Evidence Inconsistent with a Role for the Bcg Gene (Nramp1) in Resistance of Mice to Infection with Virulent Mycobacterium tuberculosis

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Summary

The superior resistance of some strains of mice over others to infection with certain intracellular pathogens, including the vaccine strain of Mycobacterium bovis, bacillus Calmette Guerin (BCG), is determined by a gene associated with a small segment of chromosome 1 designated the Ity/Lsh/Bcg locus, referred to here as the Bcg locus. DBA/2 mice containing the dominant resistant allele of the Bcg gene (Bcg'), major histocompatibility complex-compatible BALB/c mice containing the recessive susceptible allele (Bcg'), and congenic C.D2-N20 Bcg', which are genetically the same as BALB/c mice except for possessing a small piece of DBA/2 chromosome 1 containing the Bcg locus, were used to determine whether the Bcg gene determines resistance to infection with the virulent H37Rv strain of Mycobacterium tuberculosis (Mtb). According to the survival times of Bcg' and Bcg' mice infected via either the intravenous or respiratory route, Bcg' mice proved much less, rather than more, resistant to Mtb infection than Bcg' mice. Shorter survival times of Bcg' mice were associated with an inferior capacity to control Mtb growth in their lungs and to retard the development of Mtb-induced pathology in this organ. Resistance to Mtb infection was a dominant trait in the F1 progeny of Bcg' and Bcg' mice. The results show that resistance to Mtb is not determined by the resistance allele of the Bcg gene nor by the recently isolated candidate Bcg gene Nramp1, located in the Bcg locus.

Some strains of mice show a higher level of natural resistance than others to infection with certain pathogens, including Salmonella typhimurium, Leishmania donovani, and the attenuated bacillus Calmette Guerin (BCG) strain of Mycobacterium bovis (1). The genes responsible for resistance to each of these three pathogens have been mapped independently to a small region of chromosome 1 designated the Ity/Lsh/Bcg locus (2-4), and referred to here as the Bcg locus. It is generally believed that resistance to all of the pathogens mentioned is under the control of a single dominant gene. According to a number of studies, the gene is expressed in macrophages (5-7), and its dominant resistant allele provides these cells with a superior innate capacity to kill microbial pathogens or restrict their intracellular growth before the generation of specific host immunity.

A knowledge of the genetic and physical intervals defining the Bcg locus of mouse chromosome 1 was used recently to identify several candidate transcriptional units for the Bcg gene, one of which was chosen as the most likely candidate gene because it is expressed exclusively in macrophages. This candidate gene, designated Nramp1 (for natural resistance–associated macrophage protein), encodes for a polypeptide that is part of the transmembrane domain of a macrophage-specific membrane protein (8). Additional studies with a variety of BCG-resistant (Bcg') and susceptible (Bcg') strains of mice showed anti-BCG resistance to be associated with a nonconservative Gly-169-to-Asp-169 substitution within the predicted transmembrane domain 4 of Nramp1 (9-11). More recently, targeting of the Nramp1 gene by homologous recombination was used to generate mutant mice bearing a null-resistant allele of the Nramp1 gene (11). These mice proved to be susceptible to BCG, S. typhimurium, and L. donovani. An homologous gene has been shown to be expressed in human macrophages (12), thereby opening the way to investigating whether sequence variations in this gene are associated with differences in susceptibility to tuberculosis among families in endemic areas of the disease. Even so, it needs to be remembered that, in spite of the evidence that Nramp1 is the gene that determines innate resistance of mice to BCG infection, there is little evidence that this gene is responsible for determining resistance to infection with virulent strains of Mycobacterium tuberculosis (Mtb).

The purpose of this article is to show, with MHC-compatible Bcg' and Bcg' strains of mice, and with a congenic Bcg' strain, that the Bcg gene appears to be of little impor-
stance in determining resistance to infection with the virulent \( H37Rv \) strain of \( Mtb \).

**Materials and Methods**

**Animals.** Inbred, pathogen-free, 8-wk-old BALB/c (\( Bcg^r \)), DBA/2 (\( Bcg^r \)), and CD2F1 (BALB/c × DBA/2) mice were purchased from Trudeau Institute Animal Breeding Facility. Congenic CD2 Idh-1^+/tyr^-Pep3^b^ (C.D2-N20) \( Bcg^r \) breeder mice were kindly provided by Dr. Emil Skamene of the Montreal General Hospital Research Institute (Montreal, Quebec, Canada). These mice differ from BALB/c mice in possessing a small segment of chromosome 1 of DBA/2 mice containing the \( Bcg \) locus (13). All mice were free of common viral pathogens according to results of routine testing performed by the Research Animal Diagnostic and Investigative Laboratory, University of Missouri, (Columbia, MO).

**Mycobacteria.** \( M. \) \( tuberculosi s \) \( H37Rv \) (TMC #102) was obtained from the Trudeau Mycobacterial Culture (TMC) collection. It was supplied in vials as a frozen (-70°C) log phase dispersed culture in Proskauer and Beck medium (Difco Laboratories Inc., Detroit, MI) containing 0.01% Tween 80. For each experiment, a vial was thawed, subjected to 5 s of ultrasound to break up aggregates, and diluted appropriately in PBS containing 0.01% Tween 80. Mice were inoculated with \( \sim10^4 \) CFU of bacilli in 0.2 ml of PBS via a lateral tail vein, or with \( \sim5 \times 10^2 \) CFU by aerosol. Infection by aerosol was performed in a Middlebrook airborne infection apparatus (Tri Instruments, Jamaica, NY). Bacilli were enumerated at progressive times in the lungs, kidneys, livers, and spleens of infected mice by preparing homogenates of these organs in PBS containing 0.05% Tween 80 and plating 10-fold serial dilutions of the homogenates on enriched agar (Middlebrook 7H11; Difco Laboratories Inc.). Colonies were enumerated after 3–4 wk of incubation at 37°C.

**Histology.** Pieces of lungs were taken at day 70 of infection and fixed in 10% phosphate-buffered formalin. The tissues were then dehydrated and wax embedded according to routine procedures. Sections 4–6 \( \mu \)m thick were stained with Ziehl Nielsen stain for acid-fast bacilli and counterstained with methylene blue. Photomicrographs were taken with a microscope (Microphot-Fx; Nikon Inc., Melville, NY).

**Results**

\( Bcg^r \) Mice Are Much More Susceptible than \( Bcg^r \) Mice to \( Mtb \) Infection Initiated Intravenously or by Aerosol. In the first experiments, DBA/2 (\( Bcg^r \)) and MHC-compatible BALB/c (\( Bcg^r \)) mice were infected with \( 10^5 \) Mtb intravenously or with \( 5 \times 10^2 \) Mtb by aerosol, and growth of the organism in lungs, livers, spleens, and kidneys was followed over a 100-d period. Additional mice were infected to obtain survival data. The results in Fig. 1 show that, in the case of infection via the intravenous route, \( Bcg^r \) mice were substantially less, rather than more, capable than \( Bcg^r \) mice of controlling the growth of Mtb in their lungs, in that the former mice had \( \sim100 \) times more bacilli in this organ than the latter on day 100 of infection. Both strains of mice were similar, on the other hand, in terms of their ability to stabilize infection in other organs. It can be seen with both strains of mice that, after a brief period of bacillary growth in the liver, spleen, and kidneys, infection was stabilized and caused to plateau until day 100. This superior resistance of BALB/c mice was independent of the size of inoculum. Thus, DBA/2 mice inoculated intravenously with \( 10^3 \) CFU of Mtb \( H37Rv \) contained 2.5 logs more bacilli in their lungs than BALB/c mice inoculated with the same number of bacteria (7.1 ± 0.3 log for DBA/2; 4.6 ± 0.2 log for BALB/c) at day 80 of infection.

In the case of infection initiated via the respiratory route (Fig. 2), \( Bcg^r \) mice were likewise less capable than \( Bcg^r \) mice of controlling Mtb growth in their lungs. However, \( Bcg^r \) mice allowed much less initial seeding of Mtb from the lungs to the liver and spleen. In spite of this, however, Mtb grew more extensively in the livers of these mice than in the livers of \( Bcg^r \) mice after day 75 of infection. The smaller numbers of Mtb in the livers of DBA/2 mice on day 30 of the aerosol infection indicates that fewer organisms disseminated from the lungs to implant in the livers at this stage of infection. Presumably, similar levels of resistance were expressed in the livers of DBA/2 and BALB/c mice between days 30 and 70. The large increase in the number of Mtb in the livers of DBA/2 mice after day 70 might have resulted, at least in part, from continuous seeding of bacilli from the lungs, because of a massive increase in the number of bacilli in this organ from day 70 on. It could also have resulted from the expression of less resistance in the livers of DBA/2 mice after day 70.

The greater susceptibility of \( Bcg^r \) mice to Mtb infection is
Figure 2. Growth of Mtb in lungs, livers and spleens of Bcg' (DBA/2) and Bcg' (BALB/c) mice given $5 \times 10^7$ Mtb via the respiratory route. Bcg' mice were much less resistant than Bcg mice in their lungs after day 20. In addition, Bcg' mice showed a smaller number of Mtb in their livers and spleens on day 20, although their livers were less capable than those of Bcg of controlling infection from day 70 on. Means are for five mice per time point ± SD.

Figure 3. Survival times of Bcg' (DBA/2) and Bcg' (BALB/c) mice infected intravenously with Mtb as described for Fig. 1. Bcg' mice died much sooner (MST = 84 d) than Bcg' mice (MST = 290 d). There were 10 mice in each group.

Figure 4. Survival times of mice infected with Mtb aerosol as described for Fig. 3. Bcg' (DBA/2) mice died much sooner (MST = 124 d) than Bcg' (BALB/c) mice (MST = 303 d). There were 10 mice per group.

Figure 5. Growth of Mtb in lungs, livers, spleens, and kidneys of Bcg' (BALB/c), Bcg' (DBA/2) mice, and their F1 progeny (CD2-F1) after intravenous inoculation of $10^8$ bacilli. CD2-F1 mice, like BALB/c mice, were substantially more resistant to Mtb growth in the lungs. Means are for five mice per time point ± SD.

Resistance to Mtb Infection Is Not Determined by the Bcg Gene. The foregoing results show that mice that possess BALB/c mice over Bcg' DBA/2 mice is a dominant trait, growth of Mtb in the organs of these strains was compared with its growth in their F1 progeny over a 100-d period. As can be seen in Fig. 5, F1 mice were the same as BALB/c mice in displaying a superior ability over DBA/2 mice to resist the growth of Mtb in their lungs after inoculation with $10^8$ bacilli via the intravenous route. These results are supported by the survival data in Fig. 6 showing that F1 and BALB/c mice survived more than twice as long as DBA/2 mice from the $10^8$-bacilli intravenous inoculum.

Resistance to Mtb Infection Is a Dominant Trait in F1 Mice. To determine whether superior anti-Mtb resistance of Bcg' mice was even more convincingly demonstrated by the survival data shown in Figs. 3 and 4. It can be seen that after initiating infection via either route, the median survival time (MST) of Bcg' mice was substantially shorter, being less than half that of Bcg' mice.
the resistance allele of the Bcg gene are less, rather than more, resistant to infection with virulent Mtb, and that the F1 progeny of Bcg- and Bcg+ mice are resistant like Bcg+ mice. This could mean that the recessive Bcg- allele is responsible for resistance to Mtb and that it is dominant when measured against Mtb in the F1. If so, it would follow that Bcg- BALB/c mice should be more resistant to Mtb infection than congenic C.D2-N20 mice that differ from BALB/c mice in possessing a small piece of chromosome 1 of DBA/2 mice containing the Bcg- gene. The results in Figs. 7 and 8 show this not to be the case, in that Bcg- congenic C.D2-N20 mice, like BALB/c mice, were much more resistant than DBA/2 mice to Mtb infection, as measured by superior capacities to control Mtb growth in their lungs and to survive infection. It can be concluded from these results, therefore that the presence of the Bcg- allele of the Bcg gene is not needed for BALB/c mice to be more resistant to Mtb infection than Bcg+ DBA/2 mice. In other words, resistance to Mtb infection is determined by genes other than the Bcg gene.

The Superior Anti-Mtb Resistance of BALB/c Mice Is Associated with Slower Development of Lung Pathology. It has been shown in preceding sections that, in spite of their possession of the susceptibility allele of the Bcg gene, BALB/c mice are much more resistant to Mtb infection than BALB/c mice that contain the dominant resistance allele of the gene, as measured by the ability of the former mice to restrict the growth of Mtb in their lungs and survive infection. Because the difference in anti-Mtb resistance was seen only in the lungs, it was anticipated that the longer survival time of Bcg+ mice would be associated with the development of much less lung pathology. This prediction proved correct, as evidenced by the photos of 20-μm sections of lungs of BALB/c and DBA/2 mice (Fig. 9), which show that on day 70 of infection the lungs of BALB/c mice displayed fewer and smaller Mtb-induced lesions than the lungs of DBA/2 mice. At higher magnification, the lung lesions in each strain consisted of areas of intense alveolitis in which contiguous air sacs were filled with leukocytes, most of which were mononuclear. Within each lesion, acid-fast bacilli were located in collections of epithelioid macrophages occupying contiguous air sacs. These sites of infection were larger and more numerous in lung lesions of DBA/2 mice (Fig. 9) and contained larger numbers of bacilli. A histological examination of the livers and spleens of both strains of mice (not shown) revealed that Mtb infection did not cause progressive pathology. Instead, stabilization of infection in these organs was associated with small compact granulomas that did not change in size or composition over time. This is in keeping with the results of another study in this laboratory (14).
Figure 9. Evidence that the superior ability of Bcg' (BALB/c) mice over Bcg' (DBA/2) mice for controlling Mtb infection in their lungs was associated with substantially less pathology in this organ. On day 70 of infection initiated with $10^9$ Mtb via the intravenous route, there were fewer and smaller lesions in 20-μm crystal violet-stained sections of lungs of BALB/c mice (a) than in the lungs of DBA/2 mice (b). In both cases, Mtb was confined to collections of epithelioid macrophages (arrows) that were more degenerate in lung lesions of DBA/2 mice (d) than in lung lesions of BALB/c mice (c). At higher power, these sites of infection in DBA/2 mice (f) contained many more acid-fast bacilli than those in BALB/c mice (e). Samples were stained with Ziehl Nielsen counterstained with methylene blue. ×8 (a and b); ×300 (c and d); ×700 (e and f).
Discussion

This study leaves little doubt that the dominant resistant allele of the Bcg gene (Bcg'), which is responsible for the ability of certain strains of mice to better resist the growth of BCG and certain other pathogens during the preimmunity stage of infection, has little or no influence on the ability of these strains to resist infection with virulent Mtb. The results show that Bcg' DBA/2 mice are considerably less, rather than more, resistant to Mtb infection than Bcg BALB/c mice. This was the case with mice infected either via the respiratory or intravenous route. Superior anti-Mtb resistance of Bcg' mice was measured as (a) a longer survival time, (b) a superior ability to control infection in the lung, and (c) slower development of lung pathology. Resistance to Mtb infection was a dominant trait in the F1 progeny of BALB/c and DBA/2 mice, which suggested the possibility that the Bcg' rather than the Bcg allele of the Bcg gene was responsible for resistance to Mtb. However, this proved not to be the case, as evidenced by the finding that Bcg-congenic C.D2-N20 BALB/c mice were like Bcg' BALB/c mice in being more resistant than Bcg DBA/2 mice to infection with Mtb. Because the recently isolated candidate gene for the Bcg gene, Nmp1, is contained within the Bcg locus (8), it follows that Nmp1 also has little, if any, influence on resistance to Mtb infection, at least according to the way that anti-Mtb resistance was measured in this study.

It needs to be brought to mind, in this connection, that the superior resistance of Bcg' mice to BCG infection has been measured almost exclusively in the spleen as a superior ability to restrict the growth of BCG during the preimmunity first 20 d of infection (15, 16). On the other hand, that Mtb-specific Tcells. bactericidal function in response to cytokines secreted by macrophages are eventually overridden by additional antimicrobial mechanisms acquired by these cells in response to specific immunity. Indeed, the capacity to generate specific immunity to BCG seems essentially the same in Bcg' and Bcg' mice, so far as the ability of mice to resolve infection is concerned. However, this appears not to be the case for resistance to infection with Mtb, as described here. According to our results with BALB/c and DBA/2 mice infected with the same number of Mtb via the respiratory route (Fig. 3), the superior resistance of the former mice over the latter was seen in the lung after, rather than before, the onset of expression of specific immunity, as evidenced by a substantial reduction in the rate of Mtb growth in this organ from day 20 on. We are aware, however, that immunity to Mtb infection eventually is expressed by macrophages, and that the superior resistance of BALB/c over DBA/2 mice to this pathogen may depend on a superior ability of BALB/c macrophages to acquire increased mycobactericidal function in response to cytokines secreted by Mtb-specific Tcells.

Lastly, it needs to be mentioned that virulence of the H37Rv strain of Mtb for mice is similar to that of the recently isolated C strain that is responsible for a larger number of tuberculosis cases throughout the New York metropolitan area (14). On the basis of this evidence, it would seem reasonable to assume that H37Rv is still virulent for humans, in spite of its having been isolated >60 yr ago.

We acknowledge the expert technical assistance of Lynn Ryan, Debra Duso, and Ron LaCourse.
This work was supported by grants HL-51960 and AI-37844 from the National Institutes of Health, and a general tuberculosis support grant from The G. Harold and Leila Y. Mathers Charitable Foundation.

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Received for publication 7 September 1995 and in revised form 1 November 1995.

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