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MTSA-10, the Product of the Rv3874 Gene of *Mycobacterium tuberculosis*, Elicits Tuberculosis-Specific, Delayed-Type Hypersensitivity in Guinea Pigs

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In a search for new skin test reagents specific for tuberculosis, we found that the antigen encoded by gene Rv3874 of *Mycobacterium tuberculosis* elicited delayed-type hypersensitivity in *M. tuberculosis*-infected guinea pigs but not in control animals immunized with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) or *Mycobacterium avium*. The antigen, which was named MTSA-10 (for *M. tuberculosis*-specific antigen 10), is a prime candidate for a component of a new tuberculin that will allow discrimination by a skin test of latent *M. tuberculosis* infection from vaccination with BCG or from sensitization with environmental, nontuberculous mycobacteria.

The identification of persons infected with *Mycobacterium tuberculosis*—who account for one-third of the world's population—has a high priority in tuberculosis (TB) control programs, second only to the identification and treatment of infectious TB patients (1). To date, the only indicator of latent infection with *M. tuberculosis* is a positive tuberculin skin test, which measures delayed-type hypersensitivity (DTH) responses to the intradermal injection of the purified protein derivative (PPD) of tuberculin, an ammonium sulfate precipitate of filtrates of heat-inactivated, stationary-phase cultures (24). Unfortunately, the use of PPD imposes serious limitations on skin test accuracy. First, batches of PPD vary in protein composition and potency (13), making it exceedingly difficult to compare results obtained with different individuals, countries, or studies. Second, PPD is highly cross-reactive, for it shares many epitopes with antigens of other mycobacteria. Thus, the specificity of the PPD skin test (i.e., the ability of the test to correctly identify noninfected individuals) is very low for persons who have been vaccinated with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and for persons living in areas that have a high environmental burden of nontuberculous mycobacteria (3, 4).

The premise of our work is that the accurate diagnosis of TB infection by skin testing requires a new tuberculin, one consisting of defined protein antigens that are unique to *M. tuberculosis*. A new tuberculin should contain many antigens, since cocktails of multiple antigens are needed to elicit strong DTH responses (16) and to cover the broad spectrum of antigen recognition by different individuals (9) typical of TB (23). We have shown that cocktails of *M. tuberculosis* complex-specific antigens elicit DTH responses that distinguish TB infection from sensitization with nontuberculous mycobacteria (16). The challenge now is to identify antigens unique to *M. tuberculosis* to develop multiantigen cocktails that allow discrimination between *M. tuberculosis* infection and vaccination with *M. bovis* BCG.

We have initiated a search for DTH-active antigens expressed by *M. tuberculosis* but not by *M. bovis* BCG. Prior to the present work, only one antigen, ESAT-6 (26), was known to have such properties (another antigen, MPT64 [29], is absent from only some BCG substrains [15, 17], and it evokes a vigorous DTH response to vaccination with some, but not all, substrains of BCG [11]). ESAT-6, which elicits strong, TB-specific DTH responses in guinea pigs (9), in cattle (21), and in humans (22, 27), is encoded by RD1, a DNA region present in the genome of *M. tuberculosis* and virulent *M. bovis* but missing from the DNA of all substrains of *M. bovis* BCG (17). In the present work we show that a second antigen encoded by the RD1 region of *M. tuberculosis* (gene Rv3874) elicits strong, *M. tuberculosis*-specific DTH responses in guinea pigs.

The Rv3874 gene product. To identify DTH-eliciting antigens specific to *M. tuberculosis*, amino acid sequences, which were deduced from the nine putative genes in RD1 (7), were analyzed with a BLAST protein homology program to eliminate proteins having homologs encoded outside the RD1 region, since such proteins are likely to share epitopes with antigens of *M. bovis* BCG. The products of Rv3874 and Rv3879c shared no homology with proteins encoded elsewhere in the *M. tuberculosis* genome. Because of previous evidence that the Rv3874 gene is expressed and that the corresponding protein is found in the culture filtrate of *M. tuberculosis* (culture filtrate protein 10 [5]), we analyzed the Rv3874 product. The Rv3874 open reading frame was amplified from the chromosomal DNA of *M. tuberculosis* $H_{37}Rv$ by PCR and was cloned into the *Escherichia coli* expression vector pQE-30 (Qiagen) by following standard protocols (18, 19). Recombinant protein was expressed as a polyhistidine-tagged fusion protein and was purified to near homogeneity by using a three-step chromatography protocol (6). The purified protein had an apparent molecular mass of ~10 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, in agreement with a calculated M_r of 10,794 (data not shown).

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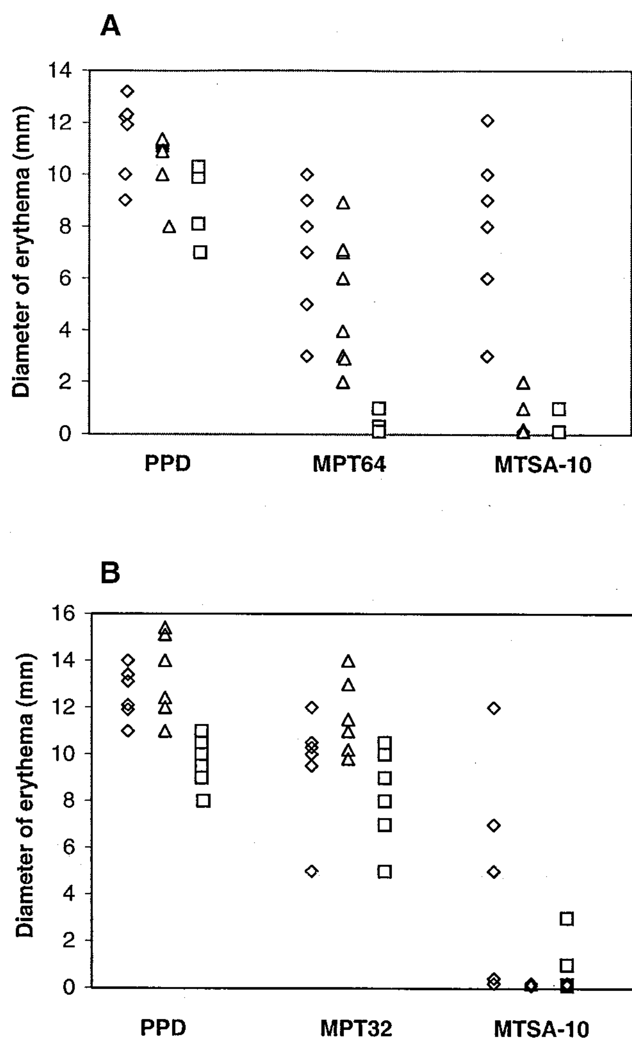

FIG. 1. The DTH response to MTSA-10 distinguishes TB infection from immunization with BCG or with *M. avium.* For skin test experiments, *M. tuberculosis* $H_{37}Rv$ was grown to mid-log phase by shaking at 37°C in liquid glycerol-alanine-salts medium with 0.05% (vol/vol) Tween 80 in a biosafety level 3 laboratory. *M. bovis* BCG Japan and BCG Pasteur were grown in Sauton's medium with 0.025% (vol/vol) tyloxapol at 37°C. *M. avium* TMC 724 was grown by shaking at 37°C in Middlebrook 7H9 (Difco) liquid medium with 2% glycerol, 10% oleic acid-albumin-dextrose-catalase supplement, and 0.05% (vol/vol) Tween 80. PPD from *M. tuberculosis* was prepared at Colorado State University by using a standard protocol (25) and was applied to a Detoxi-gel column (Pierce, Rockford, Ill.) to remove the lipopolysaccharide. Purification of native MPT32 from *M. tuberculosis* $H_{37}Rv$ was performed by the method of Dobos et al. (8). Purification of recombinant MPT64 from *E. coli* was described previously (6). Guinea pigs were aerosol infected with *M. tuberculosis* (◇) or were immunized with BCG (△) or with *M. avium* (□). Six to eight weeks later, animals were tested intradermally with 1 μg of PPD and 2 μg of purified recombinant antigens. Each point represents 1 animal. Results are expressed as diameter of erythema measured 24 h after antigen injection. (A) In a first experiment, the group sizes were six animals for *M. tuberculosis*, eight for *M. bovis* BCG Japan, four for *M. avium*, and four for saline. Antigens were PPD, MPT64 (BCG Japan produces the *M. bovis* homolog MPB64; see text), and MTSA-10. (B) In a second experiment, the group sizes were six animals for *M. tuberculosis*, six for *M. bovis* BCG Pasteur, six for *M. avium*, and five for saline. Antigens were PPD, MPT32 (BCG Pasteur does not produce MPB64; see text), and MTSA-10.

DTH responses in guinea pigs. The ability of the Rv3874 gene product to elicit DTH was determined by guinea pig skin testing. We tested four groups of female outbred Hartley guinea pigs weighing approximately 300 g each. One group was infected with *M. tuberculosis* $H_{37}Rv$ by using an aerosol-generating device (Glas-Col, Middlebrook) calibrated to deliver about 100 tubercle bacilli into the lungs of each animal. Two additional, control groups were immunized with a suspension of 10^6 cells of *M. bovis* BCG or of *M. avium* (the most common cause of nontuberculous mycobacterioses in humans [10]) by intradermal injection in the shaved abdomen. A fourth group was mock immunized by intradermal injection of phosphate-buffered saline. This four-group design was utilized in two independent experiments that included two different substrains of *M. bovis* BCG (BCG Japan and BCG Pasteur).

Six to eight weeks after sensitization, animals were injected intradermally with 2 μg of purified antigen in 0.1 ml of phosphate-buffered saline. Each animal was also injected with 1 μg of PPD to control for sensitization. Single antigens of known DTH activity were also included as sensitization controls because, due to the complex composition of PPD, low levels of sensitization may still result in a sizable reaction to PPD with little or no reaction to single antigens (our unpublished observations). MPT64 was used as a sensitization control in the experiment that utilized BCG Japan, a substrain that produces the *M. bovis* homolog MPB64 (15, 17). In a second experiment that utilized BCG Pasteur, a substrain that does not produce MPB64 (15, 17), we used MPT32 as a sensitization control. The MPT32 antigen of *M. tuberculosis* (14, 20) elicits DTH reactions both in animals sensitized with mycobacteria of the *M. tuberculosis* complex and in those sensitized with nontuberculous mycobacteria (18). Skin reactions (diameter of erythema, in millimeters) were measured 24 h after antigen injection.

TABLE 1. Mycobacterial strains used in this study

Species	Strain[a]
M. tuberculosis complex	
M. tuberculosis $H_{37}Rv$	ATCC 25618
M. tuberculosis $H_{37}Ra$	ATCC 35836
M. tuberculosis	CDC 1551
M. tuberculosis W	PHRI TN3742
M. bovis NADL	TMC 410
M. bovis Ravenel	TMC 401
M. bovis Branch	TMC 407
M. bovis BCG Pasteur	ATCC 35734
M. bovis BCG Japan	ATCC 35737
M. bovis BCG Connaught	ATCC 35745
M. bovis BCG Montreal	ATCC 35735
M. bovis BCG Russia	ATCC 35736
M. africanum	ATCC 5122
Other mycobacteria	
M. asiaticum	TMC 803
M. avium	TMC 724
M. fortuitum	TMC 1530
M. gastri	TMC 1456
M. haemophilum	ATCC 29548
M. kansasii	TMC 1201
M. malmoense	TMC 802
M. phlei	ATCC 11758
M. scrofulaceum	ATCC 1302
M. simiae	TMC 1226
M. terrae	TMC 1450
M. triviale	TMC 1453
M. ulcerans	TMC 1615

[a] ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention; PHRI, Public Health Research Institute; TMC, Trudeau Mycobacterial Collection.

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TABLE 2. Distribution of *mtsa-10* and *esat-6* among mycobacteria

Species and strain	Presence of[a]:	
	mtsa-10	*esat-6*
M. tuberculosis W	+	+
M. tuberculosis $H_{37}Ra$	+	+
M. tuberculosis $H_{37}Rv$	+	+
M. tuberculosis CDC 1551	+	+
M. africanum	+	+
M. bovis NADL	+	+
M. bovis Ravenel	+	+
M. bovis Branch	+	+
BCG Pasteur	−	−
BCG Japan	−	−
BCG Connaught	−	−
BCG Montreal	−	−
BCG Russia	−	−
M. asiaticum	−	−
M. avium	−	−
M. fortuitum	−	−
M. gastri	+	+
M. haemophilum	−	−
M. kansasii	+	+
M. malmoense	−	−
M. phlei	−	−
M. scrofulaceum	−	−
M. simiae	−	−
M. terrae	−	−
M. triviale	−	−
M. ulcerans	−	−

[a] +, presence of hybridization signal; −, absence of hybridization signal. Mycobacterial strains were cultured at 36°C in an atmosphere containing 5% CO_2 on Middlebrook 7H10 (Difco) agar plates containing 0.5% (vol/vol) glycerol and supplemented with 10% (vol/vol) albumin-dextrose-catalase. An exception was *M. haemophilum*, which was cultured on chocolate-agar plates at 30°C. The culture medium for *M. africanum* was supplemented with 0.5% (wt/vol) pyruvic acid. Cells were harvested after 4 days of culturing for fast-growing mycobacteria and 4 weeks of culturing for slow-growing mycobacteria. DNA isolation and Southern transfer hybridization were performed according to a standard protocol employed in DNA fingerprinting of *M. tuberculosis* (28).

Measurement of skin reactions to PPD and to the control single antigens (MPT64 and MPT32) indicated that all animals were sensitized (Fig. 1). As expected, the control MPT64 antigen, which is specific for the *M. tuberculosis* complex (2), induced a strong response by the TB and BCG groups, but not by the *M. avium* group (Fig. 1A). In contrast, the control MPT32 antigen, which is cross-reactive (18), induced a strong response in the TB, BCG, and *M. avium* groups (Fig. 1B).

The Rv3874 gene product elicited DTH responses in the *M. tuberculosis*-infected animals, but not in the BCG- and *M. avium*-sensitized controls (Fig. 1), indicating that the DTH response to this antigen is TB specific. In the second experiment, three animals in the TB group showed no reactivity to the Rv3874 gene product (Fig. 1B). The biological basis of such animal-to-animal variability, which has been previously observed in the DTH response of guinea pigs to other antigens of *M. tuberculosis* (9), is presently unknown.

Since the Rv3874 gene product elicits DTH responses specific for TB, we call this protein MTSA-10 (for *M. tuberculosis*-specific antigen 10) and the corresponding gene *mtsa-10*.

Genome analysis of the *mtsa-10* gene. The distribution of the *mtsa-10* gene in tuberculous and nontuberculous mycobacteria (listed in Table 1) was analyzed by Southern transfer hybridization. The presence of the *mtsa-10* gene was visualized as a single hybridization signal of approximately 4 kb (data not shown), indicating that the *mtsa-10* gene is present as a single copy in the mycobacterial chromosome. The *mtsa-10* gene was found in *M. tuberculosis* (two laboratory strains and two clinical isolates), in virulent *M. bovis*, and in *Mycobacterium africanum*. As expected, the gene was absent from all BCG substrains (Table 2). The *mtsa-10* gene was found in only 2 of 13 species of nontuberculous mycobacteria tested, suggesting a limited gene distribution. The *mtsa-10* gene was notably absent from *M. avium*, the most common human pathogen among nontuberculous mycobacteria (10). Among other nontuberculous mycobacteria causing human disease with any significant frequency (10), the *mtsa-10* gene was found in *Mycobacterium kansasii* but not in *Mycobacterium fortuitum* or *Mycobacterium scrofulaceum* (Table 2).

Since *mtsa-10* maps adjacent to *esat-6* and since the two genes are cotranscribed (5), we compared the distribution of the two genes among mycobacteria. The distribution of *mtsa-10* matched that of *esat-6* (Table 2). The results obtained with *esat-6* confirm and extend earlier studies with nontuberculous mycobacteria (12).

In conclusion, we show that the product of the *mtsa-10* gene of *M. tuberculosis* elicits a strong DTH response in guinea pigs infected with *M. tuberculosis* but not in animals immunized with *M. bovis* BCG or *M. avium*. The characterization of MTSA-10 as an *M. tuberculosis*-specific skin test antigen advances our pursuit of a new tuberculin that will allow discrimination by skin testing of latent *M. tuberculosis* infection from vaccination with BCG or sensitization with environmental, nontuberculous mycobacteria.

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