A highly immunogenic putative *Mycobacterium kansasii* lipoprotein

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The resurgence of tuberculosis, the emergence of multiple drug resistant tuberculosis, and the increasing prevalence of mycobacterial disease in AIDS patients have increased the importance of defining new mycobacterial antigens that can be utilized in the development of improved diagnostic reagents and more effective vaccines. In this report, a highly immunogenic *Mycobacterium kansasii* protein (MK35) and the gene encoding this antigen were characterized. MK35 gene probes reacted with genomic DNA from *M. avium, M. bovis* BCG, *M. intracellulare* and *M. tuberculosis* but not with DNA isolated from nine other mycobacterial species. Nucleotide sequence analysis showed that the MK35 gene encodes a 26 kDa protein which contains a consensus bacterial lipoprotein processing sequence. In addition, detergent-phase separation studies strongly suggested that MK35 is a lipoprotein. Skin test assays demonstrated that MK35 elicited a strong response in guinea pigs sensitized with *M. kansasii* but did not react in *M. tuberculosis*-sensitized guinea pigs. These results further suggest that mycobacterial lipoproteins are immunogenic antigens that should be considered in the development of new mycobacterial vaccines and diagnostic reagents.

Keywords: *Mycobacterium kansasii*, lipoproteins, mycobacterial antigens

INTRODUCTION

*Mycobacterium kansasii* is an environmental organism which has been isolated from soil, house dust, and tap water and had been commonly identified in western Europe and the United States (Davies, 1994). Infections with *M. kansasii* can cause a chronic, progressive, and sometimes fatal pulmonary disease in normal hosts (Lillo et al., 1990). Moreover, disease resulting from *M. kansasii* infections is extremely serious and life-threatening in patients with advanced immunosuppression (Sheerer et al., 1986; Shafer & Sierra, 1992). Hospital-based studies have indicated that *M. kansasii* is the second most common nontuberculous mycobacterial infection of AIDS patients in the United States (Horsburgh & Selik, 1989). In fact, two reports from hospitals in Baltimore and Los Angeles have concluded that *M. kansasii* was isolated more frequently than *M. tuberculosis* among their patients with documented HIV infection (Young et al., 1986; Levine & Chaisson, 1991).

The increasing incidence of mycobacterial diseases in immunocompromised individuals and the emergence of the highly progressive and rapidly fatal multiple drug resistant tuberculosis (MDRTB) have increased public awareness about the inadequacy of current protocols to prevent and clinically manage mycobacterial infections (Dooley et al., 1992; Reilly, 1993). An improved mycobacterial vaccine is clearly needed because the current vaccine, the bacillus Calmette-Guérin (BCG), has questionable effectiveness (Fine & Rodrigues, 1990). Colditz et al. (1994), using meta-analysis of data from relevant BCG efficacy trials, have recently concluded that the overall efficacy of BCG is only 50%. More rapid and specific methods for diagnosing mycobacterial diseases are also essential for improving patient care. Current procedures for detecting and speciating mycobacterial infections, such as tuberculin skin testing and mycobacterial cultivation, are clearly inadequate. For example, because the radiographic manifestations, pathology and clinical
features of M. kansasii and M. tuberculosis infections are similar, standard methodologies cannot rapidly distinguish between these infections (Wallace et al., 1990). The differentiation between M. kansasii and M. tuberculosis bacilli by cultivation and biochemical analysis currently requires several weeks. In an era of M. tuberculosis and the HIV pandemics, the failure to rapidly identify mycobacterial disease can have an important impact on the implementation of proper public health control measures.

The development of improved methods to prevent and detect mycobacterial disease in the future will probably be linked to the isolation of new mycobacterial antigens and the further characterization of previously identified mycobacterial immunogens. In this paper, we describe the identification and characterization of a putative mycobacterial lipoprotein, the MK35 protein from M. kansasii. The immunoreactivity of MK35 as demonstrated by serological studies and skin test assays further suggests that mycobacterial lipoproteins are immunodominant mycobacterial antigens.

METHODS

Preparation of mycobacterial DNA and antigens. The M. kansasii Bostrom strain was obtained from the culture collection at the George Washington University School of Medicine. The following mycobacterial strains were obtained from the American Type Culture Collection (Rockville, MD, USA): M. asiaticum (25274), M. avium (35714), M. bovis (27291), M. bovis BCG (27291), M. chelonae (19977), M. fortuitum (6841), M. intracellulare (13950), M. marinum (927), M. phlei (11758), M. scrofulaceum (19981), M. smegmatis (14468), M. terrae (15755), M. tuberculosis H36Ra (25177), M. tuberculosis H37Rv (27294), M. vatsiae (15463) and M. xenopi (19250). The mycobacterial antigen preparations used in these experiments were sonic extracts of actively proliferating mycobacteria grown in Long's synthetic medium (with 1% w/v, glucose) at 37 °C for three weeks. At late-exponential phase, the cells were harvested by centrifugation, washed in deionized water, sonicated and centrifuged as described previously (Rouse et al., 1990). For DNA extractions, the strains were grown in the same medium, the cells were washed, and total DNA was extracted using published protocols (Morris et al., 1988).

Production and screening of monoclonal antibody (mAb) 3869. The protocols for generating and screening anti-mycobacterial monoclonal antibodies have been previously described (Rouse et al., 1990). Briefly, Balb/c mice were injected intraperitoneally three times in one week with 10 μg per injection of an ammonium sulfate precipitate of a sonic extract from the M. kansasii Bostrom strain. Sequential bleedings of these animals indicated that serum antibody levels remained high for several months. After 5 months, the immunized mice were given an intravenous booster of 1 μg of the M. kansasii ammonium sulfate precipitate. Cell fusions were performed 3 d later by a modification of the method of Kohler & Milstein (1975). All hybridomas generated were initially screened with preparative immunoblots using the Mini-blotter 28 apparatus (Immunetics). To determine whether hybridomas recognized protein-associated epitopes, mycobacterial sonic extracts were digested to completion with proteinase K and pronase prior to the screening assay.

To evaluate the specificity of mAb 3869, the following mycobacterial sonic extracts were resolved by SDS-PAGE: M. kansasii, M. avium, M. bovis (BCG), M. chelonae, M. fortuitum, M. intracellulare, M. marinum, M. phlei, M. scrofulaceum, M. smegmatis, M. tuberculosis, M. vaccae, M. xenopi and M. vaccae, plus Escherichia coli. Immunoblots prepared from these gels were incubated with mAb 3869 and developed as previously described (Morris et al., 1991).

Production and screening of a M. kansasii agt11 expression library. Preparation of the expression library was based on established protocols (Morris et al., 1988). This methodology yielded approximately 106 individual recombinant bacteriophages. DNA restriction analyses indicated that nearly 90% of these phages contained insert fragments. Immuno-reactive bacteriophages were detected with monoclonal antibodies using protocols that were utilized to identify and isolate recombinant bacteriophages from the M. intracellulare expression library (Morris et al., 1990).

Purification of the MK35 recombinant protein. The M. kansasii recombinant protein was overproduced using the pGEMEX system (Promega), a T7-polymerase-based expression system (Studier & Moffat, 1986). Recombinant clones were over-expressed as described by the manufacturer with the modification that the BL21 (pLysS) E. coli host strain was substituted for the manufacturer's recommended host strain. Specifically, bacteriophages expressing a recombinant antigen recognized by mAb3869 were purified using LambdaSorb phage absorbent as described by the manufacturer (Promega). The mycobacterial insert fragment was isolated from low-melting-point agarose after EaeR digestion and cloned into the pGEMEX vector. The ligation mixtures were then transformed into BL21 (pLysS). The M. kansasii antigen was overexpressed by first growing the recombinant pGEMEX BL21 transformants at 37 °C to an OD600 of 0.5 in LB medium supplemented with 30 μg chloramphenicol ml−1 and 100 μg ampicillin ml−1. The cells were induced with 5 mM IPTG and incubated with aeration for an additional 3–4 h at 37 °C. After the induction period, the cells were centrifuged, resuspended in 0.05 vol. 50 mM Tris (pH 7.4), 2 mM EDTA, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF) and frozen at −70 °C.

The BL21 lysates containing the overexpressed MK35 protein were resolved using standard SDS-PAGE techniques. To visualize the separated proteins, the gels were then soaked in a fluorescent dye solution that had been prepared by diluting 1 ml of a stock solution (10 mg ml−1 in methanol) of 3,3'-dipentyl-oxacarbocyanine iodide (Molecular Probes) in 100 ml 0.1 M NaHCO3, 0.1% SDS. After 10 min, the gels were destained in water for another 10 min. The gels were exposed to UV illumination and the overexpressed protein band was excised from the gels. The recombinant protein was electroeluted from the acrylamide slices using the Elutrap System (Schleicher and Schuell). Electrophorated M. kansasii recombinant protein was precipitated with 50% saturation ammonium sulfate, resuspended in 10 mM sodium phosphate (pH 7.0) and dialysed against the same buffer at 4 °C. Eluted recombinant protein was purified by hydrophobic interaction chromatography on a Hewlett Packard 1090 series M liquid chromatograph equipped with a photodiode array detector. The column used was a Hewlett Packard phenyl 5PW (75 x 7.5 mm) equilibrated with 2 mM ammonium sulfate in 10 mM sodium phosphate (pH 7.6). Protein solutions were adjusted to 2 M ammonium sulfate and were filtered through a 0.45 μm filter before injection. Protein was eluted with a 15 min gradient from 2 mM ammonium sulfate in 10 mM sodium phosphate (pH 7.6) to 10 mM sodium phosphate. The flow rate was set at 0.5 ml min−1. Protein peaks were detected by absorbance at 280 nm.

Skin testing with purified MK35. Hartley strain guinea pigs (350 g) were sensitized by injecting into the nuchal area 2 mg of heat-inactivated bacilli from the M. kansasii Bostrom strain, the
**RESULTS**

**Isolation of a recombinant bacteriophage expressing the MK35 antigen.**

To identify novel mycobacterial antigens, a *M. kansasii* *λ*gt11 library was generated and monoclonal antibodies that recognized *M. kansasii* antigens were produced. mAb 3869 was of particular interest because immunoblot analyses demonstrated that this antibody specifically recognized *M. kansasii* sonic extract antigens and did not react with sonic extracts from 12 other mycobacterial species. Furthermore, protease sensitivity assays indicated that mAb 3869 recognized a protein-associated epitope. Initial immunoblot studies suggested that the molecular mass of the reactive antigen was 35 kDa (hence its designation as MK35) (Morris et al., 1991). Subsequent immunoblot characterizations using gradient SDS-polyacrylamide gels have demonstrated that the apparent molecular mass of the MK35 antigen is 32 kDa.

Since mAb 3869 recognized a specific *M. kansasii* protein-associated epitope, it was utilized to screen the *λ*gt11 library. A recombinant bacteriophage expressing an antigen detected by mAb 3869 was identified and purified. Lambda lysogens were then generated by infecting *E. coli* strain 1089 with antibody-reactive bacteriophage at a permissive temperature. Two mAb 3869 reactive polypeptides with molecular masses of 150 and 25 kDa were detected in *λ* lysogen lysates by immunoblotting (Fig. 1, lane 2). In subsequent immunoblot studies, the reactivity of the 150 kDa antigen with an anti-β-galactosidase antibody showed that this *M. kansasii* recombinant antigen is a β-galactosidase fusion protein (data not shown). The 25 kDa peptide that reacted with mAb 3869 is probably a breakdown product of the larger fusion protein. As controls, a lysate from an unrelated *M. avium* *λ* lysogen and *M. kansasii* sonic extract was evaluated. The control *λ* lysogen lysate did not react with mAb 3869 (Fig. 1, lane 3). In contrast, mAb 3869 recognized a 32 kDa antigen in the *M. kansasii* extract preparation (Fig. 1, lane 1).

**Immunoreactivity of MK35**

To evaluate the capacity of MK35 to induce cell-mediated responses, MK35 was tested in a guinea pig delayed-type...
Table 1. Skin test reactivity of the MK35 recombinant protein

The mean skin test reaction ± the standard deviation (mm) for three guinea pigs is shown. NR, No reaction.

<table>
<thead>
<tr>
<th>Sensitizing antigen</th>
<th>M. kansasii recombinant protein</th>
<th>Homologous sonic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. kansasii</td>
<td>15 ± 2</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>M. avium</td>
<td>6 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>6 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>NR</td>
<td>15 ± 0</td>
</tr>
<tr>
<td>Controls (not sensitized)</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

Hypersensitivity (DTH) assay. For these skin test assays, MK35–gene 10 fusion proteins were utilized because overexpression of these recombinant proteins facilitated their purification. The MK35–gene 10 protein and a gene 10 control antigen were purified as described in Methods. Three micrograms of the purified MK35 recombinant protein and the gene 10 control were injected intradermally into guinea pigs that had been sensitized with either M. kansasii, M. avium, M. intracellulare or M. tuberculosis, and into non-sensitized guinea pigs. Concurrent testing with 0.1 μg of homologous mycobacterial sonic extract confirmed that the animals had been properly sensitized. As seen in Table 1, the recombinant MK35 protein elicited a 15 mm skin test reaction in M. kansasii-sensitized animals. In contrast, the MK35 fusion protein did not evoke a response in control non-sensitized guinea pigs. The recombinant protein also induced a moderate cross-reactive response in animals sensitized with M. avium complex (6 mm) but did not elicit a detectable response in those immunized with M. tuberculosis. The gene 10 control did not induce a DTH response in either control or sensitized animals. Therefore, the cell-mediated reactions elicited by the fusion protein resulted from the antigenicity of the M. kansasii portion of this gene 10 hybrid protein.

Nucleic acid hybridization studies using MK35 gene probes

An EoRI–NcoI fragment of the MK35 coding sequence was labelled and used to probe EcoRI-digested DNA from 12 different strains of mycobacteria. As expected, the MK35 probe hybridized strongly to a 3.3 kb restriction fragment in the M. kansasii genomic sample (Fig. 2a, lane 7). Hybridization was also detected with a 3.5 kb M. avium chromosomal fragment (Fig. 2b, lane 2) and a 7.5 kb M. intracellulare fragment (Fig. 2b, lane 6) upon extended exposure. The MK35 probe weakly hybridized to a 3.0 kb restriction fragment present in all M. tuberculosis complex strains tested (Fig. 2b, lane 4).

Nucleotide sequence analysis of the MK35 gene

To facilitate characterization of the MK35 protein, the nucleotide sequence of the gene encoding it was determined. The MK35 gene sequence and deduced protein sequence are shown in Fig. 3. The sequence contains a 675 bp open reading frame, initiating at an ATG (bp 19–21) and terminating at a TAA (bp 694–696). The open reading frame encodes a putative polypeptide of 225 amino acids with an estimated size of 26 kDa. Consistent with the high G+C content of mycobacterial genes, the MK35 coding sequence is 66 mol% G+C, with an 87% G+C bias in the third nucleotide of amino acid codons. A potential Shine–Dalgarno ribosome-binding site is positioned 11 bp upstream of the initiation codon. Inverted repeat sequences that have been identified downstream of the termination codon in several mycobacterial genes and that have been postulated to facilitate transcriptional termination (Shinnick et al., 1989; Collins et al., 1990; Morris et al., 1992) were not identified in the MK35 gene sequence. The MK35 gene sequence and the

![Fig. 2](image_url). Southern blot analysis using an MK35 gene probe. (a) Mycobacterial DNA restricted with EcoRI was loaded in the following lanes: 1, M. asiaticum; 2, M. avium; 3, M. bovis; 4, M. bovis BCG; 5, M. fortuitum; 6, M. intracellulare; 7, M. kansasii; 8, M. phlei; 9, M. scrofulaceum; 10, M. smegmatis; 11, M. terrae; 12, M. tuberculosis H37Rv; 13, M. tuberculosis H37Ra. HindIII-restricted DNA molecular mass markers were loaded in lane M. (b) Hybridization was detected in the M. avium (2), M. bovis BCG (4), and M. intracellulare (6) lanes with a fivefold extended exposure.
deduced amino acid sequence were also analysed using the GCG software package (Devereux et al., 1984). No significant homologies to known non-mycobacterial genes or proteins were found.

The MK35 gene encodes a mycobacterial lipoprotein

Lipoproteins are among the most immunogenic antigens identified in mycobacteria. We have previously demonstrated that the deduced protein sequences of four known mycobacterial lipoproteins – the 19 and 38 kDa M. tuberculosis proteins and the 19 and 27 kDa M. intracellulare antigens – have similar amino-terminal sequences (Nair et al., 1992, 1993). A comparison of the MK35 protein sequence with the sequences of the other known mycobacterial lipoproteins revealed the same amino-terminal primary sequence similarities (Fig. 4). Each mycobacterial lipoprotein has a sequence motif similar to the prokaryotic consensus sequence for protein lipidation and modification (Wu & Tokunaga, 1986). For MK35, the proposed lipidation sequence LAGCS (amino acids 17–22) consists of an invariant cysteine flanked by small neutral amino acids. A hydrophobic region (possibly a signal sequence) precedes the lipidation site in each of these lipoproteins. Eleven of thirteen amino acids in this segment (amino acids 23–58) are either serine or threonine. This hydrophobic segment of MK35 (residues 4–16) characteristically consists mainly of serines and threonines followed by hydrophobic residues such as leucine, valine or alanine. The region of the mycobacterial lipoprotein (Msgpaba9), the 27 kDa lipoprotein (M. Aansusii), the 19 kDa lipoprotein (M. kansasii), and the 27 kDa lipoprotein (M. kansasii) have similar amino-terminal sequences (Nair et al., 1993a, b). For MK35, 23 of 28 amino acids in this segment (amino acids 23–58) are either serine or threonine. Triton X-114 extractions of M. kansasii sonicate extracts were performed to further examine the lipophilic nature of MK35. Proteins strongly associated with membranes (i.e. integral membrane proteins and lipoproteins) will segregate with the Triton X-114 detergent phase upon phase separation. Soluble proteins (i.e. cytoplasmic and loosely associated membrane proteins) will partition into the upper aqueous layer. To determine the detergent

Fig. 4. Comparison of the predicted amino acid sequence of the MK35 protein with the sequences of the M. intracellulare 19 kDa lipoprotein (Migant19), the M. tuberculosis 19 kDa lipoprotein (Migant19), the M. intracellulare 27 kDa lipoprotein (Migant19), and the M. tuberculosis 38 kDa lipoprotein (Migpaba). The underlined amino acid residues represent the predicted lipoprotein processing sequences. Dots represent gaps in the protein sequences which facilitate alignment of these sequences.

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solubility of MK35, *M. kansasii* sonic extracts were extracted with Triton X-114 and the phases were separated by incubation at 37 °C. The Triton X-114-soluble and aqueous phases were subjected to SDS-PAGE and then transferred to nitrocellulose. The Western blots were subsequently incubated with mAb 3869. The immunoblot analyses strongly suggested that MK35 is a hydrophobic protein because it partitioned almost completely into the detergent-soluble Triton X-114 extraction phase. Furthermore, we demonstrated that MK35 induced DTH reactions in sensitized guinea pigs. Recently, we have shown that MK35 is recognized by sera from AIDS patients with mycobacterial disease (Morris et al., 1991). This immunoreactivity of MK35 in DTH and serological assays is consistent with its classification as a mycobacterial lipoprotein.

Given the limited efficacy of the current mycobacterial vaccine, an international public health priority is the development of a more effective vaccine. The characterization of new mycobacterial immunogens should accelerate vaccine development. Because of the problems associated with a live vaccine, including stability, safety, consistency of production and limited applicability for immunocompromised individuals, it has been suggested that a mycobacterial subunit vaccine would be desirable. Recent studies have shown that a subunit vaccine may be feasible because *M. tuberculosis* culture filtrate proteins are partially protective against an *M. tuberculosis* challenge and vaccination with purified *M. leprae* proteins inhibited *M. leprae* multiplication in mouse footpads (Andersen, 1994; Gelber et al., 1994; Horwitz et al., 1995). The inherent immunogenicity of mycobacterial lipoproteins makes them potential candidates for evaluation as subunit vaccine components. Alternatively, the efficacy of BCG as a mycobacterial vaccine could be enhanced by overexpressing protective antigens in BCG. Stover et al. (1993) have shown that expression of target antigens in BCG as membrane-associated lipoproteins increases the potential to induce protective responses. Therefore, testing the protective capacity of mycobacterial lipoproteins, including MK35, as recombinant fusion proteins in BCG or a components of a subunit vaccine is warranted.

Because of the increasing importance of mycobacterial disease, there is also considerable interest in the availability of species-specific skin test or serological reagents. The selective and large DTH reactions elicited by MK35 and the seroreactivity of MK35 suggest that this protein, or peptide derivatives of it, may have potential clinical utility as immunodiagnostic reagents for the detection of *M. kansasii* infections in humans. Recent experiments have demonstrated that synthetic peptides designed from the sequences of mycobacterial lipoproteins can yield species-specific DTH responses (Vordermeier et al., 1992; Estrada et al., 1992; Mackall et al., 1993). These studies suggest that the development of species-specific diag-

**Fig. 5.** Immunoblot analysis of Triton X-114 extracted sonic extracts. The following samples were resolved by SDS-PAGE and blotted: lane 1, *M. intracellulare* sonic extract; lane 2, *M. kansasii* Triton X-114 detergent phase; lane 3, *M. kansasii* Triton X-114 aqueous phase; lane 4, *M. kansasii* sonic extract. The immunoblot was then incubated with mAb 3869 and developed as described in Methods.
nostic reagents consisting of multimeric peptide polymers may be feasible. The availability of species-specific reagents for skin testing or serodiagnosis would possibly permit the rapid differentiation of nontuberculous mycobacterial infections from those caused by\textit{M. tuberculosis}.

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