

## Characterization of the Secreted MPT53 Antigen of *Mycobacterium tuberculosis*†

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**MPT53 is a secreted protein of *Mycobacterium tuberculosis*. Southern transfer and hybridization showed *mpt53* to be conserved in the *M. tuberculosis* complex and to have homology with DNA from *Mycobacterium avium* and other nontuberculous mycobacteria. However, anti-MPT53 polyclonal antibodies detected no antigen in the culture filtrates of *M. avium* and other nontuberculous mycobacteria. MPT53 of *M. tuberculosis* induced strong, tuberculosis-specific antibody responses in guinea pigs but induced no delayed-type hypersensitivity. Involvement in immune responses during human tuberculosis was very modest.**

Proteins secreted into the extracellular environment by *Mycobacterium tuberculosis* are usually targets of immune responses in the infected host. Thus, the filtrate of *M. tuberculosis* cultures has constituted an important source of antigens that induce protective immunity and immune responses having diagnostic value (reviewed in references 1, 6, and 20). In the 1980s and early 1990s S. Nagai and his collaborators purified several proteins from culture filtrates of *M. tuberculosis* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). These proteins were termed MPT (for *M. tuberculosis*) or MPB (for *M. bovis* BCG) followed by a number indicating the relative mobility during nondenaturing polyacrylamide gel electrophoresis (13). Of the MPT and MPB proteins known to be secreted (18), many elicit immune responses that are specific for the *M. tuberculosis* complex and therefore of diagnostic value (for example, MPT64 [2], MPB70 [7], and MPT63 [12]). Others confer protective immunity (MPT44, MPT59, and MPT45, i.e., the members of the Ag85 complex [8, 17]). While all other genes encoding MPT and MPB proteins were identified and sequenced in the pregenome era, the *mpt53* gene was identified only recently (19) from the analysis of the NH<sub>2</sub>-terminal sequence of the purified protein (13) and from the genome sequence of *M. tuberculosis* (*mpt53* is Rv2878c) (5). MPT53 is a 15-kDa protein (13) that induces antibody responses in tuberculous cattle (19). In the present work, we characterized the specificity of MPT53 for the *M. tuberculosis* complex and the involvement of MPT53 in immune responses to tuberculosis (TB) in guinea pigs and in humans.

The distribution of *mpt53* among tuberculous and nontuberculous mycobacteria was determined by Southern transfer and hybridization. The gene was present in the DNA of members of the *M. tuberculosis* complex (Fig. 1, lanes 1, 4, and 8, and data not shown). Analysis of 50 clinical isolates of *M. tuber-*

*culosis* identified one isolate bearing a chromosomal deletion encompassing *mpt53* (data not shown), indicating a relative gene instability. We next investigated the distribution of *mpt53* among nontuberculous mycobacteria. Analysis of the partial *M. avium* genome sequence (<http://www.tigr.org/tdb/>) by BLAST homology programs indicated that *M. avium* contains a homolog of *mpt53* (≈80% identity with nucleotide and amino acid sequences corresponding to the extracellular MPT53 protein of *M. tuberculosis*) (data not shown). Accordingly, *mpt53* DNA hybridized weakly with DNAs extracted from reference strains and clinical isolates of *M. avium* (Fig. 1, lanes 2 and 3). *mpt53* DNA also gave weak hybridization signals with DNAs extracted from *M. intracellulare*, *M. fortuitum*, *M. haemophilum*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. scrofulaceum*, and *M. ulcerans*; it failed to hybridize with DNAs from *M. phlei* and *M. xenopi* (Fig. 1). Thus, *mpt53* homologs are broadly distributed among nontuberculous mycobacteria.

To estimate levels of secreted MPT53 and its homologs, we probed culture filtrates of tuberculous and nontuberculous mycobacteria by enzyme-linked immunosorbent assay (ELISA) using a rabbit antiserum raised against MPT53 purified from *M. tuberculosis* culture filtrates. MPT53 was detected in the culture filtrates of *M. tuberculosis* H<sub>37</sub>Rv (and nine clinical isolates [data not shown]) but not in filtrates obtained from nontuberculous mycobacteria (Fig. 2). Furthermore, binding to MPT53 of anti-MPT53 rabbit antiserum was significantly inhibited in competitive ELISA by tuberculin purified protein derivative (PPD) of *M. bovis* but not by PPD of *M. avium* (data not shown). Taken together, these results indicate that there is little, if any, secretion or synthesis of MPT53 homologs in *M. avium* and other nontuberculous mycobacteria during growth in vitro.

To investigate the immunogenicity of MPT53 during experimental and human TB, the *mpt53* gene was cloned into the pQE30 (Qiagen) expression vector of *Escherichia coli*, and the recombinant protein was purified by using a three-step chromatography protocol detailed elsewhere (3). Purified protein was >95% homogeneous, as judged by sodium dodecyl sulfate-

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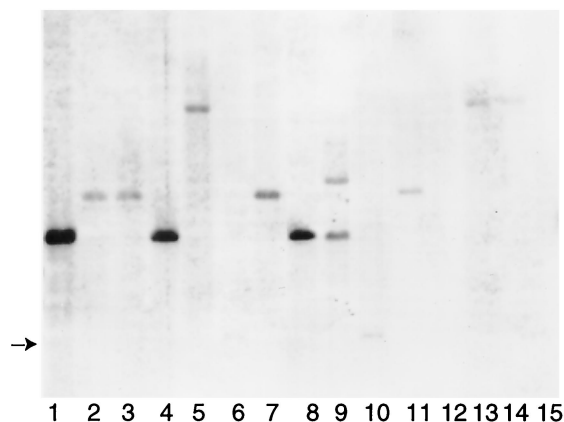


FIG. 1. Genomic analysis of *mpt53* in tuberculous and nontuberculous mycobacteria. Tuberculous and nontuberculous mycobacteria were from the Public Health Research Institute TB Center (PHRI-TB), the American Type Culture Collection (ATCC), and the Trudeau Mycobacteria Collection (TMC). Methods for culturing mycobacteria, isolating DNA, and performing Southern transfer and hybridization analyses were described elsewhere (4, 16). DNA was digested with *Pvu*II and electrophoresed on 1% agarose gels. The separated digestion products were transferred to nylon membranes and hybridized with a nonradioactively labeled 507-bp DNA fragment internal to the *mpt53* gene. After chemiluminescence detection, a single band of  $\approx 3.5$  kb was detected with DNA of the *M. tuberculosis* complex. Hybridization signals obtained with nontuberculous mycobacteria were invariably of lower intensity than those obtained with tuberculous mycobacteria. Results are shown for 3 strains of tuberculous mycobacteria and 12 strains of nontuberculous mycobacteria. Lanes: 1, *M. tuberculosis* H<sub>37</sub>Rv; 2, *M. avium* ATCC 25291; 3, *M. avium* M-64 (clinical isolate); 4, *M. africanum* TMC 5122; 5, *M. fortuitum* ATCC 1530; 6, *M. haemophilum* ATCC 29548; 7, *M. intracellulare* ATCC 13950; 8, *M. bovis* PHRI-TB 5022 (clinical isolate); 9, *M. kansasii* ATCC 12478; 10, *M. malmoense* ATCC 29571; 11, *M. marinum* ATCC 1218; 12, *M. phlei* ATCC 11758; 13, *M. scrofulaceum* ATCC 1302; 14, *M. ulcerans* ATCC 1615; 15, *M. xenopi* ATCC 19250. The arrow on the left indicates the mobility of a very weak band in lanes 6 and 10. Additional members of the *M. tuberculosis* complex tested for presence of *mpt53* were three reference strains of *M. bovis* (TMC 410, TMC 401, and TMC 407), four strains of *M. bovis* BCG (Pasteur TMC 1011, Montreal TMC 1012, Connaught TMC 1030, and Japan ATCC 35737), *M. microti* TMC 1619, and 50 clinical isolates of *M. tuberculosis* in the strain collection of the PHRI-TB Center (data not shown).

polyacrylamide gel electrophoresis followed by staining of gels with Coomassie blue (data not shown). Immunogenicity of MPT53 during experimental TB was assessed in tuberculous guinea pigs by measuring delayed-type hypersensitivity (DTH) and serum immunoglobulin G (IgG) antibodies to MPT53. Animals were aerosol infected with *M. tuberculosis* and skin tested 6 to 8 weeks after infection. The animals reacted to positive sensitization controls (PPD and the purified 45/47-kDa antigen of *M. tuberculosis*), but no DTH response was elicited by recombinant MPT53 purified from *E. coli* (Fig. 3). Some antigens of *M. tuberculosis* require posttranslational modifications, such as glycosylation, to express DTH activity (15). Since the state of MPT53 in *M. tuberculosis* cells is not known, we also measured DTH responses in tuberculous guinea pigs to MPT53 protein purified from mycobacteria (native MPT53 from *M. tuberculosis* culture filtrates [13] and *M. tuberculosis* protein purified from recombinant *M. smegmatis* [19]). Both protein preparations gave negative skin test

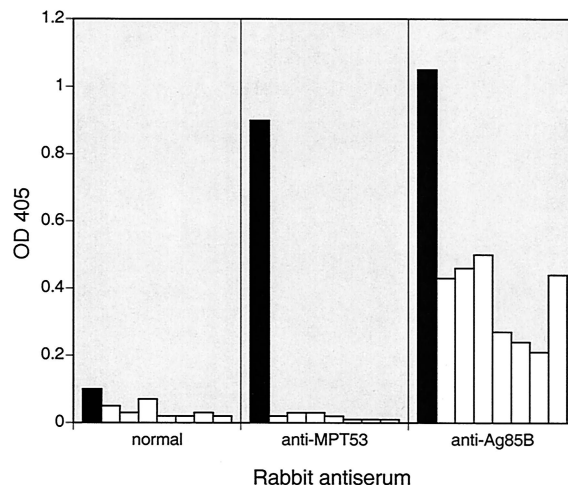


FIG. 2. Detection of MPT53 antigen in culture filtrates of mycobacteria. Culture filtrates of tuberculous and nontuberculous mycobacterial species were tested by ELISA with rabbit hyperimmune sera. Filtrates were obtained from mycobacterial cultures grown to stationary phase in Middlebrook 7H9 supplemented with 10% (vol/vol) albumin-dextrose-catalase and 0.05% (vol/vol) Tween 80 (9). ELISA was performed in polystyrene microtiter plates. The culture filtrate coating concentration was 5  $\mu$ g/ml. Rabbit antisera were used at a dilution of 1:300. Immunodetection was performed with goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Sigma) and an alkaline phosphatase substrate kit (Bio-Rad). Optical density at 405 nm (OD<sub>405</sub>) was measured. Three sets of parallel experiments were performed: with normal rabbit serum, with rabbit anti-MPT53 antiserum, and with rabbit anti-Ag85B antiserum (positive control; Ag85B is an antigen common to tuberculous and nontuberculous mycobacteria [14]). Solid bars, *M. tuberculosis* H<sub>37</sub>Rv; open bars, nontuberculous mycobacteria (from left to right, *M. avium*, *M. fortuitum*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. scrofulaceum*, and *M. ulcerans*).

results (Fig. 3). We concluded that tuberculous guinea pigs exhibit no DTH responses to MPT53. We next examined antibody responses to MPT53 in tuberculous guinea pigs. Since antigen-specific IgG antibody levels are too low at 8 to 9 weeks postinfection for detection by ELISA (our unpublished observations), anti-MPT53 IgG antibodies were measured in tuberculous guinea pigs 15 weeks after low-dose infection by aerosol. These animals exhibited high levels of anti-MPT53 antibodies in serum (Fig. 4). In contrast, sera from seven animals sensitized with *M. avium* failed to react with MPT53 (Fig. 4), suggesting that the *mpt53* homolog of *M. avium* may not be expressed. The possibility cannot be excluded, however, that some MPT53-like protein is present in *M. avium* but does not elicit a measurable antibody production. In conclusion, the above results indicate that the antibody response elicited by MPT53 in guinea pigs is specific for TB.

We next evaluated immune responses to MPT53 in human TB. Two sets of experiments were conducted. In the first experiment, we used sera from 167 patients having pulmonary TB and 75 control sera (from 16 patients having non-TB mycobacterioses, 9 patients having pulmonary diseases other than TB, and 50 healthy blood donors). Detection of serum antibodies against MPT53 was conducted by a nitrocellulose membrane-based assay devised in our laboratory for serological evaluation of antigens (11). Only a small proportion of TB patients (3 of 167; <2%) had serum IgG antibodies against

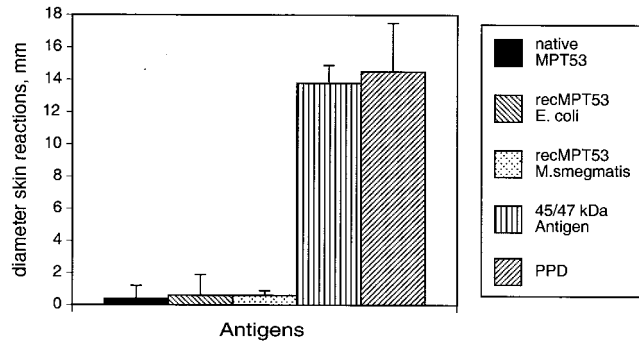


FIG. 3. DTH responses to MPT53 in tuberculous guinea pigs. Two groups of four to six outbred Hartley female guinea pigs (weighing  $\approx 300$  g) were used for skin testing. One group was infected with *M. tuberculosis* H<sub>37</sub>Rv by aerosol with a dose of  $\approx 100$  CFU per animal. A second, control group was mock sensitized by intradermal injection of phosphate-buffered saline. Six to 8 weeks after sensitization, animals were injected intradermally with 1 to 2  $\mu$ g of each preparation of purified MPT53 protein in 0.1 ml of phosphate-buffered saline. As sensitization controls, 1  $\mu$ g of purified recombinant 45/47-kDa antigen (10) and 1  $\mu$ g of *M. tuberculosis* PPD were used. Skin reactions (diameter of erythema and induration, in millimeters) were measured 24 h after antigen injection. Results obtained in tuberculous guinea pigs with recombinant (rec) MPT53 purified from *M. smegmatis* were normalized against the saline controls, since this protein preparation gave 1.5- to 3-mm background reactions in saline-treated control animals.

MPT53 (data not shown). None of the control sera reacted with the antigen. In a second set of experiments, we investigated T-cell responses to MPT53 during latent TB. MPT53 was tested for the ability to induce in vitro secretion of gamma interferon by peripheral blood mononuclear cells obtained from two asymptomatic, tuberculin skin test (TST) reactors and two TST-negative controls. No gamma interferon was secreted by peripheral blood mononuclear cells from TST reactors and negative controls in response to stimulation with MPT53 (data not shown). Taken together, these results suggest minimal, if any, involvement of MPT53 in immune responses to human TB.

The finding that MPT53 elicits strong antibody responses in experimental TB but not during human disease parallels observations made in bovine TB studies. Experimentally infected cattle mount strong antibody responses to this antigen (19; K. P. Lyashchenko, J. M. Pollock, H. G. Wiker, M. Harboe, and M. L. Gennaro, Third International Conference on *Mycobacterium bovis*, 13 to 16 August 2000, Cambridge, United Kingdom, abstr., p. 65). However, none of the naturally infected cattle had detectable anti-MPT53 antibodies in serum (19). Perhaps anti-MPT53 IgG antibodies are detectable in serum only transiently during early stages of natural infection or only when the infecting dose is higher than that usually associated with natural infection.

In summary, we found that *mpt53* homologs are present in *M. avium* and other nontuberculous mycobacteria but that the level of extracellular protein under the tested conditions of growth in vitro is below that needed for detection with a specific polyclonal antibody. The lack of detectable anti-MPT53 serum antibodies in *M. avium*-sensitized guinea pigs lends support to the idea that the *mpt53* homolog of *M. avium* may not

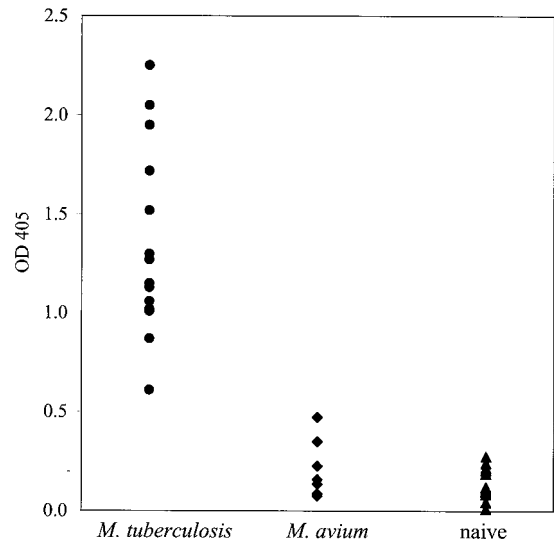


FIG. 4. Antibody responses to MPT53 in guinea pigs. Guinea pig sera were obtained from animals aerosol infected with 10 to 15 CFU of *M. tuberculosis* H<sub>37</sub>Rv 15 weeks after infection, animals sensitized by intradermal injection of  $10^7$  cells of *M. avium* 8 weeks after sensitization, and naive control animals injected intradermally with 0.1 ml of phosphate-buffered saline. ELISA was performed in polystyrene microtiter plates. The coating concentration of recombinant MPT53 purified from *E. coli* was 0.25  $\mu$ g/ml. Sera were used at a dilution of 1:50. Immunodetection was performed with rabbit anti-guinea pig IgG antibodies conjugated with alkaline phosphatase (Sigma) and an alkaline phosphatase substrate kit (Bio-Rad). Optical density at 405 nm (OD<sub>405</sub>) was measured. Each data point represents one animal. Sera obtained from *M. avium*-sensitized animals all reacted strongly with *M. tuberculosis* culture filtrates (data not shown), indicating that the animals had mounted an antibody response to infection at the time of testing.

even be expressed during infection. MPT53 induces a vigorous antibody response and no DTH in tuberculous guinea pigs. We also obtained negative evidence of cell-mediated immune responses to MPT53 in latent TB in humans. Finally, MPT53 elicits very modest antibody responses in human TB (this work) and in bovine TB (19).

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