Repertoires of Antibodies to Culture Filtrate Antigens in Different Mouse Strains Infected with Mycobacterium bovis BCG

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Two susceptible (Bcg+) mouse strains, BALB/c and C57BL/6, were compared by Western blot (immunoblot) analysis for their immunoglobulin G response to 14-day-old BCG culture filtrate (CF) following intravenous infection with live Mycobacterium bovis BCG. The two strains demonstrated a completely different antibody repertoire. BALB/c antibodies were directed against a wide range of CF antigens between 20 and about 100 kilodaltons (kDa), with a preferential recognition of the 65-kDa heat shock protein and the 32-kDa fibronectin-binding protein. C57BL/6 sera, on the other hand, showed a much more restricted antibody pattern, almost exclusively directed against three antigens with estimated molecular sizes of 37, 38, and 40 kDa. Whereas the 37- and 38-kDa antigens were also recognized by BALB/c mice, the 40-kDa antigen was very intensely stained by C57BL/6 sera only. F1 mice had the restricted antibody pattern of C57BL/6/6 after one injection of BCG and had a hybrid BALB/c-C57BL/6 phenotype following a boost injection of BCG 2 months after the initial infection. Analysis of seven recombinant inbred strains derived from the BALB/c × C57BL/6 cross and of congenic mice differing in major histocompatibility complex-coding chromosome 17 fragments suggests that a gene in the K1A region of the H-2 locus is associated with the preferential recognition of certain CF antigens. Inoculation with the same dose of killed BCG failed to elicit an antibody response to these filtrate antigens.

Protective immunity against mycobacteria is mediated by acquired populations of specifically sensitized T cells which activate macrophages by secretion of lymphokines (18). Less is known about the precise antigens involved in this acquired immunity. Some authors have hypothesized that secreted antigens are essential, because live mycobacteria are more effective than killed bacilli in evoking protective immunity (8, 24). Other authors have suggested that cell-wall-associated proteins are the major contributors to cell-mediated immunity against mycobacteria (15, 21, 23). These two hypotheses are not necessarily in contradiction with one another, as some of the cell-wall-associated proteins are also contained within early culture filtrates (CF) (1). One of these shared proteins is the 32-kilodalton (kDa) antigen, the major constituent of 14-day-old mycobacterial CF (10) and known to be an actively secreted protein with a signal peptide in the coding sequence (3).

Antibodies do not confer protective immunity against mycobacteria (25), and passive transfer of anti-mycobacterial antibodies may even enhance bacterial replication in the spleen of infected mice (13). In leprosy, it is well known that cell-mediated and humoral immunity are inversely related (2), and a similar concept has also been postulated for tuberculosis (19). Antibody responses following hypervaccini-

zation with Mycobacterium tuberculosis H37Rv soluble extract or with M. tuberculosis CF antigens are influenced by genes from the major histocompatibility complex (MHC) (17, 20). Whether MHC genes also control antibody formation during active mycobacterial infection has not been studied, to our knowledge.

By using the technique of Western blot (immunoblot) analysis, we have examined the immunoglobulin G (IgG) response against BCG CF antigens in sera from two suscep-
tible (Bcg+) mouse strains, BALB/c and C57BL/6, infected intravenously with live or killed Mycobacterium bovis BCG. Examination of BALB/c × C57BL/6 (C × B) recombinant inbred strains and MHC-congenic mice indicates that the H-2 haplotype determines to a certain extent the antibody repertoire used against these CF antigens.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, C57BL/10, (BALB/c × C57BL/6) F1, (C × DBF)F1, (C57BL/6 × BALB/c)F1, [1B × C]F1, DBA/2, and C × B D (H-2b), E (H-2a), G (H-2a), H (H-2a), I (H-2a), J (H-2b), and K (H-2b) lines were bred in the Animal Facilities at the Pasteur Institute of Brabant. BALB.B10 (H-2b), B10.D2 (H-2a), and B10.GD (K1A Ad Ee Sb Dd) MHC-congenic mice were bred from couples obtained from the Netherlands Cancer Institute. Three-month-old female mice were used unless otherwise stated.

BCG infection. Mice were inoculated intravenously with 0.5 mg (ca. 4 × 10⁶ CFU) of freshly prepared M. bovis BCG GL2 (Pasteur Institute of Brabant) which had been grown for 2 weeks as a surface pellicle on Sauton culture medium at 37.5°C (4). Mice were sacrificed by exsanguination 2, 4, 6, or eight weeks later. The sera of at least four mice per group were pooled and kept frozen at −20°C until assayed. For the boost injections, a second intravenous BCG inoculation (0.5 mg) was given 2 months after the initial one and mice were bled 4 weeks later.

Antigens. CF antigens were obtained from 7- and 14-day old cultures of M. bovis BCG GL2 (the same BCG as the one described above for infection). Proteins were concentrated by precipitation with ammonium sulfate (80% saturation). After centrifugation, the pellet was dissolved in and dialyzed against phosphate-buffered saline. The filtrate was passed through a 0.22-µm-pore-size sterilizing membrane (Flow
Laboratories, McLean, Va.) and stored at -20°C until used. The same batch of CF was used in all experiments. In parallel, BCG cellular extract was prepared by using a French press, as described previously (11).

SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of BCG CF was performed in reducing conditions on 12.5% acrylamide gels. Gels were run overnight (see Fig. 2) or for 4 hours (see other figures). After completion of SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose sheets by the method of Towbin et al. (31). Nitrocellulose was then incubated with 0.02 M Tris-0.5 M NaCl buffer (pH 7.5) (TBS) containing 2% bovine serum albumin for 1 h to prevent nonspecific absorption of proteins. It was then cut into strips (containing ≥30 μg of CF) which were incubated overnight with mouse sera or murine monoclonal antibodies (diluted 1:50) in TBS containing 1% bovine serum albumin and 0.05% Tween (TBS-T). Nitrocellulose strips were then washed twice and incubated for another 4 h with peroxidase-conjugated rabbit anti-mouse IgG antibody (Dakopatts, Copenhagen, Denmark) diluted 1:200 in TBS-T. The strips were washed again and incubated with α-chloronaphthol in the presence of hydrogen peroxide. The reaction was stopped after 15 min. A number of CF antigens were identified with monoclonal antibodies directed against M. tuberculosis filtrate antigens (HBT-1, HBT-2, HBT-3, HBT-4, HBT-7, HBT-11, HBT-12, HYT-6, HYT-27, HYT-29, HÅT-3, HÅT-5, CBA-1, CBA-4, and C38.d1) (20, 27, 33).

RESULTS

Molecular weight characterization and identification of the major antigens in BCG CF. Figure 1 shows the protein pattern observed after SDS-PAGE of a 7- and a 14-day-old M. bovis BCG GL2 CF. Protein content of 7-day-old CF was very low (4.5 μg/ml), but, following ammonium sulfate concentration, three protein bands with estimated molecular sizes of 25, 32, or 35 kDa could be clearly visualized. Filtrates from 14-day-old cultures had a higher initial protein content (25 μg/ml) and contained a number of additional protein bands. As previously reported (10, 11), the 32-kDa antigen appeared as the major component in these filtrates.

By using a battery of anti-M. tuberculosis monoclonal antibodies, a number of protein antigens could be visualized in 14-day-old CF from M. bovis BCG, demonstrating the highly cross-reactive nature of both species (Fig. 2).

Kinetics of antibody response against BCG filtrate antigens in BCG-infected susceptible BALB/c and C57BL/6 mice. Following an intravenous infection with live BCG, BALB/c sera were found to contain more IgG directed against 14-day-old CF antigens than did C57BL/6 sera (Fig. 3, lanes 3 through additional...
Antibody response was very weak at 2 weeks, peaked at 4 weeks, and slowly decreased thereafter.

Furthermore, the two strains showed a pronounced difference in repertoire which could be observed consistently when tested 4, 6, and 8 weeks after infection. BALB/c sera reacted to a wide variety of antigens with molecular sizes ranging between about 20 and 100 kDa. B6 sera reacted with a limited number of CF antigens with estimated molecular sizes of 37, 38, 40, and 82 kDa. Whereas the 37- and 38-kDa antigens were also recognized by BALB/c sera, the 40-kDa antigen was very intensely stained by B6 sera only. BALB/c sera preferentially recognized a 65-kDa protein, whereas B6 sera stained this antigen only very weakly. (The protein recognized by B6 sera after 6 weeks of infection [Fig. 3] had a slightly higher molecular size.) The 65-kDa antigen could be identified as the widely cross-reacting 65-kDa heat shock protein by using the X118 monoclonal antibody (30) with known specificity for the heat shock protein (data not shown). The 32-kDa antigen was strongly recognized by BALB/c sera after 6 weeks of infection and only weakly recognized by sera from B6 mice. Finally, a weak IgG response was observed in both strains against an antigen with an estimated molecular size of 25 kDa.

Sera taken 6 weeks after infection were also tested against a 7-day-old filtrate (Fig. 3, lanes 1 and 2). BALB/c sera showed a weak recognition of the 32-kDa antigen only. B6 sera did not react at all with this very early CF. All further studies were therefore performed with 14-day-old CF.

Antibodies against BCG extract and filtrate antigens in BALB/c and C57BL/6 mice infected with live or killed BCG. In contrast to the pronounced difference in antibody repertoire towards 14-day-old CF antigens, Western blot patterns against BCG extract antigens were very similar in sera from BALB/c and B6 mice that had been infected with live BCG 6 weeks before (Fig. 4). Antibody levels were somewhat higher in BALB/c than in B6 mice, but on the whole the same extract antigens were recognized by both strains. Extract antigens of estimated molecular sizes of 37, 38, and 45 kDa were intensely stained, whereas the 32-kDa antigen could not be visualized with antibodies in this extract. As for the CF, the 65-kDa antigen (and its degradation products) was only recognized by BALB/c but not by B6 sera. Immunization with the same dose of heat-killed BCG (1 h, 120°C) elicited a weak antibody response against BCG extract and no response at all against BCG filtrate.

Western blot analysis of (C × B)F₁, and (B × C)F₁, sera. Sera were collected from BALB/c, B6, and F₁ mice 4 weeks after one BCG injection (Fig. 5A) or 4 weeks after a boost injection of BCG (Fig. 5B). Following a primary infection with BCG, (B × C)F₁ mice showed an IgG recognition pattern comparable to that of the parental B6 phenotype, with the presence of IgG almost exclusively directed against the 37-, 38-, and 40-kDa antigens. (C × B)F₁ mice reacted only against the 40-kDa component. The 32- and 65-kDa antigens were very weakly recognized by F₁ sera. Following a second injection, BALB/c and B6 parental phenotypes did not change and mice continued to show their respective wide range and restricted antibody repertoire. (C × B)F₁ sera demonstrated a hybrid phenotype in which both the 32- and 65-kDa antigens (dominant in BALB/c) and the 37-, 38-, and 40-kDa antigens (dominant in B6) were intensely recognized. In the reciprocal (B × C)F₁ cross, antibodies to the 32-kDa antigen were found to be less intense. When comparing the male progeny of the reciprocal F₁ crosses, the antigen band with an estimated molecular size of 25 kDa was found to be preferentially stained by (C × B)F₁ sera, suggesting a possible X-linked influence.

Western blot pattern in BCG-infected C × B recombinant inbred strains. All H-2k recombinant inbred strains, i.e., C × B E, G, I, J, and K, demonstrated the preferential anti-37-, 38-, and 40-kDa responses of B6 phenotype (Fig. 6). In C × B J sera, antibodies to the 40-kDa antigen were somewhat weaker. C × B D and H mice (H-2d) did not recognize these three antigens at all. Furthermore, the antibody levels in all recombinant inbred strains were much lower than in BALB/c sera. The same restricted recognition of the 37-, 38-, and 40-kDa antigens in all five H-2k recombinant inbred strains was also observed 6 weeks after a primary infection and 4 weeks after a boost injection of BCG. Primary infection did not result in significant staining of the 65-kDa antigen, and following a boost injection, only C × B D and H (both H-2k) mice showed intense recognition of the heat shock protein (data not shown).
Western blot pattern in MHC-congenic mice. The use of congeneric mice, differing in MHC-coding chromosome 17 fragments, confirmed that the antibody repertoire is influenced by the H-2 locus. C57BL/10 mice (H-2b) recognized the 37-, 38-, and 40-kDa antigens in the same way as did C57BL/6 mice (Fig. 7). BALB.B10 mice carrying the MHC genes of B10 origin on a BALB/c background also showed this preferential recognition of those three antigens. DBA/2 mice (H-2d) demonstrated a very faint antibody response against the 32- and 65-kDa antigens and did not recognize the 37-, 38-, or 40-kDa antigens at all. B10.D2 mice having the H-2d genes of DBA/2 origin on a B10 background did not produce any antibodies to these 37-, 38-, or 40-kDa antigens either but weakly stained the 32- and 65-kDa antigens. Finally, recombinant congeneric B10.GD mice (Kd A^d E^b S^b) did not recognize the 37-, 38-, and 40-kDa antigens either but showed a substantial antibody response towards the 32-kDa antigen. Furthermore, these B10.GD mice reacted against both a filtrate antigen with an estimated molecular size of about 55 kDa and the 65-kDa heat shock protein.

DISCUSSION

Sera from BCG-infected BALB/c and C57BL/6 mice were found to contain substantial IgG levels against CF antigens. Injection of heat-killed BCG failed to elicit these antibodies, indicating that the anti-CF response was directed against components (secretion products?) of actively growing bacilli and not against degradation products of dead bacteria. Furthermore, the antibody repertoire was completely different for both strains and was apparently influenced, to a certain extent, by the H-2 haplotype.

Susceptibility to mycobacterial infections and the immune reactions they evoke have been associated with genes of the MHC, in particular with its class II antigens. In humans, the HLA-DR2-DQwl haplotype has been associated with increased susceptibility to pulmonary tuberculosis (5, 28) whereas HLA-DR3 was found to be significantly decreased in tuberculous patients (9). Also, in leprosy, human leucoocyte antigen-linked genes seem to control not the susceptibility as such but rather the type of the disease (32). In mice, H-2-linked genes are known to influence the granulomatous reaction to M. leprae (7), the antibody response following hyperimmunization with M. tuberculosis extract (17) or CF antigens (20), and the gamma interferon secretion in response to the 32-kDa antigen from BCG (16).

It is clear from the results presented here that the secretion of IgG antibodies against BCG CF antigens during active BCG infection is also influenced to a certain extent by H-2-linked genes. Thus, infected C57BL/6 mice reacted very strongly with a 40-kDa antigen and all C × B recombinant inbred strains with the H-2^d haplotype also recognized this antigen. The H-2^d haplotype, on the other hand, was associated with preferential recognition of the 32- and 65-kDa antigens. By using MHC-congeneric BALB.B10 and B10.GD mice, we were able to locate the controlling gene in the K-IA region. These results are reminiscent of data by Ivanov and Sharp (17), who showed, by using competitive inhibition tests with radiolabeled monoclonal antibodies, that following hyperimmunization with M. tuberculosis extract, the H-2^b haplotype is associated with high antibody levels towards TB71 and TB72 epitopes (located on a 38-kDa antigen of M. tuberculosis H37Rv extract).

Whether the 37-, 38-, and 40-kDa antigens are also immunodominant antigens at the T-cell level in H-2^b haplotype mice is not clear at the moment. In this respect, it must be emphasized that humoral and cellular responses may be inversely correlated during mycobacterial infections. Thus, the highest levels of anti-32-kDa antibodies were detected in sera from BALB/c mice, in which the gamma interferon response to the 32-kDa antigen (and to purified protein derivative) is known to be the lowest (16). In humans, elevated antibody titers to a 38-kDa antigen from M. tuberculosis filtrate were found in 57% of a group of tuberculosis patients but in none of the controls (12); furthermore, the highest antibody levels to the 38-kDa antigen were associated with the “susceptibility” allele DR2-DQw1 (5).

Our results show that the immunodominance of an antigen is not universal for all genetic configurations. Thus, the 65-kDa protein, which has been reported in the literature to
be a very immunodominant antigen at the B-cell level (30), induced high antibody titers during BCG infection in BALB/c, but not in C57BL/6, mice. The 65-kDa antigen also seems to be dominant in BCG-infected CBA/Ca (H-2b) and in wild mice (unpublished data). Recently, Brett et al. have reported that immunization with the recombinant 65-kDa protein resulted in specific T-cell and antibody responses in seven different H-2 congenic mouse strains and, in that study, the B10 strain also ranked as the least reactive (6).

When two mouse strains with the H-2d haplotype, BALB/c and DBA/2, were compared, it was found that besides MHC, other factors influence the antibody production to CF antigens and in particular the amount of IgG. The Bcg gene, known to express the resistant allele in DBA/2 mice (29), seems not to be involved (unpublished data on Bcg<sup>C</sup>D2 mice).

In another experimental model, i.e., murine leishmaniasis, Sadick et al. have obtained very similar results (26). Thus, lymph node cells from C57BL/6 mice produced higher gamma interferon titers against Leishmania major antigens than did cells from BALB/c mice. Moreover, C57BL/6 mice generated only a limited array of Leishmania-specific antibodies in a Western blot analysis against promastigote antigens in contrast to BALB/c mice that reacted with a large number of Leishmania antigens. It is remarkable that the restricted antibody response in B6 mice was here again directed against three antigens with molecular sizes of about 40 kDa (26). In the Leishmania model, it has been suggested that BALB/c mice preferentially activate their TH2 helper T-cell population, resulting in low levels of secretion of gamma interferon and high levels of secretion of interleukins 4, 5, and 6 (22). Whether a similar selective activation of TH2 cells also occurs in BCG-infected BALB/c mice remains to be elucidated.

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LITERATURE CITED


