C, 10 min), and frozen at −70°C in PBS. Aliquots were thawed, and viable bacteria were quantified by plating serial dilutions in PBS on 7H10 Middlebrook agar plates (Difco). Quantitative plate counts showed that BCG suspensions contained 10^7 to 10^8 CFU/ml. Stocks tested negative for reduction of nitrate and were acid-fast positive.

Pseudomonas oleovarans (ATCC 8062) was grown in 500-ml flasks containing 75 ml of heart infusion broth (Difco) overnight with continual agitation (200 rpm) at 25°C. Bacteria were washed three times with PBS by centrifugation (4,300 × *g*, 4°C, 15 min), and the final pellet was resuspended in phenol red-free Dulbecco's modified Eagle's medium (Gibco) and stored at −70°C. Quantitative plate counts showed that frozen aliquots contained 2 × 10^9 CFU/ml and displayed nitrate reductase activity.

Animal inoculations and determination of *F. tularensis* LD_{50}s for mice. Mice were given various doses of *F. tularensis* and the 50% lethal dose (LD_{50}) for the i.p. route of infection was calculated by the method of Reed and Muench (63). For BCG protection studies, approximately 10^6 CFU of BCG was inoculated i.p. 8 days before *F. tularensis* inoculation. Log protection was calculated as the difference between LD_{50}s in BCG-treated versus untreated mice.

Measurement of urinary NO_3^- excretion and NO_2^- in culture medium. Urinary NO_3^- was estimated by using the Griess reagents after reducing NO_3^- to NO_2^- with bacterial nitrate reductase (25). Briefly, approximately 40 to 100 μl of urine was collected per mouse every evening from groups consisting of five to eight mice. Urine was pooled, microcentrifuged, and stored at −30°C. Mice were maintained on NO_2^--NO_3^--free water (Millipore) and a defined NO_2^--NO_3^--free amino acid rodent mixture (Zeigler Bros., Inc., Gardners, Pa.) (25). Water was changed regularly and food was given daily. To measure NO_3^-, urine was diluted 1/30 to 1/100 with 0.2 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.3]). Frozen aliquots of *P. oleovarans*, a rich source of nitrate reductase (25), were thawed, diluted 1/10 in HEPES, and added (0.25 ml) to 0.75 ml of diluted urine. Mixtures were incubated at 37°C for 90 min in microcentrifuge tubes. Bacteria were pelleted by centrifugation (12,000 × *g*, 2 min) and a 50-μl sample of supernatant was assayed for NO_3^- with the Griess reagents. The concentration of NO_3^- in urine was calculated from a standard curve linear between 1 and 125 μM $NaNO_3$. NO_2^- in culture medium was assayed with the Griess reagents.

The concentration of creatinine in urine was assayed by the alkaline picric acid method (25).

Enumeration of bacteria in tissues. To determine the local effect of BCG and *F. tularensis* on the peritoneal cell population, peritoneal lavage was performed as previously described (23). Cell populations were characterized by differential with Wright-stained cytocentrifuged cell samples. Peritoneal cells were washed and adjusted to 1 × 10^6 macrophages/ml in Dulbecco's modified Eagle's medium (Gibco) with 5% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah). The number of *F. tularensis* organisms associated with these cells at the time of cell harvest was determined by serially diluting them in PBS and plating samples on modified Mueller-Hinton plates. Inoculated plates were incubated for 4 days at 37°C in 5% CO_2. The colonies were counted, and the number of CFU per milliliter was calculated.

Infection and treatment of peritoneal cells for anti-*Francisella* activity in vitro. Macrophages were exposed to *F. tularensis* at a multiplicity of infection of 1:100 (*F. tularensis* to macrophages). To determine infection at time 0, peritoneal cell cultures were lysed with 0.01% sodium dodecyl sulfate (SDS) and suspensions were serially diluted in culture medium and plated on modified Mueller-Hinton plates. The remaining cultures were incubated in medium or treated with dilutions of cytokines, MAbs, and N^GMMLA. After an additional 72 h at 37°C in 5% CO_2, cells were collected, lysed, diluted, and plated as indicated above to determine bacterial CFU (23).

RESULTS

BCG-mediated protection against lethal *F. tularensis* infection. We injected C3H/HeN mice i.p. with 10^6 CFU of BCG or saline 8 days prior to i.p. challenge with different doses of *F. tularensis*. The LD_{50} for saline-treated mice was <10 bacteria. Mice treated with BCG survived challenges with *F. tularensis* in excess of 600,000 bacteria. The LD_{50} of *F. tularensis* in BCG-treated mice was several-hundred-thousand-fold greater than the LD_{50} in normal mice. Inflammation alone, which also occurs during BCG inoculation, was not sufficient to protect against *F. tularensis* infection: five of five mice given Proteose Peptone 3 days prior to challenge with 100 CFU of *F. tularensis* did not survive.

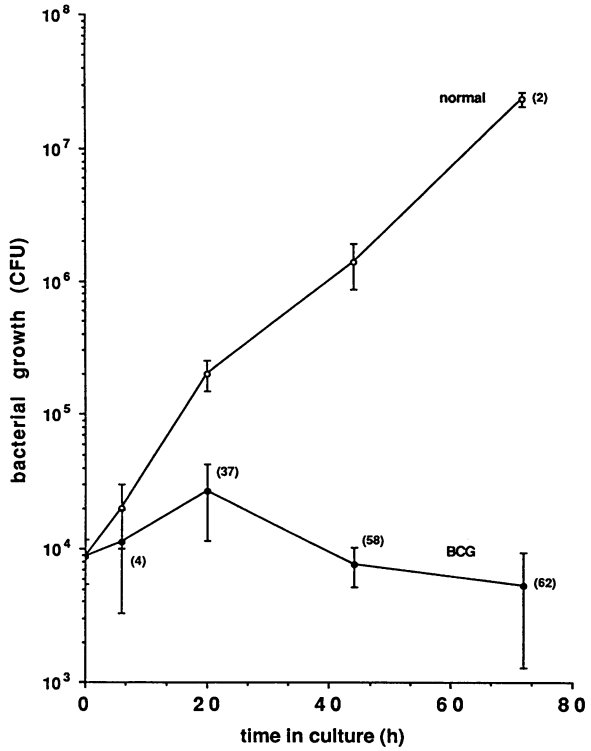

FIG. 1. In vitro NO_2^- production and *F. tularensis* growth in BCG-activated PC. Mice received an i.p. injection of 10^6 CFU of BCG or PBS (normal). Peritoneal cells (5×10^5 macrophages per 0.5 ml per tube) were harvested after 8 days and infected with 8×10^3 *F. tularensis*. Bacterial growth was determined at 6, 20, 43, and 72 h after infection. Values are means ± standard deviations for nine mice. NO_2^- release (nanomoles per 10^6 cells) is denoted in parentheses. Measurement of bacterial growth and NO_2^- release is described in Material and Methods.

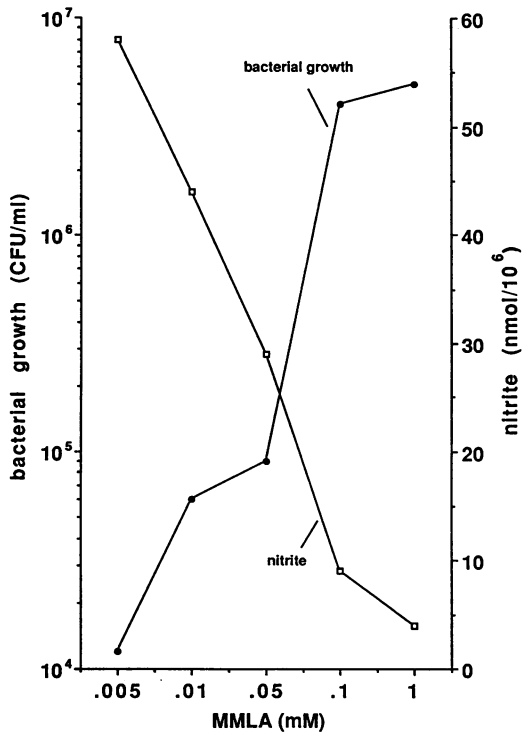

FIG. 2. Effects of N^GMMLA (MMLA) on *F. tularensis* growth and nitrite release by activated PC from BCG-infected mice. Mice were injected i.p. with 10^6 BCG, and after 8 days peritoneal cells were removed. Peritoneal cells were adjusted to 5×10^5 macrophages per 0.5 ml per tube, infected with 1×10^4 *F. tularensis*, and cultured in the presence of various amounts of N^GMMLA. After 72 h, bacterial growth and nitrite production were determined as described in Materials and Methods.

In vitro NO production and inhibition of *Francisella* growth by in vivo activated macrophages. Peritoneal macrophage cells (PC) activated in vivo during infection of mice with BCG produced >60 nmol of NO_2^- per 10^6 macrophages when exposed to *F. tularensis* in vitro, and these cells strongly inhibited the replication of *F. tularensis* (Fig. 1). The increase in numbers of *F. tularensis* recovered from normal cells after 72 h in culture was approximately 3 logs; numbers of *F. tularensis* in cultures of BCG-activated macrophages actually declined compared with the input inoculum (Fig. 1). This suppression required activation of the L-arginine–NO-dependent effector pathway (Fig. 2). The addition of N^GMMLA to cultures of BCG-activated macrophages blocked both NO_2^- and anti-*Francisella* activity in a dose-dependent fashion (Fig. 2).

Effects of MAbs to IFN-γ and TNF-α on BCG-mediated protection and intracellular destruction of *F. tularensis*. PC from mice inoculated 8 days earlier with BCG were treated with MAbs to IFN-γ or TNF-α in vitro and exposed to *F. tularensis* (Table 1). IFN-γ and TNF are both synthesized and released by peritoneal cells from BCG-infected mice; neutralization of either cytokine attenuates cytotoxicity and NO production (5). Consistent with these observations, the addition of either MAb reduced both anti-*Francisella* activity and NO_2^- production (Table 1).

To examine the role of IFN-γ and TNF-α in vivo, we inoculated mice with 10^6 CFU of BCG alone or in combination with 200 μg of anti-IFN-γ, anti-TNF-α, or an isotype-matched control antibody per mouse. We challenged these mice i.p. with graded doses of *F. tularensis* 8 days later (Table 2). Anticytokine MAbs reactive against either IFN-γ or TNF-α completely abolished BCG-mediated protection, whereas the LD_{50} for mice treated with BCG plus control antibodies was similar to that for the mice treated with BCG alone. As an additional control, a neutralizing MAb to another cytokine, IL-4, was also examined. IL-4 is ineffec-

TABLE 1. Treatment of macrophages from BCG-infected mice with anticytokine MAbs in vitro

Treatment group[a]	Bacterial growth (10^3 CFU)[b]	NO_2^- release (nmol/10^6 cells)
PC treated with:		
Medium	6	53
Anti-IFNγ	4,400	8
Anti-TNFα	500	20
Control antibody	3	60
Resident PC	47,000	1

[a] PC (5×10^5 macrophages per 0.5 ml per tube) from mice inoculated i.p. 8 days previously with 10^6 CFU of BCG were cultured with *F. tularensis* (8×10^3 CFU per tube) with or without 20 μg of MAbs per ml.

[b] Bacterial growth and NO_2^- were measured after 72 h, as described in Materials and Methods.

TABLE 2. Determination of *F. tularensis* LD_{50} in BCG-infected C3H/HeN mice

Treatment group	Dilution range (CFU/ml)[b]	LD_{50}[d]
BCG-infected mice[a] treated with:		
PBS alone	10^3–10^7 (5)[c]	630,000
Anti-IFNγ	1–10^3 (5)	4
Anti-TNFα	1–10^3 (5)	3
Anti-IL-4	10^4–10^7 (5)	290,000
Control antibody	10^3–10^7 (3)	400,000
Normal, uninfected mice	1–10^3 (5)	3

[a] Mice were inoculated i.p. with 10^6 BCG in the presence of 100 μl of PBS or PBS containing 200 μg of MAbs to IFN-γ, TNF-α, or hamster IgG (control antibody) or 500 μg of MAbs to IL-4.

[b] Eight days after BCG inoculation, mice were injected i.p. with graduated increases in inoculum size of viable *F. tularensis*.

[c] Numbers in parentheses represent the number of mice per dilution.

[d] Computation of 50% end point titration (LD_{50}) is described in Materials and Methods.

tive at stimulating NO synthesis by macrophages in culture (16), and administration of anti-IL-4 MAbs had little effect on BCG protection (Table 2).

Effects of MAbs to IFN-γ and TNF-α on excretion of urinary NO_3^- by BCG-infected mice. We measured urinary NO_3^- from C3H/HeN mice treated with BCG. Consistent with earlier reports (25, 72), the magnitude of urinary NO_3^- was dependent upon the dose of BCG. The more BCG in the i.p. inoculum, the higher the level of NO_3^- excreted. Levels of urinary nitrate on day 10 after BCG treatment were approximately 2,000 μM (10^5 CFU), 6,500 μM (10^6 CFU), and 17,000 μM (10^7 CFU), compared with 500 μM from mice treated with saline.

To determine whether IFN-γ and TNF-α regulated in vivo production of NO_3^-, we inoculated mice i.p. with 10^6 CFU of BCG alone or in combination with anti-IFN-γ, anti-TNF-α, or a control MAb. Urinary NO_3^- was measured each day after treatment (Fig. 3). Animals inoculated i.p. with 10^6 CFU of BCG alone (Fig. 3A) or with BCG and control antibody (Fig. 3D) began excreting NO_3^- above baseline levels at 3 to 4 days, and peak levels were attained by 8 to 12 days. Levels of urinary NO_3^- were 6 to 10 times greater than those found in normal mice, and urinary NO_3^- persisted at elevated levels for the duration of the experiment (23 days). In contrast, mice treated with BCG and MAbs to either IFN-γ (Fig. 3B) or TNF-α (Fig. 3C) failed to excrete urinary NO_3^- above background levels through 8 days.

The bacterial burden in PC of *F. tularensis*-challenged mice and mean time to death of these animals were determined (Table 3). MAbs to IFN-γ or TNF-α reversed the antibacterial effects of BCG treatment. A single time point was chosen for determination of bacterial growth in the peritoneum. Sixty hours after *F. tularensis* challenge, BCG-treated mice showed a half-log reduction in bacterial burden and survived indefinitely. *Francisella* organisms were not detected in the peritoneum of BCG-treated mice 7 to 9 days after exposure (data not shown). In contrast, mice treated with anti-IFN-γ or anti-TNF-α antibodies showed an approximately 3-log increase in bacterial growth, and these mice died within 5 to 7 days (Table 3). In addition, anti-IFN-γ- and anti-TNF-α-treated mice showed a threefold reduction in urinary NO_3^- excretion compared with the BCG-protected mice at day 8 (Table 3).

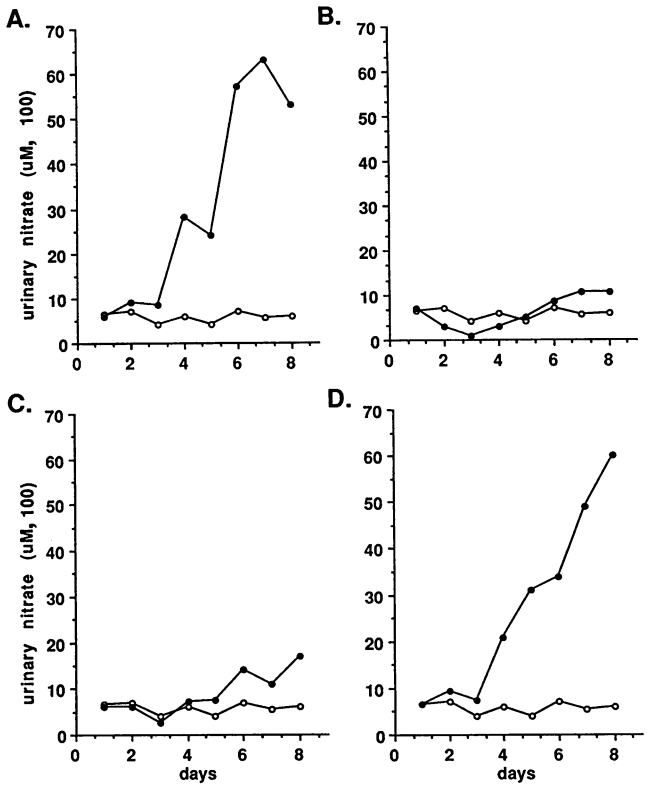

FIG. 3. Effects of anticytokines on urinary nitrate in BCG-infected mice. Mice were injected with PBS (open circles) or 10^6 BCG (closed circles) in the absence (A) or presence of 200 μg per mouse of MAbs to IFN-γ (B), TNF-α (C), or isotype control antibody (D) on day 1. Each value represents urine pooled for that day from 10 mice in each group. Measurement of urinary NO_3^- excretion is described in Materials and Methods.

The inhibitory effects of MAbs to IFN-γ and TNF-α on BCG-induced NO_3^- excretion were temporary and dependent upon both the concentration of the BCG inoculum and the duration of the MAbs in circulation. BCG-infected mice (10^6 CFU) treated with anti-IFN-γ or anti-TNF-α antibodies at the onset of infection significantly delayed excretion of NO_3^- compared with BCG treatment alone (Fig. 4). However, mice evaluated 3 weeks after treatment with BCG plus anti-IFN-γ or anti-TNF-α antibodies produced levels of NO_3^- similar to those produced by mice treated with BCG alone and were protected against a lethal i.p. challenge of 10^3 CFU of *F. tularensis*. Three out of three mice per group survived *F. tularensis* infection (data not shown).

In the previous experiments, administration of anti-IFN-γ and anti-TNF-α antibodies at the time of BCG inoculation most likely affected the induction of immunity to BCG itself; thus, protection against *F. tularensis* by BCG, a downstream event, was affected. We asked whether we could alter the expression of antimicrobial activity at a later stage, after the development of BCG immunity, and at a time we knew BCG treatment would otherwise be protective. Mice were inoculated with BCG and then treated with MAbs to IFN-γ or TNF-α at the peak of urinary NO_3^- excretion (day 8). Levels of urinary NO_3^- dropped precipitously over the next several days in these animals (Fig. 5); moreover, six out of six mice treated with anticytokine antibodies did not survive

TABLE 3. Treatment of BCG-infected mice with anticytokine MAbs and determination of *F. tularensis* growth in vivo and urinary NO_3^-[a]

Treatment group	Bacterial growth (10^3 CFU)[b]	Urinary NO_3^- (10^{-2} μM)[c]	MTTD (days)[d]
BCG-infected mice[d] treated with:			
PBS	2	30 ± 4	—
Anti-IFNγ	6,200	9 ± 3	4.6
Anti-TNFα	400	11 ± 4	7.0
Control antibody	3	36 ± 5	—
Untreated mice (PBS)	410	6 ± 3	4.0

[a] Six HeN mice were inoculated i.p. with 10^6 BCG in the presence or absence of 200 μg of antibodies to IFN-γ, TNF-α, isotype-matched (control) antibody, or PBS alone. Untreated mice are C3H/HeN mice that were injected i.p. with PBS.

[b] Eight days after BCG inoculation, six mice were injected i.p. with 10^3 *F. tularensis*, and three mice were sacrificed 60 h later. To assess bacterial burden, PC were collected and adjusted to 10^6 cells per ml, lysed in 0.01% SDS, and plated. Bacterial growth was determined 72 h later.

[c] Urine was pooled from three to five mice at time of challenge and NO_3^- was measured as described in Materials and Methods.

[d] MTTD (mean time to death) is the number of days to death after i.p. injection of the 10^3-CFU *F. tularensis* challenge (three mice per group). —, survival of 100% of the mice.

a subsequent infection with 10^3 *F. tularensis* on day 10, whereas mice who did not receive anti-IFN-γ or anti-TNF-α antibodies survived the challenge (Fig. 5).

To examine when BCG-treated mice were protected against lethal challenge with *F. tularensis*, we inoculated mice with 10^6 BCG and challenged them with *F. tularensis*

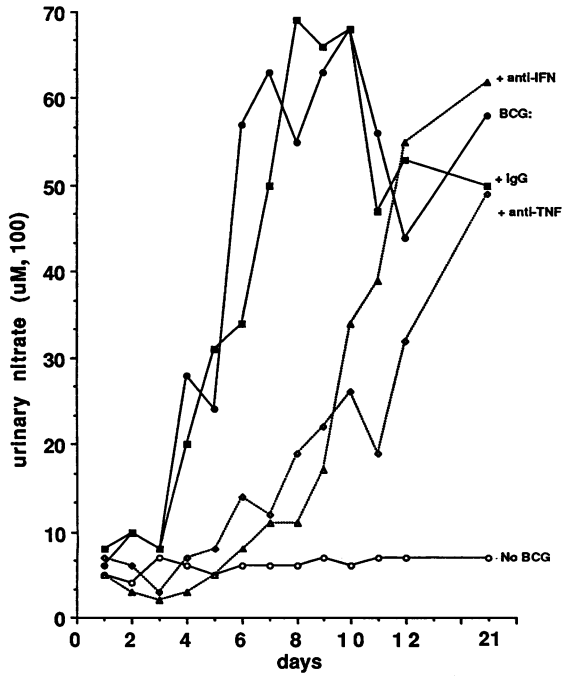

FIG. 4. Evaluation of urinary nitrate levels and protection against *F. tularensis*. Mice were injected with either PBS (No BCG) or BCG in the presence of MAbs to IFN-γ (anti-IFN), TNF-α (anti-TNF), or isotype control antibody (IgG). Each value represents urine pooled for that day from 5 mice in each group.

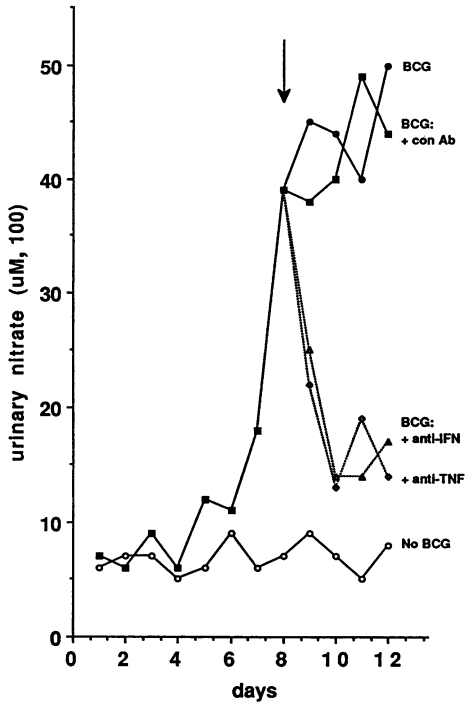

FIG. 5. Effects of MAbs to IFN-γ and TNF-α on BCG-induced urinary NO_3^- excretion after 8 days. Five mice per group were injected with BCG on day 1 and MAbs to IFNγ (anti-IFN), TNF-α (anti-TNF), or isotype control antibody (con Ab) on day 8 (arrow). Control groups consisted of mice which received only PBS (No BCG). Each value represents urine pooled for that day from five mice in each group.

(10^3 CFU) on days 1 to 7 and day 22. As shown in Table 4, BCG-treated mice were protected well before 8 days, and protection correlated with increased urinary NO_3^- excretion. With an approximate twofold increase in urinary NO_3^- levels over the first 3 days after BCG treatment, the mean time to death from *Francisella* infection increased from 4

TABLE 4. Treatment of mice with BCG for protection against *F. tularensis*

In vivo treatment group	Days after treatment[a]	Dead/total	Urinary NO_3^- (10^{-2} μM)[b]	MTTD (days)[c]
BCG-treated mice	0	3/3	6 ± 1	4.7
	1	3/3	7 ± 2	4.0
	2	3/3	9 ± 3	7.0
	3	3/3	10 ± 3	9.3
	4	0/3	21 ± 6	—
	5	0/3	23 ± 8	—
	6	0/3	44 ± 6	—
	7	0/3	52 ± 4	—
	22	0/3	39 ± 7	—
PBS-treated mice	0	3/3	6 ± 2	4.0

[a] C3H/HeN mice were inoculated i.p. with PBS or 10^6 BCG, and three mice each day were challenged i.p. with 10^3 *F. tularensis* on the days indicated.

[b] Urine was collected and pooled daily, and NO_3^- was measured as described in Materials and Methods.

[c] MTTD (mean time to death) is the number of days to death after i.p. injection of 10^3 CFU of *F. tularensis*. — indicates survival.

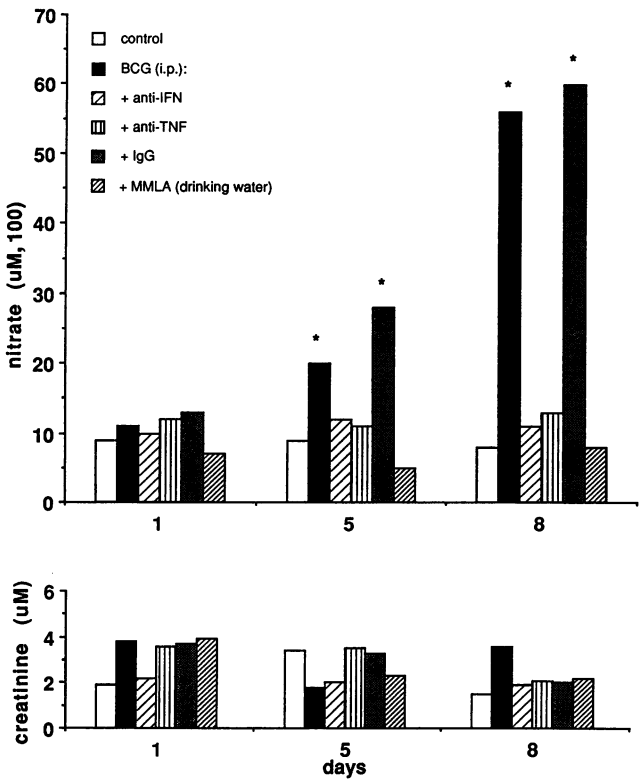

FIG. 6. Effects of anticytokines and N^GMMLA (MMLA) on urinary NO_3^- and creatinine secretion. Mice were injected with PBS (control) or 10^6 BCG in the absence or presence of 200 μg of MAbs to IFN-γ, TNF-α, or control antibody (IgG) per mouse on day 1 or BCG and ingested 50 mM MMLA in the drinking water. Each value represents urine pooled for that day from three mice in each group. Asterisks denote significant difference. Measurement of urinary NO_3^- and creatinine excretion are described in Materials and Methods.

days in mice challenged 1 day after BCG inoculation to 9 days in mice challenged 3 days after BCG inoculation (correlation coefficient = 0.873). On day 4 after BCG exposure, mice were fully protected against an i.p. inoculation of 10^3 CFU of *F. tularensis* and continued to generate greater amounts of NO_3^- in a linear fashion until a plateau was reached by 8 to 12 days.

Effects of N^GMMLA on BCG-mediated protection against *F. tularensis* and elevated levels of urinary NO_3^-. We determined whether the urinary nitrate excreted by BCG-infected mice was derived from L-arginine by administering 50 mM N^GMMLA in the drinking water at the onset of BCG infection or 8 days later. This treatment was as effective in blocking urinary NO_3^- excretion as the anticytokine antibodies were (Fig. 6). As a control for possible kidney failure at the time of infection, which would influence NO_3^- and creatinine secretion, we measured creatinine levels in urine. In contrast to urinary NO_3^- excretion, urinary creatinine levels remained relatively the same throughout the experiment, regardless of treatment (Fig. 6). Thus, changes in urinary NO_3^- were the result of increased production of this nitrogen oxide, and not an artifact of urine concentration resulting from the disease process (25). The combination of N^GMMLA in drinking water and daily i.p. injection of 10 mM N^GMMLA (to neutralize local production of NO in the peritoneum) was sufficient to block BCG-induced protection against *F. tularensis* infection (Table 5). BCG-infected mice who ingested N^GMMLA and received a daily i.p. injection of N^GMMLA did not survive a challenge of 10^3 *F. tularensis*: three out of three mice died. All BCG-infected mice who received 50 mM ammonium acetate in drinking water, as a taste control, and a daily i.p. injection of 10 mM L-arginine survived the challenge.

TABLE 5. Effects of N^GMMLA on BCG-mediated protection against *F. tularensis*

In vivo treatment group[a]	Bacterial growth (CFU/10^6 PC)[b]	Urinary NO_3^- (10^{-2} μM)[c]
BCG-infected mice treated with:		
Control	440	40
L-Arginine	38	47
N^GMMLA	140,000	6
Untreated mice (PBS)	17,000	10

[a] C3H/HeN mice were inoculated i.p. with PBS (untreated mice) or 10^6 BCG (BCG-infected mice). Three groups of BCG-infected mice, consisting of three mice per group received the following: water alone (control), 50 mM ammonium acetate in the drinking water and a daily i.p. injection of 10 mM L-arginine, or 50 mM N^GMMLA acetate and a daily i.p. injection of 10 mM N^GMMLA acetate.

[b] Seven days after BCG exposure, mice were injected i.p. with 10^3 CFU of *F. tularensis*, peritoneal cells were harvested 3 days later, and bacterial burden was determined as described in Materials and Methods.

[c] Urine was collected and pooled daily and NO_3^- was measured as described in Materials and Methods. Each value represents the amount of NO_3^- 7 days after BCG exposure for each group.

DISCUSSION

Protection against lethal infections with *F. tularensis* induced by BCG correlated with the development of activated macrophages secreting toxic nitrogen oxides and with increases in NO_3^- excretion in urine. Moreover, strong evidence for the participation of both IFN-γ and TNF-α in BCG regulation of NO synthase activity and protection against tularemia in vivo was suggested by (i) the effects of in vivo administration of anti-IFN-γ or anti-TNF-α MAbs before infection with BCG—these treatments delayed or suppressed the development of immunity to BCG and blocked induction of BCG-mediated protection against *F. tularensis*, blocked excretion of NO_3^- in urine, and blocked activation of macrophages in the peritoneal cavity, the site of BCG infection; (ii) the effects of anti-IFN-γ or anti-TNF-α MAbs after establishment of BCG-mediated protection—these treatments suppressed both protection and elevated urinary NO_3^- excretion; and (iii) the effects of natural clearance of anti-IFN-γ or anti-TNF-α MAbs over time in vivo—both BCG-mediated protection and the formation of endogenous NO_3^- were restored as the concentration of circulating anticytokine antibodies decreased in the weeks after antibody treatment. The interesting issues that the anticytokine data raise concern the source and function of IFN-γ and TNF-α during the evolution of protective immune reactions.

IFN-γ and TNF-α are produced in response to mycobacterial infection and are clearly involved in the immune response to mycobacterial infection and expression of nonspecific immunity (4, 9, 43). The inability of anticytokine-treated BCG-infected mice to survive lethal i.p. challenge with *F. tularensis* was likely due to a failure to activate

macrophage antimicrobial activities (6, 23). The immunologic stimulus for production of cytotoxic levels of NO in vitro by murine macrophages is the synergistic activity between IFN-γ and exogenous TNF or microbes and microbial products to stimulate endogenous release of TNF-α by macrophages (4, 16, 19, 23, 31, 45, 49, 52, 64, 68); microbe-stimulated TNF acts in an autocrine fashion to amplify the actual synthesis and release of NO by IFN-γ-primed cells (23, 31, 45). The data obtained anti-IFN-γ or anti-TNF-α MAb in BCG-infected mice suggest that a similar synergism exists in vivo: either of the anticytokine MAbs introduced by itself reduced the level of NO_3^- excretion by BCG-infected mice. Consistently, anti-IFN-γ exerted a greater inhibitory effect than anti-TNF, regardless of the dose administrated (data not shown). Complete neutralization of NO by anti-TNF treatment has always been difficult, as we have learned and discussed in our earlier studies on anti-TNF effects in vitro (30).

Previous reports show that the administration of anti-TNF MAbs interferes with the synthesis and secretion of both TNF-α and IFN-γ in vivo. Kindler et al. have shown that TNF accumulates in BCG-induced granulomas and declines as the granuloma regresses in situ (43). Injection of anti-TNF MAbs interfered with granuloma formation, antimycobacterial activity, and accumulation of TNF mRNA and protein. Thus, TNF released by macrophages in a developing granuloma acts in a paracrine fashion to promote TNF production (43), which ultimately influences macrophage antimicrobial activities (11, 33, 34, 43). Consistent with these observations (43), we also observed an absence of BCG-induced microgranuloma formation in the spleen and liver and a corresponding increase in BCG growth in mice treated with anti-TNF-α MAb (data not shown). Aside from a role in perpetuating its own synthesis, Bancroft and associates suggested that TNF-α has also been implicated in the induction of IFN-γ production by NK cells (7, 8). Since it takes several days for antigen-specific α and β T cells to proliferate for effective protection, an early source of IFN-γ is thought to be T-cell independent (7, 8, 20). Both NK cells and activated macrophages, as well as IFN-γ, are present in the peritoneal cavities of *Listeria*-infected mice as early as 2 days after infection (20, 56, 61). Interestingly, TNF production by macrophages infected with *Listeria monocytogenes* is required for IFN-γ production by NK cells obtained from the spleens of SCID mice (8). Exposure to BCG stimulates the early appearance of NK cells (79), and it is possible that NK cells are a source of IFN-γ that contributes to protection against tularemia during the first few days after BCG infection. Complete protection against lethal infections with *F. tularensis* occurred as early as 4 days after BCG exposure (Table 4).

TNF, then, has the potential to operate at multiple levels in vivo as follows. (i) The infectious agent (here, BCG) stimulates TNF synthesis, which in turn triggers IFN-γ production by NK cells. (ii) TNF serves as an accessory amplification factor to trigger IFN-γ-primed macrophages for production of cytotoxic levels of NO and efficient effector activity. (iii) TNF promotes additional TNF production to maintain a heightened and prolonged effector response and the organization of granulomas to isolate infective foci. The cell source(s) and different mechanism(s) of production of these cytokines during the initial phase of infection are not yet elucidated, but the central importance of these two cytokines, TNF-α and IFN-γ, for induction of nonspecific protection and NO synthase activity was clear in our studies.

To completely ablate effector activity with anti-IFN-γ or anti-TNF-α MAb, we had to deliver sufficient quantities of the antibody to the primary site of infection (here, the peritoneum); thus, the relationship between the routes of delivery for anticytokines and BCG is an important consideration (20, 56). Likewise, administration of L-arginine analogs to the site of primary infection was also important. Daily i.p. injection of N^GMMLA after *Francisella* challenge, in conjunction with the drinking water, which contained N^GMMLA, was necessary to reverse BCG-mediated protection. BCG and BCG-activated macrophages remain localized in the anatomical compartment into which BCG is injected (35); thus nonspecific resistance to intracellular pathogens (22) and extracellular tumor targets (35) is most effective when BCG is present at the same site or injected by the same route. In the present studies, additional N^GMMLA in the peritoneum was necessary to suppress anti-*Francisella* activity by the large number of cytotoxic macrophages present as a consequence of BCG infection in this compartment. In a similar fashion, daily intralesional injections of N^GMMLA aggravated cutaneous leishmaniasis in susceptible mice (50). Granger et al. observed that orally administrated N^GMMLA reduced elevated urinary NO_3^-; however, there was no change in mycobacterial burden in the peritoneum (25). In the present study, the need for supplemental i.p. injection may simply have been a matter of overcoming the potency of BCG in activating the high-output (inducible) NO synthase. Therefore, the dose of BCG, the route of administration for BCG, the nature of the BCG infection (i.e., BCG tends to remain localized at the site of BCG infection), and the site of challenge (i.e., *F. tularensis*) may all be important parameters to consider when evaluating this pathway in vivo and when evaluating the ability or inability of N^GMMLA to reduce NO_3^- and effector activity. Interestingly, we did find that both the amount of NO_3^- synthesized and the time at which peak levels are detected depend dramatically on the route of BCG administration. In comparison with other routes, i.p. injection, used in this study, leads to a rapid increase in NO_3^- excretion (days 8 to 10), whereas intravenous injection with BCG resulted in a significantly lower level of NO_3^- which peaked 2 weeks after exposure, and protection against an i.p. challenge with *F. tularensis* correlated with peak levels of NO_3^- excretion. Further studies on the metabolism of N^GMMLA in animals with chronic inflammation are warranted.

A variety of pathogens are susceptible to NO or related nitrogen oxides—*Cryptococcus* (26), *Schistosoma* (41), *Leishmania* (27, 32, 49, 52, 64), *Toxoplasma* (2), *Mycobacterium* (1, 15, 21), *Trypanosoma* (74), *Plasmodium* (53, 57), and *Francisella* (6, 23) spp.—and there are a number of suggested molecular targets and mechanisms of action on these microorganisms. For example, exposure of *Clostridium botulinum* to acidified nitrite results in a loss of iron-sulfur centers with the formation of iron-sulfur-nitrosyl complexes (62); authentic NO or NO generated by activated macrophages interacts with ferredoxin from *Clostridium pasteurianum* (70) and an iron-associated subunit of *Escherichia coli* ribonucleotide reductase (48); oxides of nitrogen diffuse into *P. falciparum*-infected erythrocytes, couple with cysteine or glutathione, and form toxic nitroso groups intracellularly (13)—nitrosothiol groups (i.e., nitroscysteine) are 1,000 times more effective at killing *P. falciparum* in vitro than sodium nitrate or nitrite or NO itself and NO forms other toxic compounds (i.e., alkylating agents, peroxynitrite, hydroxyl radicals) which inhibit the growth of many types of microbes (13, 40). NO may also inhibit microbial growth in a fashion similar to its inhibition of

mammalian cells, including tumors and NO-generating cells themselves (3, 4, 10, 18, 38, 77). Tumor cells cocultured with activated macrophages release iron, which leads to impairment of cellular replication and respiration (17, 18, 36, 38, 73). The suggested molecular targets are non-heme iron-containing ribonucleotide reductase (36, 47), 4Fe-4S prosthetic groups associated with complexes I and II in the electron transport chain (18), and aconitase in the Krebs cycle (17). Fe-S targets have since been confirmed by electron spin resonance spectroscopy studies demonstrating the formation of Fe-S-nitrosyl complexes in activated macrophages (44, 60). Alternatively, NO could also inhibit iron-dependent enzymes, such as the superoxide dismutase which protects certain protozoans (i.e., *Leishmania* spp.) against toxic oxygen species (54).

IFN-γ and TNF-α are principal players in macrophage activation by BCG in vivo, and the protection afforded by BCG against *F. tularensis* challenge ultimately depended on the physiological generation of nitrogen oxides. Although BCG-activated macrophages were the likely source of NO and effector activity in the destruction of *F. tularensis* in vivo, it should be noted that primary murine fibroblast cells can also inhibit *F. tularensis* growth in vitro when treated with IFN-γ and IL-1 (60a). In fact, many cells have the capacity to produce cytotoxic levels of nitrogen oxides: murine macrophages, fibroblasts, hepatocytes, Kupffer cells, endothelial cells, chrondrocytes, and pancreatic islets all produce NO in response to cytokines (4, 10, 12, 14, 42, 69, 77, 78). It will be interesting to definitively pinpoint the source(s) of endogenous NO generated during immune responses to BCG that are protective against *Francisella* challenge.

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