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Dependence of *Mycobacterium bovis* BCG on Anaerobic Nitrate Reductase for Persistence Is Tissue Specific

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***Mycobacterium bovis* BCG, the only presently available vaccine against tuberculosis, was obtained from virulent *M. bovis* after serial passages in vitro. The vaccine strain retained at least some of its original virulence, as it persists in immune-competent hosts and occasionally may cause fatal disease in immune-deficient hosts. Mycobacterial persistence in vivo is thought to depend on anaerobic metabolism, an apparent paradox since all mycobacteria are obligate aerobes. Here we report that *M. bovis* BCG lacking anaerobic nitrate reductase (NarGHJI), an enzyme essential for nitrate respiration, failed to persist in the lungs, liver, and kidneys of immune-competent (BALB/c) mice. In immune-deficient (SCID) mice, however, bacilli caused chronic infection despite disruption of *narG*, even if growth of the mutant was severely impaired in lungs, liver, and kidneys. Persistence and growth of BCG in the spleens of either mouse strain appeared largely unaffected by lack of anaerobic nitrate reductase, indicating that the role of the enzyme in pathogenesis is tissue specific. These data suggest first that anaerobic nitrate reduction is essential for metabolism of *M. bovis* BCG in immune-competent but not immune-deficient mice and second that its role in mycobacterial disease is tissue specific, both of which are observations with important implications for pathogenesis of mycobacteria and vaccine development.**

Mycobacterium tuberculosis claims more human lives each year than any other bacterial pathogen. *Mycobacterium bovis* BCG, the only presently available vaccine against tuberculosis, belongs phylogenetically to the *M. tuberculosis* complex. In humans, *M. bovis* BCG, like *M. tuberculosis*, forms granulomas and abscesses in various tissues. Following vaccination in immune-competent individuals, *M. bovis* BCG may persist for extended periods (34). In immune-compromised individuals the vaccine strain may even lead to fatal disease (2, 3, 10, 11, 14, 21, 27, 30, 36, 39).

Mycobacteria become firmly established within host tissues, adapting their metabolism to the available source of carbohydrates, nitrogen, and energy (4). Although the acquisition of essential nutrients by mycobacteria is an area of considerable interest, our knowledge of bacterial metabolism throughout the course of infection remains rudimentary. A recent study revealed that metabolism of fatty acids serves as a source of carbohydrates and is required for persistence of *M. tuberculosis* in mice and activated macrophages (25). Nitrate, through nitrate respiration, could provide energy for bacterial metabolism in an anaerobic environment, because anaerobic nitrate reductase (NarGHJI) couples the reduction of nitrate (NO_3) to the generation of ATP by replacing oxygen as a terminal electron acceptor (29). Anaerobic nitrate reductase coding sequences (*narGHJI*) have been identified in both obligate aerobes such as *Bacillus* and *Pseudomonas* and facultative anaerobes such as *Escherichia coli* (5, 17, 28). However, a role of this enzyme in virulence was not established. In mycobacteria, a gene cluster homologous to *narGHJI* of *Bacillus subtilis* was first identified in the course of the *M. tuberculosis* genome project (12).

Previous studies of mycobacterial nitrate reduction have been limited to its role in classification and identification of the genus *Mycobacterium*, after an extensive study 40 years ago showed that *M. tuberculosis* reduces nitrate to nitrite, whereas *M. bovis* and *M. bovis* BCG have no discernible nitrate reductase activity (7, 35). Only recent reports revived interest in a possible physiological role by showing upregulation of enzymes involved in nitrate metabolism of *M. tuberculosis* and *M. bovis* BCG under oxygen restriction in vitro (19, 20, 37). We recently showed that both *M. tuberculosis* and *M. bovis* BCG express an anaerobic nitrate reductase (NarGHJI) activity and that a Δ*narG M. bovis* BCG mutant lacked the ability to reduce nitrate under anaerobic conditions (38). The purpose of this study was to define the role of anaerobic nitrate reductase in the pathogenesis of *M. bovis* BCG.

MATERIALS AND METHODS

Bacteria. *M. bovis* bacillus Calmette-Guérin (BCG) Pasteur (Pasteur vaccine strain; Statens Serum Institute, Copenhagen, Denmark) was used in this study. 7H9 broth or 7H10 plates (Difco Laboratories, Inc., Detroit, Mich.) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADS (0.5% bovine albumin fraction V, 0.2% glucose, 140 mM NaCl) were used for culturing of mycobacteria unless indicated otherwise. Deletion of a 700-bp fragment of *narG* through allelic exchange has been described elsewhere (38).

In vitro tests. Strains were grown in 7H9 broth supplemented as indicated above for 5 to 7 days to an optical density at 600 nm of 0.7 and were washed twice with MB medium supplemented as indicated below, but without nitrate. For persistence in vitro under anaerobic conditions, strains were kept in an anaerobic jar (anaerobic conditions were achieved with the Anaero*Gen* anaerobic system from Oxoid Ltd., Basingstoke, Hampshire, England) in MB medium with 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2% glycerol, 0.05% Tween 80, 10% ADS, and 10 mM NO_3 (1 liter of MB medium contained 1 g of KH_2PO_4, 2.5 g of Na_2HPO_4, 2.0 g of K_2SO_4, and 2 ml of trace elements; 1 liter of trace elements contained

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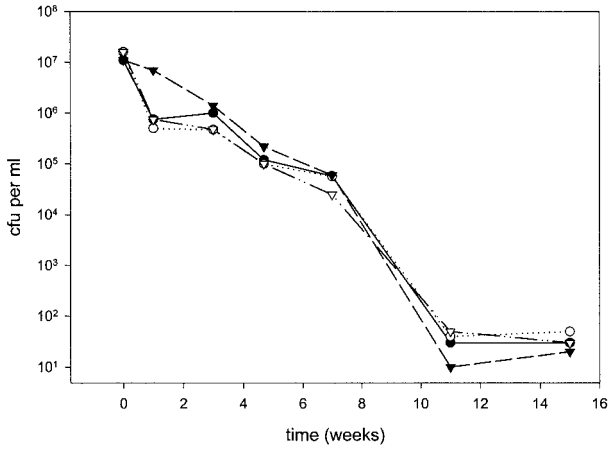

FIG. 1. Wild-type (closed symbols) and Δ*narG* mutant (open symbols) bacteria were enumerated by plating for counts of CFU. Bacteria were cultured in medium without nitrate (circles) and with nitrate (triangles) under anaerobic conditions.

40 mg of $ZnCl_2$, 200 mg of $FeCl_3 \cdot 6H_2O$, 10 mg of $CuCl_2 \cdot 4H_2O$, 10 mg of $MnCl_2 \cdot 4H_2O$, 10 mg of $Na_2B_4O_7 \cdot 10H_2O$, and 10 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$). As recommended by the manufacturer, an indicator strip was used to confirm anaerobic conditions. Bacteria were cultivated in medium without nitrate, with 10 mM nitrate, or with 10 mM nitrate plus 10 mM ammonia. At various time points, an aliquot was plated for counts of CFU.

Infection of BALB/c and SCID mice. BALB/c and SCID mice (6 to 10 weeks old) were obtained from the Tierlaboratorium of the Medical School of Hannover (Hannover, Germany). Prior to infection of mice *M. bovis* BCG and Δ*narG M. bovis* BCG were grown in 7H9 broth supplemented with Tween and ADS as described above. The infecting dose of 10^6 bacilli per mouse was established by plating aliquots for colony counts and confirmed by plating again immediately after injection of mice. Intravenous infection of mice was performed through needle puncture of the tail vein. Mice were anesthetized with ether and sacrificed by cervical dislocation. Organs were harvested at the indicated time and were homogenized in phosphate-buffered saline with 0.05% Tween 80 using a homogenizer (Ultra-Turrax T8; IKA-Werke, Staufen, Germany), diluted, and plated on 7H10 supplemented with ADS as indicated above. Three mice were sacrificed for each time point in experiments with BALB/c and SCID mice. For the last time point in experiments with SCID mice organs from three mice were harvested after mice succumbed to the infection. Animal work in this article has been approved by the ethics committee of the county government of Hannover, Germany, on 18 March 1999 (reference number 509c42502-99/163).

Histology. Small pieces of lungs and liver were fixed for 24 h in 10% buffered (pH 7.0) formalin and embedded in paraffin. Sections of 3 to 4 μm (thickness) were cut with steel knives using an ultramicrotome. The sections were stained for acid-fast bacteria with Ziehl-Neelsen stain and counterstained with hematoxylin.

RESULTS

Persistence of wild-type and Δ*narG* strains of *M. bovis* BCG under anaerobic conditions in vitro. In one study, no difference in survival under hypoxic conditions was found between *M. tuberculosis* cells cultured in medium with nitrate and those in medium without nitrate (37). Therefore, we cultured wild-type and Δ*narG* strains of *M. bovis* BCG in medium with nitrate and without nitrate under anaerobic conditions (Fig. 1). The mutant and the wild type were phenotypically indistinguishable, and bacterial viability gradually declined. After 15 weeks, cultures were almost sterile. We added ammonia as a second source of nitrogen to cultures supplemented with nitrate and repeated the experiments. Again, mutant and wild-type cells remained phenotypically indistinguishable (data not shown).

Histopathology of the lung of SCID and BALB/c mice infected with a Δ*narG* mutant of *M. bovis* BCG. To define the role of anaerobic nitrate reductase in metabolism of *M. bovis* BCG in vivo, immune-deficient SCID mice and immune-competent BALB/c mice were infected with wild-type and Δ*narG* mutant strains of *M. bovis* BCG. After 14 weeks SCID mice infected with the *M. bovis* BCG wild type succumbed to the infection. Histology revealed large coalescing lesions teeming with acid-fast bacilli replacing the entire lung and it seemed likely that mice had suffered fatal pulmonary failure (Fig. 2a). Mice infected with the Δ*narG* mutant succumbed to the infection after 37 weeks ($P < 0.05$ for difference of survival by log rank test). Histology of lungs showed multiple small lesions filled with acid-fast bacilli scattered within healthy tissue (Fig. 2b). The results obtained from SCID mice suggested that despite pulmonary inflammation, the mutant was attenuated, as tissue destruction in the lung was substantially reduced and mice lived significantly longer. However, the mutant was obviously not avirulent. It remained unclear why mice infected with the mutant strain eventually developed a fatal disease. In immune-competent mice, the ability of the mutant to cause tissue distruction was profoundly different; in fact, the mutant was avirulent. Histology showed substantial tissue destruction in lungs of mice infected with the BCG wild type (Fig. 2c), but there were no signs of tissue damage in mice infected with the mutant (Fig. 2d) at 31 weeks postinfection. Thus, although *M. bovis* BCG lacking anaerobic nitrate reductase was attenuated in both immune-deficient and immune-competent mice, it was avirulent only in mice capable of generating a specific immune response, suggesting that the role of anaerobic nitrate metabolism in the pathogenesis of *M. bovis* BCG was linked to the immune response of the host.

Bacillary load of Δ*narG* strains of *M. bovis* BCG in lungs, liver, and kidneys of mice. We further examined the phenotypic difference of wild-type and Δ*narG* strains of *M. bovis* BCG in immune-deficient and immune-competent mice by comparing the bacillary load in various organs. Immune-deficient SCID mice were infected by intravenous infection with 10^6 bacteria. The *M. bovis* BCG wild-type strain grew progressively in the lungs, liver, and kidneys. In comparison, mice infected with the mutant showed little growth of bacilli, yet we observed no reduction of bacillary count at any time during the experiment (Fig. 3). On the one hand, this could represent a dynamic steady state in which the rate of bacillary killing just balances multiplication; on the other hand, it could represent a static phase in which bacilli divide seldom or never. Whatever the mechanism is, anaerobic nitrate reductase, although it facilitates progressive growth of bacilli, is not essential for metabolism of *M. bovis* BCG in the immune-deficient host. Immune-competent BALB/c mice were also infected intravenously with 10^6 bacteria. At all times during the experiment, mice showed no signs of clinical disease. The *M. bovis* BCG wild-type strain persisted in the lungs, liver, and kidneys. In comparison, the Δ*narG* mutant was gradually cleared from these organs (Fig. 4). These results suggested that anaerobic nitrate reduction is essential for metabolism of *M. bovis* BCG in immune-competent, but not immune-deficient, mice.

Growth and persistence of wild-type and Δ*narG* strains of *M. bovis* BCG in the spleens of mice. Interestingly, the mutant was not attenuated in the spleens of infected mice. In immune-

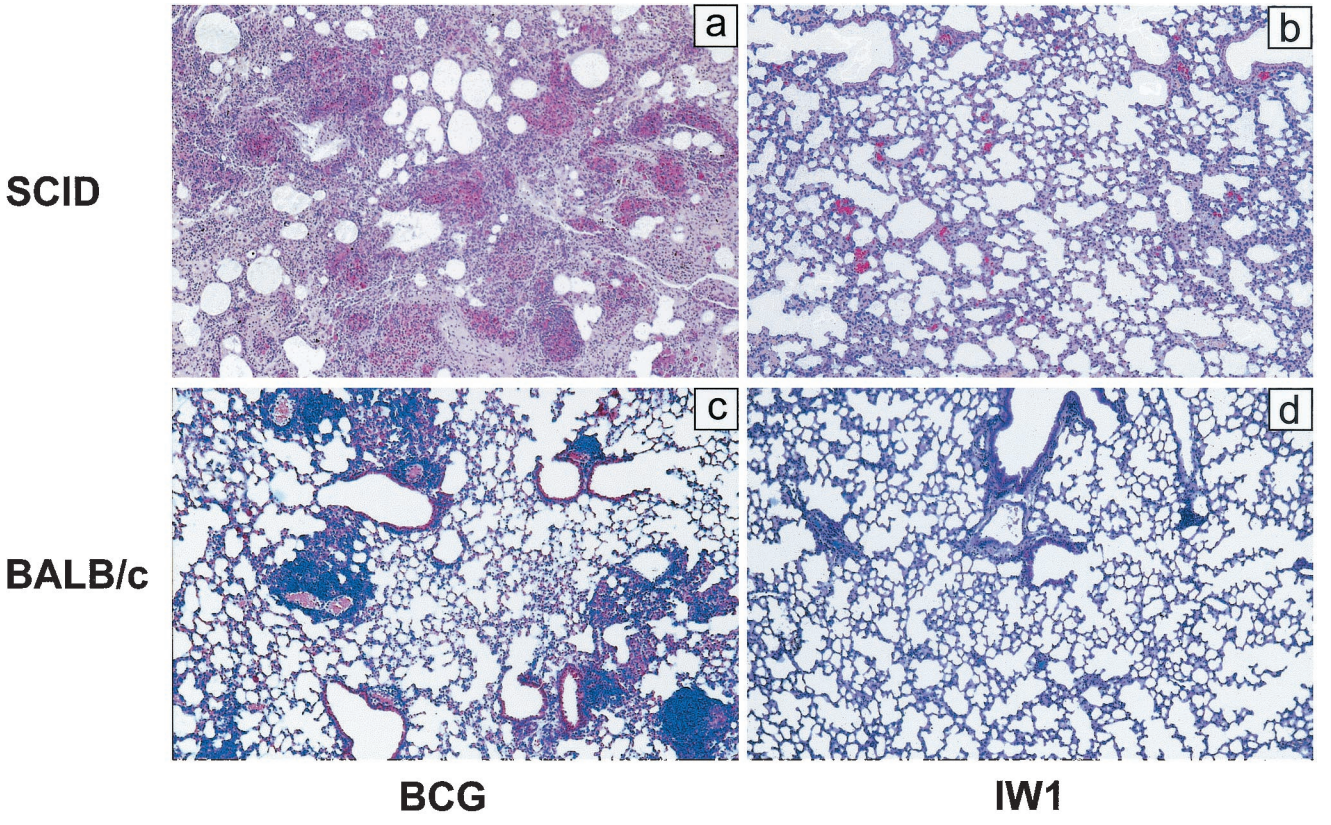

FIG. 2. Histology of the lungs of SCID mice (a and b) and BALB/c mice (c and d). Mice were infected with 10^6 CFU of wild-type (a and c) or Δ*narG* (IW1) (b and d) bacteria. SCID mice succumbed to the infection after 14 weeks (a) and 37 weeks (b), whereas BALB/c mice were sacrificed 31 weeks after infection (c and d) and sections of the lung were stained with Ziehl-Neelsen and counterstained with hematoxylin.

deficient SCID mice the mutant and the wild type grew at the same rate, eventually reaching the same plateau (Fig 5a). Equal rates of multiplication were also seen when mice were infected with 10^4 bacteria (Fig. 5b). In immune-competent BALB/c mice wild-type and Δ*narG* strains of *M. bovis* BCG persisted throughout the experiment (Fig. 5c). The spleen was enlarged both in mice infected with the wild type and in those infected with the Δ*narG* mutant (data not shown). Thus, in the spleen of both immune-competent and immune-deficient mice *M. bovis* BCG lacking anaerobic nitrate reductase activity was phenotypically indistinguishable from the *M. bovis* BCG wild type.

DISCUSSION

For understanding mycobacterial pathogenesis, the presence of essential nutrients in the host and their metabolism by the pathogen remains an area of considerable interest. This study suggests that *M. bovis* BCG uses nitrate as a key nutrient for pathogenesis, supporting bacterial metabolism in the lungs, liver, and kidneys through reduction of nitrate to nitrite. Although we observed little expansion of the Δ*narG M. bovis* BCG mutant in the lungs, liver, and kidneys of immune-deficient mice over time, the infection was not cleared from these organs, leading to a chronic infection. In immune-competent mice, by comparison, the mutant was unable to sustain an infection of these organs. Surprisingly, in the spleen anaerobic nitrate reduction appeared to be dispensable for mycobacterial metabolism. These results suggest first that anaerobic nitrate reduction is essential for metabolism of *M. bovis* BCG in immune-competent but not immune-deficient mice and second that its role in pathogenesis is tissue specific.

Dissemination and subsequent persistence of bacteria are hallmarks of an infection with *M. tuberculosis*, accounting for reactivation of a latent focus and symptomatic disease later in life. Similarly *M. bovis* BCG may lead to dissemination and persistence after vaccination of infants, even of those with an intact immune system. In fact, dissemination of *M. bovis* BCG is a normal sequel as shown by autopsies on *M. bovis* BCG-vaccinated infants and children who died of unrelated causes; histological examination of tissues demonstrated granulomas in many distant organs, including lungs, liver, kidneys, and spleen. Organisms were found up to 3 years after vaccination without evidence of clinical disease (16, 34). Moreover, reactivation of *M. bovis* BCG in a patient with AIDS 30 years after vaccination has been reported (33). In the mouse model, "persistence" has been defined as the time when the number of bacteria has reached a plateau, despite numerous culturable bacilli (24). McKinney and his colleagues (25) demonstrated that isocitrate lyase, an essential enzyme for catabolism of fatty acids, is required for persistence of *M. tuberculosis* during the chronic phase of infection and that this requirement was dependent on an intact immune response of the host. An intact immune response leads to the formation of granulomas, which

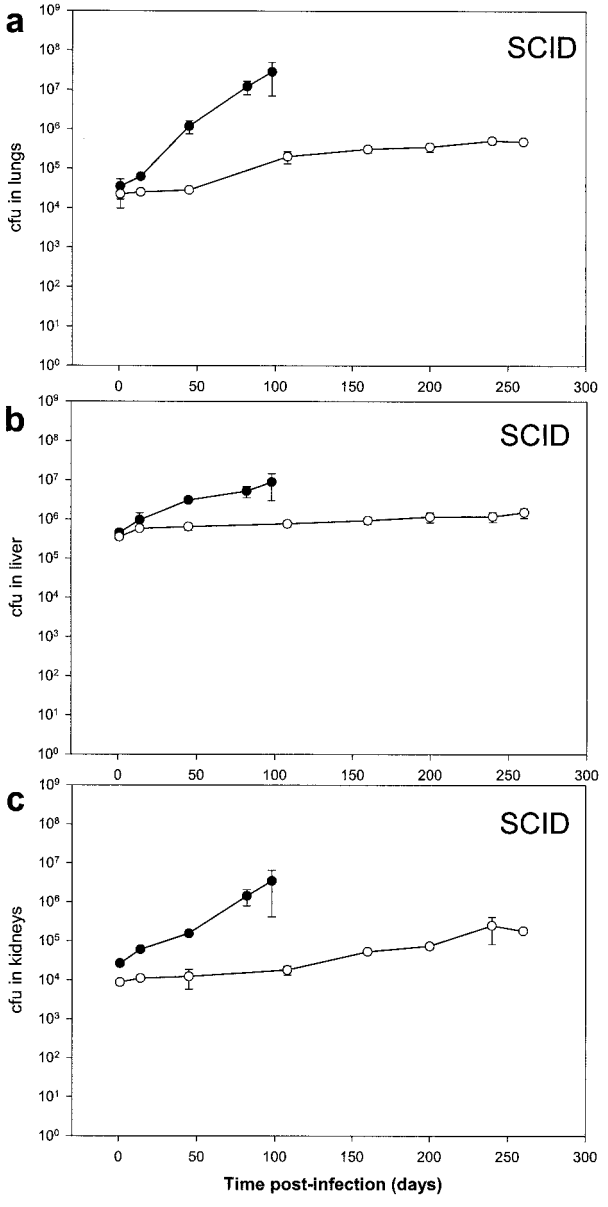

FIG. 3. Bacteria in the lungs (a), livers (b), and kidneys (c) of intravenously infected SCID mice were enumerated by plating for counts of CFU. Bacterial loads in mice infected with 10^6 CFU of Δ*narG* (open circles) or wild-type (closed circles) bacteria are shown.

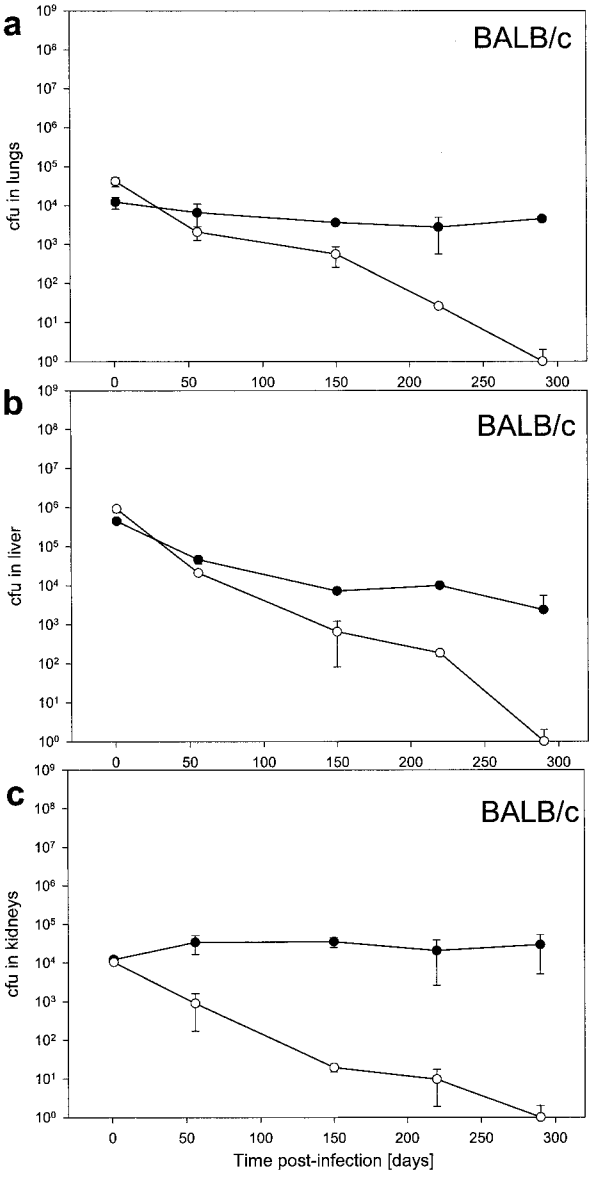

FIG. 4. Bacteria in the lungs (a), livers (b), and kidneys (c) of intravenously infected BALB/c mice were enumerated by plating for counts of CFU. Bacterial loads in mice infected with 10^6 CFU of Δ*narG* (open circles) or wild-type (closed circles) bacteria are shown.

creates a hostile environment in which restricted access to nutrients and reduced oxygen tension may force mycobacteria to adapt their metabolic activity (4). Our results show that anaerobic nitrate reduction is required for persistence of *M. bovis* BCG in the lungs, liver, and kidneys and that this requirement is dependent on an intact immune response of the host, suggesting that nitrate might play an important role in mycobacterial persistence. Polar effects on the expression of genes other than the one targeted by specific deletion have not been documented for mycobacteria. Nonetheless, in future experiments we will address the interesting issue of whether altered expression of yet unknown genes contributes to the phenotype of the *narG* mutant by complementation studies using, for example, a single-copy integrating vector for rescue of the mutant phenotype in vivo.

Apparently nitrate is sufficiently provided in the lungs, liver, and kidneys of infected animals. The reported estimates of net nitrate synthesis by mammalian tissue vary greatly and range from 0.15 to 1 mM day^{-1} (22). Within tissue, nitrate is mainly a product of spontaneous degradation of nitric oxide. Nitric oxide, in contrast, is produced enzymatically by three different nitric oxide synthetases (32). An inducible nitric oxide synthetase is expressed in response to inflammatory and proinflammatory mediators (6). A variety of cells, including hepatocytes, can be induced to synthesize nitric oxide (9). Significant amounts of nitrate are detected in the urine of mice infected with bacteria, suggesting that nitrate is available in the

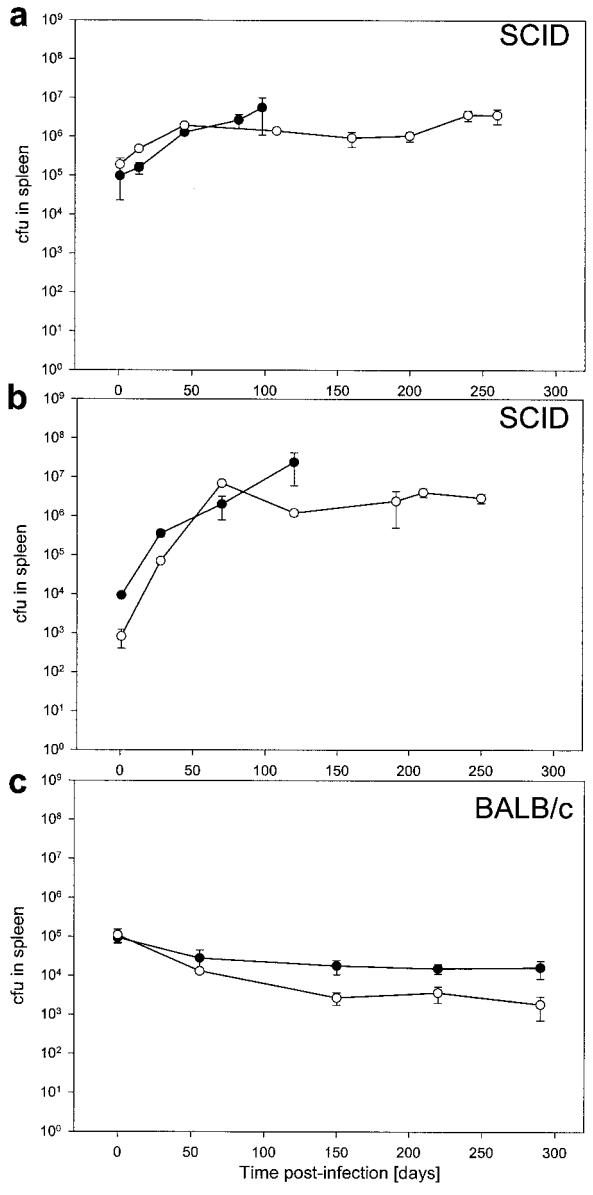

FIG. 5. Bacteria in the spleens of intravenously infected SCID (a and b) and BALB/c (c) mice were enumerated by plating for counts of CFU. Bacterial loads in mice infected with Δ*narG* (open circles) or wild-type (closed circles) bacteria are shown. Mice were infected with 10^6 CFU (a and c) or 10^4 CFU (b) of bacteria.

kidney, especially in animals undergoing an inflammatory process (8, 15). It is intriguing to speculate that the inflammatory process due to mycobacterial infection in the lungs, liver, and kidneys might increase the amount of nitrate within granulomas and thereby provide an additional supply of this nutrient for anaerobic metabolism of the pathogen. Quite unexpected was the observation that anaerobic nitrate reductase activity was dispensable for metabolism of *M. bovis* BCG in the spleens of infected mice, at least for the first 250 days of infection. We do not believe that this discrepancy occurred simply due to a better supply of the spleen with oxygen, since compared to the lung, oxygen tension of the spleen is presumably lower. In the spleen, for some reason, production of nitrate might be reduced or absent, or the organ simply provides a different set of nutrients, including a component that could replace nitrate as a substrate for mycobacterial metabolism in vivo. Or there could be a second, possibly weak nitrate reductase activity of the mutant which might be sufficient for anaerobic metabolism in the spleen and which might also account for persistence of the *narG* mutant in SCID mice.

To our knowledge, gene disruption resulting in a selectively attenuated mutant has not been described for mycobacteria. In a practical sense, dissociation of mycobacterial nitrate metabolism between the spleen and other organs might prove beneficial to the development of *M. bovis* BCG as a safe live vaccine. Survival and growth of *M. bovis* BCG is necessary for eliciting protective immune responses, as early treatment of infected mice with isoniazid to inhibit bacillary growth prevents the development of effective acquired resistance. Similarly, vaccination with nonliving or nonreplicating bacilli is less effective than vaccination with bacilli capable of in vivo growth (see reference 26 and references therein). Moreover, *M. bovis* BCG has been proposed as a multivalent vaccine vehicle, delivering viral, bacterial, or protozoan antigens (1, 13, 18, 23, 31). A Δ*narG M. bovis* BCG or a recombinant derivative expressing additional antigens, by selectively targeting the spleen and thereby inducing a protective immune response, might reduce the risk for developing disseminated disease with the vaccine strain, especially in immune-compromised individuals.

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