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

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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 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.

The diagnosis of infection caused by *Mycobacterium tuberculosis* is of increased public health concern following recent moderate increases in the number of cases in developed countries and major increases in sub-Saharan Africa associated with the spread of human immunodeficiency virus (HIV) infection (11). The only available screening method for the detection of infection is the skin reaction to the purified protein derivative (PPD) of *M. tuberculosis*. Unfortunately, both the specificity and the sensitivity of PPD testing are compromised (4). One of the reasons for the lack of specificity is previous sensitization to environmental mycobacteria (12). This factor is particularly relevant in countries with a low prevalence of mycobacterial infections and in which the *M. bovis* BCG vaccine is not routinely administered. Therefore, control of mycobacterial infections is based on the detection of infected patients and chemoprophylaxis. False-positive results may lead not only to inappropriate chemoprophylaxis but also to an overestimate of the rate of tuberculous infection within the community. In addition, there are pharmacological problems with PPD as a consequence of its poorly defined content, batch-to-batch variation, difficulty in standardization, and the different dosages and forms of administration that are used.

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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-

schweig, Germany). In this report we present evidence of the immunological activity of this recombinant antigen in humans and also in in vivo skin testing in sensitized animals which suggest that this antigen should again be considered for use in both human skin testing and serologic tests for the detection of tuberculosis.

MATERIALS AND METHODS

Antigens. The recombinant 38-kDa antigen was overproduced in *E. coli* and was purified as described previously (20). Initial experiments determined that the optimal dose for human lymphocyte stimulation in vitro was 20 μg/ml. Tuberculin PPD RT23 was obtained from Statens Seruminstitut (SSI) and was used for the determination of sensitivity in guinea pigs. For the determination of specificity of the response to the various antigens, 1 IU of PPD (standard tuberculin from the National Institutes of Health) was used. Culture filtrate from *M. tuberculosis* H37Rv and affinity chromatography-purified 38-kDa antigen were produced as described previously (1, 27).

Subjects. For T-cell assays, patients were recruited from the Department of Infection and Tropical Medicine at Northwick Park Hospital, Harrow, United Kingdom. All the patients received standard combination chemotherapy. Healthy PPD-positive controls were recruited from among laboratory and clinical colleagues. All had evidence of *M. bovis* BCG vaccination. Most had low levels of occupational exposure to tuberculosis. PPD positivity was defined as a skin test reaction of greater than 5 mm of induration to 1 tuberculin unit.

For the serological study, 350 subjects were recruited from two centers in Bombay and Madras, India. Among these subjects, pulmonary tuberculosis was diagnosed on an intention-to-treat basis for 292 subjects. Auramine staining and culture for mycobacteria were performed according to routine protocols.

Lymphocyte proliferation assay. Whole citrated blood was diluted in sterile phosphate-buffered saline (GIBCO, Paisley, United Kingdom), and the peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll (Pharmacia, Uppsala, Sweden) gradient. The cells were then washed and plated at 1.5×10^6 cells/ml in the presence of 5% type AB-positive human serum in 96-well plates for 6 days. DNA synthesis was assayed by [^{3}H]thymidine (Amersham, Buckinghamshire, United Kingdom) incorporation. For the analysis of gamma interferon (IFN-γ) production, 5×10^6 PBMCs/ml were cultured for 36 h. Supernatants were harvested and stored at −70°C until use.

IFN-γ ELISA. Maxisorp (Nunc) plates were coated with anti-IFN-γ (Mabtech AB, Stockholm, Sweden) at 2.5 μg/ml and were incubated overnight at 4°C. The plate was blocked at room temperature in phosphate-buffered saline–0.05% Tween 20–3% (wt/vol) bovine serum albumin. Each ELISA was calibrated against a standard preparation of cytokine (Genzyme, Cambridge, Mass.). The plates, which contained 100 μl of supernatant/well, were incubated for 3 h and were then washed four times. Biotinylated anti-IFN-γ (100 μl; Mabtech AB) was added at 1 μg/ml. The plates were incubated for 1 h at room temperature and were then washed five times. A total of 100 μl of streptavidin-peroxidase (1/1,000; Sigma) was added to each well, and the plates were incubated for 45 min. A final six washes was followed by the addition of K-Blue substrate (ELISA Technologies, Lexington, Ky.). The reaction was stopped and the plates were read in an ELISA-Render at 680 nm.

Direct ELISA for detection of anti-38-kDa antibody in serum. The Pathozyme (previously Immunzyme)-TB complex EIA (Omega Diagnostics, Alloa, Scotland) was used in all serological assays. Test sera were diluted 1/50, and 100 μl was added to a plate precoated with recombinant 38-kDa antigen. Defined negative serum, low-positive pooled serum, and high-positive pooled serum controls were used. These sera were incubated for 60 min at 37°C, and the plates were washed five times. A total of 100 μl of anti-human immunoglobulin G–peroxidase conjugate was then added to each well, and the plates were incubated for 30 min. A further five washes were followed by the addition of 100 μl of stabilized tetramethylbenzidine substrate and the plates were incubated for 15 min. The reaction was stopped, and the absorbance of each well at 450 nm was recorded. 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.

Guinea pigs. Female guinea pigs (weight, 300 to 350 g) of outbred strain Sss:AL bred at SSI were used for the induction of delayed-type hypersensitivity (DTH) in Copenhagen. The outbred guinea pigs used to assess the specificity of the 38-kDa antigen were raised in Siena.

Immunization. The guinea pigs were immunized intradermally in the abdomen four times with 0.1 ml of the chosen immunogen 4 weeks prior to the application of the skin tests. Immunogens were freeze-dried BCG vaccine (SSI) containing approximately 4×10^6 CFU of the reconstituted preparation per ml and glutaraldehyde-killed *M. tuberculosis* H37Rv ground in paraffin oil (M52) at 0.4 mg (semidry weight)/ml. Control groups of guinea pigs were given M52 alone. The other species of mycobacterial immunogens used were heat-killed, dried, and desiccated *M. kansasii*, *M. avium*, *M. intracellulare*, *M. fortuitum*, and *M. scrofulaceum*. Two milligrams of sterilized, heat-killed mycobacteria was suspended in 1 ml of sterile paraffin and was injected intramuscularly into each animal.

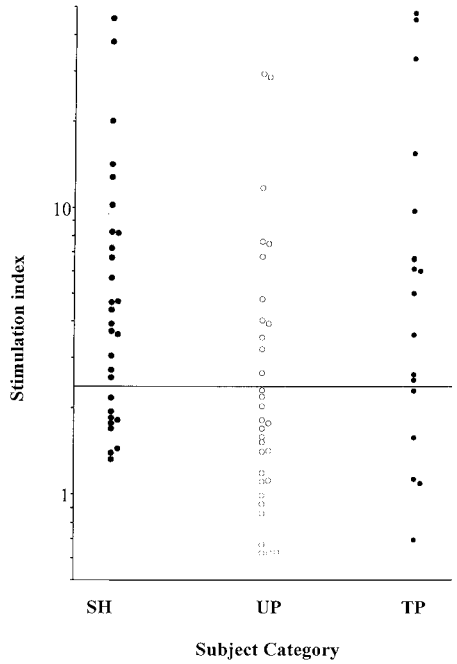

FIG. 1. Proliferative response of PBMCs to the recombinant 38-kDa antigen. Values are quoted as stimulation indices (i.e., counts per minute in the presence of antigen/counts per minute in the absence of antigen). Each value represents the mean of triplicate cultures.

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.

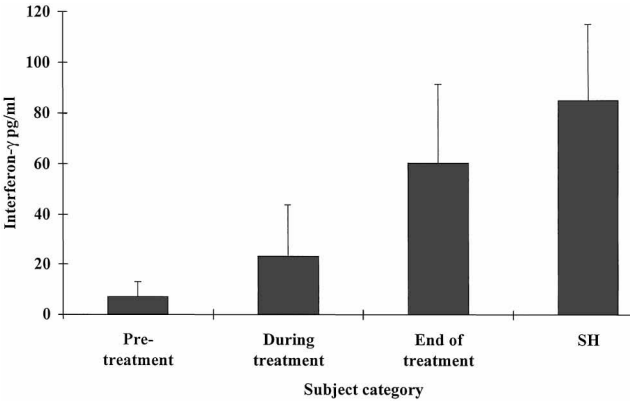

FIG. 2. IFN-γ production by PBMCs in response to the 38-kDa recombinant antigen in five patients followed longitudinally during therapy and five sensitized healthy controls. Results were obtained after a 24-h culture of 5×10^6 cells/ml.

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.

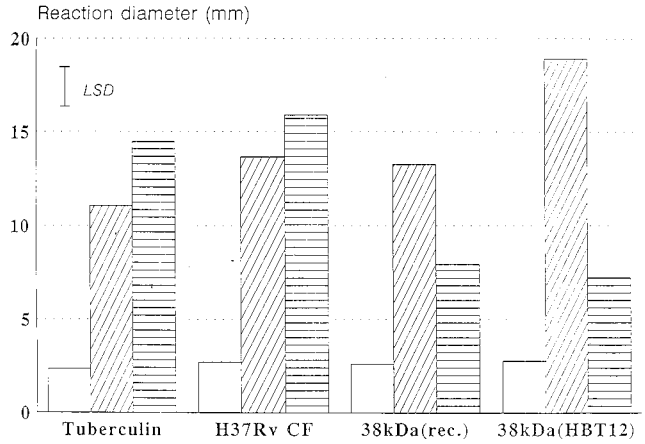

FIG. 3. Mean skin reactions to 0.2 μg of the indicated antigens (rec., recombinant) in groups of six guinea pigs immunized 4 weeks previously with M52 paraffin oil (negative controls) (□), *M. tuberculosis* H37Rv in oil (▨), or BCG vaccine (▤). Two tuberculin units of tuberculin PPD RT23 was used in 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.

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 in nine guinea pigs similarly immunized with BCG. The results are presented in Fig. 4. Although the difference in reactions between animals

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

Patient category[b]	Proportion positive[c]
Patients	
Smear +, culture +	98/125 (78.4)
Smear −, culture +	54/71 (76.0)
Total	152/196 (77.5)
Smear +, culture −	16/27 (59.3)
Smear −, culture −	26/69 (37.7)
Culture +, HIV +	7/10 (70.0)
Culture +, HIV −	112/139 (80.6)
Culture −, HIV +	4/10 (40.0)
Culture −, HIV −	26/66 (39.4)
Controls	
Healthy individuals	2/34 (5.9)
Atopic individuals	1/24 (4.2)

[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.

[b] +, positive; −, negative.

[c] Date indicate number positive/total number tested (percent positive).

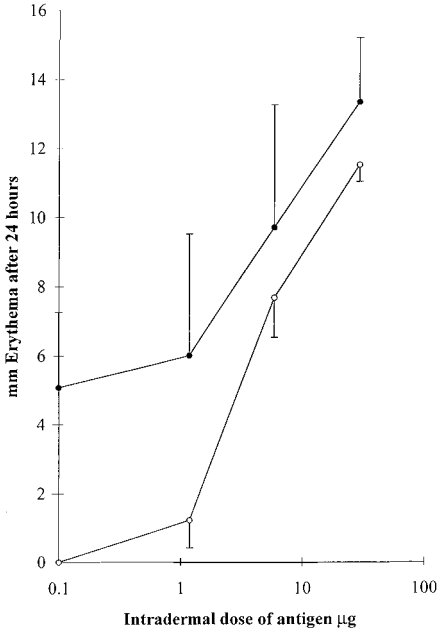

FIG. 4. Dose-response to the recombinant 38-kDa antigen. The mean diameters of erythemas in six guinea pigs immunized 4 weeks previously with *M. tuberculosis* and nine Guinea Pigs similarly immunized with BCG are presented. ●, immunization with *M. tuberculosis*; ○, immunization with BCG.

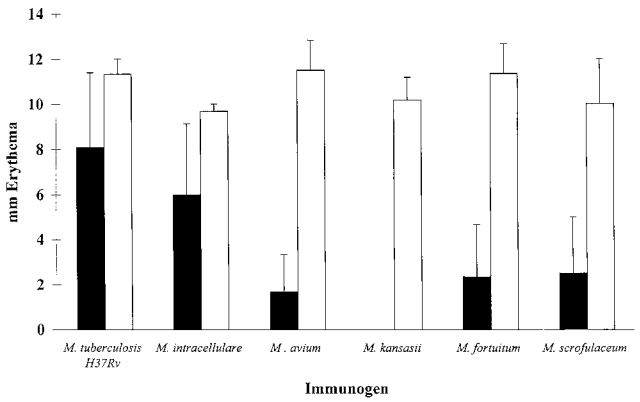

FIG. 5. Mean diameters of erythemas in 10 guinea pigs (6 in the case of *M. tuberculosis*-sensitized animals) tested with recombinant 38-kDa antigen (10 μg) or PPD (1 unit) in groups of 3 guinea pigs (6 in the case of *M. tuberculosis*-sensitized animals) immunized 4 weeks previously. Vertical bars indicate the standard error of the mean. ■, 38 k-Da antigen; □, PPD.

immunized with *M. tuberculosis* and those immunized with BCG was not statistically significant at any challenge dose of antigen, the trend at all doses was that the *M. tuberculosis*-immunized animals reacted more strongly. Thus, a challenge antigen dose of 0.1 μg induced no reaction in the BCG-immunized animals, whereas those immunized with *M. tuberculosis* retained significant reactions.

Species specificity in guinea pigs. The species specificity of the recombinant 38-kDa antigen in vivo was investigated in differently sensitized groups of outbred guinea pigs (Fig. 5). Again, the antigen induced a significant skin reaction. PPD induced a skin reaction irrespective of the sensitizing mycobacterial species, whereas the 38-kDa antigen gave rise to reactions only in *M. tuberculosis*- and *M. intracellulare*-infected animals. If the antigen dose was increased to 30 μg, this specificity was reduced (data not shown). If the antigen dose was decreased to below 10 μg, the frequency and magnitude of the skin reactions decreased in both the *M. intracellulare* and *M. tuberculosis*-sensitized groups of guinea pigs.

DISCUSSION

We have overexpressed the 38-kDa antigen of *M. tuberculosis* in *E. coli*, purified the recombinant product, and tested its sensitivity and specificity as a potential diagnostic reagent in in vitro assays of T-cell function and serology in humans and as a skin test reagent in differently sensitized groups of outbred guinea pigs.

The T-cell responses in vitro are similar to those documented previously with the native protein and confirm that this antigen provokes an immune response in the majority of exposed individuals (25, 28). In this more extensive analysis, however, the phenomenon of T-cell anergy (or reduced responsiveness in vitro) was apparent in UP. Antigen-specific (17) and epitope-specific (25) anergy in tuberculosis has been documented previously. In this study the antigen-specific anergy reverses during therapy, which is also reflected in increased antigen-specific IFN-γ production. The cause of this phenomenon is probably multifactorial, with sequestration of $CD4^+$ cells at the site of disease (3), production of anti-inflammatory cytokines such as transforming growth factor β (14), or defective antigen presentation in acute disease (19) all being likely contributors. It is debatable whether this phenomenon is relevant to the induction of DTH in vivo, because we have 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. 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-coinfected 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. tuberculosis*. There is a reaction in guinea pigs sensitized by *M. intracellulare*, but not 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. tuberculosis* specific, our genetic analysis of various *M. intracellulare* and *M. avium* strains shows the presence of a 38-kDa homolog in *M. intracellulare*, but not in *M. 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.

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