Reactivity of Purified Proteins and Polysaccharides from *Mycobacterium tuberculosis* in Delayed Skin Test and Cultured Lymphocyte Mitogenesis Assays

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Hypersensitivity responses to two highly purified and well characterized mycobacterial proteins and a preparation of mycobacterial arabinogalactan-arabinomannan were studied by using guinea pig skin tests and in vitro-cultured human lymphocyte mitogenesis stimulation assays. The purified proteins were found to elicit delayed-type hypersensitivity skin test reactions and to stimulate mitogenesis in cultured lymphocytes. The polysaccharide preparation was found to evoke delayed skin tests but not to stimulate cultured lymphocytes.

In vitro studies of delayed hypersensitivity have been stimulated in recent years by the recognition that antigen-mediated lymphocyte mitogenesis is a manifestation of delayed hypersensitivity in the cell donor. The application of this methodology to detailed study of tuberculin hypersensitivity has been limited by the fact that most available antigens, including purified protein derivative (PPD) and old tuberculin (OT), are mixtures of many antigens. This report concerns data obtained using purified mycobacterial proteins and polysaccharides of defined antigenic identities. Purified protein antigens were found to elicit delayed skin test reactions and to stimulate lymphocyte mitogenesis. Purified polysaccharide antigens were found to elicit delayed skin test reactions but not to stimulate lymphocyte mitogenesis.

**MATERIALS AND METHODS**

**Antigenic proteins.** Purified a₁ and a₂ proteins, free of detectable polysaccharide, were prepared as described previously (3). The preparations gave single bands in 7.5% polyacrylamide gel disc electrophoresis. Protein a₁ did not react in immunoelectrophoresis with the U.S.-Japan antiserum or with other available antisera. Protein a₂ gave a single line when reacted against U.S.-Japan reference antiserum 001 (11). By use of the Osserman modification of immunoelectrophoresis (17), protein a₂ was shown to identify immunologically with antigen number 6 of the U.S.-Japan nomenclature.

**Antigenic polysaccharide.** Purified arabinogalactan-arabinomannan (AGAM) was prepared as follows. As described previously, culture filtrates of *Mycobacterium tuberculosis* H₃₇Ra were concentrated, passed through a Biogel P-30 polyacrylamide gel column to remove low-molecular-weight substances, and brought to 50% saturation with ammonium sulfate (3). The ammonium sulfate supernatant was dialyzed against 0.01 M sodium phosphate buffer at pH 8.0 and concentrated by vacuum dialysis against the same buffer. It was then subjected to ion-exchange chromatography on a diethylaminoethyl (DEAE)-cellulose column (2.0 by 35 cm). Elution was carried out with 0.01 M sodium phosphate buffer at pH 8.0 with recovery of a single "drop-through" peak. By immunoelectrophoresis, this preparation was found to contain only antigens 1 and 2 of the U.S.-Japan nomenclature (11). The work of Azuma and his collaborators has shown antigens 1 and 2 to be arabinogalactan and arabinomannan, respectively (I. Azuma, F. Kanetsuna, Y. Yamamura, and A. Misaki, Presentation before the Mycobacterial Antigen Workshop, U.S.-Japan Cooperative Medical Sciences Program, San Francisco, 1971). AGAM thus prepared was found to have a ratio of spectrophotometric absorption at 260 nm to that at 280 nm of 4.8. It had polysaccharide concentrations of 0.2 mg of pentoses per ml measured with a guanosine standard and of 0.08 mg of hexoses per ml measured with an anthrone standard (5, 13). Protein, measured by the method of Lowry with a serum albumin standard, was 0.03 mg/ml (14). Analysis by analytic gel filtration was carried out for molecular weight estimation as previously described (3). The AGAM preparation was found to have an average molecular weight of 8,000 by this technique. Because of the branched structure of mycobacterial arabinogalactan and arabinomannan (Azuma et al., presentation), the actual molecular weight was probably somewhat higher.

**Skin testing.** Protein and polysaccharide antigen preparations were tested for skin test antigenicity in groups of six to ten sensitized, random-bred, adult male guinea pigs obtained from a local dealer. Animals were sensitized to mycobacterial antigens by subcutaneous injection at the nape of the neck with an emulsion of 1 ml of complete Freund adjuvant containing *M. tuberculosis* H₃₇Ra, 10 mg/ml (Difco
Laboratories), and 0.5 ml of a semipurified preparation containing a1 and a2 proteins. The a1 and a2 were added to the adjuvant because the mycobacteria in Freund adjuvant are heat-killed and a1 and a2 have been shown to be unstable at temperatures over 60°C (L. E. Ferguson and T. M. Daniel, Abstr. Amer. Rev. Rep. Dis., 1970, 101:1001). Mycobacterial polysaccharides are heat stable and need not be added to the sensitizing emulsion. Skin tests were performed by the injection of 0.1-ml volumes of antigen intradermally on the shaved flank. The diameter of induration was measured at 4, 24, and 48 h. National Institutes of Health PPD lot 5894-04 was used as a standard in skin testing animals. Commercial PPD (Parke, Davis & Co.), intermediate strength, was used in selecting donors for lymphocyte blast-cell transformation studies.

Stimulation of lymphocyte cultures. Antigen-mediated mitogenesis of cultured human lymphocytes was measured by thymidine incorporation using methods previously described (8).

RESULTS

Skin tests. Both protein preparations and the polysaccharide preparation elicited delayed-type hypersensitivity skin test reactions in sensitized animals. The evolution of the reactions to the purified antigens followed the same time course as those to PPD, suggesting they were true delayed hypersensitivity reactions. Mean skin test reaction sizes are given in Table 1. In Fig. 1, a semilogarithmic plot of the skin testing data is presented which allows comparison of the relative potency of the three antigens.

Lymphocyte mitogenesis. Results of in vitro lymphocyte mitogenesis studies of cells from tuberculin-positive and tuberculin-negative donors are presented in Fig. 2 and 3. The lymphocytes of all skin test-positive donors consistently responded to PPD by a marked increase in thymidine incorporation after 5 days of culture in vitro. The cells of two tuberculin-positive donors (Ge and Le) responded to both protein antigens a1 and a2. The cells of one (Bo) did not respond to either protein. The cells of three tuberculin-negative control subjects did not respond to PPD and did not respond to proteins a1 and a2. AGAM did not stimulate mitogenesis in the cultured lymphocytes of two PPD-positive and two PPD-negative subjects when used at two dose levels. By extrapolation from the skin testing results, the doses of AGAM used in

<p>| Table 1. Mean skin test diameters in sensitized guinea pigs |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Amt of antigen (mg)</th>
<th>Mean reaction size (mm)</th>
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</thead>
<tbody>
<tr>
<td>a2</td>
<td>0.02</td>
<td>10.0</td>
</tr>
<tr>
<td>a1</td>
<td>0.02</td>
<td>15.5</td>
</tr>
<tr>
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<tr>
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<tr>
<td>PPD</td>
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<tr>
<td>PPD</td>
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<td>9.8</td>
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</table>

![Fig. 1. Delayed-type hypersensitivity skin test dose response curve to PPD. Mean reaction sizes of reactions to PPD (solid dots) are plotted. Mean reaction sizes to purified antigens are shown on the PPD curve (open dots) allowing estimation of their relative potencies. Stated AGAM doses are total pentoses; a1 and a2 doses are total protein.](http://iai.asm.org/)

![Fig. 2. Purified protein antigen-induced mitogenesis of cultured lymphocytes. Thymidine incorporation is shown separately for the lymphocytes of each subject in control cultures (C), when stimulated by PPD (1 μg) (P), when stimulated by protein a1 (2.5 mg) (a1), and when stimulated by protein a2 (2.5 μg) (a2).](http://iai.asm.org/)
vitro were sufficiently large to provide an adequate stimulus.

**DISCUSSION**

PPD has been widely used in studies of mitogenesis in cultured lymphocytes, and the response of these cells to PPD correlates well with delayed skin test reactivity to the same antigen (7, 9, 12, 16, 18). Highly purified protein and polysaccharide antigens derived from mycobacteria have not been extensively studied, and available information suggests differences in hypersensitivity responses to proteins and polysaccharides.

**Responses to protein antigens.** Highly purified proteins of mycobacterial origin have been found to elicit positive skin test reactions (1, 3, 4, 22; L. F. Affronti, L. Grow, O. Ouchterlony, A. Lind, and M. Norlin, Abstr. Amer. Rev. Resp. Dis., 1971, 103:893; R. G. Moulton, T. M. Dietz, and S. Marcus, Abstr. Amer. Rev. Resp. Dis., 1971, 103:894). Our prior failure to demonstrate skin test antigenicity for protein \(a\) probably relates to the use of animals sensitized exclusively by the injection of bacilli which were heat killed, a treatment which probably denatures this protein (Ferguson and Daniel, Abstr. Amer. Rev. Resp. Dis., 1970, 101:1001). A purified mycobacterial protein has been demonstrated by Janicki and colleagues to stimulate blastogenesis of cultured lymphocytes from tuberculosis-positive donors (10). The protein preparation they used was demonstrated by immunoelectrophoresis to contain several antigens, probably including \(a_1\) protein. It is of interest that the lymphocytes of one of three PPD-positive donors studied did not react to \(a_1\) or \(a_2\) proteins. Presumably this patient was not sensitive to these particular antigens, although his cells did react to PPD.

**Responses to polysaccharide antigens.** Reported experience with purified mycobacterial polysaccharides examined for ability to elicit hypersensitivity reactions has been variable. The polysaccharides isolated chemically by Seibert did not evoke skin test reactions in tuberculin-positive human subjects (15, 20, 21). Chaparas and co-workers tested four tuberculin fractions in guinea pigs. The most highly purified polysaccharide fractions did not elicit skin test responses, whereas those with significant nitrogen content did (2). Root and collaborators subsequently tested one of the polysaccharide fractions which Chaparas had found to react in guinea pigs. In tuberculin-positive human subjects and patients with tuberculosis, no delayed cutaneous reactions could be elicited, suggesting an important species difference (19). Yamamura and his colleagues found that chemically purified arabinogalactan and arabinomannan did not elicit delayed skin test reactions in either patients with tuberculosis or sensitized guinea pigs (23). It has been suggested on the basis of these studies that only those polysaccharides containing protein moieties are capable of serving as skin test antigens (15, 19). However, Grappel demonstrated that a synthetic polysaccharide completely devoid of nitrogen was capable of eliciting delayed skin reactivity in sensitized guinea pigs (6). The AGAM polysaccharide preparation tested in the present study was effective in eliciting delayed-type hypersensitivity skin test reactions. It contained a small amount of protein which could have been responsible for this.

Stimulation of mitogenesis of lymphocytes from approximately one-third of tuberculosis patients was observed by Heilman and McFarland using a preparation of polysaccharide I obtained from Seibert which had a protein content similar to that of the AGAM used in this study (7). Chaparas and co-workers tested the four polysaccharide preparations referred to above with guinea pig lymphocytes and concluded that, as with the elicitation of delayed skin reactivity, transformation could be induced only with antigens containing significant protein or peptide moieties (2). Janicki and collaborators stimulated cultured lymphocytes with an electrophoretically purified polysaccharide.
ride preparation (10). Their preparation was studied immunoelectrophoretically with the same reference antiserum used in this study and likewise found to contain only antigens 1 and 2 of the U.S.-Japan nomenclature. They found essentially no lymphocyte-stimulating activity in their material. Delayed skin test studies were not included in their report. Despite its protein content, the AGAM used in this study, although a good elicitor of skin test reactions, did not stimulate mitogenesis in cultured lymphocytes.

The data presented in this study confirm the ability of mycobacterial protein antigens to elicit delayed-type hypersensitivity skin test reactions and to stimulate mitogenesis in cultured lymphocytes from hypersensitive donors using a protein preparation more highly purified than previously reported. They also support prior evidence for a dichotomy between dermal reactivity and in vitro mitogenesis with polysaccharide mycobacterial antigens.

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LITERATURE CITED


