Genetic Control of Antibody Responses Induced by Recombinant \textit{Mycobacterium bovis} BCG Expressing a Foreign Antigen

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Received 24 February 1997/Returned for modification 4 April 1997/Accepted 13 May 1997

Recombinant \textit{Mycobacterium bovis} BCG expressing foreign antigens represents a promising candidate for the development of future vaccines and was shown in several experimental models to induce protective immunity against bacterial or parasitic infections. Innate resistance to BCG infection is under genetic control and could modify the immune responses induced against an antigen delivered by such engineered microorganisms. To investigate this question, we analyzed the immune responses of various inbred strains of mice to recombinant BCG expressing \(\beta\)-galactosidase. These experiments demonstrated that BALB/c mice developed strong antibody responses against BCG expressing \(\beta\)-galactosidase under the control of two different promoters. In contrast, C57BL/6, C3H, and CBA mice produced high anti-\(\beta\)-galactosidase antibody titers only when immunized with recombinant BCG expressing \(\beta\)-galactosidase under the control of the \(pblaF\) promoter, which induced the production of high levels of this antigen. This difference in mouse responsiveness to recombinant BCG was not due to innate resistance to BCG infection, since similar immune responses were induced in \(Ity^b\) and \(Ity^c\) congenic strains of mice. In contrast, the analysis of anti-\(\beta\)-galactosidase antibody responses of \(H-2\) congenic mice in two different genetic backgrounds demonstrated that \(H-2\) genes are involved in the immune responsiveness to \(\beta\)-galactosidase delivered by recombinant BCG. Together, these results demonstrate that immune responses to an antigen delivered by recombinant BCG are under complex genetic influences which could play a crucial role in the efficiency of future recombinant BCG vaccines.

BCG, a live attenuated \textit{Mycobacterium bovis} strain, represents one of the most promising live vectors to deliver foreign antigens to the immune system. Indeed, BCG has been used in more than 3 billion people, with a very low incidence of serious side effects. Recently, the development of integrative and extrachromosomal expression vectors has enabled cloning and expression of foreign genes in BCG (2, 21, 24). A variety of viral, bacterial and parasitic antigens have been now successfully expressed in BCG (2, 7, 11, 21, 23, 24). In experimental models, recombinant BCG (rBCG) expressing foreign antigens was shown to elicit protective immunity against Lyme disease (20), pneumococcal infection (14), and cutaneous leishmaniasis (1, 4). It has also been demonstrated that oral or intranasal immunization with rBCG induces substained systemic and mucosal specific immune responses (12, 13).

Because of the high number of BCG-vaccinated people, we have recently analyzed the influence of BCG priming on immune responses induced by rBCG expressing a foreign antigen. Surprisingly, this study demonstrated that BCG priming induced a strong potentiation of the antibody response directed against a heterologous antigen delivered by rBCG (9). Therefore, vaccination with BCG is not a limitation for the use of rBCG. The present study demonstrated that there are strong differences in the capacities of different strains of mice to develop an antibody response against this antigen delivered by rBCG. The analysis of immune responses of \(Ity^b\) or \(H-2\) congenic strains of mice revealed the complexity of the genetic control of these responses.

**MATERIALS AND METHODS**

**Mice.** Female BALB/c, C57BL/6, C3H/HeOuJ, and CBA mice, 6 to 8 weeks old, were obtained from Iffa Credo (FARBRES, France). Female BALB/c, BALB/B, BALB/K, C57BL/10, B10.Br, and B10.D2 mice were obtained from Olac Ltd. (Blackthorn, Bicester, England). BALB/c mice from Olac Ltd. were used in experiments comparing the responses of BALB/c, BALB/B, and BALB/K mice. Female C.D2 [Idhi1⁰–Pep5⁰ antisense], and C.BB [Ity(N9F)9], mice, 8 weeks old, were kindly provided by F. Lantier from the Institut National de la Recherche Agronomique (Nouzilly, Tours, France). These BALB/c mice have integrated through repetitive backcrosses a fragment of chromosome 1 from \(Salmonella\)-resistant strains DBA/2 and CBA, respectively. Both strains carry the resistance allele of the \(Ity\) gene. BALB/c mice from the Institut National de la Recherche Agronomique were used in experiments comparing the responses of C.D2, C.CB, and BALB/c mice.

**Microorganisms.** The \textit{M. bovis} BCG strain Pasteur 11732P2 and the rBCG strain expressing \(lacZ\) under the promoter \(p_{bac}\) from \textit{Mycobacterium paratuberculosis}, rBCG(pAN lacZ) \(lacZ\) [previously named rBCG(lacZ) or rBCG(pAM320)], were previously described (9, 15, 24). The rBCG strain expressing \(lacZ\) under the promoter \(pblA\) was constructed by operon fusion between the \(Chv_{bacZ}\) reporter cassette of \(pm_{15}\) and the promoter \(pblA\) from the \textit{Mycobacterium fortuitum} \(\beta\)-lactamase gene, thus resulting in plasmid \(pJN30\) (22).
Higher levels of β-galactosidase activity were found in sonicated extracts of BCG expressing lacZ fused to pbba* as compared to $p_{AN}$ (2,224 ± 337 vs. 364 ± 48 U) (22).

These three BCG strains were grown as surface veils on Sauton medium, and vaccine suspensions were prepared according to the classical procedure by grinding the bacillary mass on a steel ball mill as previously described (9, 10). The vaccine suspensions were stored at −70°C until use. The number of CFU per milliliter was determined by plating suitable saline dilutions on Middlebrook 7H11 agar with or without kanamycin (10 μg/ml) and X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactoside) (1 μg/ml) to check the stability of the plasmids in rBCG strains expressing β-galactosidase.

**BCG growth in target organs.** The growth of BCG strains was monitored by the enumeration of BCG and rBCG CFU in the spleens of immunized mice, as previously described (9). At various intervals after immunization, the spleens of mice were homogenized (Stomacher Lab-Blender 80; Bioblock), and suitable dilutions were plated on Middlebrook 7H11 medium with or without kanamycin (10 μg/ml) or X-Gal (1 μg/ml) to determine the numbers of CFU of BCG and rBCG, respectively. The number of rBCG CFU was determined by counting the blue colonies on X-Gal plates.

**Analysis of antibody responses.** The mice sera were tested for antibodies against β-galactosidase by enzyme-linked immunosorbent assay (ELISA) as previously described (9). The 96-well microtiter trays (Nunc) were coated with 1 μg of purified β-galactosidase (Boehringer, Mannheim, Germany) per well in phosphate-buffered saline (PBS) containing 0.1% Tween (1 h at 37°C and 16 h at 4°C). After three washes, sera diluted in buffer (PBS, 0.1% Tween 20, 1% bovine serum albumin) were added to the wells and left for 2 h at 37°C. After three washes, the wells were treated with goat anti-mouse immunoglobulin G–alkaline phosphatase conjugate (CalTag Laboratories, San Francisco, Calif.) for 1 h, and then 1 mg of p-nitrophenylphosphate per ml was added as the substrate. After 30 min of incubation at 37°C, the plates were read photometrically at 405 nm in a micro-ELISA Autoreader (Dynatech, Marnes la Coquette, France).

The negative control consisted of pooled normal BALB/c mouse sera. Individual titers were expressed as the highest dilution that gave an absorbance twice as high as that of the negative control. Titers (± standard errors) were calculated as the arithmetic mean of the log$_{10}$ titer.

**Statistics.** ELISA titers were compared by using the Mann-Whitney U test. A P value of ≥0.05 was considered nonsignificant.

**Assay for IFN-γ.** Cell suspensions were aseptically prepared by squeezing spleens between two sterile glass slides. Cells were cultured in RPMI 1640 (Sercromed, Munich, Germany) supplemented with 10% fetal calf serum, 2 mM glutamine (Sercromed), 50 μM 2-mercaptoethanol, and antibiotics. A total of 4 × 10$^6$ cells were incubated with medium alone or with 10 μg of either β-galactosidase (Boehringer) or purified protein derivative (PPD) per ml in a 1-ml volume in flat-bottom 24-well plates (Nunc, Roskilde, Denmark). Supernatants were harvested at 96 h, and the level of gamma interferon (IFN-γ) was determined by sandwich ELISA with R4-6A2 (Pharmingen, San Diego, Calif.) as a capture antibody. Ninety-six-well microplates (Nunc) were coated with this monoclonal antibody (MAb) by overnight incubation at 4°C. The plates were then blocked with 1% bovine serum albumin (Boehringer) in PBS for 1 h at 37°C and were then incubated with supernatants for 1 h at 37°C, and then the secondary biotinylated anti-interferon-specific MAb (XMG2.2; Pharmingen) was added and left for 1 h at 37°C. The binding of the second MAb was detected with streptavidin–horseradish peroxidase (Amersham, Les Ulis, France). After three washes, the plates were developed by addition of 3,3′,5,5′-tetramethylbenzidine/substrate. After 30 min of incubation at 37°C, the plates were read photometrically at 450 nm in a micro-ELISA Autoreader (Dynatech, Marnes la Coquette, France).

**RESULTS**

**Comparison of the anti-β-galactosidase antibody responses of various strains of mice immunized with rBCG(p$_{AN}$ lacZ).** We previously showed that BALB/c mice immunized with rBCG expressing β-galactosidase under the control of the $p_{AN}$ promoter developed good humoral and cellular immune responses directed against this antigen (15). To evaluate the capacity of this rBCG strain to stimulate immune responses in various strains of mice, we therefore immunized BALB/c, C57BL/6, C3H, and CBA mice with rBCG(p$_{AN}$ lacZ) and analyzed their anti-β-galactosidase antibody responses. As shown in Fig. 2A, the binding of the second MAb was detected with streptavidin–horseradish peroxidase (Amersham, Les Ulis, France). After three washes, the plates were developed by addition of 3,3′,5,5′-tetramethylbenzidine/substrate. After 30 min of incubation at 37°C, the plates were read photometrically at 450 nm in a micro-ELISA Autoreader (Dynatech, Marnes la Coquette, France). All assays were standardized with recombinant murine IFN-γ purchased from Pharmingen. Results were expressed in picograms per milliliter.

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We previously demonstrated that BCG-primed BALB/c mice developed high antibody responses against β-galactosidase expressed by rBCG(p$_{AN}$ lacZ), as compared with mice never exposed to BCG (9). We therefore tested whether BCG priming could also affect the antibody response to rBCG(p$_{AN}$ lacZ) in strains of mice which respond poorly to this rBCG.
strain. BALB/c and C3H mice received an intravenous injection of 10^6 CFU of BCG 1 month before being immunized with rBCG(pAN lacZ). As shown in Fig. 3, priming with BCG strongly enhanced the anti-β-galactosidase antibody response of BALB/c mice, confirming our previous findings (9). In contrast, the antibody response of C3H mice was only slightly affected by this priming with BCG. These results therefore demonstrated that there is a strong difference between the capacities of BALB/c and C3H mice to produce antibodies against β-galactosidase presented by BCG, even under conditions leading to optimal immunogenicity of this antigen.

Comparison of the immunogenicities of purified β-galactosidase in various strains of mice. We previously demonstrated that antibody responses induced against an antigen delivered by recombinant attenuated Salmonella typhimurium is under genetic control (6). This genetic control was shown to be related to differences in responsiveness of different strains of mice to low doses of this antigen. We therefore analyzed if such differences in responsiveness to β-galactosidase exist between the strains of mice used in this study.

BALB/c, C57BL/6, and C3H mice were injected with various doses of β-galactosidase in the absence of adjuvant. As shown in Fig. 4, C57BL/6 mice responded poorly to β-galactosidase in comparison to BALB/c and C3H mice, which developed high antibody responses even after being injected with 1 μg of purified β-galactosidase. Similar differences in the antibody responses of C57BL/6, C3H, and BALB/c mice were observed.
after immunization with β-galactosidase mixed with alum (data not shown). These results therefore showed that differences in responsiveness to β-galactosidase cannot be responsible for the low response of C3H mice to rBCG(pAN lacZ) but can explain the poor immunogenicity of this rBCG strain in C57BL/6 mice.

Increased expression of β-galactosidase by rBCG overcomes the low responsiveness of some strains of mice to rBCG expressing β-galactosidase. The results described above clearly demonstrated that some strains of mice, such as C57BL/6 mice, are low responders to β-galactosidase and may therefore require that rBCG deliver large amounts of antigen to mount a significant level of antibody response. To test this hypothesis, various strains of mice were immunized with rBCG expressing the lacZ gene under either the pAN or pblaF* promoter. It was previously shown that a higher level of β-galactosidase activity can be found in sonicated extracts of BCG expressing lacZ fused to pblaF* as compared to pAN (22).

As shown in Fig. 5, all strains of mice tested developed higher anti-β-galactosidase antibody responses after immunization with rBCG(pblaF* lacZ) than after immunization with rBCG(pAN lacZ). Moreover, after three injections of rBCG (pblaF* lacZ), the four strains of mice tested developed antibody responses higher than 4 log10 units. Increasing the level of
β-galactosidase produced by rBCG therefore overcame the genetic unresponsiveness of some strains of mice to rBCG expressing β-galactosidase.

**Genetic control of interferon production by spleen cells from mice immunized with rBCG.** The previous results show that antibody responses to β-galactosidase delivered by rBCG are genetically controlled. To analyze if such genetic control could also affect T-cell responses, we then analyzed the capacities of three different strains of mice to produce IFN-γ after rBCG immunization. As shown in Fig. 6, a strong production of IFN-γ was observed after PPD stimulation of spleen cells from BALB/c, C3H, and C57BL/6 mice immunized with wild-type BCG or rBCG, whereas normal mouse spleen cells did not respond to PPD (data not shown). After in vitro stimulation with β-galactosidase, significant levels of IFN-γ production were also found in supernatants of spleen cells from BALB/c mice immunized with rBCG(pAN lacZ) or rBCG(pblaF* lacZ). Interestingly, C3H mice immunized with rBCG(pAN lacZ) or rBCG(pblaF* lacZ) produced very high levels of IFN-γ, in contrast to immunized C57BL/6 mice, which did not produce any significant amounts of this cytokine.

**Influence of the Bcg gene on immune responses to β-galactosidase expressed by rBCG.** The natural resistance of mice to BCG is controlled by a locus called Ity, Lsh, and Bcg, for S. typhimurium, Leishmania donovani, and M. bovis (16, 18, 19). Therefore, to determine whether this gene could control the induction of humoral and cellular immune responses to an antigen expressed by rBCG, we have compared the anti-β-galactosidase responses of Ity congenic strains of mice.

Three Ity congenic strains of mice were used: BALB/c (Ity), C.D2 Idh1-Pep3b (Ity), and C.CB (Ity) mice. C.D2 mice have incorporated a 30-centimorgan segment of chromosome 1, from Idh1 to Pep3b (17). C.CB mice have incorporated a shorter fragment of chromosome 1, including the Idh1, Crog, and Vil markers (6, 14a). Both strains carry the resistance allele of the Ity gene, as tested by in vivo injections of S. typhimurium, M. bovis, and L. donovani (17).

As shown in Fig. 7, these three congenic strains of mice developed comparable antibody responses after immunization with rBCG(pAN lacZ) or rBCG(pblaF* lacZ). Higher anti-β-galactosidase antibody titers, however, were induced in mice immunized with rBCG(pblaF* lacZ). Similar proliferative responses and IFN-γ production were observed after stimulation of Ity and Ity’ spleen cells with PPD or β-galactosidase (data not shown). Together, these results indicate that the Ity gene is not responsible for the difference in immunogenicities of rBCG observed in various inbred strains of mice.

**Influence of H-2 genes on anti-β-galactosidase antibody responses induced by rBCG.** In order to determine if the difference in responsiveness to rBCG of BALB/c versus C57BL/6 or C57BL/10 mice is linked to H-2 genes, we then analyzed the immune responses of H-2 congenic mice in BALB/c and C57BL/10 backgrounds.

As shown in Fig. 8, BALB/c (H-2k), BALB/K (H-2d), and BALB/B (H-2b) mice developed high antibody responses after immunization with rBCG(pblaF* lacZ), whereas, as previously observed, lower responses were obtained in mice immunized with rBCG(pAN lacZ). After immunization with rBCG(pblaF* lacZ), no difference between the responses of the three congenic strains of mice was found. Slightly higher anti-β-galactosidase levels, however, were obtained in BALB/K mice immunized with rBCG(pAN lacZ) as compared to BALB/c and BALB/B mice.

Similar results were obtained with H-2 congenic B10 strains of mice, which developed higher anti-β-galactosidase antibody responses after immunization with rBCG(pblaF* lacZ) than after rBCG(pAN lacZ) injection (Fig. 9). In both cases, significantly higher responses were observed in B10.Br (H-2k) mice than in C57BL/10 (H-2d) and B10.D2 (H-2b) mice. Antibody responses obtained in B10 lines were much lower than those obtained in BALB/c strains of mice. In particular, after immunization with rBCG(pAN lacZ), these results clearly indicate that both H-2 and non-H-2 genes play a role in the responsiveness to β-galactosidase delivered by rBCG. They also indicate the complexity of the genetic control of immune responses directed to rBCG, since H-2b mice, depending upon their ge-
netic background, may behave as low (C3H) or high (B10.Br) responders to β-galactosidase delivered by rBCG.

**DISCUSSION**

In the present study, we have analyzed the antibody responses of various inbred strains of mice immunized with rBCG expressing β-galactosidase under the control of two different promoters. After immunization with rBCG(pAN lacZ), a strong difference between the levels of anti-β-galactosidase antibodies produced by these different strains of mice was observed. The low immunogenicity of rBCG(pAN lacZ) in some strains of mice could be related to a decreased BCG multiplication in the target organs in these mice. Indeed, multiplication of BCG in the reticuloendothelial tissues of the mouse is controlled by the Bcg locus, which exists in two allelic forms, resistant (Bcgr) and susceptible (BcgS) (18, 19). After intravenous injection of small doses of BCG, the mean numbers of CFU recovered from the spleens of sensitive strains 4 weeks after infection were at least 10 times higher than those in resistant ones (8). A rapid elimination of rBCG in the Bcgr C3H mice could explain the low antibody response of this mouse strain to β-galactosidase, by decreasing the amount of antigen delivered to lymphoid organs. However, whereas C57BL/6 and BALB/c mice...
This result could explain the difference observed in the present immunity in C57BL/10 mice, but not in BALB/c mice (26).

cation was shown to generate efficient acquired protective demonstration in C57BL/6 mice after two or three rBCG injections.

After a first injection, a more rapid elimination of rBCG can be better in both BALB/c and C57BL/6 mice than in C3H mice. The analysis of BCG recovery in the spleens of these mice at various times after infection demonstrated that while rBCG grows to MalE and responds to very low doses of this protein (6).

(5).

To test directly the influence of the Bcg gene on the antibody responses to rBCG, we have used Ity congenic strains of mice. These experiments clearly demonstrated that there are no significant differences in the capacities of Ity' and Ity congenic mice to produce anti-β-galactosidase antibodies or to develop a T-cell response following immunization with rBCG(pAN lacZ).

In a previous study, we have observed that genetic factors modulate the immune responses to the MalE antigen delivered by recombinant attenuated S. typhimurium (6). The major genetic influence was shown to be exerted by H-2 genes, which control the responsiveness of different strains of mice to low doses of MalE (6). In the present study, we demonstrated that mouse strains differ also by their capacities to respond to low doses of β-galactosidase. The lack of response of C57BL/6 mice to limited amounts of β-galactosidase can therefore explain the low immunogenicity of rBCG(pAN lacZ) in this mouse strain, as compared to BALB/c or C3H mice, which produced good anti-β-galactosidase responses even after immunization with low doses of this antigen. In agreement with this hypothesis, following immunization with rBCG(pblaF* lacZ), which produced approximately sixfold-larger amounts of β-galactosidase than rBCG(pAN lacZ) (22), all strains of mice developed strong antibody responses. However, the anti-β-galactosidase antibody responses of C57BL/6 and C57BL/10 mice remained significantly lower than that of BALB/c mice, showing that increasing the amount of foreign antigen delivered by rBCG does not totally overcome the genetic difference between these mouse strains.

To further analyze the mechanisms of this genetic control of responsiveness to rBCG, we have compared the antibody responses of H-2 congenic mice in two different backgrounds corresponding, in both cases, to Bcg/k mice. These experiments clearly show that H-2 genes play a major role in the control of these responses. Indeed, whereas C57BL/10 (H-2d) and B10.D2 (H-2d) mice responded very poorly to rBCG(pAN lacZ), B10.Br (H-2k) mice developed antibody responses comparable to those of BALB/c mice. The antibody response of B10.Br mice to rBCG(pblaF* lacZ) was also significantly higher than those of C57BL/10 and B10.D2 mice. This effect could indeed be related to the observation that C3H mice responded well to low doses of β-galactosidase. Such an effect of H-2 haplotype was also observed with H-2 congenic BALB/c mice, but only after immunization with rBCG(pAN lacZ). Together, these results indicate that H-2 genes play an important role in the induction of immune responses directed against antigens delivered by rBCG but that this effect can be evidenced only if the amount of antigen delivered to the immune system is suboptimal. This last hypothesis is in very good agreement with our recent findings that C57BL/6 mice developed very good antibody responses to the MalE antigen delivered by rBCG (data not shown). Indeed, this mouse strain is a high responder to MalE and responds to very low doses of this protein (6).

These last findings can therefore explain the differences in responsiveness of BALB/c and C57BL/6 mice. However, since C3H mice also respond to low doses of β-galactosidase, they cannot account for the lack of responsiveness of this strain to rBCG(pAN lacZ). However, the analysis of interferon production in rBCG-immunized C3H mice clearly demonstrated that these mice developed a very strong β-galactosidase-specific T-cell response. The lack of induction of an antibody response in C3H mice by rBCG(pAN lacZ) may therefore reflect the

are both Bcg5 (8), rBCG(pAN lacZ) induced good anti-β-galactosidase levels in BALB/c but not in C57BL/6 mice. The analysis of BCG recovery in the spleens of these mice at various times after infection demonstrated that while rBCG grows better in both BALB/c and C57BL/6 mice than in C3H mice after a first injection, a more rapid elimination of BCG can be demonstrated in C57BL/6 mice after two or three rBCG injections. Interestingly, although both C57BL/10 and BALB/c mice bear the susceptible phenotype of innate resistance, BCG vaccination was shown to generate efficient acquired protective immunity in C57BL/10 mice, but not in BALB/c mice (26). This result could explain the difference observed in the present study between BCG elimination in BCG-immune BALB/c and C57BL/6 mice. Differences in T-cell responses induced by BCG in these two strains of mice were also recently demonstrated (5).

FIG. 9. Antibody responses of H-2 congenic B10 lines immunized with BCG expressing β-galactosidase. On day 0, C57BL/10 (H-2b), B10Br (H-2k), B10.D2 (H-2d), and B10D2 (H-2k) mice (five per group) were immunized intravenously with 5 x 10^6 CFU of rBCG(pAN lacZ) or rBCG(pblaF* lacZ). On days 28 and 56, mice received intravenous boost injections of 10^6 CFU of these recombinant BCG strains. Mice were bled at various times after injections, and antibody responses were determined by ELISA. Results are given as mean log_{10} titer ± standard errors for individual serum samples.
induction in this strain of a strong CD4+ immune response highly polarized towards a Th1 phenotype.

Together, the results of the present study demonstrated that the induction of immune responses against an antigen delivered by rBCG is under complex genetic influences. Some of these factors are certainly specific to the antigen expressed by the rBCG. Indeed, using rBCG expressing the outer surface protein A antigen of Borrelia burgdorferi, Stover et al. (20) have obtained high antibody responses in C3H mice, whereas BALB/c mice required boosting to develop comparable responses. It is interesting that these two mouse strains immunized with rBCG vaccines expressing this antigen as a lipoprotein developed strong specific antibody responses (20), which is in good agreement with our recent finding that increasing the genetic control of innate resistance to BCG infection in genetically resistant and susceptible mouse strains. J. Immunol. 129:2179–2185.


ACKNOWLEDGMENTS

We thank F. Lantier for his generous gift of C.D2 and C.CB mice and S. Pires for typing the manuscript.

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