INFECTION AND IMMUNITY, Sept. 2002, p. 4826–4832 Vol. 70, No. 9
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.9.4826–4832.2002

virB-Mediated Survival of *Brucella abortus* in Mice and Macrophages Is Independent of a Functional Inducible Nitric Oxide Synthase or NADPH Oxidase in Macrophages

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Received 19 April 2002/Returned for modification 28 May 2002/Accepted 6 June 2002

The *Brucella abortus virB* locus is required for establishing chronic infection in the mouse. Using in vitro and in vivo models, we investigated whether *virB* is involved in evasion of the bactericidal activity of NADPH oxidase and the inducible nitric oxide synthase (iNOS) in macrophages. Elimination of NADPH oxidase or iNOS activity in macrophages in vitro increased recovery of wild-type *B. abortus* but not recovery of a *virB* mutant. In mice lacking either NADPH oxidase or iNOS, however, *B. abortus* infected and persisted to the same extent as it did in congenic C57BL/6 mice up until 60 days postinfection, suggesting that these host defense mechanisms are not critical for limiting bacterial growth in the mouse. A *virB* mutant did not exhibit increased survival in either of the knockout mouse strains, indicating that this locus does not contribute to evasion of nitrosative or oxidative killing mechanisms in vivo.

Brucella species are facultatively intracellular pathogens whose host range includes livestock, wild animals, and humans. Following infection of susceptible hosts, bacteria localize to tissues of the reticuloendothelial system and survive intracellularly within professional macrophages. The mechanisms by which brucellae are able to avoid killing by macrophages are unknown. The ability of *Brucella abortus* to persist in the macrophage suggests that it is able to avoid or withstand phagocyte killing mechanisms, such as lysosomal enzymes and products of the oxidative burst (3).

The *B. abortus virB* locus, a region on chromosome II with homology to type IV secretion systems, is required for establishing and maintaining persistent infection in the mouse model (11, 22). This locus has also been found to be required for intracellular survival in macrophages and HeLa cells (17, 30, 39). Recent evidence suggests that the *virB* locus mediates secretion of an unknown effector(s) required for establishment of an intracellular compartment favorable for bacterial replication in HeLa cells (5, 10). This hypothesis is supported by the finding that expression of the *Brucella suis virB* operon is induced intracellularly (4). To determine whether *B. abortus* is able to avoid intracellular degradative pathways, the trafficking of *B. abortus* was studied in nonphagocytic cells (12–14, 32–35). Results of these studies suggest that *B. abortus* avoids fusion with lysosomes and localizes to a compartment containing proteins characteristic of rough endoplasmic reticulum and autophagosomes.

Studies of trafficking in macrophages suggest that the intracellular fate of *B. abortus* in these cells appears to be distinct from the well-described localization in nonphagocytic cells (2). Recent work suggests that *B. abortus* and *B. suis* survive in a compartment that acidifies but has delayed fusion with lysosomes (2, 37). Thus, as in nonphagocytic cells, *Brucella* appears to delay or modify the maturation of its vacuolar compartment. In addition, *B. abortus* has been found to inhibit bactericidal functions of phagocytes, including phagolysosomal fusion, neutrophil degranulation, and the oxidative burst (2, 3, 28).

Brucella spp. share the ability to modulate the composition of intracellular compartments with several other intracellular bacterial pathogens, including *Legionella*, *Mycobacterium*, and *Salmonella*. *Salmonella enterica* serovar Typhimurium prevents assembly of the NADPH oxidase complex on phagosomes, which allows it to avoid the toxic products of the oxidative burst, and this activity is dependent on the *Salmonella* pathogenicity island 2 type III secretion system (40). Interestingly, an ultrastructural study examining the localization of NADPH oxidase in *B. abortus*-infected macrophages found that vacuoles containing *B. abortus* tended not to be associated with NADPH oxidase (18), suggesting that *B. abortus* might employ similar mechanisms for survival within phagocytes. In the present study, we sought to determine whether the *virB* locus contributes to evasion of NADPH oxidase- and inducible nitric oxide synthase (iNOS)-mediated control of bacterial growth during *B. abortus* infection of cultured macrophages and of mice.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains which we used are *B. abortus* 2308 and its isogenic mutant BA41, which has an insertion of mTn*5*Km-2 at nucleotide 1232 of the *B. abortus virB* locus (GenBank accession number AF226278). This insertion is located 59 bp downstream of the *virB1* gene and is polar on the expression of downstream genes in the *virB* operon (data not shown). Strains were cultured on tryptic soy agar (TSA) (Difco/Becton-Dickinson, Sparks, Md.) or in tryptic soy broth at 37°C on a rotary shaker. Bacterial inocula used for infection of mice were cultured on potato infusion agar, as growth on this medium prevents appearance of spontaneous rough mutants (1).

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Antibiotics, when required, were added at the following concentrations: carbenicillin, 100 mg/liter; kanamycin, 100 mg/liter; and chloramphenicol, 5 to 30 mg/liter. All work with live *B. abortus* was performed at biosafety level 3.

Cell lines. Two clones of the mouse macrophage-like cell line J774, J774.16 and J774.D9, were cultured in Dulbecco's modified Eagle's medium (Gibco, Rockville, Md.) supplemented with 10% heat-inactivated equine serum, 1% nonessential amino acids, and 1 mM glutamine (DMEMsup). The ability or inability of these cell lines to generate oxygen radicals via the hexose monophosphate shunt (oxidative burst) after induction with phorbol myristyate acetate (Sigma) was confirmed as described elsewhere (9, 19).

For macrophage killing assays, 24-well microtiter plates were seeded with macrophages at a concentration of 5×10^5 cells/well in 0.5 ml of DMEMsup and incubated overnight at 37°C in the presence of 5% CO_2. For experiments with activated macrophages, 10 U of recombinant murine gamma interferon (IFN-γ) (Endogen, Woburn, Mass.) per ml was added to the medium 24 h before infection, and this concentration of IFN-γ was maintained throughout the assay by replacing the medium every 24 h. Bacteria were grown for 48 h and then diluted in DMEMsup, and about 5×10^7 bacteria in 0.02 ml of DMEMsup were added to each well containing macrophages. The microtiter plates were centrifuged at $250 \times g$ for 5 min at room temperature in order to synchronize the infections. Cells were incubated for 20 min at 37°C in the presence of 5% CO_2, free bacteria were removed by three washes with phosphate-buffered saline (PBS), and the zero-time samples were taken as described below. The washing solutions were collected, and extracellular bacteria were quantified by dilution in sterile PBS and plating on TSA. DMEMsup containing 50 μg of gentamicin per ml was added to the wells, and the cells were incubated at 37°C in the presence of 5% CO_2. After 1 h, the DMEMsup containing 50 μg of gentamicin per ml was replaced with medium containing 25 μg of gentamicin per ml. Wells were sampled at zero time and 24 and 48 h after infection by aspirating the medium, lysing the macrophages with 0.5 ml of 0.5% Tween 20, and rinsing each well with 0.5 ml of PBS. Viable bacteria were quantified by dilution in sterile PBS and plating on TSA. All experiments were performed independently in triplicate at least three times, and the standard error for each time point was calculated.

Isolation and infection of mouse peritoneal macrophages. As a previous study revealed that only low levels of NO were produced by proteose peptone-elicited murine peritoneal macrophages infected with *B. abortus*, we used a method that allowed us to obtain macrophages that produced both iNOS and NADPH oxidase (24). Elicited peritoneal macrophages were isolated from C57BL/6 mice by peritoneal lavage 4 days after injection of 5 mM sodium periodate, which stimulates both the phagocyte respiratory burst and inducible NO synthase (15). Cells were seeded into 96-well plates at a concentration of 3×10^5 cells/well and allowed to adhere to the plastic overnight. For activation, cells were incubated overnight in the presence of 20 U of murine recombinant interferon (IFN-γ; Endogen) per ml and 1 pg of *S. enterica* serovar Typhimurium lipopolysaccharide (LPS) (Sigma, St. Louis, Mo.) per ml. Cells were used for macrophage killing assays as described above for macrophage cell lines. For inhibition of iNOS, 250 μM N^G-L-monomethyl arginine (MMLA) (Sigma) was maintained in the tissue culture medium throughout the assay. NO production by peritoneal macrophages was assayed by incubating macrophage supernatants with an equal volume of Griess reagent (Sigma) and measuring absorbance at 570 nm to determine the nitrite concentration.

Infection of mice. All mice used in this study were derived from the C57BL/6 strain. C57BL/6 mice and C57BL/6-Cybb^{tm1} mice carrying a targeted knockout of the gene encoding gp91 (gp91^{phox}) of NADPH oxidase (36) were obtained from Jackson Laboratory (Bar Harbor, Maine). C57BL/6 Ai-[KO]iNOS N5 mice carrying a targeted knockout of the *Nos2* gene encoding iNOS were obtained from Taconic (Germantown, N.Y.) (26). Mice were kept in microisolator cages with sterile bedding and water and irradiated feed in a biosafety level 3 facility. For infection experiments, groups of five knockout mice and age-matched controls were inoculated intraperitoneally (i.p.) with 0.2 ml of PBS containing 5×10^5 to 10×10^5 CFU of *B. abortus*. At the appropriate times, mice were euthanized by CO_2 asphyxiation, and the spleens and livers were collected aseptically at necropsy. A portion (one-quarter) of the spleen and the liver of each mouse were fixed in phosphate-buffered formalin and processed for sectioning and staining with hematoxylin and eosin. The remaining three-quarters of the spleen was homogenized in 3 ml of PBS, and serial dilutions of the homogenate were plated on TSA and TSA containing kanmycin for enumeration of CFU. The competitive index was calculated by dividing the mean ratio of mutant CFU to wild-type CFU recovered from spleens by the ratio of the mutant CFU to the wild-type CFU in the inoculum. The experimental groups each contained four or five knockout mice and four or five age-matched controls that were 6 to 10 weeks old. Hematoxylin- and eosin-stained sections of the spleen and liver from each mouse were coded and scored for pathological changes by a veterinary pathologist. All animal experiments were approved by the Texas A&M University Laboratory Animal Care and Use Committee and were conducted in accordance with institutional guidelines.

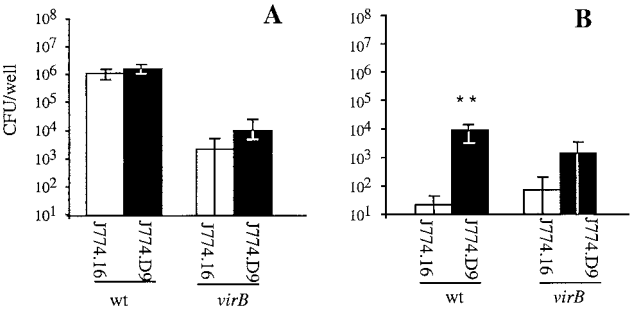

FIG. 1. Survival of *B. abortus* 2308 and isogenic *virB* mutant BA41 in J774.16 cells (open bars), which generate an oxidative burst upon activation, and J774.D9 cells (solid bars), which lack a complete NADPH oxidase. Cells were incubated for 24 h without IFN-γ (A) or with IFN-γ (B) and subsequently were infected with *B. abortus* 2308 or BA41. The numbers of intracellular CFU of *B. abortus* were determined 48 h postinoculation. Each bar indicates the mean ± standard deviation for a total of six data samples (from two independent experiments, each containing triplicate samples). Data were analyzed by using a Student's *t* test. Statistically significant differences between survival in J774.16 cells and survival in J774.D9 cells are indicated by asterisks ($P < 0.05$). The two J774 clones had equal numbers of cell-associated *B. abortus* CFU at zero time (data not shown). wt, wild type.

RESULTS

Intracellular survival of *B. abortus* in macrophages unable to produce superoxide. In order to determine whether the oxidative burst is required for control of *B. abortus* infection by macrophages, we compared the data for survival of *B. abortus* 2308 and its isogenic *virB* mutant in two clones of the J774 cell line that differ in the ability to produce an oxidative burst upon activation. J774.16 cells produce superoxide (O_2^-) upon activation with IFN-γ, while J774.D9 cells do not produce detectable levels of O_2^- due to a lack of expression of the *cybb* gene encoding the cytochrome b_{558} (gp91) subunit of NADPH oxidase (19). In the absence of IFN-γ activation, there was no significant difference ($P > 0.05$) in the numbers of *B. abortus* CFU (either mutant or wild type) recovered from J774.16 and J774.D9 cells at 48 h after infection (Fig. 1A). The numbers of *virB* mutant BA41 CFU recovered from both J774 clones at 48 h were approximately 1% the numbers of the wild-type CFU recovered, in agreement with previous reports (30, 39). The numbers of cell-associated CFU of *B. abortus* 2308 and the *virB* mutant determined at zero time were similar, suggesting that there were no differences in internalization of the two strains (data not shown).

In cells activated with IFN-γ (Fig. 1B), significantly greater numbers of *B. abortus* 2308 CFU were recovered from J774.D9 cells than from J774.16 cells ($P < 0.005$), suggesting that production of superoxide by activated macrophages is a mechanism that limits intracellular growth of *B. abortus*. If the *virB* locus contributes to avoidance of oxidative killing in activated cells, we would expect the *virB* mutant to exhibit increased survival in cells unable to produce superoxide. Although a difference in the numbers of *virB* mutant BA41 CFU recovered

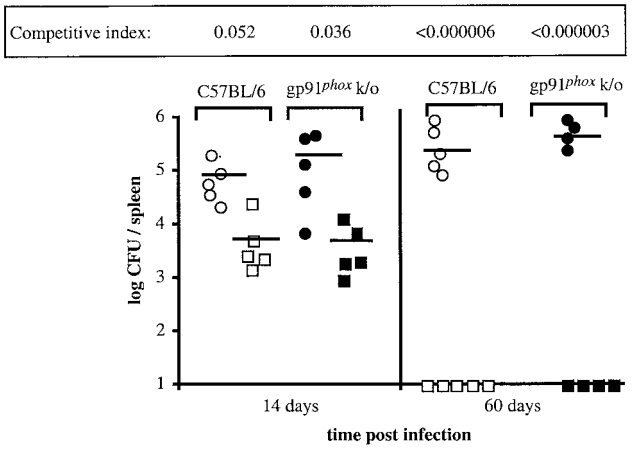

FIG. 2. Effect of NADPH oxidase on growth of *B. abortus* 2308 and the *virB* mutant in mouse spleens. Groups of 10 C57BL/6 mice and 10 gp91^{phox} knockout mice were inoculated i.p. with a mixture containing equal amounts of *B. abortus* 2308 and the *virB* mutant. The CFU of each strain were enumerated in the spleens of five mice from each group at 14 days and 60 days postinoculation. For each mouse, the results are indicated by one circle (log CFU of the wild type) and one square (log CFU of the *virB* mutant). The open circles indicate the log CFU of *B. abortus* 2308 recovered from the spleens of C57BL/6 mice, and the open squares indicate the log CFU of the *virB* mutant recovered from the spleens of C57BL/6 mice. The solid circles indicate the log CFU of *B. abortus* 2308 recovered from gp91^{phox} knockout mice, and the solid squares indicate the log CFU of the *virB* mutant recovered from the spleens of gp91^{phox} knockout mice. The means of the data are indicated by horizontal lines. The competitive index was calculated by dividing the mean ratio of mutant CFU to wild-type CFU recovered from spleens by the ratio of the mutant CFU to the wild-type CFU in the inoculum. k/o, knockout.

from the two cell lines was observed at 48 h after infection (Fig. 1), this difference was not significant. Thus, these data did not suggest that the the *virB* locus protects *B. abortus* against oxidative killing by macrophages in vitro.

gp91^{phox} gene knockout mice are able to control replication of both *B. abortus* wild type and the *virB* mutant to the same extent as C57BL/6 mice. In order to determine whether NADPH oxidase may play a role in controlling *B. abortus* infection in vivo and whether the *virB* locus may play a role in evasion of this killing mechanism in the host, we performed mixed-infection experiments with C57BL/6 mice and with mice carrying a targeted knockout of the *cybb* gene encoding the gp91 cytochrome *b* subunit (gp91^{phox}) of NADPH oxidase (36). Mixed infections were used in order to provide an internal control for the level of infection with wild-type *B. abortus* in each animal. Each mouse was injected i.p. with a mixed inoculum containing approximately 5×10^5 CFU of *B. abortus* 2308 and 5×10^5 CFU of BA41 (*virB*), and the CFU of each challenge strain were enumerated in spleen homogenates 14 and 60 days postinfection. If NADPH oxidase is required for control of *B. abortus* in vivo, the gp91^{phox} knockout mice should harbor higher total numbers of *B. abortus*. If *virB* is required for evasion of oxidative killing in this model, the ratio of the CFU of the *virB* mutant to the CFU of the wild type would be expected to increase in the knockout mice relative to the ratio in C57BL/6 mice.

Figure 2 shows that at 2 weeks postinfection, although the total number of *B. abortus* CFU recovered from the spleens of gp91^{phox} knockout mice was slightly higher than the total number of *B. abortus* CFU recovered from the spleens of C57BL/6 mice, the two groups did not differ significantly ($P = 0.30$), suggesting that NADPH oxidase is dispensable for control of *B. abortus* infection in vivo. Similarly, at 60 days postinoculation, we observed no significant difference between the two strains in terms of the number of wild-type or *virB* mutant bacteria recovered from the spleens. Furthermore, the ratios of *virB* mutant BA41 CFU to wild-type strain 2308 CFU also did not differ significantly at either time point, arguing against a role for the *virB* locus in evasion of this killing mechanism in mice. A control experiment in which C57BL/6 and gp91^{phox} knockout mice were infected individually with *B. abortus* 2308 or BA41 (*virB*) resulted in numbers of wild-type and mutant bacteria in the spleens similar to the numbers obtained in the mixed-infection experiment. This result confirmed that wild-type *B. abortus* was not able to rescue the *virB* mutant in vivo by providing *virB*-mediated functions in *trans* (data not shown).

TABLE 1. NO production by periodate-elicited murine peritoneal macrophages at 24 h postinfection[a]

Inoculum	Nitrite concn (μM/10^5 cells) in cell supernatants pretreated with:			
	None	MMLA[b]	IFN-γ + LPS[c]	IFN-γ + LPS + MMLA
Uninfected	3 ± 2	3 ± 2	102 ± 14	5 ± 0.3
B. abortus 2308	11 ± 6	17 ± 10	570 ± 390	43 ± 13
B. abortus virB mutant	20 ± 5	12 ± 6	244 ± 104	37 ± 16

[a] The nitrite concentration in 0.05 ml of cell supernatant was measured spectrophotometrically at 570 nm after incubation with Griess reagent. The values are averages ± standard deviations for triplicate wells.
[b] MMLA (250 μM) was added to cells after infection with *B. abortus*.
[c] Cells were activated 16 h before infection with 20 U of IFN-γ per ml and 1 pg of LPS per ml.

Inhibition of iNOS in activated peritoneal macrophages increases intracellular survival of the *B. abortus* wild type but not the *virB* mutant. Like NADPH oxidase, iNOS (or NOS2) is a bactericidal enzyme whose activity increases in response to macrophage activation. We next sought to determine whether the *virB* locus mediates evasion of NO-mediated killing. The J774 cell lines used for previous experiments did not produce NO upon activation with IFN-γ and *B. abortus* infection (data not shown). Therefore, in order to determine whether NO produced by the macrophages is bactericidal, we measured the effect of inhibition of iNOS on survival of the *B. abortus* wild type and *virB* mutant BA41 in elicited peritoneal macrophages from C57BL/6 mice. Production of NO by macrophages was quantified by using the Griess reagent that measures nitrite, a reaction product of NO. Table 1 shows that periodate-elicited peritoneal macrophages produced only low levels of nitrite when they were infected with either the *B. abortus* wild type or the *virB* mutant and that these low levels were not reduced by treatment of cells with the iNOS inhibitor MMLA. These data document that *B. abortus* infection does not activate iNOS in these cells. However, activation of macrophages with 20 U of murine IFN-γ per ml and 1 pg of LPS per ml prior to infection increased the production of NO both by uninfected cells and by *B. abortus*-infected cells. NO production by activated macro-

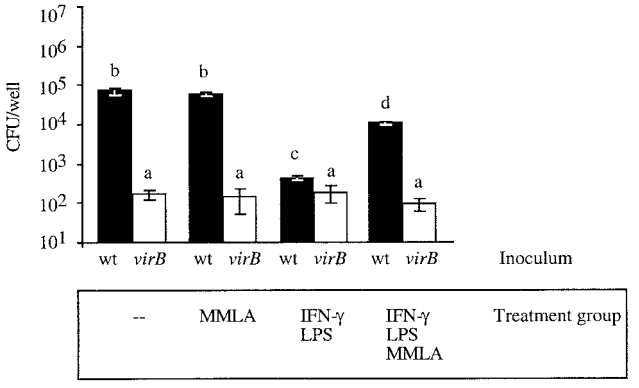

FIG. 3. Survival of *B. abortus* 2308 and the isogenic *virB* mutant BA41 in elicited murine peritoneal macrophages. Macrophages were either not treated (–), treated with the iNOS inhibitor MMLA, activated with 20 U of murine IFN-γ per ml and 1 pg of LPS per ml, or activated and treated with MMLA. The solid bars indicate the number of intracellular CFU of *B. abortus* 2308 per well, and the open bars indicate the number of CFU of BA41 (*virB*) at 24 h postinoculation. Each bar indicates the mean ± standard deviation for triplicate samples from a single experiment. Statistical analysis was performed by using a Student's *t* test. The same letter above bars indicates that differences were not statistically significant ($P > 0.05$). Different letters above bars indicate that differences were statistically significant at $P < 0.05$. wt, wild type.

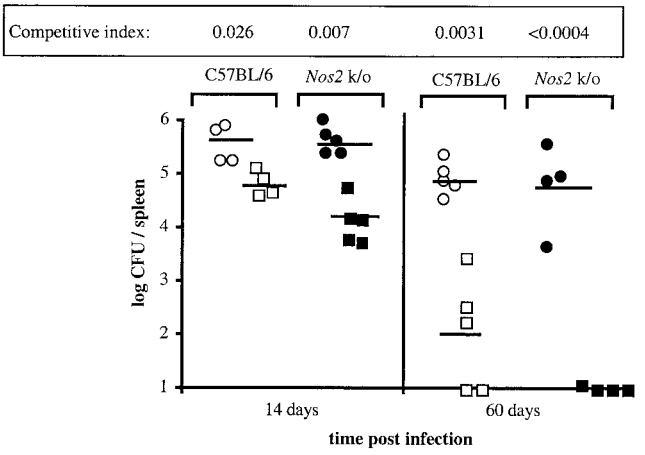

FIG. 4. Effect of iNOS on growth of *B. abortus* 2308 and the *virB* mutant in mouse spleens. Groups of 10 C57BL/6 mice and 10 *Nos2* knockout mice were inoculated i.p. with a mixture containing equal amounts of *B. abortus* 2308 and the *virB* mutant. The CFU of each strain were enumerated in the spleens of five mice from each group at 14 days and 60 days postinoculation. For each mouse, the results are indicated by one circle (log CFU of the wild type) and one square (log CFU of the *virB* mutant). The open circles indicate log CFU of *B. abortus* 2308 recovered from the spleens of C57BL/6 mice, and the open squares indicate log CFU of the *virB* mutant recovered from the spleens of C57BL/6 mice. The solid circles indicate log CFU of *B. abortus* 2308 recovered from *Nos2* knockout mice, and the solid squares indicate log CFU of the *virB* mutant recovered from the spleens of *Nos2* knockout mice. The means of the data are indicated by horizontal lines. The competitive index was calculated by dividing the mean of mutant CFU recovered from spleens/wild-type CFU recovered from spleens by the ratio of the mutant to the wild type in the inoculum. k/o, knockout.

phages could be inhibited by MMLA treatment, indicating that the NO is the product of iNOS activity (Table 1).

In nonactivated macrophages, inhibition of iNOS did not increase the number of either *B. abortus* 2308 or *virB* mutant BA41 CFU recovered from cells 24 h after inoculation (Fig. 3). However, in activated macrophages, inhibition of iNOS with MMLA led to a 10-fold reduction in the amount of NO produced and resulted in a 10-fold increase in the number of wild-type *B. abortus* CFU surviving intracellularly (compared to nontreated cells; $P < 0.005$) (Fig. 3). In contrast, the *virB* mutant was unable to grow within elicited peritoneal macrophages, irrespective of IFN-γ activation (Fig. 3). No difference in the number of cell-associated CFU was observed for different samples, suggesting that similar numbers of bacteria were internalized in all of the treatment groups (data not shown). These data indicate that NO generated by activated macrophages limits intracellular growth of *B. abortus*, but the finding that the *virB* mutant was not rescued by MMLA treatment of the macrophages suggests that the *virB* genes do not act in evasion of NO.

iNOS knockout mice control growth of a *virB* mutant better than C57BL/6 mice control growth of the mutant. To determine whether the in vitro role of iNOS in limiting intracellular growth of *B. abortus* is relevant in the host, we compared the abilities of C57BL/6 mice and congenic *Nos2* knockout mice to limit *B. abortus* infection. Mice were inoculated i.p. with a mixture containing approximately 5×10^5 CFU of *B. abortus* 2308 and 5×10^5 CFU of BA41 (*virB*), and the CFU of each challenge strain in the spleens were enumerated 14 and 60 days postinfection. If iNOS is required for control of *B. abortus* in vivo, the *Nos2* knockout mice should harbor higher total numbers of *B. abortus* CFU in their spleens than the C57BL/6 mice harbor. If *virB* is required for evasion of NO in this model, the ratio of the number of CFU of the *virB* mutant to the number of CFU of the wild type is expected to increase in the *Nos2* knockout mice relative to the ratio in the C57BL/6 mice.

At 14 days postinoculation, spleens from *Nos2* knockout mice and C57BL/6 controls contained equivalent total numbers of *B. abortus* ($P = 0.58$). Similarly, at 60 days, no difference in total bacterial load was observed between the two groups of mice ($P = 0.79$), suggesting that the knockout mice are able to control the infection with *B. abortus*. Examination of histological sections of spleens and livers of *Nos2* knockout mice and controls revealed similar pathological findings in the two mouse strains (data not shown).

Analysis of the mixed-infection data revealed that at 14 days postinoculation, the *Nos2* knockout mice contained significantly less of the *virB* mutant than the wild-type controls contained, which was represented by a lower competitive index ($P = 0.01$) (Fig. 4). After 60 days, the two strains of mice were infected with equivalent numbers of the wild-type bacteria. A slight difference between the *Nos2* knockout and C57BL/6 mice in the numbers of the *virB* mutant in the spleens was observed 60 days postinoculation. Three of the four *Nos2* knockout mice had cleared the *virB* mutant, while four of the five C57BL/6 mice still harbored detectable numbers of the attenuated strain. However, the differences observed were not statistically significant ($P = 0.06$). These data suggest that while the *Nos2* knockout mice and the control mice harbored similar numbers of wild-type *B. abortus* CFU, the *Nos2* knock-

out mice were able to clear the attenuated *virB* strain more rapidly than the C57BL/6 controls. Taken together, these results indicate that iNOS activity is not required for control of *B. abortus* infection in the mouse and that the *virB* locus does not play a role in evasion of or resistance to NO.

DISCUSSION

The function of the *B. abortus virB* locus is essential for intracellular survival, both in cultured cells and in the mouse model (17, 22, 30, 39). Disruption of the *virB* locus results in failure to traffic to a cellular compartment of HeLa cells that permits replication (5, 10). However, it is not clear how *virB* acts to enable replication of *B. abortus* in the macrophage, the cell type in which *B. abortus* is thought to persist in the host. Evidence obtained by other investigators suggests that *B. abortus* is able to inhibit phagosome-lysosome fusion and that phagosomes containing *B. abortus* do not contain NADPH oxidase (2, 18, 28). In order to elucidate the mechanism by which the *virB* genes enable *B. abortus* to survive in the macrophage, we investigated whether the *virB* locus enables *B. abortus* to evade killing by the macrophage NADPH oxidase and by iNOS.

To determine whether *virB* mediates evasion of NADPH oxidase, we examined the intracellular survival of *B. abortus* 2308 and an isogenic *virB* mutant in in vitro and in vivo NADPH oxidase deficiency models. The kinetics of bacterial replication and killing were examined in two J774 macrophage clones that differed in the ability to produce the oxidative burst. When the cells were not activated with IFN-γ prior to inoculation with *B. abortus*, cells of J774.16, the cell line capable of generating an oxidative burst, and J774.D9 cells, which are unable to generate superoxide, contained equivalent numbers of *B. abortus* 2308 cells and of *virB* mutant cells (Fig. 1). However after activation of these cells with IFN-γ, the J774.D9 cells contained 2 logs more of *B. abortus* 2308 and slightly more of the *virB* mutant than the clone J774.16 cells contained. This result suggests that in activated macrophages oxygen-dependent mechanisms are important for killing of *B. abortus*, but it did not provide evidence for *virB*-mediated evasion of NADPH oxidase. Similarly, when we performed competitive infections of gp91^{phox} knockout mice and the inbred parent strain, we found no significant difference in the ratios of the *virB* mutant to the wild type recovered from the two mouse strains (Fig. 2). This result suggests that *virB* does not play a role in evasion of oxidative killing in vivo. Interestingly, the total numbers of *B. abortus* CFU recovered from the two mouse strains did not differ significantly, suggesting that in vivo NADPH oxidase is not required to control *B. abortus* replication. This result is in agreement with the findings of Ko et al. (25), which showed that reactive oxygen intermediates are not required for control of *B. abortus* infection in naïve mice.

Inhibition of iNOS in activated peritoneal macrophages increased survival of *B. abortus* 2308 in vitro, showing that NO limits bacterial replication in these cells. However, the *virB* mutant was unable to grow in these cells, independent of the activation state or NO production. In *Nos2* knockout mice, *B. abortus* 2308 replicated to the same extent in the spleen as it replicated in the parent C57BL/6 strain. However, lower numbers of CFU of the *virB* mutant were recovered from the iNOS knockout mice than from the C57BL/6 mice, suggesting that the knockout mice are able to clear the attenuated mutant more rapidly. At 60 days postinfection, more of the iNOS knockout mice than of the C57BL/6 mice had cleared the attenuated *virB* mutant, although the difference between the numbers of CFU was not significant ($P = 0.06$) (Fig. 3). If the *virB* genes are involved in evasion of NO, we would expect to recover higher rather than lower numbers of CFU of the *virB* mutant from the knockout mice. Thus, these data show that iNOS is dispensable for control of *B. abortus* infection in vivo and argue against a role for the *virB* locus in evasion of nitrosative killing in vivo. Splitter and colleagues have found that high levels of NO production in a macrophage cell line correlate with increased survival after prolonged incubation, so taken together, our findings may indicate that NO production may in some way be advantageous to the persistence of *B. abortus* in vivo (41).

Several investigators have shown that in vitro, either opsonization of bacteria with specific antiserum or activation of cells with IFN-γ is required in order to detect generation of O_2^- and NO by macrophages when they are challenged with *B. abortus* or *B. suis* (16, 20, 21, 24). Based on these observations, it has been suggested that these defense mechanisms may play a role in acquired immunity (20). Although we did not observe differences in the numbers of *B. abortus* CFU in wild-type and knockout mice at 60 days postinfection, well after the onset of adaptive immunity, our data do not rule out the possibility that oxidative and nitrosative killing mechanisms may play a role in resistance to reinfection or infection in vaccinated hosts.

The lack of a requirement for both NADPH oxidase and iNOS in control of *B. abortus* infection contrasts with data obtained for acute *S. enterica* serovar Typhimurium infection, in which gp91^{phox} knockout mice exhibit increased organ loads and succumb rapidly to infection with an attenuated isolate, while onset of mortality in iNOS knockout mice occurs approximately 1 week later (27). However, our findings resemble those obtained with a murine model of pulmonary tuberculosis, in which IFN-γ was also required for containment of bacterial infection but neither NADPH oxidase nor iNOS was a critical mediator controlling bacterial multiplication (6–8). Similarly, Ko et al. found that whereas IRF-1 knockout mice succumbed rapidly to *B. abortus* infection, both gp91^{phox} and *Nos2* knockout mice had only slightly higher organ loads of *B. abortus* than the parent mouse strains (25). Since both tuberculosis and brucellosis are chronic infections, the host may use a common IFN-γ-dependent mechanism for controlling these infections that is independent of NADPH oxidase and iNOS.

The J774.D9 cells activated with IFN-γ did not produce NO (data not shown), but they controlled replication of *B. abortus* significantly better than nonactivated macrophages controlled replication (Fig. 1). These data suggest that macrophage activation by IFN-γ may induce expression of killing mechanisms that are independent of iNOS and NADPH oxidase. In the case of *Mycobacterium avium* infection, it has been shown that activation of macrophages by IFN-γ increases acidification and alters maturation of the *M. avium*-containing vacuole (38). Similarly, treatment of *S. enterica* serovar Typhimurium-infected macrophages with IFN-γ has been shown to increase fusion of *Salmonella*-containing vacuoles with lysosomes (23). IFN-γ has also been shown to regulate intracellular cholesterol

distribution, which has been shown to influence entry of *B. suis* into macrophages (29, 31). Like *M. avium* and other intracellular pathogens, *B. abortus*, *B. suis*, and *Brucella melitensis* have been shown to alter endocytic trafficking to establish a compartment favorable for intracellular replication. The *virB* locus has been shown to be required for modulation of intracellular trafficking in HeLa cells (5, 10). We have found that in nonactivated macrophages, the numbers of *B. abortus* wild-type CFU recovered are 1 to 2 logs higher than the numbers of *virB* mutant CFU recovered at 48 h (Fig. 1 A). However, after IFN-γ activation, the number of wild-type CFU is similar to the number of *virB* mutant CFU. Given the role of the *virB* genes in intracellular trafficking of *Brucella* spp. (5, 10), it is tempting to speculate that as in *M. avium*, IFN-γ activation may alter endocytic trafficking in a manner that prevents the action of the *B. abortus* VirB proteins from establishing a replicative niche in the macrophage. Further experiments will be required to understand the mechanism by which the *B. abortus virB* locus mediates intracellular replication of this organism in macrophages.

ACKNOWLEDGMENTS

We thank N. Buchmeier and B. Bloom for providing the J774.16 and J774.D9 cell lines and A. Bäumler for critical reading of the manuscript.

This work was supported by PHS grant AI50553 to R.T.

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Editor: D. L. Burns