Characterization of a *Mycobacterium leprae* Antigen Related to the Secreted *Mycobacterium tuberculosis* Protein MPT32

BRIGITTE WIELES,†† MIRANDA VAN AGTERVELD,† ANNEKE JANSON,† JOSEPHINE CLARK-CURTNISS,‡ TOBIAH RINKE DE WIT,§ MORTEN HARBRE,¶ AND JELLE THOLE†

Department of Immunohaematology and Blood Bank, Leiden University Hospital, 2333 AA Leiden,† and Department of Immunology, Erasmus University Rotterdam, 3000 DR Rotterdam,§ The Netherlands; Department of Biology and Molecular Microbiology, Washington University, St. Louis, Missouri 63130‡; and Institute of Immunology and Rheumatology, University of Oslo, N-0172 Oslo 1, Norway¶

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Secreted proteins may serve as major targets in the immune response to mycobacteria. To identify potentially secreted *Mycobacterium leprae* antigens, antisera specific for culture filtrate proteins of *Mycobacterium tuberculosis* were used to screen a panel of recombinant antigens selected previously by leprosy patient sera. Four potentially secreted antigens were identified by this approach, and one was recognized by antibodies specific for MPT32, a secreted *M. tuberculosis* protein. The DNA coding for the *M. leprae* antigen, which we have designated 43L, was isolated and characterized and found to encode a 25.5-kDa protein that is preceded by a consensus signal peptide of 39 amino acids. The N-terminal amino acid sequence of 43L shows 50% homology with the 20 known N-terminal amino acids of MPT32, and 47% homology was found with the N terminus of a 45/47-kDa antigen complex identified in *Mycobacterium bovis* BCG. These findings indicate that 43L represents an antigen related to MPT32 and the *M. bovis* BCG 45/47-kDa complex and that 43L is likely to be a protein secreted by *M. leprae*. Purified recombinant 43L protein is recognized by antibodies and T cells from healthy contacts and leprosy patients, illustrating that secreted proteins are of importance in the immune response to *M. leprae*.

In recent years, recombinant DNA technology has become an important tool in the identification of *Mycobacterium leprae* antigens that are involved in the spectrum of immune responses that can be induced upon infection with this intracellular parasite (30). Among these antigens are heat shock proteins (HSPs), which are predominantly localized in the cytoplasm. HSPs, a large number of which were selected by using monoclonal antibodies, are prominent antigens in both T-cell and B-cell responses to *M. leprae* (13, 14, 31). In addition to HSPs, secreted antigens have been shown to constitute important target structures in the immune response towards *M. leprae* (8, 10, 25).

Upon infection with *M. leprae*, protective immunity as well as immunopathology leading to disease may be induced, and both processes are believed to be dependent on antigen-specific T-cell recognition. It is still a controversial matter whether particular *M. leprae* antigens may either provide protection or induce immunopathology. A role for actively secreted antigens in protective immunity has been proposed on the basis of the observation that living attenuated mycobacteria provide a higher degree of immunity than heat-killed bacteria (27) and a limited degree of protection with secreted antigens was obtained in an animal model for tuberculosis (18). Recently, it has been demonstrated that live *Mycobacterium tuberculosis* predominantly provokes T-cell responses to antigens secreted by the bacteria during growth (2). Several antigens that could be involved have been identified in culture filtrates of *M. tuberculosis* and have been designated antigen 85 complex, MPT32, MPT51, MPT53, and MPT63, and a 3- to 9-kDa protein (3, 15, 28).

Since *M. leprae* cannot be cultured in vitro, it is difficult to analyze putative products that are specifically secreted by this species. To circumvent this problem, we have used sera that are specific for secreted antigens of *M. tuberculosis* to identify potentially secreted *M. leprae* homologs among a panel of more than 20 different recombinant antigens that were previously selected by using antibodies from leprosy patients (22). DNA clones expressing four different recombinant antigens were identified by the sera directed against secreted *M. tuberculosis* proteins. One of these was shown to encode an antigen related to the *M. tuberculosis* secreted protein MPT32.

**MATERIALS AND METHODS**

**Bacterial strains, phages, plasmids, and cosmids.** The bacterial strains, phages, plasmids, and cosmids used in this study are listed in Table 1. Strain POP2136 was used as a host for plasmid pEX2 and derivatives, and strain χ2819 was used as a host for an *M. leprae* genomic library cloned into the cosmid pHCT79 (5). Strain JM109 was used as a host for plasmids pUC13 and derivatives. Bacterial strains were grown in LB medium and LB agar (11) supplemented with ampicillin (100 μg/ml) and tetracycline (10 μg/ml).

**Antibodies.** Serum samples from 21 borderline tuberculous and tuberculous (BT/TT) leprosy patients, 17 lepromatous and borderline lepromatous (LL/BL) leprosy patients, and 10 healthy contacts (HCs) were from endemic leprosy regions in Ethiopia. These sera were used in a 1:100 dilution. Polyclonal rabbit antibodies directed against secreted proteins of *M. tuberculosis* were K12 and K13 directed against MPT3, K14 and K15 directed against MPT51, K16 and K17 directed against MPT53, K23 directed against total secreted proteins of *M. tuberculosis*, K95 directed against MPT64, and K180 and K190 directed against MPT32 (15). These sera were used in a 1:200 dilution. Horseradish peroxidase-
labelled anti-rabbit and anti-human immunoglobulins were obtained from Sigma Chemical Co., St. Louis, Mo.

Production of recombinant protein. Expression of cro-lacZ hybrid genes from pEX2 derivatives was induced as described previously (26). For T-cell proliferation assays, lysates of Escherichia coli were centrifuged (10 min, 12,000 × g) and the pellets, enriched in fusion proteins, were resuspended in Iscove's modified Dulbecco's medium and stored at −20°C until use.

Expression of glutathione hydrolase genes from pGEX derivatives was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to a log-phase culture with an optical density at 600 nm of 0.5. The culture was maintained for another 3 h at 37°C. Cells were pelleted by spinning the culture for 30 min at 1,000 × g. Cells were resuspended to an optical density at 600 nm of 5 in lysis buffer (30% sucrose, 50 mM Tris-HCl [pH 8], 100 mM KCl, 1 mM dithioerythritol, 5 mM EDTA). Ten percent sarcosyl solution (10% N-lauryl sarcosine in H2O) was added to a final concentration of 0.2%. After mixing and sonication (six times for 30 s at 80 W in a Branson sonifier B-12 sonicator), the cell extract was frozen at −20°C until use. In order to purify the fusion protein, we performed affinity chromatography, using a glutathione-binding column according to the manufacturer's instructions (Pharmacia P-L Biochemicals, Inc., Milwaukee, Wis.). Purification of the native 43L antigen after cleavage of the glutathione fusion product with thrombin was performed as described elsewhere (23). Proteins were >90% pure, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were stored at −20°C until use.

SDS-PAGE and immunoblotting. Production of recombinant proteins in E. coli lysates was analyzed by SDS-PAGE on 7.5 or 12% acrylamide gels and by Western blotting (immunoblotting). SDS-PAGE was done according to the method of Laemmli (9), and electrophoresis was performed at 70 mA. Either the gels were stained with Coomassie brilliant blue or the proteins were transferred to nitrocellulose membranes (type BA83; 0.2-μm-pore-size filters; Schleicher & Schuell, Dassel, Germany) in a Bio-Rad transfer apparatus according to the recommendations of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Immunoblotting of the membranes with pooled patient sera or monoclonal polyclonal rabbit antisera was done as described previously (1). Patient sera were used in a 1:100 dilution. The monoclonal polyclonal rabbit antisera were used in a 1:200 dilution.

DNA analysis and sequencing. Restriction endonucleases were used as specified by the manufacturer (Promega Corporation, Madison, Wis.) Standard procedures were used for preparation of plasmid and cosmid DNAs. Southern blotting and hybridization were performed as described previously (11) with nylon membranes (Hybond-N+; Amersham International, Amersham, Buckinghamshire, United Kingdom). Hybridization buffer and hybridization conditions used were as specified for the Hybond-N+ membranes. Washing was performed under conditions of medium stringency, one time for 10 min at room temperature with 2× SSPE (1× SSPE is 10 mM Na2HPO4, 0.18 M NaCl, and 1 mM EDTA [pH 7.0]) followed by one time for 10 min at 65°C with 1× SSPE. Probe labelling with α-32P was done as specified by the manufacturer (Promega Corporation) with the use of a random-primed labelling kit. The EcoRI DNA insert from αgt11 recombinant clone L14 was subcloned into the EcoRI site of the expression vector pEX2 by PCR as described by Thole et al. (25).

Nucleotide sequence analysis was performed on plasmid and cosmid DNAs that had been purified by CsCl-gradient centrifugation (11), by the dyeoxy chain-termination method (21) with the T7 sequencing kit (Pharmacia). The synthetic oligonucleotide primer 8625-2 that is complementary to the DNA sequence 12 to 26 bp upstream of the EcoRI site of pEX2 was used to sequence the M. leprae region fused to the cro-lacZ gene in the pEX2/L14 derivative. A set
of primers based on the *M. leprae* insert gene sequence was subsequently synthesized to determine the 5′ part of the sequence of the clone pEX2/L14. To obtain a cosmid carrying the complete gene corresponding to L14, an *M. leprae* cosmid library (5) was screened by hybridizing with the 1.0-kb *EcoRI* insert of pEX2/L14. *PstI* fragments from a positive cosmid that hybridized with the 1.0-kb insert of pEX2/L14 were subcloned into pUC13, and a resulting pUC13 derivative (pUC13/43L) containing the complete 43L gene was used to determine the sequence of the 3′ part of the gene. To obtain expression of a glutathione fusion carrying the complete gene, unidirectional subcloning into the BamHI and EcoRI sites of expression vector pGEX-1AT was performed with a BamHI- and EcoRI-digested PCR fragment obtained by using a BamHI site containing a forward primer (CGCGGATTCGATCCCGCCGCTTGGCGCG) complementary to the first codons of the mature 43L gene and an *EcoRI*-containing primer (GGCGAATTCGCTCTACAA CACCGATAACGA) complementary to sequences downstream of the putative stop codon of 43L, with genomic *M. leprae* DNA as a template. All primers were synthesized on a Cyclone DNA synthesizer (Biosearch Inc., Millipore Corporation, Bedford, Mass.).

**Proliferation assays.** Lymphocyte stimulation tests were performed by testing freshly isolated peripheral blood mononuclear cells. In a 96-well round-bottomed microtiter plate, 100,000 cells per well were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% human serum and the optimal concentration of antigen. The antigen used was the *E. coli* lysate extract from pEX/L14 in 1:100 and 1:5,000 dilutions. For testing the recombinant purified native 43L protein, 150,000 to 200,000 cells per well were used and the purified antigen was added at a concentration of 10 μg/ml. Cultures were set up in triplicate and incubated at 37°C in a fully humidified atmosphere containing 5% CO₂ for 6 days. For the last 18 h, 1.0 μCi of [³H]thymidine (specific activity, 5.0 μCi/mmol; NEN, Boston, Mass.) was added to each well and cells were finally harvested full automatically on glass fiber filters. [³H]thymidine incorporation was assessed by liquid scintillation spectroscopy on a β-plate reader.

**RESULTS**

**Identification of putative *M. leprae* secreted proteins.** Previously, 50 recombinant clones were selected from an *M. leprae* λgt11 expression library by pooled leprosy patient serum samples (22). These clones were grouped by hybridization studies and were shown to express at least 22 novel antigenic determinants. By immunoblotting, we tested whether antigens expressed by 25 representative members of this large panel of clones were recognized by antisera K23 directed against total culture filtrate proteins of *M. tuberculosis*. The gene products of five λgt11 clones (L2, L7, L14, L24, and L44) were recognized (Fig. 1, L7 and L14). This indicated that these clones produce potentially secreted *M. leprae* antigens. The reactivity with antigens produced by L7 and L44 was not unexpected, since these clones express parts of the *M. leprae* B component of the secreted antigen 85 complex (25). To further characterize the antigens expressed by L2, L14, and L24, they were tested for recognition by monospecific polyclonal rabbit antibodies directed to individual secreted proteins of *M. tuberculosis*. This revealed that the antigen expressed by L2 was recognized by antibodies to MPT53, a 15-kDa protein. The antigen expressed by L14 was recognized by antibodies directed to MPT32, a 41-kDa protein, whereas the antigen expressed by L24 was not recognized by any of the tested antisera against individual secreted proteins of *M. tuberculosis*. In this study, we have focused on the protein expressed by L14.

**Subcloning of L14 in expression systems.** In order to generate large quantities and to purify the antigen expressed by L14, the 1-kb EcoRI PCR fragment of L14 was subcloned into the EcoRI sites of pEX2 and pGEX-1AT. The insert...
orientation was checked by comparing fusion protein sizes of the Cro-β-galactosidase and the glutathione fusion products with the expected protein size from the original Agt11 clone. A characteristic doublet can be seen in both Coomassie-stained SDS-PAGE and in Western blots probed with anti-MPT32 and anti-M. tuberculosis total secreted protein (Fig. 1). Recombinant native complete antigen shows a similar doublet band of 27/32 kDa (see the description below and Fig. 4). Although much smaller, these bands could correspond to the observed double bands reported for the corresponding 45/47-kDa complex antigens of Mycobacterium bovis BCG (20).

Molecular analysis of pEX2/L14 and derivatives. The complete M. leprae DNA insert of pEX2/L14 was sequenced and found to be 1,080 bp long. The analysis of this DNA fragment revealed the presence of an open reading frame (ORF) predicting a protein consisting of 230 amino acids fused to β-galactosidase. To obtain the complete gene, the EcoRI insert of the L14 clone was used as a probe to screen a cosmid library. Two cosmids clones, designated pYA1108 and pYA1109, were isolated. A 4.3-kb PstI fragment from pYA1108 hybridized with the 1.0-kb fragment of clone L14, and a PstI fragment with a similar size was detected in genomic M. leprae DNA. The 4.3-PstI fragment from pEX2/L14, a similar gene in M. tuberculosis was cloned into pUC13, resulting in plasmid pEX2/L14 (1080). The position of the ORF is indicated by a open box.

FIG. 2. Schematic presentation of the sequenced M. leprae insert DNA of clones pEX2/L14 and pYA1108. Sizes of the inserts are shown in base pairs (parentheses). The position of the ORF is indicated with a open box.

FIG. 3. Nucleotide sequence of clone pYA1110. The ORF encoding a 25.5-kDa protein is indicated in single-letter amino acid code, and the putative signal sequence is in italics. A putative Shine-Dalgarno ribosomal binding site and possible −10 sequences are underlined. M. start codon; −, putative stop codon.
does not contain this N-terminal region, we conclude from these data that we have identified and characterized an *M. leprae* antigen related to MPT32 and the *M. bovis* BCG 45/47-kDa complex. We have designated the *M. leprae* antigen 43L, corresponding to the nomenclature for MPT32 (43T) listed in a recent compilation of mycobacterial protein antigens (30).

To purify native recombinant 43L, PCR-amplified *M. leprae* DNA encoding the mature protein was subcloned into the expression vector pGEX-1Xa and purified from the expressed glutathione fusion protein after cleavage with thrombin as described in Materials and Methods. Native mature 43L migrates with a mobility of 27 and/or 32 in SDS-PAGE, corresponding to the calculated molecular mass of the mature protein of 25,553 Da (Fig. 4). The molecular mass of 43L thus is considerably smaller than the estimated molecular masses of 41 kDa for MPT32 and of 45/47 kDa for the *M. bovis* BCG 45/47-kDa complex.

**Immunological analysis of the secreted *M. leprae* protein.** In order to test the B-cell response to the 43L protein in leprosy patient sera, immunoblotting was performed with purified native 43L and serum samples from LL/BL patients, BT/TT patients, and HCs. Of the 17 tested LL/BL patient samples, 15 were shown to strongly recognize recombinant 43L. Eleven of 21 BT/TT patient serum samples gave a moderate reaction, and a weak reactivity with 43L was found in 3 of 10 tested healthy contacts. A representative number of these reactivities are shown in Fig. 6. The data indicate that 43L is recognized by the majority of the tested LL/BL patients but that the reactivity in TT/BL patients and HCs is generally much weaker.

In order to assess T-cell recognition of this antigen, proliferation of peripheral blood mononuclear cells derived from HCs, LL/BL patients, and BT/TT patients was tested in the presence of a semipurified lysate containing the fusion protein expressed by pEX2/L14. Despite the semipurified nature of the antigen preparations, significant proliferation was found in 4 of 21 HCs, in 4 of 29 BT/TT patients and in 1 of 18 LL/BL patients tested (data not shown). In concordance with these data, purified native 43L is recognized by peripheral blood lymphocytes from two of two tested HCs and two of three tested BT/TT or LL/BL patients (Table 2). The T-cell and antibody recognition indicates that antigen 43L is recognized by HCs and by patients throughout the leprosy spectrum.

**TABLE 2. Proliferation T-cell response to the purified 43L protein by peripheral blood mononuclear cells**

<table>
<thead>
<tr>
<th>Protein source</th>
<th>HC A</th>
<th>HC B</th>
<th>BT A</th>
<th>BT B</th>
<th>BL A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>293</td>
<td>612</td>
<td>1,995</td>
<td>738</td>
<td>1,362</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>22,086</td>
<td>30,855</td>
<td>18,262</td>
<td>2,124</td>
<td>43,266</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>17,840</td>
<td>19,022</td>
<td>17,745</td>
<td>25,364</td>
<td>52,181</td>
</tr>
<tr>
<td>43L</td>
<td>14,953</td>
<td>6,286</td>
<td>12,772</td>
<td>1,323</td>
<td>10,814</td>
</tr>
</tbody>
</table>

* Responses from three tested patients (BT and BL) and two HCs are shown. *M. leprae* sonicate, *M. tuberculosis* sonicate, and purified 43L protein were tested at a concentration of 10 μg/ml. For the patients, 200,000 fresh isolated cells were tested and 150,000 cells were tested for the HCs.
DISCUSSION

In this study, we present structural and immunological analyses of the M. leprae antigen 43L that show clear structural and immunological relatedness with the secreted M. tuberculosis protein MPT32 and structural homology with the M. bovis 45/47-kDa complex antigen.

In a search for secreted proteins of M. leprae, we have identified a number of M. leprae recombinant antigens reacting with rabbit antibodies directed against secreted proteins of M. tuberculosis. The antigen 43L has been shown to cross-react with an antiserum against MPT32, a secreted protein of M. tuberculosis.

DNA sequence analysis indicates that the gene for 43L encodes a mature protein of 248 amino acids. The N terminus of the mature protein shows 50% homology with the N terminus of MPT32 (15) and 47% homology with the N terminus of the 45/47-kDa complex of M. bovis BCG (20). No significant homologies were found in screening the complete protein sequence with other proteins using GenBank/EMBL data banks. Furthermore, the protein contains three Asp-Pro-Asn-Ala repeats at positions 301, 321, and 351. The function of these repeats is unknown. Interestingly, the mature protein is preceded by a signal peptide of 39 amino acids. The presence of a signal sequence strongly supports the notion that 43L is secreted like its M. tuberculosis counterpart MPT32.

The appearance of a doublet on SDS-PAGE and immunoblots is consistent with the multiple bands observed for the 45/47-kDa protein complex of M. bovis BCG (20). Posttranslational modifications of a protein encoded by a single gene could explain the doublet. An alternative explanation could be partial degradation. A homolog of 43L in M. tuberculosis, MPT32, is found to be the most unstable protein of all secreted proteins tested thus far (15). Southern blot analysis of digested total genomic DNAs of M. leprae and M. tuberculosis indicates that the gene coding for 43L has one copy in M. leprae and that a homolog is indeed present in M. tuberculosis, as a single-copy gene. The presence of similar but less-related genes, as postulated for the 45/47-kDa complex of M. bovis BCG, cannot be excluded (20). The N-terminal sequence of M. tuberculosis MPT32 shows a distinct difference from the N-terminal sequence of the M. bovis BCG 45/47-kDa protein at residues 1, 3, and 11 (Fig. 4). This is considerably more than usually observed when corresponding amino acid sequences of M. tuberculosis and M. bovis BCG are compared. This observation of extensive homology but distinct difference between the two N-terminal sequences may also point to the existence of more than one gene.

The size of the 43L protein as deduced from sequence analysis of two independently cloned genes reveals a molecular mass of 25.5 kDa for the mature protein. Correspondingly, the native protein 43L appears on SDS-PAGE as a protein with a molecular mass of approximately 27/32 kDa. This molecular mass is considerably smaller than those reported for MPT32 of M. tuberculosis (41 kDa) and for the 45/47-kDa complex of M. bovis BCG (15, 20). This difference in molecular mass may be accounted for by differential posttranslational modification, yielding a larger product in the case of naturally expressed protein in M. leprae than for the expression in E. coli. In this regard, it should be noted that evidence for glycosylation of mycobacterial proteins has recently been presented for the M. tuberculosis 19-kDa antigen (7). This issue can be further investigated after expression of 43L in a mycobacterial host (12).

Secreted proteins are believed to be important in the immune response to M. leprae. The secreted proteins analyzed so far are shown to elicit a T-cell response and a B-cell response in leprosy patients and HCs. This is shown for the antigen 85 complex (19, 25). Secreted proteins of M. leprae have been difficult to identify, largely due to the inability to culture M. leprae in vitro. The current study demonstrates that this problem can be circumvented by using antisera directed against secreted proteins of M. tuberculosis. This approach is limited by the use of rabbit antisera directed to M. tuberculosis. Secreted proteins of M. leprae that display less or no homology with M. tuberculosis will therefore not be detected. Furthermore, the rabbit’s immune system might be unresponsive to certain antigens. Therefore, among the 25 recombinants tested, there may be additional secreted proteins to clones identified in this study.

Immune responses to secreted proteins might be particularly important in the initial phase of infection. M. leprae is capable of surviving intracellularly in resting macrophages. Secreted proteins of M. leprae may induce immune responses in an early stage because of their accessibility to the immune system. In this early stage, they can be processed and presented in combination with major histocompatibility complex molecules on the surface of macrophages, thereby stimulating T cells that can initiate activation of the resting macrophages and causing removal of intracellular bacteria. The observation that putative secreted M. leprae protein 43L indeed elicits T-cell and B-cell responses in a number of HCs, BT/TT patients, and LL/BL patients supports this hypothesis. This study confirms the assumption that secreted M. leprae proteins may induce both cellular and humoral immune responses in patients and controls, regardless of the ability of these individuals to respond to total M. leprae (17). Several of the most prominent mycobacterial antigens identified thus far are HSPs. Such HSPs are evolutionarily conserved and share homology with eukaryotic HSPs. Consequently, the immune system recognizing mycobacterial HSPs may cross-react with human HSPs. The antigen recognized by T and B cells described in this study may be important in the light of vaccine development, since it would not provoke an autoimmune reaction, possibly as a result of its expression being restricted to mycobacteria.

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