Influence of the \textit{Bcg} Locus on Natural Resistance to Primary Infection with the Facultative Intracellular Bacterium \textit{Francisella tularensis} in Mice

HANA KOVÁŘOVÁ,¹* LENKA HERNYCHOVÁ,¹ MARIÁN HAJDUCH,²
M. ŠIROVÁ,³ AND ALES MACELA¹

Institute for Immunology and Radiobiology, Parkyně Military Medical Academy, 500 01 Hradec Králové,¹ Department of Pediatrics, Faculty of Medicine, Palacky University and Faculty Hospital, 775 15 Olomouc,² and Institute of Microbiology, Academy of Science of the Czech Republic, 142 20 Prague,³ Czech Republic

Received 3 September 1999/Returned for modification 26 October 1999/Accepted 10 December 1999

The implication of the \textit{Bcg} locus in the control of natural resistance to infection with a live vaccine strain (LVS) of the intracellular pathogen \textit{Francisella tularensis} was studied. Analysis of phenotypic expression of natural resistance and susceptibility was performed using mouse strains congenic at the \textit{Bcg} locus. Comparison of the kinetics of bacterial colonization of spleen showed that B10.A.Bcg(r) mice were extremely susceptible during early phases of primary sublethal infection, while their congenic C57BL/10N \textit{[Bcg(s)]} counterparts could be classified as resistant to \textit{F. tularensis} LVS infection according to the 2-log-lower bacterial CFU within the tissue as long as 5 days after infection. Different phenotypes of \textit{Bcg} congenic mice were associated with differential expression of the cytokines tumor necrosis factor alpha, interleukin-10, and gamma interferon and production of reactive oxygen intermediates. These results strongly suggest that the \textit{Bcg} locus, which is close or identical to the \textit{Nramp1} gene, controls natural resistance to infection by \textit{F. tularensis} and that its effect is the opposite of that observed for other \textit{Bcg}-controlled pathogens.

The establishment of protective immunity to intracellular bacterial pathogens involves an early innate immunity followed by an acquired immune response (12). Resistance or susceptibility to infection results from the interplay between genetic variability in the host response and the pathogen’s virulence. Genetic control of natural resistance is phenotypically expressed by the ability of a host to restrict the rate of early intracellular growth of a pathogen before development of an effective T-cell-mediated immunity (13, 33). One of the best-studied susceptibility genes is \textit{Nramp1}. It maps to the 30-centimorgan (cM) locus, formerly named \textit{bcg}, located on chromosome 1 in mice (34). Gene knockout and transgenesis provided final verification that the \textit{Nramp1} gene is implicated in resistance to taxonomically and antigenically unrelated microorganisms like \textit{Mycobacterium bovis}, \textit{Salmonella typhimurium}, and \textit{Leishmania donovani} (19, 35). In mice, \textit{Nramp1} is expressed by macrophages in two allelic forms, resistant (\textit{r}) and susceptible (\textit{s}); the latter has the 169Gly-to-169Asp substitution, which confers susceptibility to infection (28). Expression of the \textit{Bcg(r) \{Nramp1(r)\}} allele has many in vitro pleiotropic effects associated with priming or activation of macrophages in response to interferon gamma (IFN-\gamma), lipopolysaccharide (LPS), or other bacterial components. It is probable that these observations can be relevant to resistance and susceptibility to in vivo infections also (5, 6). It has been shown that the \textit{Nramp1} protein is a member of an ancient family of proteins with a typical structural organization of ion transporters and channels (8). These findings, together with the identification of bacterial homologues of cation transporters, suggest a model in which competition for divalent metal cations between host and parasite may be related to host resistance or susceptibility to the pathogen (1, 4).

\textit{Francisella tularensis} is a gram-negative, facultatively intracellular bacterium and the etiological agent of tularemia, a disease of a variety of animal hosts (30). An attenuated vaccine strain (LVS) of \textit{F. tularensis}, developed as a human vaccine, is more or less virulent for various strains of mice. Experimental murine tularemia is thus considered an excellent model of human disease, including mechanisms of antibacterial resistance. As in other intracellular bacteria, protein antigens of \textit{F. tularensis} LVS induce T-cell-mediated immunity, which contributes significantly to host protection and is responsible for ultimate clearance of the bacterium (32). Cytokines such as IFN-\gamma and tumor necrosis factor alpha (TNF-\alpha) are key regulators, since neutralization of these cytokines in vivo increased the severity of the \textit{Francisella} infection while systemic administration of these cytokines reduced it (16). The cooperation of IFN-\gamma and TNF-\alpha in the induction of the synthesis of NO and the possible regulation of iron homeostasis and pH appear to be important in limiting the survival of \textit{Francisella} within macrophages (17). The mechanisms of innate immunity that follow after internalization of the bacterium into macrophages deserve further study, as they may have a strong impact on the development of specific immunity and the protection of the host.

Studies on the genetic basis of resistance to murine tularemia have shown that the phenotype of resistance is inherited in a dominant manner and the mode of inheritance is complex, involving multiple genetic loci (3). Comparing an \textit{F. tularensis} LVS-susceptible C3H \textit{[Lps(n/d) Bcg(r)]} murine model with C57BL/10N \textit{[Lps(n) Bcg(s)]} mice, which are noticeably resistant to tularemia, we have previously shown that early onset of TNF-\alpha production and/or production of reactive oxygen intermediates (ROI) contributes significantly to resistance against tularemia (23, 27). To ascertain whether the \textit{Bcg} locus, one of the possible candidates in these different genetic backgrounds,
might be implicated in the control of natural resistance to tularemia, we performed an analysis of phenotypic expression of early resistance and susceptibility using mouse strains derived from C57BL/10 mice and congenic at the Bcg locus. Comparison of the kinetics of bacterial colonization of the spleen showed that B10.A.Bcg(r) mice, compared to their F. tularensis LVS-resistant Bcg(s) counterparts, were extremely susceptible during the early phases of primary sublethal infection. Different phenotypes of Bcg congenic mice were associated with differential expression of the cytokines TNF-α, interleukin-10 (IL-10), and IFN-γ and differential production of ROI. These results strongly suggest that the Bcg locus, which is close or identical to the Nramp1 gene, controls natural resistance to infection by F. tularensis and that its effect is the opposite of that observed for other Bcg-controlled pathogen.

MATERIALS AND METHODS

Mice. Specific-pathogen-free 6- to 8-week-old female C57BL/10N [Bcg(s)] mice were obtained from Charles River Deutschland (Sulzdorf, Germany), and the congenic B10.A.Bcg(r) strain (11) was obtained initially from E. Skamene (McGill University, Montreal, Canada) and bred and maintained in our laboratories in conventional facilities. This congenic strain was constructed by the backcross NX system using resistance to Mycobacterium bovis BCG Montreal as a selective agent. B10.A.Bcg(r) mice are identical with the C57BL/10N [Bcg(s)] strain except for the 30-cM segment of chromosome 1 that contains the Bcg locus which the Bcg(s) gene, associated with resistance to M. bovis BCG, is located.

Experimental infection in mice. The infectious inoculum of F. tularensis LVS (ATCC 29304; American Type Culture Collection, Manassas, Va.) was prepared as described previously (27). Bcg(s) or Bcg(r) mice (three to six per group) were inoculated subcutaneously (s.c.) in the left hind leg with 0.1 ml of physiological saline containing 102 CFU of F. tularensis LVS. The degree of infection was assessed by determining the number of CFU in the spleen at predetermined time intervals. Briefly, the homogenate of the spleen of each individual mouse was prepared in sterile physiological solution by sieving, further serially diluted in the same buffer, and plated in triplicate samples onto Mueller-Hinton solid medium. The number of F. tularensis LVS CFU was determined after a 72-h incubation of the plates at 37°C.

Cell cultures. Spleens from mice sacrificed at predetermined time intervals were removed, and cell suspensions were prepared in RPMI 1640 culture medium (SEBAK Biologische Forschungs-GmbH, Aidenbach, Germany) supplemented with 50 μg of glutamine (USOL, Prague, Czech Republic)/ml, 0.25% of gentamicin (Sigma, St. Louis, Mo.)/ml, and 5% fetal calf serum (FCS; Bioveta, Ivanovice, Czech Republic). Cells were then dispensed in duplicate into 24-well flat-bottom tissue culture plates at 2 × 106 cells per well in 1.0 ml of culture medium and were incubated at 37°C under 5% CO2. Media were changed every other day. The supernatants were aspirated from the wells, and 150 μl of dimethyl sulfoxide was added to extract reduced formazan. The absorbance (A490) per well was determined using a Dynex MRX (Dynatech Laboratories).

NO assay. NO in 24-h-conditioned macrophage culture supernatants was measured as amounts of nitrite, a stable product of NO decay, using Griess reagent (20).

Analysis of data. Statistical analysis was performed using unpaired two-tailed Student’s t tests to compare Bcg congenic strains of mice at each time point, and the 95% confidence limit was assumed to establish the level of significance.

RESULTS

Bacterial load in spleens of congenic Bcg(s) and Bcg(r) mice. To examine whether natural resistance or susceptibility to infection with F. tularensis LVS is controlled by the Bcg locus, we compared C57BL/10N [Bcg(s)] mice with their congenic counterparts B10.A.Bcg(r), which differ in the chromosome segment containing the studied locus. Mice of each strain were infected s.c. with a sublethal dose of F. tularensis LVS (102 CFU/mouse), and the number of bacteria in the spleen was determined at different times after infection. As shown in Fig. 1, the number of bacteria increased rapidly in the first 3 days and reached a maximum 5 days after infection. After this time, the infection underwent resolution and was under the detection limit of the CFU assay in the spleens of C57BL/10N [Bcg(s)] and B10.A.Bcg(r) mice for 14 and 21 days, respectively. Nevertheless, in the course of the early period, B10.A.Bcg(r) mice showed significantly higher bacterial loads in the spleen than did C57BL/10N [Bcg(s)] mice. The coefficient of increase, c, expressed as a ratio of the mean number of bacterial CFU in the spleen at 5 days after infection and the CFU injected initially (log10 CFU/1010 CFU0), yields a value of 4.5 for B10.A.Bcg(r) mice and 2.6 for C57BL/10N [Bcg(s)] mice. The former group is classified as susceptible, while the latter is classified as resistant according to the above criteria. Based on our results (data not shown) as well as other observations (9, 14) demonstrating for various inbred mouse strains and the routes of F. tularensis LVS infection that bacterial growth curves in the livers and spleens are practically congruent, we have measured the bacterial burden in spleens only and consider the values sufficient and a reliable measure of the ability of a host to control bacterial growth.

Cytokine responses in infected Bcg congenic mice. Since the cytokines IFN-γ and TNF-α are required for early nonspecific-phase immunity as well as for T-cell-mediated immunity in mice infected with F. tularensis LVS (15, 18), we addressed the question of whether upon infection of congenic Bcg(s) and Bcg(r) mice, these cytokines are produced at different levels. Besides IFN-γ and TNF-α, mentioned above, we also measured the level of IL-10, the cytokine that exerts activity contrasting to that of TNF-α. Bcg(s) and Bcg(r) mice were infected with equal and relatively low initial bacterial doses that allowed analysis of phenotypic expression of resistance and susceptibility during the course of infection. As depicted in Fig. 2a, significantly higher levels of TNF-α were found in the supernatants of C57BL/10N [Bcg(s)] PEC than in the supernatants of B10.A.Bcg(r) PEC from day 3 after in vivo infection. Analysis of IL-10 showed (Fig. 2b) a significant difference in endogenous levels between congenic Bcg(s) and Bcg(r) mice, which may represent one of many pleiotropic effects of the Bcg locus (5). The initially high endogenous IL-10 levels decreased in supernatants of C57BL/10N [Bcg(s)] PEC, and IL-10 reached minimal levels on day 5 after infection (significance level, P < 0.05). In contrast, the low endogenous levels of IL-10 observed in B10.A.Bcg(r) supernatants were increased 2.5-fold (significance level, P < 0.05) during the course of infection. IFN-γ responses to infection were measured in supernatants of spleen cells, and the results are shown in Fig. 2c. While production...
occurs 5 to 7 days after infection in both congenic mouse strains, B10.A.Bcg(r) splenic cells showed significantly higher 
(up to threefold-higher) IFN-γ levels than the spleen cells of C57BL/10N [Bcg(s)] mice.

**Relationship of Bcg resistant or susceptible genotype of macrophages with the production of ROI and nitrite.** Table 1 shows the kinetics of ex vivo production of ROI and nitrite by congenic macrophages. While B10.A.Bcg(r) macrophages constitutively produced a small amount of nitrite, no significant differences between congenic macrophages in the amount of nitrite induced by infection were observed. In contrast, oxidative metabolism during the course of LVS infection, revealed by ROI production mediated by macrophage electron transport pathways, generated significant differences between the two mouse strains. An early increase, more pronounced in F. tularensis LVS-resistant C57BL/10N [Bcg(s)] macrophages on days 3 and 5, was followed by suppression on day 7. On the contrary, at this later time, B10.A.Bcg(r) macrophages produced high levels of ROI.

**DISCUSSION**

The genetic basis of host response to infection provides a major regulatory interface that influences the ability to restrict and eliminate pathogens. Variation in the resistance or susceptibility of mice to infection with F. tularensis has been recognized and shown to be a multifactorial trait (3). To observe the possible contribution of the Bcg locus, we performed a study of phenotypic expression of natural resistance and susceptibility using mice congenic at this single gene interval. Our results have demonstrated that B10.A.Bcg(r) mice, carrying the resistant allele of the Bcg locus and known to express higher levels of growth-inhibiting activity toward mycobacteria than C57BL/10N [Bcg(s)] mice (21), are susceptible to primary infection with F. tularensis LVS, as shown by a significant early increase in bacterial CFU within the tissue. In contrast, their congenic C57BL/10N [Bcg(s)] counterparts could be classified as resistant to infection. We have utilized a model with low doses of bacteria in order to investigate the capacity of genetic systems of natural resistance to infection, which might be hidden when a high antigenic load is applied. The fact that the size of the infective inoculum is critical and must be considered in determining genetic resistance or susceptibility has been noted in BCG infection. When high doses (>10⁷ CFU) were used, an inverse relationship between the inoculum size and the rate of BCG multiplication could be observed. These results were interpreted to be the effect of specific cell-mediated immunity, which develops with a short latency period and is responsible for the control of bacterial counts in mice infected with large inocula (13). The data presented, taken together with our previous results (23, 27; H. Kovárová, unpublished data) obtained with other strains of mice sharing the Bcg alleles, suggest that the Bcg locus exerts an influence on the early phase of primary F. tularensis infection.

It is well established that the effector cell responsible for the phenotypic expression of the Bcg locus in mice is the macrophage (34). Studies on the mechanisms of host defense against F. tularensis point to the macrophage as the effector cell ultimately responsible for resistance to tularemia (15), although the relevant contribution of other cells cannot be neglected (9, 10). The mechanism by which C57BL/10N [Bcg(s)] macrophages exert enhanced cytokidal or cytostatic activity toward F. tularensis LVS is not clear. Since many pleiotropic effects of the Bcg locus (6) are likely to be mediated by affecting the redox-sensitive cellular signaling pathways (7, 26), we expect that one of the possible mechanisms by which the Bcg locus determines relative resistance or susceptibility to infection is differential cytokine expression. C57BL/10N [Bcg(s)] PEC appear to be superior to B10.A.Bcg(r) PEC in the production of TNF-α and ROI early in the response to infection. This is consistent with the findings of our previous studies (23) demonstrating a possible association between resistance to infection and TNF-α production coupled with cellular redox regulation. While the up-regulation of the proinflammatory cytokine TNF-α in Bcg(s) mice is accompanied by a decrease in levels of the anti-inflammatory cytokine IL-10, the kinetics of IL-10 in Bcg(r) mice is, in contrast, increased. Adverse changes

**FIG. 1.** Growth of F. tularensis LVS in the spleens of Bcg congenic B10.A.Bcg(r) and C57BL/10N [Bcg(s)] mice. Mice were inoculated s.c. with 10⁵ CFU of F. tularensis LVS, and bacterial growth was monitored for 7 days (day 0 represents plate counts for the initial inoculum). The means for three to six mice per group per time point are shown, and the level of variability is expressed using standard errors of the means. Where error bars are not visible, the standard error was within the area occupied by the symbol. Significance levels of differences between both mouse strains at a given time point were determined by a t test and are indicated as follows: ++, P < 0.01; +, P < 0.05. Similar significant differences were obtained in two separate experiments.
spleen cell cultures. Mice were infected s.c. with $10^2$ CFU of Bcg(s) and C57BL/10N [Bcg(s)] were infected s.c. with $10^2$ CFU of F. tularensis LVS, and the cells were harvested on the indicated days of infection (day 0 represents noninfected mice). Cell-free supernatants were assessed by enzyme-linked immunosorbent assay. Data are means ± standard errors of the means for four to six mice per group per time point in two separate experiments. The significance levels of differences between the two mouse strains at a given time point were determined by a t test and are indicated as follows: * $P < 0.05$; ** $P < 0.01$.

Table 1. Production of ROI and nitrite in adherent peritoneal cell cultures from Bcg congenic B10.A.Bcg(r) and C57BL/10N [Bcg(s)] mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day postinfection</th>
<th>ROI (% of control)</th>
<th>Nitrite level ($10^{-5}$ M nitrite/2 x $10^9$ AC)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/10N [Bcg(s)]</td>
<td>0 (Noninfected)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>134 ± 8.9*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>143 ± 6.7*</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>60.3 ± 5.8**</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>B10.A.Bcg(r)</td>
<td>0 (Noninfected)</td>
<td>100</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>96.3 ± 6.7</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>120.6 ± 4.1</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>213.3 ± 8.8</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

*a Mouse strains B10.A.Bcg(r) and C57BL/10N [Bcg(s)] were infected s.c. with $10^2$ CFU of F. tularensis LVS, PEC were harvested on the indicated days after infection, and adherent cells (AC) were assessed for the production of ROI and nitrite.

FIG. 2. Cytokine secretion in cell cultures from Bcg congenic B10.A.Bcg(r) and C57BL/10N [Bcg(s)] mice. TNF-α (a) and IL-10 (b) levels were measured in spleen cell cultures. Mice were infected s.c. with $10^2$ CFU of F. tularensis LVS, and the cells were harvested on the indicated days of infection (day 0 represents noninfected mice). Cell-free supernatants were assessed by enzyme-linked immunosorbent assay. Data are means ± standard errors of the means for three to six mice per group per time point. Two or three separate experiments were performed with similar results. The significance levels of differences between the two mouse strains at a given time point were determined by a t test and are indicated as follows: * $P < 0.05$; ** $P < 0.01$.

Noted in the expression of TNF-α and IL-10 under the influence of the Bcg locus are of crucial importance at the initial stages of inflammation. Our current experiments indicate (data not shown) that observed ex vivo differences in early resistance to infection and differential expression of TNF-α levels can be well reproduced in vitro using Bcg congenic macrophage lines.

As already mentioned, B10.A.Bcg(r) mice, but not their Bcg(s) counterparts, are capable of reaching high levels of IFN-γ and ROI 5 and 7 days after infection, respectively. This points towards possible interaction between macrophages and other cells, such as T cells or NK cells, which represent major producers of IFN-γ. Regarding the time course of infection, this effect coincides with the time when the initial events of cell-mediated acquired immunity occur (2, 25). It is likely that B10.A.Bcg(r) mice, which are naturally susceptible to F. tularensis LVS, preserve the mechanism to polarize cytokine response in favor of Th1 cells. This suggestion is supported by the previously described observation that enhanced antigen processing and presentation in macrophages carrying the Bcg(r) [Nramp1(r)] allele are functionally associated with an enhanced Th1 response (31).

Although we are not able to distinguish whether the Nramp1 gene or another gene located in Bcg locus is involved in the control of the replication of F. tularensis LVS, recent results on the mechanism of the Nramp gene family in the control of host natural resistance indicate that the expression of the Nramp1 gene might be related to the innate phase of response to F. tularensis LVS infection. It has been demonstrated that the phagocyte-specific Nramp1 protein is localized in late-endosomal and lysosomal compartments (29) and that it regulates the intraphagosomal replication of live mycobacteria by altering phagosomal pH (22). Nramp1(r)-expressing macrophages display considerably enhanced acidification compared to the macrophages carrying the susceptible allele of the gene. This effect appeared to be associated with an enhanced ability of mycobacterial phagosomes to fuse with vacuolar-type ATPase-containing late endosomes and/or lysosomes. Our observation indicates that expression of the mutant allele Bcg(s) of the Bcg locus (the Nramp1 gene) could be hostile to F. tularensis LVS, while the resistant allele could mediate a permissive environment after internalization of the pathogen. This finding is in contrast to those for other microorganisms such as M. bovis, S. typhimurium, and L. donovani (13, 19, 35). Furthermore, it has been demonstrated, using agents that block acidification, that F. tularensis LVS does require an acidic environment for intracellular growth. This acidification can be linked to the availability of nutrient iron (17).

The nature of anti-Francisella natural resistance and susceptibility operating under the influence of the Bcg locus (the Nramp1 gene) at the level of phagosomal-phagolysosomal pH regulation and iron flux is currently under scrutiny.

ACKNOWLEDGMENTS

This work was supported by the Grant Agency of the Czech Republic (grant 310/99/1185 to H.K.), the Ministry of Defense (grant MO66020398130 to H.K.), and the Ministry of Education, Youth and Sports (grant VZ J14/98 151100001 to M.H.).

We are grateful to E. Skamene of McGill University for providing us with B10.A.Bcg(r) mice. We thank K. L. Elkins of the Center for...
REFERENCES