Isolation and Restriction Site Maps of the Genes Encoding Five Mycobacterium tuberculosis Proteins

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A series of recombinant phage expressing five Mycobacterium tuberculosis antigens were isolated. Restriction maps for these antigens were deduced, and the size of the expressed proteins was determined.

During the course of an infection with Mycobacterium tuberculosis, it is the immune response to the mycobacterial antigens that plays a key role in determining immunity from infection as well as the pathogenesis of disease (reviewed in references 3, 6, and 15). Monoclonal antibodies have been used recently in studies to identify and characterize individual mycobacterial antigens (2, 5, 9, 10, 12; H. D. Engers et al., Letter, Infect. Immun. 48:603–605, 1985; H. D. Engers et al., Letter, Infect. Immun. 51:718–720, 1986). The targets of these antibodies were analyzed in two World Health Organization (WHO)-sponsored workshops, and seven M. tuberculosis protein antigens were identified (summarized by Engers et al. [Letters, 1985, 1986]). The apparent molecular sizes of these proteins on sodium dodecyl sulfate-polyacrylamide gels are 12, 14, 19, 23, 38, 65, and 71 kilodaltons (kDa). To study these proteins further, we have used the WHO-characterized monoclonal antibodies as specific probes to detect recombinant phage that express the proteins in Escherichia coli. To do this, we used a pool of the monoclonal antibodies provided by the WHO (see below) and published procedures (8, 16) to screen about 2 × 109 recombinant phage from a recombinant DNA library in which mycobacterial DNA fragments had been inserted into the EcoRI site of the λgt11 expression vector (16).

The pool contained a 1:1,000 dilution of each of the samples (IT-1 through IT-33) included in a 1985 WHO workshop. In particular, the pool contained antibodies that recognized protein antigens with apparent molecular sizes of 12 kDa (IT-3), 14 kDa (IT-1, 4, and 20), 19 kDa (IT-10, 12, 16, and 19), 23 kDa (IT-17 and 29), 38 kDa (IT-15, 21, and 23), 65 kDa (IT-13, 31, and 33), and 71 kDa (IT-11). The original designations of these antibodies and appropriate references can be found in the workshop report (Engers et al., Letter, 1986). The WHO workshop report (Engers et al., Letter, 1986) also contains a summary of the results from several laboratories on the isolation of M. tuberculosis genes. Some of the results reported here were included in that report.

We isolated 157 phage that produced antigens that reacted with the pool of antibodies. To determine which phage produced which of the seven mycobacterial antigens, the recombinants were plaque purified and then tested for reactivity with individual monoclonal antibodies in a spot assay (16). Twenty-two recombinants failed to react with any of the antibodies in the spot test and were then assayed for reactivity with the antibodies, using a more sensitive plaque assay (16). Recombinants that reacted with one but not all of the monoclonal antibodies specific for a given protein were also retested with the plaque assay.

Overall, the recombinants fell into six sets of reactivity patterns, five of which corresponded to the expression of five of the seven target mycobacterial proteins (Table 1). For the 12-kDa protein, two phage were isolated that produced antigen that reacted with antibody IT-3 (pattern A) and hence express at least a portion of this antigen. Three of the antibodies (IT-1, 4, and 20) reacted with a 14-kDa antigen. We isolated 19 phage expressing antigen that reacted with all three antibodies (pattern B), 2 phage expressing antigen that reacted with IT-1 and IT-4 but not IT-20 (pattern C), and 1 expressing antigen that reacted with IT-1 and IT-20 but not IT-4 (pattern D). In a more sensitive test using an immunoblot procedure, the two clones of pattern C were found to react with all three antibodies, although weakly with IT-20 (data not shown). These results suggest that the IT-4 antibody reacts with a different epitope on the 14-kDa molecule than do the other two antibodies, in agreement with immunocompetition studies which indicate that each of the antibodies recognizes a different epitope (Engers et al., Letter, 1986). Antibodies IT-10, 12, 16, and 19 reacted with a 19-kDa antigen, and three phage were isolated which expressed this antigen (pattern E). Each reacted with all four antibodies. Antibodies IT-13, 31, and 33 reacted with a 65-kDa protein, and 28 of the recombinants produced antigen that reacted with all three antibodies (pattern F) while 10 recombinants produced antigen that reacted with only one or two of the antibodies (patterns G, H, I, and J). This pattern of reactivity suggests that the three antibodies detect different epitopes on the same molecule. Ninety-one phage (pattern K) were isolated which produced antigen that bound to the IT-11 monoclonal antibody (71-kDa antigen). The sixth type of reactivity pattern (L) was displayed by a single phage which produced an antigen that bound to all of the monoclonal antibodies, although it bound to the antibodies of the immunoglobulin M subclass more strongly than to those of the immunoglobulin G subclass. This suggests that the phage produces an antigen that nonspecifically interacts with the mouse immunoglobulins.

Using a similar approach, Young et al. (16) isolated six clones that produced antigen that reacted with IT-13 (65-kDa antigen), three that reacted with IT-19 (19-kDa antigen), and six that reacted with IT-20 (14-kDa antigen) from the λgt11-
M. tuberculosis library. Clones reactive with the IT-3 (12-kDa) and IT-11 (71-kDa) antibodies have also been isolated (R. Husson and R. Young, personal communication).

The failure to isolate phage that expressed the 23- or 38-kDa antigens could be due to (i) the inability of the available antibodies to recognize their target antigen or corresponding fusion proteins in this type of assay (i.e., somewhat denatured antigens); (ii) the target epitope residing at or near the amino terminus of the protein such that the inclusion of this epitope in a fusion protein is a rare event; (iii) the proteins or corresponding fusion proteins being unstable in E. coli; or (iv) the concentrations of the antibodies directed against these two proteins in the initial pool of antibodies being too low to permit detection of the recombinant-produced antigens. At present, we cannot distinguish among these possibilities.

We characterized the recombinants further by determining (i) the restriction enzyme cleavage site maps for the mycobacterial DNA inserts and (ii) the size of the antigen produced by the recombinants. All or up to five representative recombinants were analyzed for the phage expressing each of the mycobacterial antigens. DNA was isolated by using published procedures (7), and the restriction enzyme cleavage site maps were deduced (Fig. 1). One of the key features of these maps is that all of the analyzed phage which produced antigen that reacted with a given antibody or set of antibodies contained the same region of the mycobacterial genome. This suggests that each antigen is produced by a single region of the genome (most likely by a single gene) and that the epitopes to which the antibodies are binding are found on only one antigen and not two or more similarly sized antigens. (A caveat here is that since only 4 of the 91 IT-11 reactive clones were analyzed, other genes encoding an IT-11 reactive epitope might have been missed due to the small sample size.) Interestingly, the clone (XSK2) that reacted with IT-1 and 20, but not IT-4, contained, as expected, a smaller portion of the presumed 14-kDa-antigen-coding region than the clones that reacted with all three antibodies. However, a precise mapping of the IT-4 epitope must await a detailed analysis of the 14-kDa-antigen-coding region.

A series of Mycobacterium leprae protein antigens having sizes similar to those of the M. tuberculosis protein antigens were also identified in the WHO-sponsored workshops (Engers et al., Letters, 1985, 1986). Of the cloned and mapped genes (17), only the genes for the M. leprae and M. tuberculosis 65-kDa antigens appear to share restriction enzyme cleavage sites. This is not too surprising given that, at the genomic DNA level, these two species display only about 30% homology (1) and that restriction site maps sample only a small portion of the sequences. The 65-kDa antigens, on the other hand, appear to be unusually highly conserved proteins in the mycobacteria (11, 14).

The sizes of the products produced by each of the mapped recombinants were analyzed in immunoblots in which crude lysates of cells expressing the recombinants were probed with the monoclonal antibodies (Fig. 2). For the recombinants expressing the 12-kDa antigen, only the fusion proteins that migrated at about 120 kDa were detected (Fig. 2, lanes A). However, for the 19-kDa (Fig. 2, lanes C), 65-kDa (lanes D), and 71-kDa (lanes E) antigens, we identified clones that expressed the proteins as molecules which migrated with the same apparent molecular sizes as the authentic mycobacterial proteins, as well as clones that expressed these antigens as fusion proteins. These results suggest that the translation and posttranslational modification (if any) of these three antigens is similar in E. coli and M. tuberculosis.

The 14-kDa reactive antibodies, on the other hand, detected a protein in four of the five recombinants tested that migrated with an apparent molecular size of 25 kDa (Fig. 2, lanes B) instead of the expected 14 kDa. (The other, XSK2, expressed a fusion protein of ~120 kDa.) This suggests either that this gene is expressed differently in E. coli than in M. tuberculosis (e.g., utilizes different initiation or termination triplets) or that the two gene products are processed differently in the two species.

Given the ease with which large amounts of E. coli can be grown, the recombinants described here may be a reasonable source of defined mycobacterial antigens for use in the analysis of the immunogenicity and antigenticity of individual proteins during naturally occurring infections. Such studies with recombinant-produced M. leprae and M. bovis BCG antigens have been reported (4, 11, 13). The recombinants are also sources of mycobacterial genes that can be transferred to other expression vectors for efficiently expressing the proteins or to vaccine vehicles to analyze possible roles of the proteins in immunity from infection. These studies may lead to better-defined reagents for the immunodiagnosis or immunoprophylaxis of tuberculosis.

### Table 1. Patterns of reactivity of the recombinants with the monoclonal antibodies

<table>
<thead>
<tr>
<th>Pattern</th>
<th>12 kDa: IT-3</th>
<th>14 kDa: IT-1</th>
<th>14 kDa: IT-4</th>
<th>14 kDa: IT-20</th>
<th>19 kDa: IT-10</th>
<th>19 kDa: IT-12</th>
<th>19 kDa: IT-16</th>
<th>19 kDa: IT-19</th>
<th>65 kDa: IT-13</th>
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<th>65 kDa: IT-33</th>
<th>71 kDa: IT-11</th>
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<td>91</td>
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</table>

* Both produced antigen that reacted with IT-1, IT-4, and IT-20 (weakly) in an immunoblot assay.
FIG. 1. Restriction endonuclease cleavage site maps of the M. tuberculosis genes that encode the 12-, 14-, 19-, 65-, and 71-kDa antigens. For each gene, the upper thick line represents the deduced map of the mycobacterial genome, and the thin lines below it represent the individual phage that are identified to the right of the corresponding line. The vertical lines at the ends of the thin lines represent the EcoRI sites that flank the mycobacterial DNA in these recombinants. The arrows next to the phage designation depict the direction of lacZ transcription. All recombinants expressing a particular antigen produced material that reacted with each of the antibodies recognizing that antigen except XSK2, which reacted with IT-1 and IT-20 but not IT-4. Restriction sites: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; S, SalI.

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ADDENDUM IN PROOF

After submission of the present study R. Husson and R. A. Young reported the restriction maps of these M. tuberculosis genes (Proc. Natl. Acad. Sci. USA 84:1379-1383, 1987 [cited above as a personal communication]).

FIG. 2. Immunoblot analysis of the proteins expressed by the recombinants. Crude lysates of cells expressing the recombinant phage and proteins were made as described by Huynh et al. (8), and the immunoblots were done as previously described (14). Of each pair of lanes, lane 1 contains an extract of cells expressing one of the recombinant antigens and lane 2 contains an extract of cells expressing the parental Xgt11 phage. (A) XSK3 extract, antibody IT-3 (12 kDa); (B) XSK13 extract, antibody IT-4 (14 kDa); (C) XSK183 extract, antibody IT-12 (19 kDa); (D) XSK119 extract, antibody IT-13 (65 kDa); (E) XSK89 extract, antibody IT-11 (71 kDa).

Their restriction maps are in reasonable agreement with ours.

LITERATURE CITEd


