Isolation, Characterization, and Biological Properties of a Tuberculin-Active Peptidoglycan Isolated from the Culture Filtrate of Mycobacterium tuberculosis

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A water-soluble tuberculin-active peptidoglycan (TAPG) with a molecular weight of ca. 28,000 to 30,000 was isolated from the culture filtrate of Mycobacterium tuberculosis. TAPG was approximately four to five times more potent than tuberculin purified protein derivative S in guinea pigs sensitized with M. tuberculosis or M. bovis (freeze-dried BCG). It showed little or no cross-reactivity at a dose of 0.1 to 0.4 μg in guinea pigs sensitized with M. kansasii, M. scrofulaceum, M. intracellulare, or M. avium. TAPG did not show any adjuvant activity when injected in guinea pigs in a water-in-oil emulsion containing ovalbumin.

TAPG, in Freund incomplete adjuvant, proved to be an effective immunogen for inducing delayed hypersensitivity in guinea pigs. Chemical analysis of TAPG showed that it contains proline, glutamic acid, alanine, diaminopimelic acid, tyrosine, threonine, glucosamine, and the reducing sugars, arabinose and galactose. In immunoelectrophoretic studies with reference M. tuberculosis H37Rv antiserum, TAPG did not show any precipitin bands.

Tuberculin purified protein derivative (PPD) is capable of eliciting a delayed hypersensitivity reaction in humans and animals infected with Mycobacterium tuberculosis, but, due to its lack of specificity, it also elicits delayed hypersensitivity cross-reactions in humans and animals infected with mycobacteria other than M. tuberculosis or M. bovis. These cross-reactions obscure the interpretation and limit the significance of tuberculin skin testing as an aid to the diagnosis of tuberculosis (8). Mycobacterial antigens that contain few or no cross-reacting components are therefore needed to differentiate between tuberculous and nontuberculous infections.

Although we have previously reported (10) on the isolation of "specific" tuberculin peptides from PPD, the need for specific antigens with tuberculin activity is still great. The present studies were, therefore, undertaken to find out whether specific antigens could be isolated from the culture filtrate of M. tuberculosis after its PPD contents had been removed.

MATERIALS AND METHODS

Isolation of a tuberculin-active peptidoglycan (TAPG) from the culture filtrate of M. tuberculosis. The outline of the method used for the isolation of a peptidoglycan fraction from the culture filtrate of M. tuberculosis Johnston is shown in a flow diagram (Fig. 1).

Precipitation of PPD. From the culture filtrate of M. tuberculosis, tuberculin PPD was precipitated by employing the method described by Landi (15). Essentially, the method consists of growing M. tuberculosis Johnston on the synthetic medium of Long and Seibert (17) for 6 weeks at 37°C. The culture is then steamed at 100°C for 3 h and filtered through a Berkfeld candle. Tuberculin PPD is precipitated from the culture filtrate by trichloroacetic acid, at a final concentration of 4%, and separated by centrifugation.

PTA precipitate. To the trichloroacetic acid supernatant, phosphotungstic acid (PTA) was added to give a 0.5% final concentration, resulting in a heavy white precipitate, which was sedimented by centrifugation at 17,300 × g for 30 min. A saturated solution of barium hydroxide was added slowly to the precipitate and stirred until the pH of the suspension was 7.2. After centrifugation at 30,900 × g for 30 min, the supernatant was collected and neutralized with 0.1 N H₂SO₄. The acid precipitate was removed by centrifugation and then discarded. The addition of 10 volumes of acetone to the supernatant resulted in the formation of a white precipitate, which was washed twice with acetone, dried in vacuo, and designated "PTA precipitate."

Fractionation of PTA precipitate by ion-exchange chromatography on Dowex 50W-X8. The ion-exchange chromatographic method using volatile buffers was chosen for the fractionation of the PTA precipitate. Dowex 50W-X8 resin was prepared by the method of Moore and Stein (20). Six hundred milligrams of PTA precipitate was dissolved in 5 ml of 0.2 M pyridine-acetic acid buffer (pH 3.1). The sample was carefully layered on the top of the resin bed. The column (100 by 2.5 cm) was connected to a gradient vessel. The mixer and the reservoir contained 500 ml each of 0.2 M pyridine-acetic acid (pH 3.1) and 2 M pyridine-acetic acid buffer (pH 5), respectively. The
flow rate was adjusted to 40 ml/h, and 5-ml volumes were collected and monitored by ninhydrin analysis after alkaline hydrolysis. For each peak the contents of the tubes showing a positive ninhydrin reaction were pooled. The pooled fractions were designated A1, A2, A3, and A4, and were dried at 45 to 50°C in a rotary flash evaporator.

Chromatography of fraction A1 on Sephadex G-25. Fraction A1 from Dowex 50W-X8 column was fractionated on a column (2.5 by 100 cm) of Sephadex G-25 at room temperature. The elution process was carried out by using 0.02 M ammonium bicarbonate buffer with the buffer flowing upward through the column at a rate of 25 ml/h. The eluate was collected in 5-ml volumes and analyzed for ultraviolet absorbancy at a wavelength of 280 nm. The resulting fractions, S25-1 and S25-2, were dried at 45 to 50°C in a rotary flash evaporator.

Chromatography of fraction S25-1 on Sephadex G-50. Fraction S25-1, which showed tuberculin activity in sensitized guinea pigs, was rechromatographed on Sephadex G-50 by using 0.02 M ammonium bicarbonate as eluant. The eluate was analyzed for ultraviolet absorbancy at 210 nm. Two fractions, G50-1 and G50-2, were obtained, of which only fraction G50-1 showed tuberculin activity and was designated TAPG.

Immunoelectrophoretic analysis of TAPG. Immunoelectrophoresis was carried out as described by Janicki et al. (13). Reference H37Rv culture filtrate antigen lot no. 602 CF and anti-H37Rv antisera were obtained through the United States-Japan Cooperative Medical Science Program from the Geographic Medicine Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., and PPD was supplied by Connaught Laboratories Ltd. Six milliliters of buffer agar (pH 8.6) containing 0.55 g of barbital, 3.50 g of sodium barbitol, 7.5 g of Ionagar no. 2, and 0.1% of sodium azide per liter of distilled water was spread on the surface of glass slides (75 by 50 mm). Five microliters of the appropriate antigens at 25 mg/ml was placed in each well, and electrophoresis was carried out at room temperature for 90 min by using a constant current of 3 mA per slide.

After electrophoretic separation, 0.1 ml of the reference antiserum or 0.1 ml of a 2.5-mg/ml solution of concanavalin A was added to each trough, and the slides were incubated in a water-saturated atmosphere at room temperature and examined daily, for a period of 4 to 7 days.

Analytical methods. TAPG was hydrolyzed for 18 h at 100°C in 6 N HCl, and the amino acid composition was determined with a Beckman 121 amino acid analyzer.

Neutral sugars were identified by paper chromatography on Whatman no. 1 filter paper in N-butanol-pyridine-water (6:3.4, vol/vol/vol), after hydrolysis in 2 N HCl at 100°C. Amino sugars were identified by paper chromatography after hydrolysis for 6 h at 100°C in 4 N HCl.

Molecular weight determination. The molecular weight of TAPG was determined with Sephadex G-75 chromatography (3). The proteins used as reference standards were trypsin, chymotrypsinogen, pepsin, and pepsinogen.

Sensitization of guinea pigs. A sensitizing emulsion was prepared by mixing equal parts of a saline suspension of appropriate heat-killed mycobacteria such as M. kanssasi, M. avium, or M. intracellulare, M. avium, or M. tuberculosis Johnstone with 85% Bayol F-15% Arlacel (vol/vol). Guinea pigs (Connaught) weighing ca. 300 to 350 g were injected intracutaneously with this emulsion in two simultaneous doses of 0.1 ml each into the shaved skin of each animal over the lumbar spine. The total sensitizing dose was 0.05 mg (dry weight). For sensitization of guinea pigs with M. bovis BCG, the animals were injected intracutaneously with two simultaneous doses of 0.1 ml of a buffered saline suspension of freeze-dried BCG lot 293-1 with a viable count 1.48 x 10^7 cells per ml of reconstituted vaccine. The total sensitizing dose contained 0.2 mg of BCG (wet weight).

Guinea pigs were also sensitized by subcutaneous injection of Freund incomplete adjuvant (FIA) containing 0.2 mg of TAPG or PPD. Control animals were injected with 0.2 mg of TAPG or PPD in normal saline.

Skin testing. The guinea pigs were closely clipped and depilated with Neet (Whitehall Laboratories Ltd., Toronto, Ontario). After the back and sides before the intracutaneous tests were done. Four animals were used for each test. The skin test antigens used were: TAPG, PPD-S (distributed by the Bureau of Biologics, Food and Drug Administration, Bethesda, Md.), in phosphate-buffered solution containing 1 mg of PPD-S per ml and 0.5% [wt/vol] phenol as a preservative, PPD-A (International Standard of tuberculin PPD of M. avium origin supplied by the International Laboratory for Biological Standardization, Statens Serum Institute, Copenhagen) (M. avium origin), PPD-Y (M. kansasii origin), PPD-G (M. scrofulaceum origin), and PPD-B (M. intracellulare origin) (PPD-Y, PPD-G, and PPD-B supplied by Connaught Laboratories; prepared by the trichloroacetic acid precipitation method).

Two dosages of 0.1 and 0.4 µg (dry weight), or as otherwise specified, of the material on test and of the homologous or heterologous PPD antigens were assigned in duplicate in a random fashion on eight positions on each animal. A 0.1-ml dose was injected intracutaneously with disposable plastic syringes. At 24 h after injection, the size of each skin reaction (erythema) was measured with a ruler, and the results were recorded as the sum of the longitudinal and transverse diameters in millimeters. The estimate of potency was made by the method of Long et al. (16).

Adjuvant activity of TAPG. Guinea pigs, weighing ca. 300 to 350 g, were injected in the hind footpad with 0.2 ml of water-in-oil emulsion made up of equal parts of a 50-mg/ml saline solution of ovalbumin (5x crystalized, Calbiochem) and of 2 mg of TAPG per ml in FIA consisting of 85% Bayol-15% Arlacel A.

The control guinea pigs were injected either with 0.2 ml of a water-in-oil emulsion made up of 0.5 ml of FIA and 0.5 ml of 50 mg of ovalbumin per ml or with a saline solution containing 25 mg of ovalbumin per ml.

After 6 weeks, the guinea pigs were tested for delayed hypersensitivity by injecting 0.1 ml of a 100-µg/ml solution of ovalbumin. The method used for testing
delayed hypersensitivity to ovalbumin was similar to that already described for testing TAPG tuberculin activity.

To study the effect of adjuvants on antibody response, we bled the guinea pigs and titrated the antibodies by enzyme-linked immunosorbent assay (ELISA) as described by Engvall and Perlmann (9).

RESULTS

Ion-exchange chromatography of PTA precipitate. The PTA precipitate was fractionated on a Dowex 50W-X8 column (Fig. 1). The chromatographic elution pattern of PTA precipitate is represented in Fig. 2. The PTA precipitate can be resolved into four major fractions designated A1, A2, A3, and A4 (Fig. 2). These fractions were tested for their biological activity, and only fraction A1 showed tuberculin activity in guinea pigs sensitized with BCG or M. tuberculosis.

Sephadex gel chromatography. Fraction A1 was chromatographed on Sephadex G-25 column, and three fractions were obtained (Fig. 3). Only the first fraction, S25-1, which was eluted with the void volume of the column, showed tuberculin activity in guinea pigs sensitized with BCG or M. tuberculosis.

Further purification of fraction S25-1 on Sephadex G-50 was carried out, and only the first fraction (TAPG) was found to be tuberculin active (Fig. 4).

Biological activity of TAPG: tuberculin activity. The results of testing for tuberculin activity of TAPG in guinea pigs sensitized with M. tuberculosis or M. bovis BCG are shown in Table 1. In guinea pigs sensitized with heat-killed BCG, the potency of TAPG was comparable to PPD-S, whereas in guinea pigs sensitized with freeze-dried BCG or M. tuberculosis, TAPG was about four to five times more potent than PPD-S (Table 1). TAPG did not elicit any skin reactions in normal guinea pigs.

Table 2 shows the results of tuberculin activity of TAPG in guinea pigs sensitized with M. kansasii, M. scrofulaceum, M. intracellulare, or M. avium. Whereas PPD-Y, PPD-B, PPD-G, and PPD-A showed a strong tuberculin reaction in homologously sensitized guinea pigs at a dose of 0.1 µg, TAPG showed little or no tuberculin reaction at doses of 0.1 and 0.4 µg (Table 2).

Induction of delayed hypersensitivity to TAPG or PPD. Guinea pigs were sensitized with 200 µg of TAPG or PPD in FIA. Animals were skin tested 6 weeks later. The guinea pigs sensitized with TAPG in FIA gave a skin reac-
tion response of 38 and 33.5 mm with 2 µg and 0.4 µg of TAPG, respectively (Table 3). This delayed response was also elicited by PPD, but the reactions were smaller than those from equivalent doses of TAPG. The guinea pigs sensitized with PPD in FIA show a reaction size of 19.6 mm to 14.3 with 2 µg to 0.4 of PPD and a reaction size of 14.0 to 8.3 mm with the same dosages of TAPG. The guinea pigs sensitized with TAPG or PPD in saline showed little or no skin reaction.

Induction of antibody response by PPD and TAPG. Guinea pigs immunized with PPD in FIA and with TAPG in FIA were bled, and the sera were tested for the production of antibodies.

When the sera were tested by immunoelectrophoresis by using PPD and TAPG as antigens, no precipitin bands were detected. ELISA showed the presence of a low level of antibody titer (10 ELISA units) in sera of guinea pigs sensitized to TAPG in FIA compared to >100 ELISA units detected in the sera of guinea pigs sensitized to PPD in FIA. Guinea pigs injected with PPD and TAPG in saline did not show the production of antibodies either by immunoelectrophoresis or by ELISA.

Adjuvant activity: delayed hypersensitivity response to ovalbumin. Studies were carried out to determine whether TAPG had any effect on the delayed hypersensitivity response to ovalbumin. The average skin reaction responses to 10 µg of ovalbumin in guinea pigs sensitized with ovalbumin and FIA or with ovalbumin and FIA and TAPG were only slightly different, indicating that TAPG does not have an adjuvant activity on the immune response to ovalbumin in FIA (Table 4).

Adjuvant effect on antibody response to ovalbumin. Studies were carried out to determine whether TAPG had any effect on antibody...
GUPTA AND LANDI

**TABLE 3. Induction of delayed hypersensitivity response in guinea pigs sensitized by TAPG or PPD in FIA**

<table>
<thead>
<tr>
<th>Guinea pig sensitization</th>
<th>Skin reaction (mm, diam)*</th>
<th>TAPG</th>
<th>PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAPG + FIA</td>
<td>38.0</td>
<td>33.5</td>
<td>18.2</td>
</tr>
<tr>
<td>PPD + FIA</td>
<td>14.0</td>
<td>8.3</td>
<td>19.6</td>
</tr>
<tr>
<td>TAPG + saline</td>
<td>9.0</td>
<td>6.2</td>
<td>4.2</td>
</tr>
<tr>
<td>PPD + saline</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Average of four readings in four guinea pigs of the sum of the greater and lesser diameters of erythema.

**TABLE 4. Adjuvant effect of TAPG on immune response of guinea pigs to ovalbumin in FIA**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Delayed hypersensitivity reaction* to 10 μg of ovalbumin (mm, diam)</th>
<th>Antibodies against ovalbumin (ELISA units ×10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin + FIA</td>
<td>19.3</td>
<td>1</td>
</tr>
<tr>
<td>Ovalbumin + FIA +</td>
<td>20.3</td>
<td>1</td>
</tr>
<tr>
<td>TAPG</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin in saline</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Average of three readings in three guinea pigs of the sum of greater and lesser diameters of erythema.

**DISCUSSION**

From the culture filtrate of *M. tuberculosis* TAPG was isolated which showed high tuberculin activity in guinea pigs sensitized with *M. tuberculosis* or *M. bovis* BCG and little or no cross-reactivity in guinea pigs sensitized with various other mycobacteria. The presence of diaminopimelic acid indicates that it may be a constituent of the cell wall and may have been liberated by autolysis during the 6-week cultivation period or released from the organism during steaming for 3 h.

In recent years, a number of workers (Adam et al. [1, 2]; Hiu [11]; Migliore-Samour and Jolles [18, 19]; Stewart-Tull et al. [24]) have reported the isolation of water-soluble peptidoglycans, with adjuvant activity, from the cell walls and culture filtrates of mycobacteria. Although the chemical composition and biological activity of these peptidoglycans seem to be similar, no attempt has been made by these workers to establish the antigenic similarity or dissimilarity of these fractions. TAPG differed from these peptidoglycans in (i) amino acid compositions (TAPG showed a high proportion of proline); (ii) tuberculin activity (TAPG showed a high tuberculin activity in guinea pigs sensitized with *M. tuberculosis* or BCG); and (iii) adjuvant ac-

**Fig. 5.** Drawing of immunoelectrophoretic studies of TAPG. The upper well contained PPD, the central well contained United States-Japan reference culture filtrate lot 002 (002 CF), the lower well contained TAPG (GP), and the troughs contained reference antiserum lot 002.
From our preliminary experiments, TAPG did not show any adjuvant activity.

From the culture filtrate of tubercle bacilli, Seibert et al. (23) obtained a glycoprotein fraction which was known as polysaccharide 1 in their early experiments (22). TAPG differs from this glycoprotein in that it did not show a precipitation band with the reference H₃7Rv antiserum, although according to Daniel and Affronti (7), polysaccharide 1 fraction contains three antigens; namely, antigens 1 and 2 and one unidentified anodal antigen of the United States-Japan reference system, (Janicki et al. [13]).

TAPG, which shows high tuberculin activity in guinea pigs sensitized with M. tuberculosis or M. bovis BCG, is devoid of precipitins. This observation is contrary to the findings of previous workers (Baer and Chaparas [4]; Baker et al. [5]; Inque [12]; Kaourilsky et al. [14]; Morisawa et al. [21]) who had observed that precipitins were associated with fractions possessing tuberculin activity.

The results of our studies also show that TAPG injected in FIA is an effective immunogen for inducing delayed hypersensitivity in guinea pigs against the sensitizing antigen. This delayed response was also positive with PPD, but the skin reaction response with TAPG was more intense than that elicited by PPD. This induction of delayed hypersensitivity to TAPG or PPD also differentiates TAPG from other adjuvant active glycoproteins (1, 2, 11, 18, 19, 24), and it is also possible that TAPG may play an important role in immunity to tuberculosis.

Objections can be raised that the reactions induced by TAPG in guinea pigs sensitized with this antigen in FIA may be Arthus reactions. Although we have not performed any histological studies of these reactions, we feel that these reactions are not Arthus reactions due to the following reasons. (i) No early inflammatory response was seen in animals sensitized with TAPG or PPD in FIA. (ii) Immunoelectrophoretic studies did not show the presence of antibodies in the sera of guinea pigs sensitized with TAPG or PPD. (iii) ELISA showed the presence of very low levels of antibodies in guinea pigs sensitized with TAPG and FIA (10 ELISA units) compared to 100 ELISA units detected in the sera of guinea pigs sensitized with PPD and FIA.

It is pertinent to point out that Casvantz and Youmans (6) have reported that PPD in combination with mycobacterial ribonucleic acid adjuvant can sensitize guinea pigs.

ACKNOWLEDGMENTS

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LITERATURE CITED


