Antigenic Specificity of the Mycobacterium leprae Homologue of ESAT-6

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The sequence of the Mycobacterium leprae homologue of ESAT-6 shows only 36% amino acid correspondence to that from Mycobacterium tuberculosis. Anti-M. leprae ESAT-6 polyclonal and monoclonal antibodies and T-cell hybridomas reacted only with the homologous protein and allowed identification of the B- and T-cell epitopes. The protein is expressed in M. leprae and appears in the cell wall fraction. Thus, M. leprae ESAT-6 shows promise as a specific diagnostic agent for leprosy.

The global impact of effective chemotherapy for leprosy has resulted in the diminution of cases from approximately 10 million in 1985 to about 800,000 today (27). However, there is no evidence as yet of any reduction in the number of new cases (28), and we know little about the transmission of leprosy or the time elapsed between infection and disease. The single greatest need from leprosy research is definitive diagnostic tools to help understand transmission and allow early detection of disease. The recent completion of the sequencing of the genomes of Mycobacterium tuberculosis (4) and Mycobacterium leprae (5) provides the opportunity to identify leprosy-specific antigens. An analogous approach applied to Mycobacterium bovis BCG allowed the identification of deleted genes and the development of antigens that can distinguish between M. tuberculosis infection and vaccination with BCG (17). Among those antigens were two low-molecular-weight M. tuberculosis culture filtrate proteins, ESAT-6 and CFP10 (2, 10), both encoded by genes in the RD1 region, a genetic segment that has been deleted from all strains of BCG. When tested together in a gamma interferon assay of peripheral blood mononuclear cells from M. tuberculosis-infected and BCG-vaccinated individuals, the sensitivity and specificity of the response were 84 and 100%, respectively, with no responses in purified protein derivative-negative individuals (1).

Although previous studies have identified a number of M. leprae proteins (7, 11, 19) and peptides (6, 26) capable of inducing gamma interferon responses in leprosy patients, a comparative analysis of the M. tuberculosis and M. leprae genomes should reveal new specific antigens, potential diagnostic and epidemiological tools for leprosy. In this report, comparative analysis of the M. leprae and M. tuberculosis ESAT-6 homologues suggests that the M. leprae product holds promise in this respect.

Comparison of the sequences of ESAT-6 from M. leprae and M. tuberculosis. Whereas M. tuberculosis contains 14 members of the ESAT-6 family (23), the M. leprae genome shows evidence of only 4 (5, 8). A comparison of the alignment of the sequences of the 95-amino-acid (aa)-length ESAT-6 protein from M. tuberculosis (22) with its counterpart from M. leprae showed 36% homology overall (Fig. 1). Although there was identity between 9 out of 13 amino acids (69% homology) in the region bounded by aa 34 and 46, this is the only instance with more than 4 consecutive, identical amino acids. The rest of the sequence shows only one or two identical amino acids, interrupted by conserved and nonconserved stretches.

Cloning and production of recombinant M. leprae ESAT-6. The DNA sequence encoding the full-length M. leprae ESAT-6 protein (designated ML0049) (5, 8) was cloned from M. leprae genomic DNA using Vent Pfu DNA polymerase (Promega, Madison, Wis.). PCR amplification was carried out with the forward primer 5’-CATATGATACAGGCGTGGCAC-3’ and reverse primer 5’-AAAGCTTTCCGGTGGAACATACT-3’ designed to introduce Ndel and HindIII sites to the 5’ and 3’ ends of the open reading frame. The pBluescript vector (Stratagene, La Jolla, Calif.) was digested with the restriction endonuclease-

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>M. leprae</td>
<td></td>
</tr>
<tr>
<td>p1</td>
<td>MIQAWHFFPL</td>
</tr>
<tr>
<td>p2</td>
<td>QGAVNELG52</td>
</tr>
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<td>LVQAIHSAE</td>
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<td>TEGNIHALGD</td>
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<td>M. tuberculosis</td>
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</tr>
<tr>
<td>p1</td>
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</tr>
<tr>
<td>p6</td>
<td>YQGQQKWDA</td>
</tr>
</tbody>
</table>

* Overlapping peptides.

**Boldface type indicates amino acid identity between M. leprae and M. tuberculosis ESAT-6.
as NdeI and HindIII, ligated to the PCR products, and transformed into competent Escherichia coli TOP10 cells. The ex gene was subcloned into the expression vector PET 23b(+) (Novagen, Madison, Wis.) and transformed into BL21(DE3) pLys S cells by the heat shock method. Single colonies expressing ESAT-6 were grown in Luria-Bertani medium with ampicillin and induced with isopropyl β-D-thiogalactopyranoside. Recombinant ESAT-6 (rESAT-6) found in inclusion bodies was solubilized in 8 M urea in 20 mM Tris–HCl buffer, loaded onto a nickel-nitrilotriacetic acid resin column, and eluted with imidazole (20). The purity of the recombinant protein was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16); lipopolysaccharide content was less than 0.2 ng/mg. Recombinant protein was purified over a protein G-Sepharose affinity column. The reactivity of the antisera to the homologous and heterologous forms of ESAT-6 was determined by ELISA. Polyclonal antisera raised against M. leprae rESAT-6 showed no reactivity to any of the heterologous ESAT-6 peptides (p1, aa 1 to 20) or to the last two C-terminal peptides (p8, aa 71 to 90; p9, aa 81 to 95) or to either of the heterologous M. tuberculosis immunodominant peptides (p1, aa 1 to 20; p6, aa 50 to 70). It reacted most intensely to p2 (aa 11 to 30), slightly more weakly to p3 through p6 (p3, aa 21 to 40; p4, aa 31 to 50; p5, aa 41 to 60; p6, aa 51 to 70), and weakly to p7 (aa 61 to 80). These data are in accord with analysis of the M. leprae ESAT-6 peptides using the Jameson-Wolf antigenic index (12) and the Kyte-Doolittle hydrophilicity plot (15). The M. tuberculosis ESAT-6-specific polyclonal sera showed no reactivity to any of the M. leprae ESAT-6 peptides but reacted well to both of the homologous ESAT-6 peptides (p1 and p6) (Table 2). Of the five MAbs produced, all of which reacted strongly to the homologous ESAT-6 as determined by Western blotting, four (1C7.2F1, 2F4.2C4, 7G7.2A5, and 8C9.2B5) recognized the single (p2) peptide, suggesting that p2 contains the dominant B-cell epitope.

Detection of native M. leprae ESAT-6 in subcellular fractions of M. leprae. M. tuberculosis ESAT-6 is found mainly as a secreted protein in culture filtrate (22). However, M. leprae is derived from the spleens and livers of experimentally infected armadillos, rendering it very difficult to detect the protein in the tissue milieu. Instead, the subcellular fractions of M. leprae (18) were solubilized, and the proteins were subjected to Western blotting (Fig. 4). ESAT-6 was observed in the cell wall but not in the cytosol or membrane fractions.
Identification of T-cell epitopes on M. leprae ESAT-6. BALB/c mice (H-2b) were immunized into the hind footpad with 40 μg of M. leprae rESAT-6 in incomplete Freund's adjuvant, and lymph node cells were restimulated in vitro with dendritic cells pulsed with 0.5 μg of rESAT-6/ml fused with the T-cell fusion partner BWα-β (25), and plated. Clones that grew in individual wells were screened using dendritic cell antigen-presenting cells with 0.5 μg of rESAT-6/well. Hybrids were tested for the production of interleukin 2 (IL-2) (13), and positive hybridomas were further tested for their response to the nine overlapping peptides (p1 to p9) of M. leprae ESAT-6. Clone 2A3 responded to peptide p4, and clones 3D8 and 6B7 responded to the C-terminal peptide, p9. None of the M. leprae ESAT-6-specific T-cell hybridomas responded to either of the two M. tuberculosis ESAT-6 peptides, p1 or p6. Using B-cell lymphoma lines bearing either I-A^d (M12.B5), I-E^a (M12.A2) (9), or both (A20) (14), all three of the T-cell hybridomas were further tested for their response to the nine overlapping peptides (p1 to p9) of M. leprae ESAT-6. Clone 2A3 responded to peptide p4, and clones 3D8 and 6B7 responded to the C-terminal peptide, p9. None of the M. leprae ESAT-6-specific T-cell hybridomas responded to either of the two M. tuberculosis ESAT-6 peptides, p1 or p6. Using B-cell lymphoma lines bearing either I-A^d (M12.B5), I-E^a (M12.A2) (9), or both (A20) (14), all three of the T-cell hybridomas were shown to recognize their respective peptides using the I-A^d restriction element (Table 3). In contrast, with M. leprae ESAT-6, only one peptide was shown to stimulate T cells from the H-2^b haplotype (BALB/c strain) mice, the N-terminal peptide (aa 1 to 20), whereas mice of H-2^a and H-2^d haplotypes recognized a separate internal peptide, aa 51 to 70 (3).

Conclusion. Comparative analysis of the complete genome sequences of M. leprae and M. tuberculosis established that gene deletion and decay have resulted in the formation of 1,116 nonfunctional pseudogenes, resulting in an elimination of many key metabolic activities of M. leprae (5). Thus, M. leprae barely maintains its existence with a minimal gene set (24, 30). There are an estimated 135 functional coding sequences in the M. leprae genome that show no similarity to any known genes, and some of these, if found to actually produce a functional immunogenic protein, may be useful in the development of new epidemiological and diagnostic tools. The M. leprae version of ESAT-6, in light of its exceptional specificity, may qualify, although its promise was revealed by pursuing a different principle: proteins that share sizeable correspondence to the M. tuberculosis counterpart but prove to have immunological specificity.

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REFERENCES
Use of an ordered cosmid library to deduce the genomic organization of 


