Genes for the major protein antigens of *Mycobacterium tuberculosis*: The etiologic agents of tuberculosis and leprosy share an immunodominant antigen

*Robert N. Husson*† and *Richard A. Young*†

†Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142; †Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; and ‡Division of Infectious Diseases, Department of Medicine, The Children’s Hospital, Boston, MA 02115

Communicated by Maclyn McCarty, November 7, 1986 (received for review July 18, 1986)

**ABSTRACT** *Mycobacterium tuberculosis* genes encoding immunologically relevant proteins were isolated by systematically screening a λgt11 recombinant DNA expression library with a collection of murine monoclonal antibodies directed against protein antigens of this pathogen. These antibodies, previously characterized by a World Health Organization workshop on monoclonal antibodies against mycobacteria, were used to isolate DNA sequences encoding five major protein antigens of this pathogen. To evaluate the extent of crossreactivity between these *M. tuberculosis* antigens and antigens of *Mycobacterium leprae*, recombinant antigens were probed with monoclonal antibodies directed against the protein antigens of these bacilli. One of the antigens, a 65-kDa protein, has determinants common to *M. tuberculosis* and *M. leprae*. We find not only that this antigen is recognized by mouse monoclonal antibodies but that it is the major protein recognized by anti-*M. tuberculosis* rabbit sera. The 65-kDa proteins of *M. tuberculosis* and *M. leprae* appear to play a role in the humoral and cell-mediated immune response to these pathogens.

Tuberculosis, recognized as the major cause of infectious mortality in Europe and the United States in the 19th and early 20th centuries (1), remains a significant global health problem. In the United States there are over 20,000 new cases of tuberculosis annually, and the steadily declining incidence of tuberculosis established in preceding decades appears to have changed course, reaching a plateau in 1985 and showing an actual increase in 1986 (2, 3). Worldwide, tuberculosis remains widespread and constitutes a health problem of major proportions, particularly in developing countries. 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 (4).

Tuberculosis is caused by *Mycobacterium tuberculosis*, *M. africanum*, or *M. bovis*, the “tubercle bacilli” of the family Mycobacteriaceae. The mycobacteria are aerobic, acid-fast, non-spore-forming, non-motile bacilli with high lipid contents and slow generation times. Among the other mycobacteria, *M. leprae*, the etiologic agent of leprosy, is the only major pathogen (5). Other species, however, are capable of causing disease (6). Members of the *M. avium*-intracellulare complex are important pathogens among individuals with the acquired immunodeficiency syndrome (AIDS). Certain groups of individuals with AIDS have a markedly increased incidence of tuberculosis as well (7).

Diagnostic and immunoprophylactic measures for mycobacterial diseases have changed little in the past half century. Tuberculin, developed by Koch as a cure for tuberculosis in the late 1800s and subsequently used as a skin-test reagent, is a crude mixture of antigens from *M. tuberculosis* cultures (8). Enrichment of the protein fraction of this material in the 1930s produced the purified protein derivative (PPD) (9), which is still used to diagnose exposure to tuberculosis. Bacillus Calmette–Guérin (BCG), an avirulent strain of *M. bovis* (10), has been used as a vaccine against tuberculosis for more than 50 years. Though numerous studies found that it had protective efficacy against tuberculosis (reviewed in ref. 11), a major trial of BCG in India indicated that this vaccine was not protective against tuberculosis in this setting (12).

Isolation and characterization of major antigens of *M. tuberculosis* and of other mycobacteria should lead to the development of more effective tools for the diagnosis and prevention of tuberculosis and other mycobacterial diseases.

In 1984 and 1985 the World Health Organization (WHO) sponsored two workshops to characterize murine monoclonal antibodies against mycobacteria (13, 14). Fifty-six monoclonal antibodies developed by several independent investigators were analyzed in multiple laboratories, and the reactivity of the monoclonal antibodies with several species of mycobacteria was determined. Some of these antibodies recognized determinants unique to a single mycobacterium; however, many were found to crossreact with antigens from multiple species. Ten of the anti-*M. leprae* antibodies recognized four protein antigens, of 12, 18, 36, and 65 kDa. These, and additional monoclonal antibodies against a 28-kDa antigen (15), were used to isolate *M. leprae* genes encoding the five antigens (16). Nineteen of the anti-*M. tuberculosis* antibodies bound to seven protein antigens, of 12, 14, 19, 23, 38, 65, and 71 kDa. We have used these anti-*M. tuberculosis* monoclonal antibodies to screen a λgt11 recombinant DNA expression library of *M. tuberculosis* in order to isolate genes for the protein antigens of this bacillus. We report here the isolation of genes for five major protein antigens of *M. tuberculosis* and their use to investigate the antigenic relationship between *M. tuberculosis* and *M. leprae*.

**MATERIALS AND METHODS**

**Phage and Bacterial Strains.** Bacteriophage λgt11 and *Escherichia coli* strain Y1090 have been described (17, 18).

**M. tuberculosis Recombinant DNA Library.** Construction of the *M. tuberculosis* recombinant DNA library in λgt11 has been described (19). The library has a titer of 2 × 10¹⁰ plaque-forming units per ml and contains approximately 40% recombinants with an average insert size of 4 kilobases.

**Monoclonal Antibody Probes.** Murine monoclonal antibodies to protein antigens of *M. tuberculosis*, *M. leprae*, and other mycobacteria were generously supplied by the World Health Organization (WHO). These antibodies were screened for reactivity against *M. tuberculosis* and *M. leprae* antigens, and the reactivity of these reagents was verified by Western blot analysis (20).

**Tuberculin and PPD.** Bacterial antigens and the purified protein derivative (PPD) were generously supplied by the World Health Organization (WHO). The PPD consists of at least two distinct antigens, each with at least two components (21, 22).

**BCG.** Bacterial antigens and the avirulent strain of *M. bovis* (BCG) were generously supplied by the World Health Organization (WHO). The BCG vaccine was developed in the 1920s by Calmette and Guérin (23). It has been used as a vaccine against tuberculosis for more than 50 years. Though numerous studies found that it had protective efficacy against tuberculosis (reviewed in ref. 11), a major trial of BCG in India indicated that this vaccine was not protective against tuberculosis in this setting (12).
Health Organization and independent investigators. Among these antibodies, those used in the studies reported here were IT-3 (20), IT-10 (21), IT-11 (14), IT-12 (22), IT-13 (23), IT-15 (23), IT-16 (14), IT-17 (15), IT-19 (23), IT-20 (23), IT-21 (22), IT-23 (23), IT-27 (14), IT-29 (14), IT-31 (14), IT-33 (24), ML06-A13 (25), L7-15 (26), SA1.D2D (identical to IT-17), SA1.B11H (15), F47-9-1 (13), ML04-A (25), C1.1 (13), IIIH9 (identical to IT-33), IIIE9 (24), IIIC8 (24), IIIC8 (24), T2.3 (13), Y1-2 (13), SA2.D7C (gift of D. B. Young) and ML30A (25). All monoclonal antibodies were used at approximately 1:200 to 1:300 dilution in 50 mM Tris–HCl, pH 8/150 mM NaCl/0.05% Tween 20.

Rabbit Anti-M. tuberculosis Serum. Anti-M. tuberculosis hyperimmune serum, produced by repeated immunization of rabbits with M. tuberculosis H37Rv culture filtrate, was generously provided by J. Bennedsen (Statens Seruminstitut, Copenhagen). This serum was used at 1:100 dilution.

Screening of the Agt11 M. tuberculosis Library with Antibody Probes. Screening was as described (19), except that 1% bovine serum albumin was used in place of 20% fetal bovine serum to decrease background. Positive plaques were detected with a biotinylated secondary antibody system (Vectastain, Vector Laboratories, Burlingame, CA) or with an alkaline phosphatase-conjugated secondary antibody system (Proteoblot, Promega Biotec, Madison, WI).

Probing of Arrays of Agt11 DNA Clones with Antibody Probes. Two hundred microliters of a saturated culture of E. coli Y1090 was added to 2.5 ml of molten Luria–Bertani (LB) soft agar, poured onto 100-mm plates containing 1.5% LB agar, and allowed to harden at room temperature for 10 min. One hundred microliters of phase plate stock containing \(10^8\) of the agt11 DNA clones of interest was placed into alternate wells of 96-well tissue culture plates. A multipronged transfer device was placed briefly in the wells containing phage and then touched lightly to the surface of the plate. Plates were then incubated at 42°C for 3 hr, at which point clear plaques \(5\) mm in diameter were visible. Plates were then overlayed with nitrocellulose filters saturated with 10 mM isopropyl \(\beta\)-thiogalactopyranoside and incubated at 37°C for 3.5 hr. Processing of filters for detection of antigen was as described for screening the agt11 library.

Recombinant DNA Manipulation. DNA from recombinant agt11 clones was isolated and mapped with restriction endonucleases by standard techniques (27).

Filter Hybridization of Insert DNA. Arrays of agt11 clones were created as described above and incubated at 42°C for 5 hr. The plates were then overlayed with nitrocellulose filters and placed at 4°C for 1 hr. Probe DNA was labeled with \(^{32}P\) by nick-translation. Filter hybridization was performed as described (27). Hybridization conditions were 50% (vol/vol) formamide/5x SSPE (1x SSPE is 0.18 M NaCl/10 mM Na2HPO4/1 mM Na2EDTA, pH 7.0)/1x Denhardt’s solution/0.02% (wt/vol) Ficoll/0.02% (wt/vol) polyvinylpyrrolidone/0.02% (wt/vol) bovine serum albumin)/0.3% NaDodSO4 at 42°C for 16 hr, followed by washing in 2x SSPE/0.2% NaDodSO4 at 45°C.

RESULTS

Gene Isolation and Characterization. Monoclonal antibodies directed against protein antigens of M. tuberculosis were used individually to probe a agt11 M. tuberculosis recombinant DNA library. The specific antibody probes used and the size of antigen recognized are as follows: IT-3 (12 kDa); IT-20 (14 kDa); IT-19 and IT-27 (19 kDa); IT-17 and IT-29 (23 kDa); IT-15, IT-21, and IT-23 (38 kDa); IT-13 (65 kDa); and IT-11 (71 kDa) (14). This DNA library was previously constructed with M. tuberculosis genomic DNA fragments such that all protein-coding sequences would be represented and expressed (19). Signal-producing clones were isolated using antibodies directed against protein antigens of 12, 14, 19, 65, and 71 kDa. In each case similar numbers of clones were isolated in screens of \(10^6\) recombinant plaques. DNA clones encoding the 23-kDa and 38-kDa antigens could not be detected with these antibodies.

The insert DNAs of the recombinant DNA clones were mapped with restriction endonucleases. Fig. 1 shows the genomic DNA restriction maps deduced for the genes encoding each of the five M. tuberculosis antigens and illustrates how each of the cloned DNAs aligns with that map. All clones isolated with monoclonal antibodies directed against any single antigen align with a single genomic DNA segment. This result indicates that all clones were isolated because they express the protein of interest rather than an unrelated polypeptide containing a similar or identical epitope. In addition, this result suggests that each antigen is the product of a single gene.

![restriction map](image-url)
The orientation of each DNA insert in the recombinant clones was determined by restriction analysis. Only among the clones for the 65-kDa antigen were the inserts found in both possible orientations relative to the direction of lacZ transcription in agt11. This result suggests that this protein can be expressed in E. coli from signals independent of those provided by lacZ. Similar results have been obtained for recombinant DNA clones encoding the 65-kDa antigens of M. bovis (28) and M. leprae (16).

**Recombinant Antigens Recognized by Rabbit Serum.** To assess the response of another experimental animal to antigen preparations of M. tuberculosis, we examined the reactivity of rabbit anti-M. tuberculosis hyperimmune serum with recombinant antigens. This serum produced positive signals with agt11 clones encoding each of the five M. tuberculosis epitopes that had been isolated with murine monoclonal antibodies (Fig. 2). Particularly strong signals were observed with the 65-kDa and 71-kDa antigens (Fig. 2). These results demonstrate that mice and rabbits can mount an antibody response to the same protein antigens of M. tuberculosis.

We find that clones for the five M. tuberculosis antigens are detected at similar frequencies in the agt11 recombinant DNA library, using monoclonal antibodies. Thus, the relative number of each of the five genes isolated with polyclonal serum antibodies should reflect the relative titer and diversity of the serum antibodies to each of the five antigens.

To determine whether any of the five M. tuberculosis antigens are relatively immunodominant in the rabbit humoral immune response to M. tuberculosis, we performed the following experiment. The M. tuberculosis agt11 recombinant DNA library was screened with the rabbit serum, and 40 signal-producing clones were isolated. These 40 clones were arrayed and probed with monoclonal antibodies directed against each of the five recombinant M. tuberculosis protein antigens. Remarkably, 17 of the 40 clones (43%) reacted strongly with at least one of the four anti-65-kDa monoclonal antibodies tested. An additional six clones (15%) reacted strongly with the anti-71-kDa monoclonal antibody IT-11. These results indicate that a large proportion of the anti-M. tuberculosis antibody present in this rabbit serum is directed against the 65-kDa antigen of M. tuberculosis and suggest that it is a dominant antigen for the rabbit humoral immune response. Seventeen of the clones did not react with any of the monoclonal antibodies tested, suggesting that the rabbit serum may identify M. tuberculosis proteins not recognized by the murine antibodies.

**Antigenic Relatedness of M. tuberculosis and M. leprae Proteins.** Evidence that M. tuberculosis and M. leprae share immunologically important antigens led us to investigate the exact nature of the immunological relatedness among recombinant protein antigens of M. tuberculosis and M. leprae. For each of five M. tuberculosis and four M. leprae protein antigens, a single recombinant DNA clone containing most or all of the gene of interest was used to express antigen in the following experiment. The recombinant phage clones were arrayed on a lawn of E. coli Y1090, which was then grown and induced for antigen expression. Antigen immobilized on nitrocellulose filters was then probed with 26 individual anti-M. tuberculosis and anti-M. leprae monoclonal antibodies. Fig. 3 shows the array of DNA clones used and the results obtained with the anti-M. tuberculosis antibodies IT-1, IT-3, IT-11, IT-16, and IT-31, which recognize proteins of 14, 12, 71, 19, and 65 kDa, respectively. Table 1 details the results of these experiments, showing the reactivity of antigen expressed from individual recombinant DNA clones with each of the monoclonal antibodies.

Several conclusions can be drawn from the results shown in Table 1. Among the 11 monoclonal antibodies that recognize a 65-kDa antigen, 7 react with the 65-kDa protein from both mycobacteria (IT-31, C1.1, IIH9 (identical to IT-33),

**FIG. 2.** Arrays of antigens from mycobacterial recombinant DNA clones probed with rabbit hyperimmune serum. Sixteen cloned agt11 recombinants were arrayed on lawns of E. coli Y1090. The phage were grown, antigen expression was induced, and the antigens were blotted and probed with serum as described in the text. The code of the recombinant DNA clones shown on the numbered filter is as follows: 1, Y3275; 2, Y3274; 3, Y3279; 4, Y3277; 5, Y3247; 6, Y3272; 7, Y3150; 8, Y2254; 9, Y3147; 10, Y3163; 11, Y3179; 12, Y3191; 13, Y3252; 14, Y3178; 15, Y3180; 16, Y3143; 17, agt11. Clones 1, 5, 6, 7, 9, and 16 are M. tuberculosis recombinants described in the text. Clones 10, 11, 14, and 15 are M. leprae recombinants expressing epitopes of the 18-, 28-, 36-, and 65-kDa antigens, respectively (28). Clones 2, 3, 4, 8, 12, and 13 are uncharacterized recombinants from the a gt11 M. tuberculosis and M. leprae libraries. Clone 17 is a nonrecombinant a gt11 control.

**FIG. 3.** Arrays of recombinant mycobacterial antigens probed with monoclonal antibodies to assess antigen crossreactivity. Cloned a gt11 recombinants were arrayed on lawns of E. coli and probed with monoclonal antibodies as described in the text. The array of clones is identical to that shown in Fig. 2. Antibody probes and the antigen sizes recognized are as follows: 1, IT-11 (71 kDa); 2, IT-31 (65 kDa); 3, IT-16 (19 kDa); 4, IT-1 (14 kDa); 5, IT-3 (12 kDa).
IIC8, T2.3, Y1-2, SA2.D7C, one antibody reacts only with the *M. tuberculosis* protein (IT-13), and two antibodies react only with the *M. leprae* protein (IIIE9 and IIIC8). One antibody, ML30A, crossreacts with an antigen in *E. coli* and could not specifically identify antigen-producing clones. These results indicate that the 65-kDa protein antigens of *M. tuberculosis* and *M. leprae* are homologues and share a number of epitopes. In addition to these shared epitopes, however, both 65-kDa antigens have epitopes that are specific for one species relative to the other.

No crossreactivity was observed between other antigens of these two mycobacterial species. Because monoclonal antibodies recognize a single epitope and because only one or a few antibodies were available for each antigen, it is not clear whether the 65-kDa proteins are the only homologous protein antigens of *M. tuberculosis* and *M. leprae*. Among the other antigens for which λgt11 clones have been isolated, however, only the 18-kDa antigen of *M. leprae* and the 19-kDa antigen of *M. tuberculosis* are of similar size. To determine whether these two antigens are related, we examined the homology of the DNA sequences that encode these antigens. At conditions of moderate stringency, where the DNAs encoding the *M. tuberculosis* and *M. leprae* 65-kDa antigens hybridized strongly, no hybridization was observed between the insert DNA sequences that encode these antigens.

Table 1. Reactivity of monoclonal antibodies with recombinant protein antigens

<table>
<thead>
<tr>
<th>ANTIBODIES</th>
<th>DNA CLONES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. tuberculosis</em></td>
</tr>
<tr>
<td></td>
<td>12kD</td>
</tr>
<tr>
<td>Y3275</td>
<td>+</td>
</tr>
<tr>
<td>Y3247</td>
<td>-</td>
</tr>
<tr>
<td>Y3147</td>
<td>-</td>
</tr>
<tr>
<td>Y3150</td>
<td>-</td>
</tr>
<tr>
<td>Y2272</td>
<td>-</td>
</tr>
<tr>
<td>λgt11</td>
<td>-</td>
</tr>
</tbody>
</table>

Arrays of recombinant antigens of *M. tuberculosis* and *M. leprae* were probed with monoclonal antibodies directed against antigens of these species. Clones were scored as positive only if the signal produced was clearly greater than the background signal produced by the nonrecombinant λgt11 clone included in each array. KD, kDa.
DNA of Y3147 (an *M. tuberculosis* 19-kDa clone) and Y3179 (an *M. leprae* 18-kDa clone), indicating no significant homology between the DNA sequences of the insert DNAs of these two clones. This result suggests that the *M. tuberculosis* 19-kDa protein and the *M. leprae* 18-kDa protein are unlikely to be homologous.

**DISCUSSION**

Recombinant DNA clones encoding five major protein antigens of *M. tuberculosis* were isolated with an extensive collection of well-characterized murine monoclonal antibodies. These five proteins were also found to be major antigens in the rabbit humoral immune response to *M. tuberculosis*. One of these antigens, the 65-kDa protein, is shared with the other mycobacterial pathogen *M. leprae*.

Several lines of evidence indicate that the 65-kDa antigen is an immunodominant protein antigen of *M. tuberculosis*. Eleven of the 25 different *M. tuberculosis* and *M. leprae* monoclonal antibodies examined in this study recognized the 65-kDa recombinant antigen from one or both mycobacteria. In addition, almost half of the recombinant DNA clones isolated with rabbit polyclonal anti-*M. tuberculosis* serum express the 65-kDa antigen, reflecting the predominance of antibody to this antigen in this serum.

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 (28). The 65-kDa antigen is present in PPDs of *M. tuberculosis*, *M. bovis*, and other mycobacteria (28). Finally, helper-T-cell clones reactive with recombinant 65-kDa protein have been isolated from patients with tuberculosis (29, 30), indicating that this antigen is involved in the cell-mediated as well as the humoral immune response to tuberculosis. Because a large number of T-cell clones were isolated that do not appear to react with the 65-kDa antigen, it is possible that antigens equally or more important to T-cell immunity remain to be identified.

Among the major antigens of the leprosy bacillus, the 65-kDa antigen appears to elicit antibody and T-cell responses similar to those observed for the *M. tuberculosis* antigen. Both serum antibodies (26) and T cells (31) directed against the 65-kDa *M. leprae* antigen have been observed in patients with leprosy. In addition, T-cell clones from leprosy patients have been found to respond to recombinant 65-kDa protein of *M. bovis* (32), as well as to PPDs from both *M. bovis* BCG and *M. leprae* (33). It is interesting to note that in vaccine trials in Asia and Africa, BCG provided significant protection against leprosy, ranging from 20% to 80% (reviewed in ref. 34). An intriguing possibility is that the *M. bovis* BCG 65-kDa antigen is involved in engendering the immune protection provided by this vaccine against *M. leprae* as well as against *M. tuberculosis*.

There is evidence that the 19-kDa and 71-kDa antigens of *M. tuberculosis*, in addition to the 65-kDa antigen, are involved in the immune response to this bacillus. Helper-T-cell clones from tuberculosis patients have been isolated that respond to the recombinant 19-kDa protein (29). The 71-kDa antigen is recognized by the humoral immune system of both mice and rabbits, and we have shown that antibody to this antigen is a prominent component of hyperimmune anti-*M. tuberculosis* rabbit serum.

The isolation of genes for major protein antigens of *M. tuberculosis* should permit the development of improved reagents for diagnosis and immunoprophylaxis of tuberculosis. For example, proteins encoded by some of these DNA sequences may be a source of specific serodiagnostic and skin-test antigens, reagents that would be valuable for monitoring disease transmission and the effectiveness of vaccination or therapy. Finally, it is now possible to introduce into viruses, and possibly into cultivable mycobacteria, genes that specify polymyotides that may provide immunological protection in order to produce more specific and effective vaccines.

We thank Barry Bloom, Vijay Mehra, Tore Godal, Douglas Young, Juraj Ivaný, Thomas Shimnick, Steven Shoemaker, Jim Watson, Doug Sweetser, and Bobby Cherayil for advice and stimulating discussion. We are also grateful to the World Health Organization and the individual investigators for their gifts of monoclonal antibodies. This work was supported by grants from the National Institutes of Health (AI23545), the World Health Organization Program for Vaccine Development, the World Health Organization/World Bank/United Nations Development Program Special Program for Research and Training in Tropical Diseases, and the Heiser Program for Research in Leprosy.