Evaluation of the Recombinant 38-Kilodalton Antigen of *Mycobacterium tuberculosis* as a Potential Immunodiagnostic Reagent


MRC Tuberculosis and Related Infections Unit, Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London, W12 0NN, Department of Infection and Tropical Medicine, Imperial College School of Medicine, Northwick Park Hospital, Harrow, HA1 3UJ; United Kingdom; Statens Serum Institut, 2300 Copenhagen 5, Denmark; IRIS, Chiron Biocine, 53100 Siena, Italy; Tuberculosis Research Centre, ICMR, Chetput, Madras 600031, India; and Group of TB Hospitals, Sewri, Bombay 400015, India; and Gesellschaft für Biotechnologische Forschung mbH, Abteilung Geneexpression, D-38124 Braunschweig, Germany

Received 3 September 1996/Returned for modification 8 October 1996/Accepted 26 November 1996

The diagnosis of infection caused by *Mycobacterium tuberculosis* is of increased public health concern following increases in the number of cases in developed countries and major increases in developing countries associated with the spread of human immunodeficiency virus (HIV) infection. The specificity of purified protein derivative skin testing for the detection of infection is compromised by exposure to environmental mycobacteria. Examination of sputum detects the most infectious patients, but not those with extrapulmonary disease. The 38-kDa antigen of *M. tuberculosis* contains two *M. tuberculosis*-specific B-cell epitopes. We overexpressed the gene for this antigen in *Escherichia coli* and evaluated the recombinant product in vitro assays of T-cell function and as a target for the antibody response in humans. The sensitivity and specificity of the antigen as a skin test reagent were also assessed in outbred guinea pigs. We found that 69% of healthy sensitized humans recognize the antigen in vitro, as manifested by both cell proliferation and the production of gamma interferon. Untreated patients initially have a lower frequency of response (38%); this recovers to 72% during therapy. A total of 292 patients (20 with HIV coinfection) and 58 controls were examined for production of antibody to the 38-kDa antigen by using a commercially available kit. The sensitivity of the test in comparison with that of culture was 72.6%, and the specificity was 94.9%. The antigen was also tested for its ability to induce skin reactions in outbred guinea pigs sensitized by various mycobacterial species. The antigen provoked significant skin reactions in *M. tuberculosis*-, *M. bovis* BCG-, and *M. intracellulare*-sensitized animals. The significance of these findings and the usefulness of this antigen in immunodiagnosis are discussed.

Species-specific antigens of the *M. tuberculosis* complex are theoretically attractive immunodiagnostic reagents that are potentially able to distinguish infection of pathogenic potential from cross-reactive sensitization by environmental mycobacteria. The immunodominant 38-kDa lipoprotein antigen of *M. tuberculosis* is a phosphate-binding protein (6). It was first isolated as a component of antigen 5 by affinity chromatography and was reported to be specific to the *M. tuberculosis* complex (9). However, a limited clinical trial of this preparation as a skin test reagent with 14 individuals suggested that the apparent species specificity might be compromised (8). At about the same time the same antigen was discovered to contain at least two species-specific B-cell epitopes (7), and the defining monoclonal antibodies (TB71 and TB72) were developed for use in the serological diagnosis of tuberculosis in a competition enzyme-linked immunosorbent assay (ELISA) (26). The same monoclonal antibodies were used in affinity purification of a preparation which, contrary to the previous findings, was active in T-cell proliferation assays with T cells from humans (28). Further serological evidence (1) and in vivo evidence obtained from studies with inbred guinea pigs (13) suggested that the species specificity of the 38-kDa antigen may have been underestimated by the previous studies with antigen 5. The gene for the 38-kDa antigen has been cloned and overexpressed in *Escherichia coli* (20). The recombinant product is available to the research community via the World Health Organization’s Recombinant Protein Bank (Braun-
were harvested and stored at 2°C and desiccated.

The cutoff level for a positive result was defined as the optical density (OD) of the low-positive control/1.5. This was typically at about an OD of 0.25. All other results were considered negative.

Skin tests. The guinea pigs were given intradermal injections of 0.1 ml of antigen solution. The dose was varied according to the individual experiment. The results of the reactions were read after 24 h by two independent blinded observers, each of whom measured two transverse diameters of the erythema; the quoted result is the mean.

Statistical analysis. Contingency analysis was performed by the Fisher exact test of probability. Continuous nonparametrically distributed data were analyzed by the Wilcoxon signed rank test.

RESULTS

Lymphocyte proliferation and IFN-γ production in humans.

The PBMC responses of 29 sensitized healthy subjects (SH; 16 males and 13 females; average age, 34.9±2.1 years) and 37 biopsy- or culture-positive untreated patients (UP; 26 males and 11 females; average age, 29.5±2.0 years) were examined. Eighteen of the patients were retested (TP) halfway (approximately 3 months) during treatment. The results are presented in Fig. 1. The response frequencies, defined by a stimulation index of >2.5, were 69% for SH, 38% for UP, and 72% for TP. The difference between SH and UP is significant (P<0.01), as is the difference between UP and TP (P<0.02). All subjects tested responded to concanavalin A at all time points. There was therefore evidence of antigen-specific decreased responsiveness in vitro in the patient group whose response reversed during chemotherapy.

The ability of the recombinant antigen to elicit IFN-γ production, believed to be important in the genesis of DTH responses (18), was examined in five SH and five UP. The patients’ responses were retested during and at the end of chemotherapy. The results are presented in Fig. 2. Production of IFN-γ was significantly higher in SH in comparison with that in UP (P<0.05). During treatment there was an increase in IFN-γ production in UP, such that the difference from SH ceased to be statistically significant.
Sensitivity and specificity of enzyme immunoassay for the 38-kDa antigen. A total of 292 patients with suspected pulmonary tuberculosis were selected on an intention-to-treat basis. Twenty of 225 patients tested were positive for antibodies for HIV. Fifty-eight controls (34 healthy subjects and 24 atopic individuals) were also examined. The results are presented in Table 1. On the basis of an intention-to-treat basis, the overall sensitivity of the test was 63.1%, increasing to 73.5% when only patients with culture-confirmed tuberculosis were considered. The sensitivity was not impaired by concurrent HIV infection in the small number of patients tested. The specificity of the test was 94.9%.

Induction of DTH in outbred guinea pigs. The biological activity of the recombinant 38-kDa antigen was studied in differently sensitized groups of guinea pigs (Fig. 3). The results indicated that the antigen induced highly significant skin reactions in guinea pigs sensitized with mycobacterial antigens, but not in nonsensitized animals. The patterns of reactivity to the recombinant antigen and affinity chromatography-purified antigen were similar, with both preparations giving significantly higher reactions for animals sensitized with M. tuberculosis than for those sensitized with BCG.

Dose-response in guinea pigs. The dose-response to the 38-kDa antigen was determined in six guinea pigs immunized 4 weeks previously with M. tuberculosis and nine guinea pigs similarly immunized with BCG. The results are presented in Fig. 4. Although the difference in reactions between animals

![Graph](http://jcm.asm.org/)

**FIG. 3.** Mean skin reactions to 0.2 μg of the indicated antigens (rec., recombinant) in groups of six guinea pigs immunized 4 weeks previously with M52 paraffin oil (negative controls) (C), M. tuberculosis H37Rv in oil (2), or BCG vaccine (III). Two tuberculin units of tuberculin PPD RT23 was used in this experiment. Any differences between columns larger than the vertical bar marked LSD (least significant difference) are significantly different (P < 0.05).

- **CF,** culture filtrate from M. tuberculosis H37Rv; 38-kDa (HBT12), monoclonal antibody HBT12-purified 38-kDa antigen.

![Graph](http://jcm.asm.org/)

**FIG. 4.** Dose-response to the recombinant 38-kDa antigen. The mean diameters of erythemas in six guinea pigs immunized 4 weeks previously with M. tuberculosis and nine Guinea Pigs similarly immunized with BCG are presented. ●, immunization with M. tuberculosis; ○, immunization with BCG.

![Graph](http://jcm.asm.org/)

**FIG. 2.** IFN-γ production by PBMCs in response to the 38-kDa recombinant antigen in five patients followed longitudinally during therapy and five sensitized healthy controls. Results were obtained after a 24-h culture of $5 \times 10^6$ cells/ml.

![Graph](http://jcm.asm.org/)

**TABLE 1.** Proportion of subjects with positive enzyme immunoassay results with the recombinant 38-kDa antigen

<table>
<thead>
<tr>
<th>Patient category</th>
<th>Proportion positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td></td>
</tr>
<tr>
<td>Smear +, culture +</td>
<td>98/125 (78.4)</td>
</tr>
<tr>
<td>Smear -, culture +</td>
<td>54/71 (76.0)</td>
</tr>
<tr>
<td>Total</td>
<td>152/196 (77.5)</td>
</tr>
<tr>
<td>Smear +, culture -</td>
<td>16/27 (59.3)</td>
</tr>
<tr>
<td>Smear -, culture -</td>
<td>26/69 (37.7)</td>
</tr>
<tr>
<td>Culture +, HIV +</td>
<td>7/10 (70.0)</td>
</tr>
<tr>
<td>Culture +, HIV -</td>
<td>112/139 (80.6)</td>
</tr>
<tr>
<td>Culture -, HIV +</td>
<td>4/10 (40.0)</td>
</tr>
<tr>
<td>Culture -, HIV -</td>
<td>26/66 (39.4)</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>2/34 (5.9)</td>
</tr>
<tr>
<td>Atopic individuals</td>
<td>1/24 (4.2)</td>
</tr>
</tbody>
</table>

*a* A positive result is defined as an OD (greater than the OD for the low-positive control/1.5. The positive control serum for this assay was a pooled preparation from patients known to have tuberculosis. The high-positive result typically gave an OD of $>1.0$, and the low-positive result typically gave an OD of 0.5. The cutoff OD, therefore, was typically about 0.33. Background binding (i.e., to non-antigen-coated wells) typically gave rise to an OD of about 0.1.

*+,* positive; *−,* negative.

* Date indicate number positive/total number tested (percent positive).*
observed a poor correlation between the magnitude of the PBMC response to PPD in vitro and the diameter of induration in vivo (data not shown). In addition, there is now evidence that the initiation of DTH requires the type 2 cytokine interleukin-4 (2). The native 38-kDa antigen induces an excess of interleukin-4 in patients in comparison with SH (22), and so it may be well suited for skin testing.

The sensitivity and specificity of the serological test accord well with those in previous analyses with the native 38-kDa antigen (15) and competition assays based on the TB72 epitope (5, 26), which is \( M. \) \( \text{tuberculosis} \) specific (7). The sensitivity is highest for the smear-positive, culture-positive group and lowest for the smear-negative, culture-negative group. It is possible, however, that some of the patients in the latter groups had either inactive tuberculosis or other respiratory conditions mimicking tuberculosis. Of note are the 71 patients (24.2% of the total) who were smear negative and culture positive. Fifty-four (76%) of these patients were antibody positive, and the antibody test has the most diagnostic value for these patients, potentially resulting in earlier treatment.

Among the small number of patients tested, HIV coinfection did not impair the sensitivity of the test. This is similar to the findings of others with other antigens (16, 24). The value of serology in the diagnosis of extrapulmonary tuberculosis has already been proposed (26). Although the present study concentrated on pulmonary disease, an excess of extrapulmonary disease is associated with HIV infection (21). This can be difficult to diagnose microbiologically, and this serological test could be evaluated for a larger group of HIV-infected patients. A previous evaluation with a different preparation of the antigen in African patients with HIV coinfection did, however, report decreased specificity and sensitivity (about 50%) in comparison with those for non-HIV-infected patients (23).

The recombinant antigen is active as a skin test reagent in outbred guinea pigs sensitized with both BCG and \( M. \) \( \text{tuberculosis} \). There is a reaction in guinea pigs sensitized by \( M. \) \( \text{intracellulare} \), but not for four other common environmental mycobacterial species (Fig. 5), a clear improvement in specificity compared with that of the PPD skin test. Although the TB72 and TB71 B-cell epitopes are \( M. \) \( \text{tuberculosis} \) specific, our genetic analysis of various \( M. \) \( \text{intracellulare} \) and \( M. \) \( \text{avium} \) strains shows the presence of a 38-kDa homolog in \( M. \) \( \text{intracellulare} \), but not in \( M. \) \( \text{avium} \), which correlates well with the skin test results presented here (10). This compromise in specificity may have contributed to the apparently disappointing performance of antigen 5 in a pilot human skin test study (8). However, the groups tested could have been more rigorously defined and were small in number, and the mycobacterial species responsible for environmental sensitization may differ according to geographical location. In addition, other contaminants may have been present in antigen 5. We believe that large quantities of pure recombinant antigen are now available and that a phase I clinical trial involving rigorously defined groups of differently sensitized individuals is worthwhile, and we are instigating such a study.

ACKNOWLEDGMENTS

We thank R. N. Davidson of Northwick Park Hospital for allowing us to include the patients under his care in this study. We also thank Omega Diagnostics, Alloa, Scotland, for supplying the Pathozyme-TB complex serology kits.

REFERENCES

1. Andersen, A. B., Z.-L. Yuan, K. Haslov, B. Vergmann, and J. Bennedsen. 1986. Interspecies reactivity of five monoclonal antibodies to \( M. \) \( \text{tuberculosis} \) as examined by immunoblotting and enzyme-linked immunosor-