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Genes for Immunodominant Protein Antigens Are Highly Homologous in *Mycobacterium tuberculosis*, *Mycobacterium africanum*, and the Vaccine Strain *Mycobacterium bovis* BCG

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The relatedness of immunodominant protein antigens in *Mycobacterium tuberculosis*, *M. africanum*, and *M. bovis* BCG was investigated by comparing the genes that encode major protein antigens in *M. tuberculosis* with their counterparts in the other two mycobacteria. Genes encoding homologs of *M. tuberculosis* major protein antigens were isolated from *M. africanum* and *M. bovis* BCG by constructing λgt11 recombinant DNA expression libraries and screening them with murine monoclonal antibodies and DNA probes. The antibodies were directed against four major protein antigens of *M. tuberculosis* with molecular masses of 71, 65, 19, and 14 kilodaltons. The isolated *M. africanum* and *M. bovis* BCG DNA clones were mapped with restriction endonucleases, and the maps of the mycobacterial genes were confirmed by Southern analysis of mycobacterial genomic DNA. The restriction maps of DNA containing the four genes in *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG are identical, indicating that the immunodominant proteins that they encode are highly homologous in the three mycobacteria. Thus, the immunity against tuberculosis engendered by *M. bovis* BCG vaccination could be provided, at least in part, by the immune response to these homologous antigens.

Several human diseases are caused by mycobacteria, aerobic bacilli with high lipid contents and slow generation times. Tuberculosis is caused by *Mycobacterium tuberculosis*, *M. africanum*, or *M. bovis*, the tubercle bacilli of the family *Mycobacteriaceae*. The World Health Organization estimates that there are ten million new cases of active tuberculosis per year, with an annual mortality of approximately three million (13). Leprosy, caused by *M. leprae*, afflicts approximately 13 million individuals (1). Members of the *M. avium-M. intracellulare* complex have become important pathogens among individuals with acquired immunodeficiency syndrome (25). Certain groups of individuals with acquired immunodeficiency syndrome have a markedly increased incidence of tuberculosis as well (19).

Isolation and characterization of major antigens of *M. tuberculosis* and other mycobacteria should lead to the development of more effective tools for the diagnosis and prevention of tuberculosis and other mycobacterial diseases. Diagnostic and immunoprophylactic measures for mycobacterial diseases have changed little in the past half century. Tuberculin is a crude mixture of antigens from *M. tuberculosis* cultures (15), and enrichment of the protein fraction of this material produces the purified protein derivative (20) which is used to diagnose exposure to tuberculosis. Bacillus Calmette-Guérin (BCG), an avirulent strain of *M. bovis* (2), is used extensively as a vaccine against tuberculosis. Though numerous studies have found that it has protective efficacy against tuberculosis (for a review, see reference 17), BCG has failed to protect against tuberculosis in several trials (26) for reasons that are not entirely clear (8).

Genes have been isolated for five immunodominant protein antigens of *M. tuberculosis* (10). Among these antigens are proteins that appear to play a role in the humoral and cell-mediated immune responses to tuberculosis. To determine how similar the homologous protein antigens are in *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG, we have compared the restriction endonuclease maps of four of the genes that encode these antigens in these three mycobacteria. At this level of analysis, the genes are similar, if not identical, in all three mycobacteria.

MATERIALS AND METHODS

Bacteriophage and bacterial strains. λgt11 and *Escherichia coli* Y1089 and Y1090 have been described previously (29, 30). *M. tuberculosis* (Erdmann), *M. africanum* (TMC 5122), and *M. bovis* BCG (Copenhagen vaccine strain) were the source of the DNA used in these experiments. The mycobacteria were grown as previously described (28).

Construction and screening of recombinant DNA libraries. Construction of the *M. africanum* and *M. bovis* BCG recombinant DNA libraries in λgt11 was accomplished by a procedure described previously (28). Briefly, mycobacterial DNA was sheared to an average length of 4 to 6 kilobase pairs with 200 passages through a 25-gauge needle that had been bent at multiple sites. *Eco*RI sites were methylated, and the ends of the DNA fragments were cut flush with T4 DNA polymerase. *Eco*RI linkers were added to the ends and digested with *Eco*RI, and excess linkers were removed by gel filtration. The linkered DNA was fractionated on a 1% agarose gel, and DNA in the size range of 3 to 8 kilobase pairs was electroeluted. The fractionated DNA was suspended in TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) and ligated with dephosphorylated λgt11 arms (Promega Biotec). The libraries were amplified once on Y1090 (*hsdR* mutant) cells (Promega Biotec). Both libraries have a titer of 5 × 10^{10} PFU/ml and contain approximately

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TABLE 1. λgt11 DNA clones and reactivity of their expression products with anti-*M. tuberculosis* monoclonal antibodies[a]

λgt11 clone	Recombinant protein reactive with monoclonal antibody[b]	Recombinant protein size (kDa)
M. bovis BCG		
PL1-101	IT11 (71)	71
PL1-102	IT11 (71)	71
PL1-105	IT13 (65), MLIIC8 (65), IT33 (65)	170[c]
PL1-106	IT13 (65), MLIIC8 (65), IT33 (65)	170[c]
PL1-501	IT19 (19)	130[c]
PL1-502	IT20 (14)	130[c]
M. africanum		
PL1-614	IT11 (71)	71
PL1-308	IT13 (65), MLIIC8 (65), IT33 (65)	65

[a] The clones were isolated as described in the text and tested for their ability to express an antigen reactive with a variety of monoclonal antibodies.
[b] Values in parentheses are the sizes (in kilodaltons) of *M. tuberculosis* protein antigens recognized by the monoclonal antibodies.
[c] Fusion protein produced with β-galactosidase.

90% recombinants, with an average insert size of 4 kilobase pairs.

Antibody screening of the λgt11 recombinant DNA libraries was performed as previously described (28). Detection of signal-producing plaques was performed with an alkaline phosphatase-conjugated secondary antibody system (Protoblot; Promega Biotec), according to the instructions of the manufacturer.

DNA isolation and manipulation. Mycobacterial DNA was isolated as previously described (28). DNA from recombinant λgt11 clones was isolated and mapped with restriction endonucleases by standard techniques (6). Nick translation of the DNA probes, Southern blotting, and DNA-DNA filter hybridization were performed as described by Davis et al. (6). Hybridization was done in a solution of 50% (vol/vol) formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $Na_{1.5}H_{1.5}PO_4$, 1 mM disodium EDTA, pH 7.0), 0.1 mg of sonicated salmon sperm DNA per ml, and 0.3% sodium dodecyl sulfate (SDS) at 42°C for approximately 16 h, followed by washing in a solution of 2× SSPE and 0.2% SDS at 45°C.

Antibody probes. Murine monoclonal antibodies directed against protein antigens of *M. tuberculosis* were generously supplied by the World Health Organization and independent investigators. The antibodies used here were IT-11 (H. D. Engers and workshop participants, Letter, Infect. Immun. **51**:718–720, 1986), IT-13 (3), IT-19 (3), IT-20 (3), IT-33 (9), and MLIIC8 (9; H. D. Engers and workshop participants, Letter, Infect. Immun. **48**:603–605, 1985). All monoclonal antibodies were used at a dilution of approximately 1:200 to 1:300 in 50 mM Tris hydrochloride (pH 8)–150 mM NaCl–0.05% Tween 20. Rabbit hyperimmune anti-*M. tuberculosis* antiserum, produced by repeated immunization with *M. tuberculosis* H37Rv culture filtrate, was a gift of J. Bennedsen (Statens Seruminstitut, Copenhagen, Amsterdam). This serum was used at a dilution of 1:200.

Analysis of recombinant antigens. The ability of λgt11 clones to express specific antigens was tested by arraying purified phage on lawns of *E. coli* Y1090, transferring antigen to nitrocellulose filters, and probing the bound antigen with monoclonal antibodies as previously described (10). The size of the expressed antigen was determined by Western blot (immunoblot) analysis. A lysogen of *E. coli* Y1089 was created with each of the clones (11), production of foreign protein was induced, and a lysate of the lysogen was subjected to SDS-polyacrylamide gel electrophoresis. The fractionated protein was transferred to nitrocellulose filters by electroblotting as previously described (24). The filters were then probed with monoclonal antibodies, and the bound antibody was revealed with a secondary antibody detection system (Protoblot; Promega Biotec).

RESULTS

To investigate the relatedness of immunodominant protein antigens in *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG, our strategy was to compare genes that encode major protein antigens in *M. tuberculosis* with their counterparts in *M. africanum* and *M. bovis* BCG. *M. tuberculosis* genes that encode a variety of immunodominant proteins have been isolated (9). To study the homologous genes in the other tubercle bacilli, two complementary approaches were used. Recombinant DNA expression libraries were constructed with *M. africanum* and *M. bovis* BCG genomic DNA, and DNA clones that expressed antigens recognized by anti-*M. tuberculosis* monoclonal antibodies were isolated and characterized. Independently, by Southern analysis, the *M. tuberculosis* genes were used to probe the structure of the homologous genomic DNA loci in *M. africanum* and *M. bovis* BCG.

Construction of λgt11 expression libraries. Recombinant libraries of *M. africanum* and *M. bovis* BCG genomic DNA were constructed in the phage expression vector λgt11. To ensure that all protein-coding sequences would be represented and expressed, the libraries were constructed with large numbers of DNA fragments containing randomly generated endpoints. Both the *M. africanum* and *M. bovis* BCG recombinant DNA libraries contained approximately 10^6 recombinant clones, and the frequency of recombinant clones in the libraries was greater than 90%.

Gene isolation and characterization. Monoclonal antibodies directed against four protein antigens of *M. tuberculosis*, rabbit anti-*M. tuberculosis* hyperimmune serum, and *M. tuberculosis* DNA clones were used independently to probe the *M. africanum* and *M. bovis* BCG libraries for antigen-producing clones. After the phage clones had been purified, the monoclonal antibodies were used to confirm the identity of antigen expressed from each of the recombinant clones. To do this, the isolated phage were arrayed on lawns of *E. coli* Y1090, the expressed protein was transferred to nitrocellulose, and the immobilized protein was probed with antibodies. The specific monoclonal antibody probes used and the size of *M. tuberculosis* antigens recognized are as follows: IT-11 (71 kDa), IT-13 (65 kDa), IT-19 (19 kDa), and IT-20 (14 kDa). Clones that produce signals with each of the four monoclonal antibodies were isolated from the *M. bovis* BCG library (Table 1). Similarly, clones capable of expressing proteins recognized by monoclonal antibodies specific for the 71- and 65-kDa antigens of *M. tuberculosis* were isolated from the *M. africanum* DNA library (Table 1). Recombinant DNA clones encoding the *M. africanum* homologs of the 19- and 14-kDa *M. tuberculosis* antigens were not isolated. The size of the specific antigen expressed from each clone in *E. coli* was then determined by SDS-polyacrylamide gel electrophoresis and Western blot analysis (Fig. 1). These results are summarized in Table 1.

Restriction endonuclease maps were constructed for the insert DNAs of the recombinant *M. africanum* and *M. bovis* BCG clones and compared with the maps previously deduced for the homologous *M. tuberculosis* DNA clones (10)

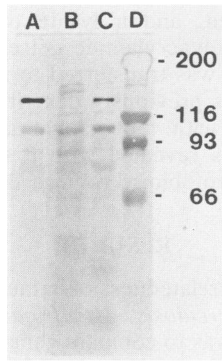

FIG. 1. Western blot of mycobacterial antigens produced in *E. coli* Y1089. Lysogens containing λgt11 phage were constructed and induced to express mycobacterial proteins. Lysates of these induced lysogens were fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with monoclonal antibodies. Lane 1, Lysate of Y1089 lysogen of PL1-501 probed with monoclonal antibody IT19; lane 2, control lysate of Y1089 probed with monoclonal antibody IT20; lane 3, lysate of Y1089 lysogen of PL1-502 probed with monoclonal antibody IT20; lane 4, prestained molecular weight markers. The sizes of the molecular weight markers are given in kilodaltons.

(Fig. 2). Restriction sites were examined in the DNA containing the gene for the following antigens: 11 for the 71-kDa antigen, 13 for the 65-kDa antigen, 6 for the 19-kDa antigen, and 3 for the 14-kDa antigen. No restriction site polymorphisms were detected for any of these DNAs. These data indicate that the cloned DNA fragments from *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG encoding the 71- and the 65-kDa antigens contain highly homologous sequences. Although fewer restriction sites were examined for the cloned *M. tuberculosis* and *M. bovis* BCG fragments encoding the 19- and 14-kDa antigen homologs, these DNA fragments also appear to contain very similar sequences. In each of the cases studied, it appears that considerable homology exists both within the genes encoding antigens and in the surrounding DNA.

Direct analysis of genomic DNA. To confirm and extend the restriction endonuclease maps derived from cloned mycobacterial DNAs, *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG genomic DNA was examined by Southern analysis. Radiolabeled *M. tuberculosis* DNA fragments encoding each of the four protein antigens were used to probe genomic DNA digested with either *Kpn*I and *Xho*I (71-, 65-, and 19-kDa gene probes) or *Kpn*I, *Xho*I, and *Bam*HI (14-kDa gene probe). The results obtained with a probe for the gene encoding 65-kDa antigen are shown in Fig. 3. The data confirm the analysis of cloned DNA fragments from *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG and provided information about the genomic restriction maps of *M. africanum* DNA surrounding the genes encoding 19- and 14-kDa antigens. For each of the restriction enzymes used, these three mycobacteria have identical restriction sites at and surrounding the genomic loci encoding each of the four protein antigens. The results also indicate that each antigen

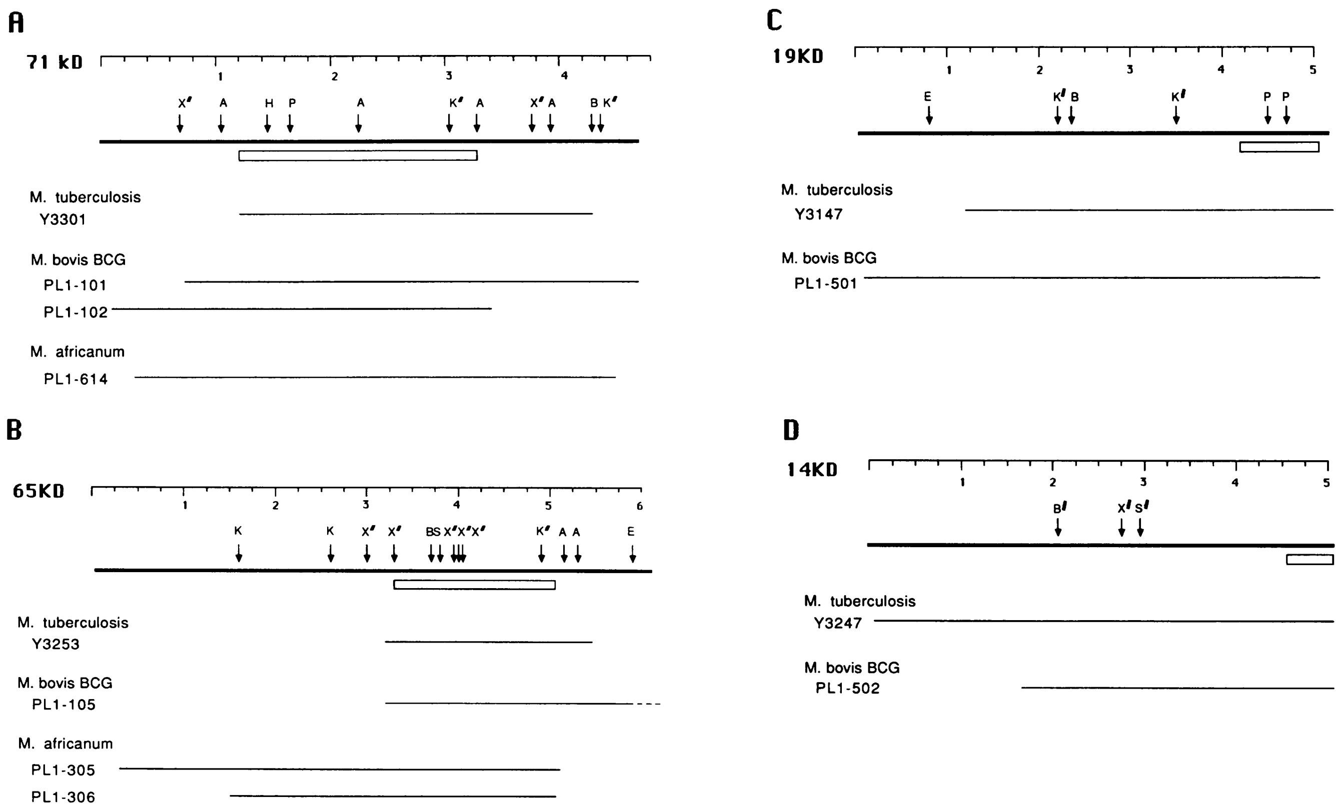

FIG. 2. Restriction maps of mycobacterial DNA encoding the protein antigens of 71 (A), 65 (B), 19 (C), and 14 (D) kDa (KD). These restriction maps summarize data obtained from mapping clones and from analysis of genomic DNA restriction digests. The scale is in kilobase pairs. The approximate position of the antigen-coding sequence is shown as a box. The insert DNAs of recombinant clones encoding the protein antigens are represented by the thin horizontal lines. Restriction enzymes: A, *Sal*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*I; S, *Sac*I; X, *Xho*I. The primes indicate restriction endonuclease cleavage sites that were confirmed by genomic Southern analysis.

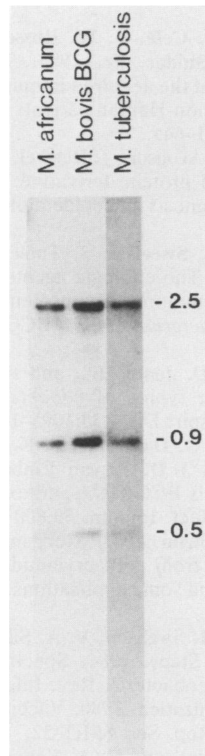

FIG. 3. Southern analysis of mycobacterial genomic DNA. *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG DNAs were digested with *Kpn*I and *Xho*I and probed with the labeled insert DNA of the *M. tuberculosis* clone Y3253, which contains the gene encoding the 65-kDa antigen. The 0.9- and 0.5-kilobase bands are predicted by the restriction map obtained from the clones, and the 2.5-kilobase band represents DNA that extends to the right of the starred *Kpn*I site in Fig. 1B. A smaller amount of *M. tuberculosis* DNA was loaded than of *M. africanum* and *M. bovis* BCG DNA, and the lane containing *M. tuberculosis* DNA was overexposed for clarity.

is the product of a single gene, because the pattern obtained with each probe was that expected for a single locus with sequences complementary to the probe.

DISCUSSION

Differences in *M. tuberculosis* and *M. bovis* BCG have been detected by phage typing (22) and by restriction fragment-length polymorphisms (4, 5). The investigation described here was designed to determine whether differences could be detected in the genes that encode several protein antigens. The evidence indicates that *M. tuberculosis*, *M. africanum*, and the vaccine strain *M. bovis* BCG contain highly homologous genes encoding four immunologically relevant protein antigens.

Additional evidence indicates that the genomic DNA sequences of tubercle bacilli are highly conserved, particularly sequences that encode immunodominant protein antigens. Genomic DNA hybridization studies indicate that *M. tuberculosis* and *M. bovis* BCG share greater than 95% DNA homology overall (12). Recent comparison of the sequences of *M. tuberculosis* and *M. bovis* BCG DNAs containing the 65-kDa antigens reveals that the antigen-coding sequences are identical in these two mycobacteria and that nucleotide sequences flanking the gene differ by no more than 1% (21). Thus, *M. tuberculosis* and *M. bovis* BCG DNA 65-kDa antigens are identical in their amino acid sequences.

Each of the four *M. tuberculosis* antigens whose homologs were examined in this study has been implicated in some aspect of the immune response to infection. Considerable evidence indicates that the 65-kDa antigen plays a role in the human immune response to tuberculosis. Antibodies directed against this protein can be detected in the serum of patients with tuberculosis (23), and the 65-kDa antigen is present in purified protein derivatives of *M. tuberculosis*, *M. bovis*, and other mycobacteria (23). Furthermore, helper-T-cell clones reactive with recombinant 65-kDa antigen have been isolated from patients with tuberculosis (7, 14, 16, 18), indicating that this antigen is involved in the cell-mediated, as well as the humoral, immune response to tuberculosis. The 19-kDa antigen has also been shown to be involved in the human cell-mediated immune response to *M. tuberculosis*; helper-T-cell clones that respond to the recombinant 19-kDa protein have been isolated from tuberculosis patients (16, 18). Mice and rabbits mount an antibody response to the 14- and the 71-kDa antigens (27), and antibody to the 71-kDa antigen is a prominent component of hyperimmune anti-*M. tuberculosis* rabbit sera (10, 27).

The conservation of genes encoding these four antigens in *M. tuberculosis*, *M. africanum*, and *M. bovis* BCG, together with evidence that the *M. tuberculosis* antigens play a role in the humoral and cellular immune response, suggests the possibility that these antigens are common targets in the immune response to mycobacterial infection or immunization. If so, these antigens could contribute to the immune protection conferred by BCG against tuberculosis in humans and are among the candidates that should be considered for a subunit vaccine against tuberculosis.

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