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Influence of Mouse Strain and Vaccine Viability on T-Cell Responses Induced by *Mycobacterium bovis* Bacillus Calmette-Guérin

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C57BL/6 and BALB/c mice were vaccinated with either live or heat-killed *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) organisms, and splenic T cells were used to screen the stimulatory potential of fractionated somatic and secreted mycobacterial proteins by production of gamma interferon (IFN-γ). Maximum responses were obtained with fractionated secreted proteins of *Mycobacterium tuberculosis*. There was no single dominant antigen, but five regions of mycobacterial proteins induced high concentrations of IFN-γ. However, only two of the five regions stimulated T cells from both mouse strains: two were exclusively recognized by T cells from BALB/c mice, and one was exclusively recognized by T cells from C57BL/6 mice. T cells from mice vaccinated with heat-killed *M. bovis* BCG organisms failed to respond to fractionated secreted proteins but recognized several somatic antigen fractions. As late as 1 year after primary vaccination, memory T cells responded to similar protein regions, and IFN-γ production was intensified by secondary infection. Our data confirm a central role for secreted proteins in immunity to mycobacteria. Moreover, we demonstrate that a major set of mycobacterium-reactive T cells is stimulated only by vaccination with live but not with heat-killed *M. bovis* BCG organisms. Because a major impact of genetic host factors on antigen recognition was observed, we favor the use of live carrier organisms which secrete mycobacterial proteins over subunit vaccines as an improved antituberculosis vaccine.

Tuberculosis is primarily caused by the facultative intracellular bacterium *Mycobacterium tuberculosis* and, to a small degree, by *Mycobacterium bovis* and *Mycobacterium africanum* (26). Globally, it is the leading cause of death among infectious diseases. The only available vaccine so far is the attenuated *M. bovis* bacillus Calmette-Guérin (BCG [9]) vaccine. Although BCG ranks among the most frequently used vaccines in the world, its efficiency in adults is unpredictable and ranges from 0 to 80% protection (24). A recent meta-analysis indicates that BCG reduces the average risk of tuberculosis by 50% (10). Several new vaccination strategies are being discussed, including subunit vaccines and recombinant bacteria or viruses (20, 30). Thorough knowledge of host-parasite interactions and characterization of potentially protective or harmful T-cell antigens are essential prerequisites for any rational approach.

We have previously shown that the T-cell response in tuberculosis patients is not directed toward one or few dominant antigens but rather is directed toward a multitude of mycobacterial proteins (13, 41). However, individual differences in antigen recognition patterns complicated the identification of dominant protein regions in these studies. After infection of mice with *M. tuberculosis*, T cells also respond to numerous mycobacterial proteins (4, 36), and proliferation and target cell lysis are stimulated by several mycobacterial protein fractions (36). In vitro, gamma interferon (IFN-γ) is produced by T cells which confer protection against challenge with *M. bovis* BCG (29, 31). The indispensable role of IFN-γ in vivo has been demonstrated in antibody neutralization experiments and by the fatal outcome of *M. bovis* BCG and *M. tuberculosis* infections in IFN-γ or IFN-γ-receptor gene knockout mice (6, 11, 12, 16, 25).

Vaccination of mice with live mycobacteria induces long-lasting immunity (7, 34), and animals are protected against challenge with virulent *M. tuberculosis* as long as 2 years after immunization with *M. bovis* BCG (33). However, little is known about development and survival of protective memory T lymphocytes. Antigen persistence, claimed to be of crucial importance, could be afforded by chronic infection. Nonreplicating vaccines are generally less efficient than live microorganisms, and a protective role has been proposed for secreted mycobacterial proteins as metabolites of bacteria living inside host cells (34). Vaccination of mice with heat-killed *M. tuberculosis* organisms fails to protect against lethal challenge infection (35), although evidence for protection with heat-killed *Mycobacterium w* has been provided (42). Recently, protection has been achieved by vaccination of mice or guinea pigs with culture filtrate proteins prior to challenge with virulent *M. tuberculosis* (2, 21, 37).

Inbred mouse strains differ in a multitude of genetic factors. The *bcg* gene controls the susceptibility or resistance of mice to mycobacterial infections during the first weeks postinfection (18, 43). The gene product of *Nramp*, a candidate gene for *bcg*, seems to affect macrophage antibacterial activities and/or binding to extracellular matrix proteins (17, 47). Although C57BL/6 and BALB/c mice are naturally susceptible (bcg^s) to infection with *M. bovis* BCG (18), presentation of antigenic peptides to T lymphocytes should vary because both mouse strains express different major histocompatibility complex (MHC) haplotypes. Peptides binding to individual MHC class I molecules are characterized by specific amino acids in anchor positions (39), and a similar principle holds true for MHC class II molecules (15).

In this report, three major aspects of the immune response caused by vaccination with *M. bovis* BCG were investigated in a mouse model. (i) T-cell responses to secreted and somatic mycobacterial antigens were compared for two bcg^s mouse strains, and a major impact of host genetic differences was observed. (ii) Vaccination with either live or heat-killed *M. bovis* BCG organisms revealed that a nonviable vaccine is in-

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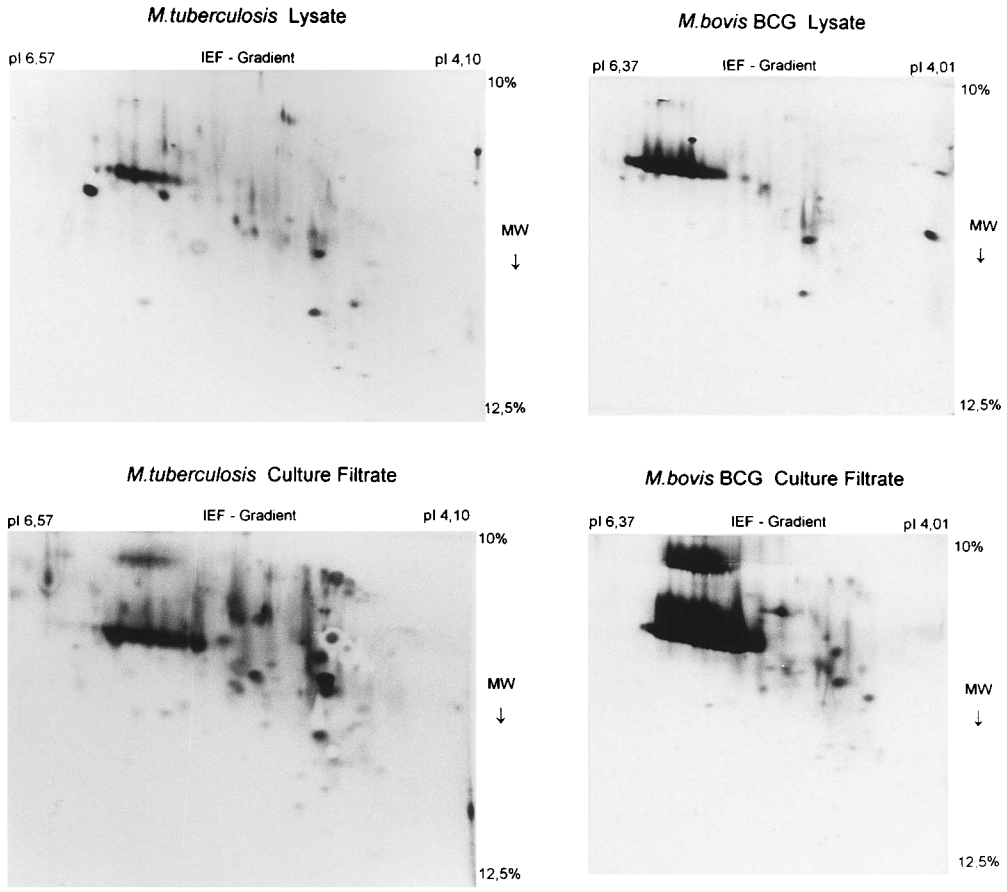

FIG. 1. *M. tuberculosis* and *M. bovis* BCG lysate and culture filtrate proteins after two-dimensional separation. *M. tuberculosis* proteins (left) and *M. bovis* BCG proteins (right) were separated in the first dimension by IEF. One hundred micrograms of lysates (upper row) and 100 μg of culture filtrates (lower row) were loaded on IEF gels and separated further on native polyacrylamide gradient gels (10 to 12.5%) by molecular weight (MW). Proteins were visualized with Coomassie brilliant blue and then by silver staining.

sufficient for activating T cells which recognize secreted proteins. (iii) Comparison of antigen recognition patterns during active and memory immunity showed that the initial T-cell repertoire remained stable over a long time.

MATERIALS AND METHODS

Mice and vaccination. C57BL/6 and BALB/c mice were bred and maintained under specific-pathogen-free conditions at the University of Ulm. Eight- to 12-wk-old male mice were used for primary infections; from 1×10^6 to 3×10^6 *M. bovis* BCG (strain Chicago, ATCC 27289) organisms were injected intravenously in a volume of 0.2 ml into the tail vein. For secondary infections, 6.6×10^6 bacteria were injected by the same route 1 year after the first infection. Individual inocula were confirmed by plating an aliquot of the bacterial suspension on Middlebrook agar plates (Difco, Detroit, Mich.) supplemented with 10% OADC enrichment (Difco). Heat-killed *M. bovis* BCG bacteria were injected intravenously at a concentration equivalent to 7×10^7 organisms. Mice were killed by cervical dislocation, and spleens were removed aseptically.

Bacteria and bacterial antigens. *M. bovis* BCG organisms were cultured from the homogenized spleen of an infected mouse and grown in Dubos broth base (Difco) containing 10% Dubos medium albumin (Difco) until mid-log phase. Aliquots were stored at −70°C. Numbers of viable bacteria were determined by plating serial 10-fold dilutions on Middlebrook agar plates. For infection, an aliquot was thawed, washed in phosphate-buffered saline (PBS), briefly sonicated, and diluted as required. Heat-killed *M. bovis* BCG bacteria were produced by autoclaving and subsequent washing in PBS. Autoclaved bacterial suspensions were filtered through steel mesh sieves to remove clumps and were sonicated prior to use. For production of bacterial lysates, *M. tuberculosis* H37Rv and *M. bovis* BCG organisms were grown in Dubos medium with albumin at 37°C for 4 weeks. Bacteria were harvested by centrifugation, washed once with PBS, and killed by treatment with 70% ethanol (2 h) and irradiation with UV light (2 h). After washing, bacteria were resuspended in a small volume of 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$. An equal weight of glass beads (0.18 mm in diameter; B. Braun Melsungen, Melsungen, Germany) and a cocktail of protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride; all from Boehringer Mannheim, Mannheim, Germany) were added, and the organisms were disrupted in a glass bead mill (Braun). Crude lysates were treated with DNase-RNase (Sigma, St. Louis, Mo.) and centrifuged at 107,000 × *g* for 30 min. Culture filtrate proteins were produced as described previously (13). In short, *M. tuberculosis* and *M. bovis* BCG organisms were grown in zinc-free Sauton's medium at 37°C for 3 weeks. Proteins >10 kDa were concentrated from the medium through an Amicon YM-10 membrane (Amicon, Beverly, Mass.). Protein concentrations were determined with the bicinchoninic acid protein test (Pierce, Rockford, Ill.). Aliquots were stored at −20°C.

Two-dimensional PAGE. In the first dimension of two-dimensional polyacrylamide gel electrophoresis (PAGE), proteins were focused according to their pIs as described previously (19) with slight modifications. In short, 100 μg of proteins was loaded on denaturing gels containing 8 M urea (Bio-Rad, Richmond, Calif.) and 10% ampholytes (consisting of 60% Servalyte 4 to 6, 20% Servalyte 5 to 7, and 20% Servalyte 3 to 10; Serva, Heidelberg, Germany). Isoelectric focusing (IEF) gels were run in glass tubes over 6,000 V · h. After equilibration in 60 mM Tris-HCl (pH 8.8) containing 10% glycerol for 45 min, IEF gels were stored at −20°C. For the second dimension, linear 10 to 12.5% native polyacrylamide gradient gels were employed, because addition of sodium dodecyl sulfate (SDS) is detrimental for the subsequent use of proteins in tissue culture. The final gel size (8.5 by 11 cm) was fitted exactly to the master plate of the Blotelutor electroelution device (Biometra, Göttingen, Germany) with its grid of 24 by 20 fractions. Molecular weight standards were not used because they do not migrate according to their size in the native system.

Protein transfer. An electroelution device, Blotelutor, was used to transfer 480 distinct liquid protein fractions from two-dimensional gels as described earlier (19). Fractions were stored at −20°C. Before use, each fraction was diluted with 130 μl of plain Iscove's modified Dulbecco's medium (Biochrom, Berlin, Germany); duplicates of 20 μl were transferred into round-bottom 96-well tissue culture plates (Nunc, Roskilde, Denmark) and sterilized with UV light. The

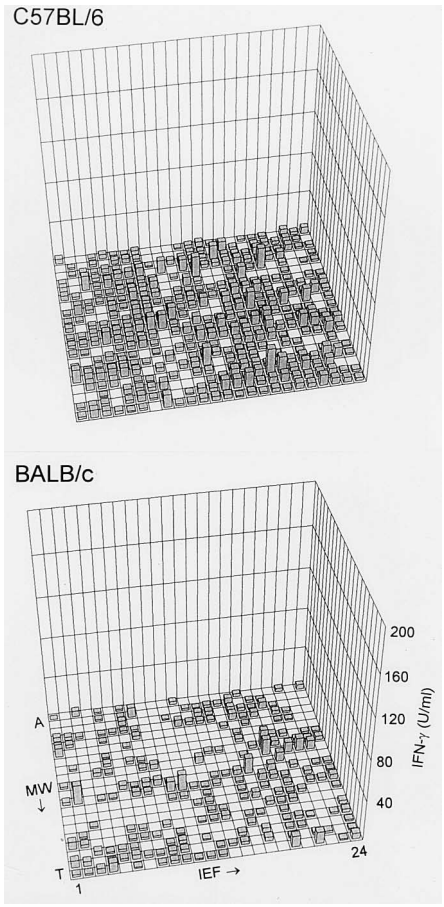

FIG. 2. IFN-γ profiles of T cells from naive C57BL/6 and BALB/c mice. T cells from the spleens of naive C57BL/6 (top) and BALB/c (bottom) mice were enriched and stimulated with fractionated *M. tuberculosis* culture filtrate proteins for 5 days in vitro. The concentration of IFN-γ in culture supernatants was determined by ELISA; the concentration scale is given on the ordinate of the lower figure. Each square on the base grid represents one protein fraction. All fractions were tested in duplicates, and the arithmetic mean is shown. Fraction numbers 1 (pI 6.57) to 24 (pI 4.10) mark the IEF gradient; fractions A (10% acrylamide) to T (12.5% acrylamide) indicate the molecular weight (MW) gradient. The bromophenol blue marker front was located just below fraction T. Controls were performed with unfractionated mycobacterial proteins and ConA (all used at 0.6 μg/ml). Concentrations of IFN-γ for medium alone, *M. tuberculosis* lysate, *M. tuberculosis* culture filtrate, *M. bovis* BCG lysate, *M. bovis* BCG culture filtrate, or ConA were 1, 3, 6, 4, 6, and 11 U/ml, respectively, for C57BL/6 and 1, 3, 1, 4, 5, and 40 U/ml, respectively, for BALB/c.

protein concentrations of individual fractions could not be determined, but we know from previous studies that a direct correlation between abundance of proteins and dominance of T-cell antigens does not exist (13, 14, 41).

In vitro T-cell stimulation. Spleens from infected animals were passed through steel mesh sieves to obtain single-cell suspensions. Erythrocytes were lysed with 0.17 M ammonium chloride, and the remaining spleen cells were washed three times in medium. Depletion of macrophages and B cells was performed by filtration over nylon wool columns. Fluorescence-activated cell sorter analysis of enriched cells showed that they were made up of 80 to 85% T lymphocytes and 10 to 15% B lymphocytes (data not shown). T cells were resuspended in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 100 U of penicillin-streptomycin per ml (Gibco, Paisley, Great Britain), 2 mM glutamine (Gibco), 5×10^{-5} M 2-mercaptoethanol (Gibco), and 1 μg of indomethacin per ml (Sigma). Antigen-presenting cells from the spleens of naive, syngeneic mice were prepared and irradiated with 3,000 rads. Cell concentrations were separately adjusted to approximately 1×10^6 T cells per ml and 2×10^6 to 3×10^6 antigen-presenting cells per ml. T cells and antigen-presenting cells were mixed at equal volumes and cultured in a total volume of 220 μl per well (200 μl of cells plus 20 μl of antigen). Controls were performed with unfractionated *M. tuberculosis* lysate, *M. bovis* BCG lysate, *M. tuberculosis* culture filtrate, *M. bovis* BCG culture filtrate (all titrated from 1 μg to 0.03 μg per well), and concanavalin A (ConA; Sigma [0.5 to 0.12 μg per well]) as a T-cell mitogen. T cells were stimulated for 2 days (ConA-treated cultures) or 5 days (antigen fractions and controls), supernatants (150 μl per well) were removed, and duplicate samples were pooled and stored at −20°C for cytokine determination.

IFN-γ ELISA. Culture supernatants were screened for IFN-γ as described previously (14, 44). In short, a double-sandwich enzyme-linked immunosorbent assay (ELISA) was performed with the two specific monoclonal antibodies R4-6A2 (45) and AN18-17-24 (38). Murine recombinant IFN-γ (kind gift of G. Adolf, Ernst Boehringer-Institut für Arzneimittelforschung, Vienna, Austria) was diluted in complete medium from 100 U/ml to 0.2 U/ml and used to determine a standard curve. Cytokine concentrations were calculated with ELISA LITE software (Meddata, Inc., New York, N.Y.). The detection limit of the ELISA was 0.8 to 1.5 U of IFN-γ per ml.

RESULTS

Comparison of two-dimensionally separated proteins of *M. tuberculosis* and *M. bovis* BCG reveals a high degree of similarity. To facilitate identification of single mycobacterial protein antigens, *M. tuberculosis* and *M. bovis* BCG lysates (soluble somatic proteins) and culture filtrates (secreted proteins) were separated by two-dimensional gel electrophoresis (Fig. 1). Upon comparison of *M. tuberculosis* and *M. bovis* BCG protein gels, distribution patterns of respective lysates and culture filtrates appeared similar. Although major differences between proteins of *M. tuberculosis* and *M. bovis* BCG organisms were not detected, several strain-specific proteins were seen in each preparation. By comparison of somatic or secreted proteins from *M. tuberculosis* and *M. bovis* BCG, a number of proteins which were either exclusively secreted or retained in the cytoplasm could be identified.

T lymphocytes from two *bcg*^{s} mouse strains recognize different mycobacterial protein regions. The capacity of T cells from two *bcg*^{s} mouse strains (C57BL/6 and BALB/c) to respond to fractionated mycobacterial proteins was analyzed. To exclude the influences of potentially cross-reactive or mitogenic fractions, T cells from naive mice were enriched and stimulated with fractionated *M. tuberculosis* culture filtrate proteins (Fig. 2). Although T cells from both C57BL/6 and BALB/c mice produced low amounts of IFN-γ in response to various protein fractions, specific antigen recognition patterns were not observed and major influences by nonspecific stimulation can be excluded. Groups of C57BL/6 and BALB/c mice were then vaccinated with live *M. bovis* BCG organisms and sacrificed 5 weeks later. IFN-γ patterns of splenic T cells from both C57BL/6 and BALB/c mice were compared (Fig. 3). As expected, no single dominant antigen was identified, but a multitude of proteins stimulated T-cell responses. While T-cell proliferation did not yield clear antigen recognition patterns (data not shown), determination of IFN-γ from culture supernatants allowed the identification of five distinct protein regions. Figure 4 illustrates these regions. Fractionated *M. tuberculosis* culture filtrate proteins induced maximum IFN-γ production, but T cells were also stimulated by numerous fractions from *M. tuberculosis* lysates, *M. bovis* BCG lysates, and *M. bovis* BCG culture filtrates. Comparison of IFN-γ profiles revealed that stimulatory regions were not identical for the two mouse strains and that differences were most obvious after stimulation with *M. tuberculosis* culture filtrate fractions. Out of the five stimulatory areas, only region I (proteins with high molecular weights and pIs between 5 and 6 [fractions 6 to 17, A to F]) and region III (proteins with low molecular weights and neutral pIs [fractions 1 to 4, R to T]) induced IFN-γ production by T cells from both mouse strains. On the other hand, three regions were exclusively recognized by T cells from either mouse strain. Two regions (region IV, proteins with medium molecular weights [fractions 14 to 19, D to J] and region V, proteins with low molecular weights [fractions 15 to

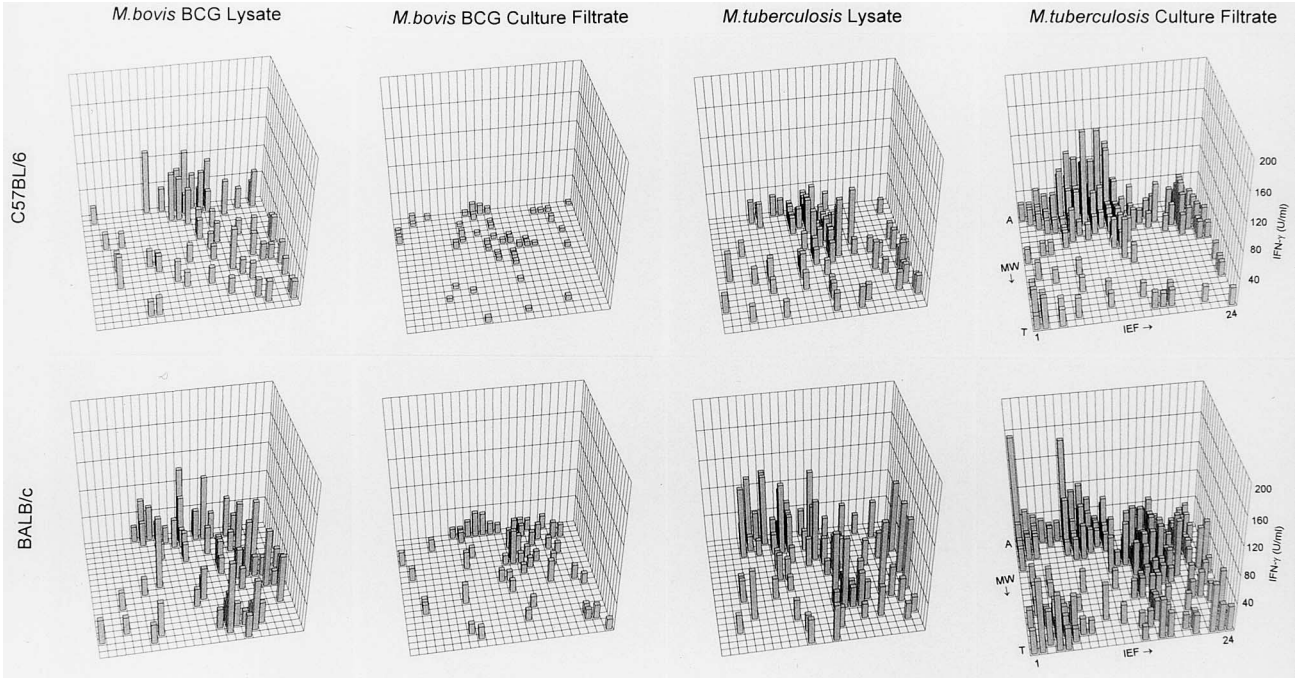

FIG. 3. IFN-γ profiles of T cells from C57BL/6 and BALB/c mice vaccinated with live *M. bovis* BCG. T cells from the spleens of C57BL/6 (upper row) and BALB/c (lower row) mice infected with live *M. bovis* BCG 5 weeks previously were enriched and cultured with fractionated mycobacterial proteins for 5 days in vitro. The concentration of IFN-γ in culture supernatants was determined by ELISA; the concentration scale is given on the ordinate of the figures on the right-hand side. Graphs were individually obtained from representative experiments. Fraction numbers 1 (pI 6.57 or 6.37) to 24 (4.10 or 4.01) mark the IEF gradient; fractions A (10% acrylamide) to T (12.5% acrylamide) indicate the molecular weight (MW) gradient. Further details are given in the legend to Fig. 2. Controls were performed with unfractionated mycobacterial proteins and ConA (all used at 0.6 μg/ml). Typical concentrations of IFN-γ detected in culture supernatants for medium alone, *M. tuberculosis* lysate, *M. tuberculosis* culture filtrate, *M. bovis* BCG culture filtrate, or ConA were 19, 60, >80, >80, and >80 U/ml, respectively, for C57BL/6 and 9, >80, >80, >80, and >80 U/ml, respectively, for BALB/c.

20, N to S]) induced IFN-γ production by T cells from BALB/c mice, whereas region II (proteins with high molecular weights and highly acidic pIs [fractions 20 to 24, A to F]) was stimulatory only for T cells from C57BL/6 mice. Each of the five regions recognized by specific T cells comprised a number of protein spots. It is difficult to match stimulatory fractions with individual protein spots on corresponding gels, and thus far, we have no information whether any of the known mycobacterial antigens (48) are contained within the circumscribed protein regions. In some cases, IFN-γ production was detected in response to fractions without any visible protein staining (e.g., in region III). Although this is most likely a question of sensitivity, a similar phenomenon has been described for a secreted mycobacterial protein which was not detectable by routine protein staining techniques but nevertheless represented a major T-cell antigen in guinea pigs (40). Our findings confirm that *M. bovis* BCG-reactive T cells from both C57BL/6 and BALB/c mice not only respond to proteins of the immunizing organism *M. bovis* BCG but also recognize cross-reactive proteins of *M. tuberculosis*. Moreover, our data reveal a genetic impact on antigen recognition, because only two of the five protein regions identified were stimulatory for T cells from both mouse strains.

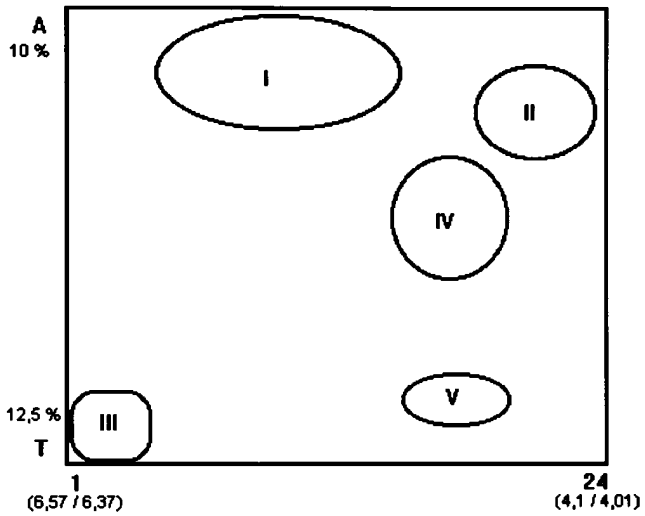

FIG. 4. T-cell-stimulating regions in fractionated mycobacterial proteins. Fractions 1 to 24 indicate the IEF gradient, and fractions A to T indicate the molecular weight (MW) gradient of a two-dimensional protein gel. Region I comprises fractions 6 to 17, A to F; region II comprises fractions 20 to 24, A to F; region III comprises fractions 1 to 4, R to T; region IV comprises fractions 14 to 19, D to J; and region V comprises fractions 15 to 20, N to S. Further description of these regions is given in the text.

Vaccination with heat-killed *M. bovis* BCG is insufficient for activating T-cell responses to secreted antigens. To examine the effect of a nonreplicating vaccine on T-cell responses to mycobacterial antigens, groups of C57BL/6 and BALB/c mice were immunized with heat-killed *M. bovis* BCG organisms. Successful T-cell activation was verified by challenge for delayed-type hypersensitivity reactions with purified protein derivative (Statens Serum Institute, Copenhagen, Denmark), and small responses were measured (data not shown). To allow comparison with live vaccination, mice were killed after 5 weeks, and splenic T cells were cultured with fractionated mycobacterial proteins. Although immunization with heat-

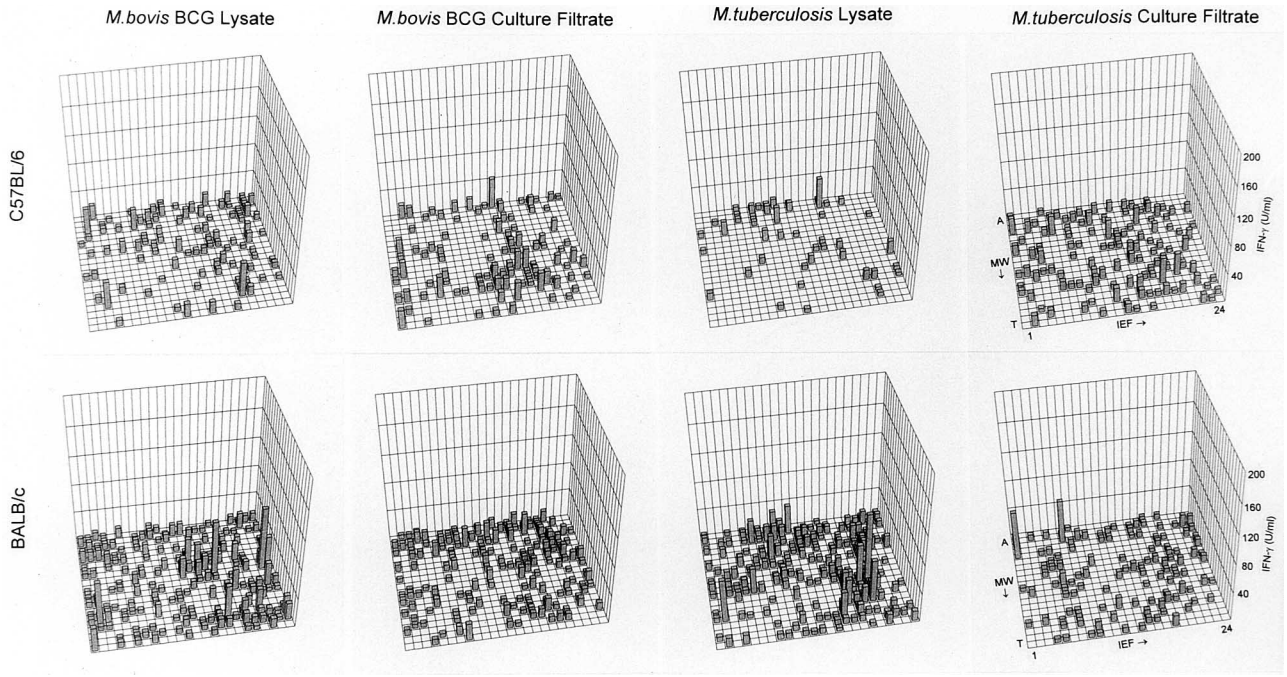

FIG. 5. IFN-γ profiles of T cells from C57BL/6 and BALB/c mice vaccinated with heat-killed *M. bovis* BCG organisms. T cells from spleens of C57BL/6 (upper row) and BALB/c (lower row) mice vaccinated with heat-killed *M. bovis* BCG organisms 5 weeks previously were enriched and cultured with fractionated mycobacterial proteins for 5 days in vitro. The concentration of IFN-γ in culture supernatants was determined by ELISA; the concentration scale is given on the ordinate of the figures on the right-hand side. Fraction numbers 1 (pI 6.57 or 6.37) to 24 (4.10 or 4.01) mark the IEF gradient; fractions A (10% acrylamide) to T (12.5% acrylamide) indicate the molecular weight (MW) gradient. Further details are given in the legend to Fig. 2. Controls were performed with unfractionated mycobacterial proteins and ConA (all used at 0.6 μg/ml). Concentrations of IFN-γ detected in culture supernatants for medium alone, *M. tuberculosis* lysate, *M. tuberculosis* culture filtrate, *M. bovis* BCG lysate, *M. bovis* BCG culture filtrate, or ConA were 3, 27, 43, 29, 27, and 22 U/ml, respectively, for C57BL/6 and 4, 26, 13, 16, 30, and 23 U/ml, respectively, for BALB/c.

killed *M. bovis* BCG organisms induced weaker responses than live vaccination, IFN-γ was produced in response to unfractionated mycobacterial proteins. IFN-γ profiles of T cells from C57BL/6 and BALB/c mice are shown in Fig. 5. IFN-γ production was weakly stimulated by numerous protein fractions, particularly by those with low molecular weights. However, in contrast to T-cell reactions after live vaccination, fractionated *M. tuberculosis* culture filtrate proteins were not preferentially recognized. Except for protein fractions of region V, which stimulated T cells from BALB/c mice in fractionated *M. tuberculosis* and *M. bovis* BCG lysates, none of the strain-specific protein regions induced IFN-γ production. These findings show that a specific though weak immune responses was induced after vaccination of mice with heat-killed *M. bovis* BCG organisms. Yet, although T cells from such animals were stimulated by a number of somatic proteins, they were not efficiently activated to respond to secreted mycobacterial proteins. Thus, T cells which show maximum responses to secreted proteins after live immunization are not activated by vaccination with dead bacteria.

Specific antigen recognition is long lasting and intensified by secondary infection. Antigen recognition patterns of *M. bovis* BCG-reactive memory T cells were compared with those seen during the phase of active immunity. Two groups of C57BL/6 mice were infected with live *M. bovis* BCG organisms. One group was reinfected with live *M. bovis* BCG organisms 1 year later and sacrificed 6 days postinfection, whereas the second group was left untreated and used to analyze T-cell responses in immune animals. Viable *M. bovis* BCG organisms were not detected in seven of eight immune animals, as measured by plating liver homogenates on Middlebrook agar plates. The liver of the remaining animal contained as few as 100 *M. bovis* BCG organisms. In both experimental groups, splenic T cells were prepared and cultured with fractionated *M. tuberculosis* culture filtrates (because these yielded distinct T-cell stimulation patterns after primary infection), and IFN-γ profiles are shown in Fig. 6. The reaction patterns of T cells from both immune and reinfected animals were strikingly similar, but higher concentrations of IFN-γ were detected after secondary infection. Of the three protein regions, I, II, and III, which were recognized by T cells from C57BL/6 mice after primary infection with *M. bovis* BCG (described above), only regions I and II were highly stimulatory for memory T cells; region III induced little IFN-γ production. These data show that T-cell-mediated immunity to *M. bovis* BCG is long lasting and that antigen recognition patterns remain stable over time. After secondary infection, specific memory T cells were rapidly reactivated to secrete high amounts of IFN-γ. We conclude that clonal selection of protective T cells does not occur and that the initial repertoire of antigen specificity is maintained in the memory T-cell population.

DISCUSSION

M. tuberculosis and *M. bovis* BCG organisms preferentially live inside macrophages. Because protective immune responses crucially depend on recognition of infected host cells by specific T lymphocytes (28), identification of mycobacterial T-cell antigens is a major aim for the design of new vaccines. Separation on two-dimensional gels uncovered a high degree of similarity between proteins from *M. bovis* BCG and those from *M. tuberculosis*, as has been demonstrated previously (1). Although many proteins appeared to be either exclusively secreted or retained in the cytoplasm, a number of distinct pro-

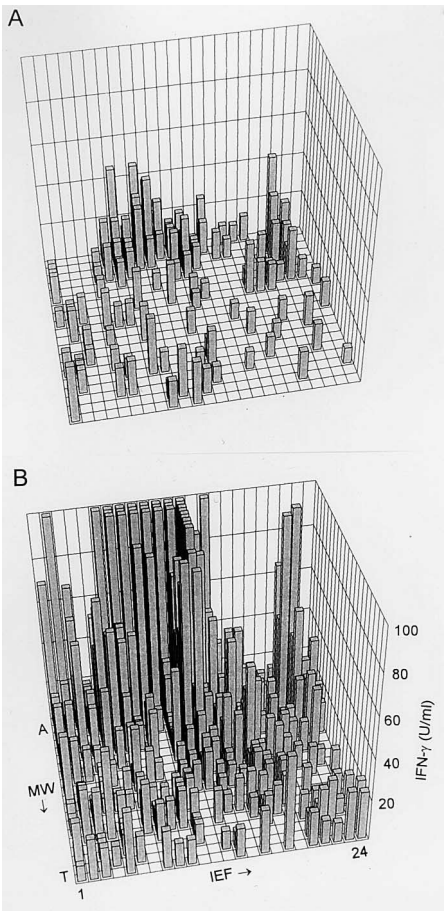

FIG. 6. IFN-γ profiles of T cells from immune and reinfected C57BL/6 mice. Two groups of C57BL/6 mice were infected with live *M. bovis* BCG organisms. One year later, one group was reinfected with a high dose of live *M. bovis* BCG organisms and sacrificed 6 days later (B); the second group was left untreated and used as control immune animals (A). T cells from spleens were enriched and cultured with fractionated *M. tuberculosis* culture filtrates for 5 days in vitro. The concentration of IFN-γ in culture supernatants was determined by ELISA. The concentration scale is given on the ordinate or the lower figure (note the reduced IFN-γ scale compared with previous figures). Fraction numbers 1 (pI 6.57) to 24 (pI 4.10) mark the IEF gradient; fractions A (10% acrylamide) to T (12.5% acrylamide) indicate the molecular weight (MW) gradient. Further details are given in the legend to Fig. 2. Controls were performed with unfractionated mycobacterial proteins and ConA (all used at 0.6 μg/ml). Concentrations of IFN-γ detected in culture supernatants for medium alone, *M. tuberculosis* lysate, *M. tuberculosis* culture filtrate, *M. bovis* BCG lysate, *M. bovis* BCG culture filtrate, or ConA were 5, 10, 35, 35, 39, and 26 U/ml, respectively, for immune animals and 4, >80, >80, >80, >80, and >80 U/ml, respectively, for reinfected animals.

teins were identified in both preparations. This is probably due to the fact that secreted proteins were prepared from 3-week-old mycobacterial cultures in which a limited degree of autolysis had already occurred, although it was macroscopically not yet apparent (3). Therefore, our preparation of secreted proteins is biochemically not as defined as the short-term culture filtrates used in several other studies (2, 4, 5, 21). Yet because of the chronic course of tuberculosis, somatic and secreted proteins are likely to be present as a combination in vivo, although the relative contribution of somatic or secreted proteins to stimulation of specific T cells may change with the stage of infection. The secreted proteins were prepared from mycobacteria which had been cultivated in vitro; hence, the protein composition might vary from those of the proteins secreted in the infected host. It is known that host stress factors can either induce or suppress the expression of bacterial proteins (27) and that a number of bacterial virulence genes are selectively induced in vivo, as has been demonstrated for *Salmonella typhimurium* (32). Although we are aware of this problem, no assay system is available so far to selectively identify the mycobacterial proteins which are synthesized within the infected host.

We have demonstrated that *M. bovis* BCG-induced immune responses do not focus on one or few dominant antigens but that a multitude of mycobacterial proteins are stimulatory for T cells. This finding is consistent with observations in the human and murine systems by this and other laboratories (13, 36, 41). Studies employing human peripheral T lymphocytes from tuberculosis patients and healthy contacts consistently showed individual heterogeneity in terms of proliferation with mycobacterial antigens (13, 41). Therefore, in the present report, two inbred mouse strains (C57BL/6 and BALB/c) were used as a model system for host genetic differences which might influence T-cell recognition of mycobacterial proteins. Both strains are susceptible to infection with mycobacteria (bcg^s) and therefore should not display differences in their *bcg*-controlled innate responses (18). Nevertheless, the additional influence of host genetic factors, including MHC haplotypes, cannot be excluded (8, 22), and differential susceptibility to *M. bovis* BCG has been observed in both normal and α/β T-cell-deficient C57BL/6 and BALB/c mice (12a). Among the multiple protein fractions that stimulated IFN-γ production, five regions were preferentially recognized. However, only two of these five regions were immunodominant and stimulated T cells from both mouse strains, whereas the other three regions were exclusively recognized by T cells from either C57BL/6 or BALB/c mice. Among other factors, the influences of the MHC haplotype on presentation of antigenic peptides may be involved in differential T-cell recognition. It has been shown that T cells from C57BL/6 and BALB/c mice are stimulated by different epitopes of the 30- to 31-kDa protein of *M. bovis* BCG and that recognition of mycobacterial antigens by serum antibodies varied between mouse strains expressing different MHC haplotypes (22, 23).

Infection with *M. bovis* BCG takes a chronic course in mice, and long-lasting immunity develops (7). In order to study the development of memory T cells, mice were kept without chemotherapy in our experiments. As late as 1 year postinfection, few *M. bovis* BCG organisms were detected in the spleen of one of eight immune animals, whereas no bacteria were found in the other seven animals. Antigen recognition patterns of long-term immune animals were almost identical to those obtained 5 weeks postinfection, although lower IFN-γ concentrations were induced. This observation indicates that the initial repertoire of mycobacterium-reactive T cells was maintained in vivo and argues against clonal selection of few protective memory T cells. However, our data are in contrast to those from a report claiming that the antigen recognition capacity of memory T cells is narrowed to mycobacterial protein fractions with low molecular weights (5). The mice used in this study were immunized with *M. tuberculosis* and received chemotherapy, which could have influenced the development of memory T cells. Secondary infection induced rapid reactivation of specific T cells, as measured by elevated IFN-γ production. The antigen recognition profiles, however, remained virtually unchanged. We conclude that the capacity of the immune system to respond to a challenge infection with *M. bovis* BCG is not impaired with age, at least in terms of T-cell reactivity.

Heat-killed *M. bovis* BCG organisms were used to study the antigen reactivity of T cells activated by a nonviable vaccine.

Successful T-cell activation was proven by challenge for delayed-type hypersensitivity reactions, but the magnitude of T-cell responses was not comparable to that during live infection. Culture of splenic T cells from both C57BL/6 and BALB/c mice with fractionated *M. bovis* BCG and *M. tuberculosis* lysates and culture filtrates showed that only a few somatic proteins stimulated IFN-γ production and, moreover, that recognition of secreted proteins did not occur. It is generally accepted that vaccination with killed *M. tuberculosis* organisms does not prime protective T cells and that delayed-type hypersensitivity reactions are diminished (7, 35). Upon in vitro restimulation with mycobacterial proteins, a lower degree of T-cell reactivity is induced (4). In this context, the secreted mycobacterial proteins have been claimed to be involved in the generation of protective T cells and the establishment of immunity (34). It has been shown recently that mice can be protected against challenge with *M. tuberculosis* by vaccination with mycobacterial culture filtrate proteins and that protection is equal to immunization with live *M. bovis* BCG (2). In addition to confirming previous findings, our data indicate that T cells which show maximum responses to secreted proteins after live immunization are not activated by vaccination with dead bacteria. This failure could explain why killed-bacteria vaccines are less-efficient inducers of protection.

Our data support the suggestion that live *M. bovis* BCG organisms are suitable carrier organisms for an improved antituberculosis vaccine (24, 46); vaccination with live *M. bovis* BCG organisms induced long-lasting immunity and primed cross-reactive T cells which recognized *M. tuberculosis* proteins and produced high concentrations of IFN-γ. Furthermore, the initial repertoire of antigen specificity was maintained in memory T cells and the immune system of aged animals was rapidly reactivated upon challenge with *M. bovis* BCG. We propose that a subunit vaccine consisting of only one or few mycobacterial proteins is insufficient for tuberculosis control because of the impact of MHC variation and other genetic differences on T-cell responses as demonstrated here and previously in the human system (13, 41). A living carrier system which delivers secreted mycobacterial proteins seem more appropriate.

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