

Use of *Mycobacterium tuberculosis* Complex-Specific Antigen Cocktails for a Skin Test Specific for Tuberculosis

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The tuberculin skin test currently used to diagnose infection with *Mycobacterium tuberculosis* has poor diagnostic value, especially in geographic areas where the prevalence of tuberculosis is low or where the environmental burden of saprophytic, nontuberculous mycobacteria is high. Inaccuracy of the tuberculin skin test often reflects a low diagnostic specificity due to the presence in tuberculin of antigens shared by many mycobacterial species. Thus, a skin test specific for tuberculosis requires the development of new tuberculins consisting of antigens specific to *M. tuberculosis*. We have formulated cocktails of two to eight antigens of *M. tuberculosis* purified from recombinant *Escherichia coli*. Multiantigen cocktails were evaluated by skin testing guinea pigs sensitized with *M. bovis* BCG. Reactivity of multiantigen cocktails was greater than that of any single antigen. Cocktail activity increased with the number of antigens in the cocktail even when the same amount of total protein was used for cocktails and for each single antigen. A cocktail of four purified antigens specific for the *M. tuberculosis* complex elicited skin test responses only in BCG-immunized guinea pigs, not in control animals immunized with *M. avium*. These findings open the way to designing a multiantigen formulation for a skin test specific for tuberculosis.

Identification of individuals infected with *Mycobacterium tuberculosis*, who account for approximately one-third of the world's population (36), is of paramount importance for the control of tuberculosis (TB). TB control programs are usually established on the basis of the proportion of infected individuals in a given community (10). Moreover, infection with *M. tuberculosis* often constitutes an indication for prophylactic chemotherapy against TB, especially in individuals at risk of rapid progression to disease (6). The method currently used to detect infection with *M. tuberculosis*, the tuberculin skin test, is based on measuring delayed-type hypersensitivity (DTH) responses (local skin induration and erythema) to the intradermal injection of purified protein derivative (PPD) of tuberculin (reviewed in references 5 and 6). Unfortunately, the tuberculin skin test has low diagnostic specificity, because PPD contains antigens that are shared by many mycobacterial species (8, 11). Thus, a positive test result is not necessarily associated with *M. tuberculosis* infection but may also be caused by immune cross-reactions in individuals vaccinated with the bacille Calmette-Guérin (BCG) attenuated strain of *M. bovis* or sensitized with nontuberculous mycobacteria (5, 6). Cross-reactions greatly complicate interpretation of skin test results in subjects living in or originating from geographic areas with a high environmental load of nontuberculous mycobacteria (4–6). Thus, there is a need to develop new reagents that are specific for TB to overcome the limitations of the current tuberculin skin test.

Development of new TB-specific tuberculins requires identification of *M. tuberculosis*-specific antigens that elicit DTH responses in TB. Since measurement of DTH activity is usually part of the characterization of *M. tuberculosis* antigens, many

antigens active in DTH-based assays have been described (for a partial list, see references 15, 23, 26 to 28, and 38). However, efforts to identify a potent, species-specific antigen that could replace PPD for skin testing have been disappointing. For example, in a study performed with human volunteers (35), only few PPD-positive subjects responded to MPT64, an *M. tuberculosis* complex-specific antigen that elicits strong DTH responses in tuberculous guinea pigs (28). We argue that a single antigen, however potent, is bound to be inadequate for skin testing because (i) one antigen may contain too few epitopes to recruit to the site of antigen injection the number of DTH effector T cells necessary to obtain a response measurable by skin testing and (ii) antigen recognition in TB is broad and highly variable from individual to individual (21, 32). Thus, multiple antigens should be required to detect infection with *M. tuberculosis* by skin testing.

To evaluate cocktails of multiple antigens for skin testing, we chose antigens found in the filtrate of *M. tuberculosis* cell cultures because culture filtrate antigens are usually potent in DTH-based immunoassays (15, 23, 26, 27). Culture filtrate antigens were purified as recombinant proteins from *Escherichia coli* cells and tested in combination for DTH responses in guinea pigs sensitized with *M. bovis* BCG, an avirulent member of the *M. tuberculosis* complex. To assess specificity for the *M. tuberculosis* complex in skin test, multiantigen cocktails were also tested in control guinea pigs immunized with *M. avium*, a nontuberculous mycobacterial species commonly found in the environment. We report that (i) skin test activity of a cocktail is greater than that of any single antigen and increases with the number of antigens in the cocktail, even when the same amount of total protein is used for the cocktail and for each single antigen, and (ii) a cocktail of *M. tuberculosis* complex-specific antigens elicits DTH responses in BCG-immunized guinea pigs but not in *M. avium*-immunized animals. These findings indicate that the use of multiantigen cocktails should yield a new, specific skin test for TB.

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TABLE 1. Recombinant protein antigens of *M. tuberculosis* used in this study

Antigen	Molecular mass (kDa)	Reference
KatG	80	16
MPT32	45/47	18
38 kDa	38	1
Ag85B	30	25
MTC28	28	23
MPT51	27	29
MPT64	23	37
MPT70	22	34
19 kDa	19	3
MPT63	16	24

MATERIALS AND METHODS

Bacterial strains and products. *E. coli* strains were grown in standard liquid and solid media (31). *M. bovis* BCG Japanese ATCC 35737 and *M. avium* ATCC 25291 were obtained from the American Type Culture Collection. The Japanese strain of BCG was chosen because it produces at high levels the MPB64 antigen (19), whose *M. tuberculosis* homolog, MPT64, was used in this study. Mycobacteria were grown at 37°C in rotating bottles in 7H9 medium enriched with 0.05% Tween 80 and standard albumin-dextrose additive. PPD produced from *M. tuberculosis* (PPD-CT-68) was purchased from Connaught Laboratories Inc. (Swiftwater, Pa.). PPDs from *M. bovis* and from *M. avium* were purchased from Kursk Biofactory (Kursk, Russia).

Gene cloning and protein purification. Ten genes encoding *M. tuberculosis* culture filtrate proteins (Table 1) were cloned in the pQE30 (Qiagen) plasmid vector of *E. coli* as described previously (23, 24). Recombinant proteins were expressed as NH₂-terminally polyhistidine-tagged fusion proteins and purified to near homogeneity from *E. coli* cells by using a three-step protocol consisting of sequential chromatography with metal chelate affinity, size exclusion, and anion-exchange columns, as reported elsewhere (7).

Guinea pig sensitization. Groups of six female guinea pigs of the outbred strain Hsd:DH (Harlan Sprague Dawley) weighing 300 to 350 g were sensitized by intradermal injection in the abdomen with 10⁷ live *M. bovis* BCG Japanese or *M. avium* cells in 0.2 ml of phosphate-buffered saline (PBS), pH 7.2.

Skin tests. Five to eight weeks after sensitization, animals were shaved on the back and injected intradermally with 2 µg of each purified antigen in 0.1 ml of PBS or with 0.5 to 8 µg of multiantigen cocktails in 0.1 ml of PBS, as indicated. Each animal was also injected with 10 tuberculin units (TU) of PPD as a control for sensitization. Skin reactions (diameters of erythema, in millimeters) were independently measured 24 h after antigen injection by two investigators. Single purified antigens and antigen cocktails were tested in three or four separate experiments. Results were expressed as means of diameters of erythema ± standard deviations.

RESULTS

Skin test reactivity of recombinant antigens of *M. tuberculosis*. Ten purified recombinant proteins of *M. tuberculosis* (Table 1) were tested for tuberculin-like activity and specificity to the *M. tuberculosis* complex, using two groups of guinea pigs, one sensitized with *M. bovis* BCG and the other sensitized with the nontuberculous species *M. avium*. Measurement of skin reactions to PPDs from *M. bovis* and *M. avium* indicated that similar degrees of sensitization were obtained in the two groups of animals (Table 2), a prerequisite for interpretation of results obtained with purified antigens. All 10 purified antigens elicited DTH responses of similar intensities in the BCG-immunized group (Table 2). In contrast, DTH responses in the *M. avium*-immunized guinea pigs differed from antigen to antigen. Some were equally active in the BCG- and *M. avium*-immunized animals, while others displayed little, if any, reactivity in the *M. avium*-immunized group. The specificity index (SpI) (Table 2), which was obtained by dividing sizes of skin reactions in the BCG-immunized group by those obtained in the *M. avium*-immunized group, measured specificity of antigen for the *M. tuberculosis* complex. Six antigens were cross-reactive (SpI ~ 1 [Table 2]). The presence of homo-

TABLE 2. Guinea pig DTH responses to purified proteins of *M. tuberculosis*

Antigen	Diam (mm) of skin test reactions in guinea pigs immunized with ^a :		SpI ^b
	<i>M. bovis</i> BCG	<i>M. avium</i>	
Tuberculins			
PPD-t	8.9 ± 1.3	8.0 ± 1.4	1.11
PPD-b	12.1 ± 1.3	9.5 ± 1.5	1.27
PPD-a	8.0 ± 1.5	12.6 ± 1.4	0.63
Cross-reactive antigens			
19 kDa	7.0 ± 2.0	9.3 ± 1.6	0.75
MPT51	6.2 ± 0.5	5.0 ± 0.7	1.24
Ag85B	7.9 ± 2.1	8.9 ± 0.8	0.89
38 kDa	6.4 ± 0.9	5.0 ± 0.8	1.28
MPT32	7.2 ± 1.3	7.8 ± 1.7	0.92
KatG	7.4 ± 1.2	4.1 ± 0.9	1.80
Specific antigens			
MPT63	7.5 ± 1.6	0.5 ± 1.0	15.00
MPT70	7.6 ± 1.2	1.0 ± 1.2	7.60
MPT64	7.6 ± 1.0	0.5 ± 1.0	15.20
MTC28	6.6 ± 1.4	0.8 ± 1.5	8.25

^a Means ± standard deviations of diameters of erythema after intradermal injection of 2 µg of each purified antigen in six guinea pigs immunized with *M. bovis* BCG and six guinea pigs immunized with *M. avium*, as described in Materials and Methods; 10-TU doses of PPD from *M. tuberculosis* (PPD-t), *M. bovis* (PPD-b), and *M. avium* (PPD-a) were used as controls for sensitization.

^b Determined by dividing mean diameters of skin reactions measured in BCG-immunized guinea pigs by those in *M. avium*-immunized animals.

gous proteins in *M. avium* or the demonstration of shared T-cell epitopes has been reported for some of these antigens by us (23) and others (14, 17, 20, 30). Four antigens (MPT63, MPT64, MTC28, and MPT70) elicited DTH responses 8 to 15 times stronger in BCG- than in *M. avium*-immunized guinea pigs (Table 2). Specificity for tubercle bacilli in guinea pig tests has already been reported for three of these four antigens by us (MTC28) (23) and others (MPT64 and MPT70) (2, 12, 26). The quantitative assessment of skin test reactivity and specificity for the *M. tuberculosis* complex obtained for each antigen in this set of experiments provided baseline values to evaluate multiantigen cocktails.

Activity of multiantigen cocktails. We next formulated multiantigen cocktails and compared the activity of cocktails with that of each antigen in the cocktails by skin testing BCG-immunized guinea pigs. In this set of experiments, we used six antigens (MPT63, MPT64, MTC28, MPT32, MPT51, and 38 kDa) to formulate cocktails containing two, four, and six antigens. Single antigens and antigen cocktails were all tested at the same amount (2 µg) of total protein. Skin test reactivity of the cocktails was greater than that of any single antigen and increased with the number of antigens in the cocktail (Fig. 1), even though the amount of each antigen in the cocktail decreased. This finding suggests an additive, perhaps even cooperative, effect of multiple T-cell epitopes on the development of DTH responses elicited by antigen cocktails.

Specificity of multiantigen cocktails. We next set out to assess skin test specificity of multiantigen cocktails for tuberculous mycobacteria. We formulated three cocktails. Cocktail A contained four *M. tuberculosis* complex-specific antigens (MPT63, MPT64, MTC28, and MPT70) (Table 2), cocktail B contained four cross-reactive antigens (MPT51, MPT32, Ag85B, and KatG) (Table 2), and cocktail C contained all eight

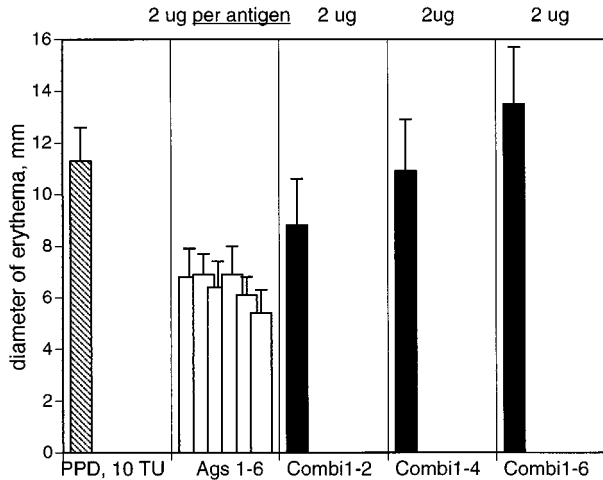


FIG. 1. Skin test reactivity of purified recombinant antigens of *M. tuberculosis* tested singly and in combinations in guinea pigs immunized with *M. bovis* BCG. Six guinea pigs were sensitized as described in Materials and Methods. Five weeks after sensitization, animals were intradermally injected with 10 TU of PPD and 2 μ g of purified recombinant antigens, singly or in combination. Results are expressed as the means (plus standard deviations) of the diameters of erythema measured 24 h after antigen injection. Ags 1-6, six antigens injected singly (1, MPT63; 2, MPT64; 3, MTC28; 4, MPT32; 5, MPT51; 6, 38 kDa); Combi1-2, two-antigen cocktail (antigens 1 and 2); Combi1-4, four-antigen cocktail (antigens 1 through 4); Combi1-6, six-antigen cocktail (antigens 1 through 6).

antigens present in cocktails A and B. Each of the three multiantigen cocktails was evaluated at different concentrations (from 0.5 to 8 μ g) by skin testing guinea pigs sensitized with BCG and with *M. avium*. Both animal groups responded to PPD from *M. bovis* and PPD from *M. avium* with similar-size skin reactions (Fig. 2A). In sharp contrast, BCG-immunized, but not *M. avium*-immunized, animals mounted DTH responses to the specific cocktail A (Fig. 2B). Both groups of animals gave DTH responses similar in strength to that of the cross-reactive cocktail B (Fig. 2C). Cocktail C, a mixture of specific plus cross-reactive antigens, was of intermediate specificity, as it elicited slightly stronger responses in the BCG-immunized animals than in the group immunized with *M.*

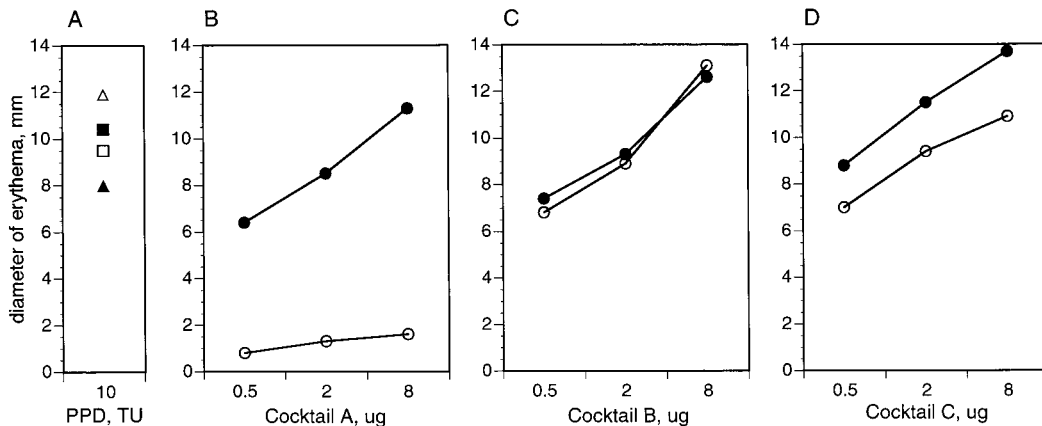


FIG. 2. Skin test reactivity of multiantigen cocktails in guinea pigs immunized with *M. bovis* BCG (solid symbols) and with *M. avium* (open symbols). Six guinea pigs were sensitized and skin tested as described in Materials and Methods. In this set of experiments, animals were skin tested with 10 TU of PPD and increasing amounts (0.5 to 8 μ g) of multiantigen cocktails. Results are expressed as means of the diameters of erythema measured 24 h after antigen injection. Cocktail A, four *M. tuberculosis* complex-specific antigens (MPT63, MPT64, MTC28, and MPT70); cocktail B, four cross-reactive antigens (MPT51, Ag85B, MPT32, and KatG); cocktail C, eight-antigen cocktail (antigens in cocktails A plus B). ■, □, *M. bovis* PPD; ▲, △, *M. avium* PPD; ●, ○, cocktails of purified antigens.

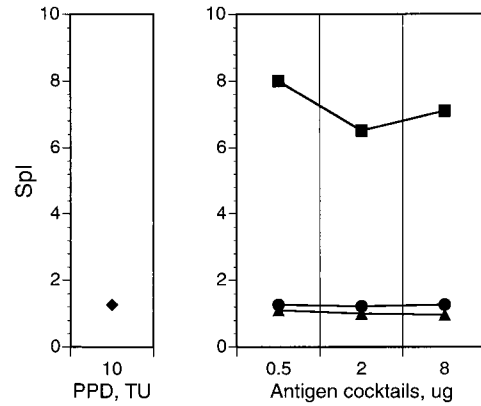


FIG. 3. Specificity for tuberculous mycobacteria of multiantigen cocktails tested at different doses. Protocols of guinea pig sensitization and skin testing with PPD and multiantigen cocktails were as described in the legend to Fig. 2. Spi was calculated as described in Table 2, footnote b. ◆, PPD; ■, cocktail A (*M. tuberculosis* complex specific); ▲, cocktail B (cross-reactive); ●, cocktail C (antigens in cocktails A plus B).

avium (Fig. 2D). Thus, the specificity of the antigen cocktail is that of its components.

Results shown in Fig. 2 also indicated that skin reactivity of cocktails was dose dependent, with cocktails used in the range of 2 to 8 μ g of total protein eliciting skin reactions similar in size to those elicited by 10 TU of PPD (compare Fig. 2B to D to Fig. 2A). In contrast, cocktail specificity for the *M. tuberculosis* complex was dose independent (Fig. 3). This property is important, because high doses of the immunoreagent may be required in skin tests for accurate discrimination between *M. tuberculosis*-infected and noninfected individuals.

DISCUSSION

The findings described in this report establish that (i) cocktails of purified antigens of *M. tuberculosis* are significantly more active in skin tests than any of the single antigens in the cocktail and (ii) a cocktail retains the specificity to tubercle bacilli in the cocktail. Involvement of many antigens in DTH responses to infection with tubercle bacilli

(all 10 antigens tested in the present study [Table 2]), together with the above-described properties in skin tests of multiantigen cocktails, provide a basis for the rational design of a skin test specific for TB that uses cocktails of purified, *M. tuberculosis* complex-specific antigens.

A requirement for multiple, rather than single, purified antigens as skin test reagents can be due to several factors. First, multiantigen formulations can presumably recruit many antigen-specific T cells to the site of antigen injection to afford a skin reaction of the appropriate size. Second, numerous antigens may be required to overcome problems related to genetic restriction in antigen recognition (9) that causes some individuals to react to certain antigens and not to others.

Formulations of purified antigens offer several advantages over PPD. First, PPD is a highly cross-reactive antigen that does not always allow distinction between tuberculous infection, infection with nonpathogenic mycobacteria, and vaccination with BCG. The present study indicates that use of *M. tuberculosis* complex-specific antigens can yield a skin test that discriminates between tuberculous infection and infection with *M. avium*, a nonpathogenic mycobacterial species commonly found in the environment. Further experimentation may be needed to evaluate skin test specificity of cocktails containing *M. tuberculosis* complex-specific antigens vis-à-vis sensitization with additional nontuberculous mycobacteria. Similar principles can also be applied to the design of multiantigen cocktails that may possibly discriminate between BCG vaccination and infection with virulent tuberculous mycobacteria by selecting antigens, such as ESAT-6 (33) and MPT64, that are produced by virulent mycobacteria but are absent in all (ESAT-6) or some (MPB64) BCG substrains (13, 19, 22). A second advantage is that the use of purified recombinant antigens should facilitate manufacturing and quality control of skin test reagents.

Antigens of *M. tuberculosis* should be purified as recombinant proteins, since the large-scale requirement for skin test reagents (many million doses are used each year worldwide) is incompatible with purification of native protein from *M. tuberculosis* cells. Recombinant proteins purified from *E. coli* should be suitable reagents for diagnostic skin testing, since several recombinant antigens were found indistinguishable in guinea pig skin tests vis-à-vis the corresponding native proteins by us (MPB70, MPT63, Ag85B, and MPT51) (our unpublished observations) and others (MPT64) (28). Should optimal activity of certain antigens require posttranslational modification of protein, which might occur in *M. tuberculosis* but not in *E. coli*, alternative recombinant DNA techniques in fast-growing, nonpathogenic mycobacteria could be adopted.

The present study strongly suggests that a multiantigen cocktail should be more effective than PPD or single antigens as a reagent for TB skin testing. The choice of antigens to formulate optimal immunodiagnostic cocktails for human use will have to be guided by ex vivo and in vivo human studies.

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