Tuberculin Activity of Mycobacterial Fractions Obtained by Chromatography

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Commercial purified protein derivatives (PPD), old tuberculin (OT), the bacillar extract, and the culture filtrate of Mycobacterium tuberculosis H37Ra were submitted to Sephadex G-25 and diethylaminoethyl (DEAE)-cellulose chromatography. The ability of the fractions obtained to elicit delayed dermal hypersensitivity in M. tuberculosis H37Ra-infected guinea pigs was studied. Skin tests with Sephadex fractions in M. tuberculosis H37Ra-infected guinea pigs showed that the tuberculin activity was localized in the first fraction. All other Sephadex fractions were nonessential and nonspecifically irritating. Fractions from chromatography of Sephadex G-25 fraction 1 on DEAE-cellulose columns showed that all but the first were able to elicit delayed hypersensitivity reactions. There was a variability in the capacity to elicit the tuberculin reaction according to the fraction injected and the stage of tuberculosis infection in guinea pigs. Compared to the others, the seven lots of commercial PPD were variable in composition and content. They contained both essential and nonessential materials for the tuberculin reaction. Sephadex fraction 1 would appear to be a better tuberculin as it excludes nonessential nonspecifically irritating elements and contains the complement able to elicit the tuberculin reaction. Its methodological simplicity would be economically advantageous.

Tuberculin is usually prepared from cell-free mycobacterial culture medium by precipitation with 50% ammonium sulfate. Such purified protein derivatives (PPD) contain varying amounts of reactive and inert materials and therefore require biological standardization (4). Dermal hypersensitivity produced by these relatively crude preparations only indicate prior exposure to mycobacteria. They provide little information concerning the stage or activity of disease in tuberculosis patients.

Data are presented here comparing delayed dermal hypersensitivity produced by commercial tuberculins (PPD and old tuberculin) and by chromatographically prepared Mycobacterium tuberculosis H37Ra fractions (bacillary extract and culture filtrate). These data confirm the complex mixture in present commercial tuberculins. Some of these materials in PPD are inert in eliciting dermal hypersensitivity and are additionally nonspecifically irritating. It was further demonstrated that commercial tuberculins vary in content and composition from lot to lot. Chromatographically prepared tuberculin (Sephadex G-25 fraction 1) appeared to be a "purer" tuberculin.

MATERIALS AND METHODS

- Tuberculins. The following tuberculins were studied. (i) PPD: random lots were obtained from two pharmaceutical companies (Parke Davis & Co. and Merck, Sharpe & Dohme). In addition, two large unsterilized lots were purchased from Parke Davis & Co. (ii) Old tuberculin (OT): this was prepared by the California Department of Agriculture (as described by Method for Production of Mammalian Tuberculin for the Official Testing of Cattle, Bureau of Animal Health, Division of Animal Industry, Sacramento, Calif.) from M. tuberculosis. (iii) Bacillary extract (BE) and culture filtrate (CF): M. tuberculosis H37Ra was grown for 8 weeks at 37°C on Sauton fluid medium, 150 ml per Roux bottle, and then was harvested by centrifugation. The cells, approximately 10 g (wet weight) per bottle, were washed three times with sterile distilled water and stored at -70°C. Bacillary extracts were prepared by ultrasonic disruption (Branson Sonifier, model S110, Stamford, Conn.) of a 50% cell suspension in 0.005 M sodium phosphate buffer, pH 7.6. A water-jacketed sonic treatment chamber through which ice water was circulated maintained a reduced temperature during the procedure.

A 95% disruption of cells was effected by sonic treatment for 3 min at 3 ma, followed by 20 min at 9 ma. Following this treatment, particulate material
was removed by centrifugation for 1 hr in the cold at 32,000 × g (Lourdes Betafuze, Brooklyn, N.Y.). The yellow opalescent supernatant fluid was clarified by passage through a 0.46-μm pore-size membrane filter (Millipore Corp., Bedford, Mass.); the sterile extract was stored at −70 C.

The supernatant fluid (CF) from the *M. tuberculosis* H37Ra culture was evaporated at 4 C to one-tenth to one-fifteenth its original volume and stored at that temperature.

**Column chromatography.** Sephadex G-25 (fine beads; Pharmacia Fine Chemicals, Piscataway, N.J.) was packed in Pharmacia columns under 15 to 20 cm of hydrostatic pressure. Two columns, one 2.5 by 30 cm, the other 5.0 by 40 cm, were used. Different volumes and concentrations of mycobacterial products were loaded on these columns. The volume of a sample never exceeded 15% of the bed volume. Hydrostatic pressures on the columns were adjusted to give flow rates of 100 ml/hr. Elution from a column was effected with double-distilled water, deionized water, or 0.005 M sodium phosphate buffer, pH 7.6. Eluted fractions were monitored at 280 nm (Beckman model 32 Fraction Collector, model DB-G spectrophotometer, and Ten Inch Recorder). Collections varied from 2.5- to 15-ml samples depending upon the size of the column. All chromatography was done at 4 C. The fractions were then lyophilized and stored in vacuo.

Diethylaminoethyl (DEAE)-cellulose (Whatman D52 Microgranular, Reeve Angel & Co., Inc., Clifton, N.J.) was brought to pH 8.0 with 0.005 N HCl and then washed repeatedly with 0.005 M sodium phosphate buffer, pH 8.0. It was packed in columns of 2.5 by 20 and 6 by 10 cm. The material to be chromatographed was dissolved in a minimum volume of starting buffer and loaded in a volume not exceeding 15% of the bed volume. A series of eight buffers was used for frontal elution; all contained 0.005 M sodium phosphate, pH 8.0, but they differed in NaCl content: 0, 0.005, 0.1, 0.15, 0.25, 0.5, 1.0, and 2.0 M. Eluted fractions were monitored as above at 280 nm, dialyzed in the cold until free of chloride, lyophilized, and stored in vacuo.

**Skin tests in guinea pigs.** Twenty adult guinea pigs were infected intraperitoneally with 60,000 to 80,000 viable units of *M. tuberculosis* H37Rv. The backs of the animals were closely clipped 6 weeks postinfection, and the nine Sephadex fractions from BE were applied 24 hr later. The fractions had been reconstituted in 0.005 M phosphate buffer, pH 7.6, without Tween 80 to approximately 1× and 2× the concentration of 1:5 OT in 0.1 ml, used in routine laboratory testing. The intradermal tests, using disposable plastic tuberculin syringes, were done in triplicate on three different animals with both concentrations. The test site of each antigen concentration was rotated in the three animals. Each animal received six test injections plus the standard OT test as the positive control. Three negative control guinea pigs each received ten skin tests (nine 2× concentrated Sephadex fractions and one OT control). Readings of the induration in millimeters were made in two diameters at 4, 8, 24, 48, and 72 hr. The results were arithmetically averaged and plotted.

For DEAE-cellulose fraction studies, a pilot study was done to determine the proper antigen dose by weight. Fifteen guinea pigs were intraperitoneally infected 6 weeks previously with 80,000 viable units of *M. tuberculosis* H37Rv. Triplicate testing was done. Each animal received nine skin tests (two DEAE-cellulose fractions each in concentrations of 200, 100, 50, and 25 μg/0.1 ml and OT). Measurements in two diameters were taken at 4, 8, 24, 48, and 72 hr. The results showed that the 100-μg dose caused the most easily readable skin induration for the dose.

For the test study, fifty adult guinea pigs were infected intraperitoneally with 60,000 to 80,000 viable units of *M. tuberculosis* H37Rv. Triplicate skin tests were done on three different animals on the 3rd, 5th, 14th, 28th, 60th, and 84th day of the infection, again rotating the skin test sites. Each test group consisted of six infected animals with each animal receiving six 0.1-ml skin tests (five DEAE-cellulose fractions and one OT control). The amount of antigen injected per skin test was 100 μg.

**RESULTS**

**Chromatography.** Sephadex G-25 chromatography of H37Ra BE, concentrated CF, and OT resulted in similar chromatograms (Fig. 1). Initially, there was a homogeneous peak of high-molecular-weight material eluted from the column, followed by a variable number of low-molecular-weight materials. The reverse was true for BE.

Figure 2 shows the Sephadex G-25 chromatography of seven lots of commercial PPD. Variations were apparent; peaks varied in

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**Fig. 1.** Sephadex G-25 chromatography of *M. tuberculosis* H37Ra products and OT. A, H37Ra bacillary extract; B, H37Ra culture filtrate; C, H37Ra culture filtrate; D, OT, 30 ml. The ordinates represent absorbancy which does not register above 1.0.
height or number, indicating variations in the concentrations and composition of the various fractions. Variations were observed not only between lots from different pharmaceutical companies, but between lots from the same company. From Fig. 2, it appeared that Parke Davis & Co. PPD contained a larger quantity of high-molecular-weight fraction 1 than Merck, Sharpe & Dohme PPD, even when much less material was loaded on the columns as shown by comparing chromatograms C with those of E and F.

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FIG. 2. Sephadex G-25 chromatography of commercial PPD. A, P-D lot 789129A, 75 mg; B, P-D lot 989128A, 74.5 mg; C, P-D lot 963079B, 0.005 mg; D, P-D lot 960279D, 1.5 mg; E, MSD lot 0692J, 0.016 mg; F, MSD lot 78497B, 0.08 mg; G, MSD lot 76905B, 0.0016 mg. P-D is Parke-Davis & Co., MSD is Merck, Sharpe & Dohme. The abscissae represent volume eluted. The ordinates represent absorbancy which does not register above 1.0.

FIG. 3. DEAE-cellulose chromatograms of mycobacterial Sephadex fraction 1. A, H37Ra bacillary extract, 581 mg; B, H37Ra culture filtrate, 200 mg; C, OT, 100 mg; D, P-D PPD lot 989129A, 67.2 mg; E, P-D lot 989129A, 12 mg. P-D is Parke-Davis & Co. The abscissae represent volume eluted. The ordinates represent absorbancy which does not register above 1.0. Breaks are placed at nonsignificant points to shorten the graph.

only on the high-molecular-weight material eluted from the Sephadex columns, since this constituted the material essential for delayed hypersensitivity. DEAE-cellulose chromatography of Sephadex fraction 1 from the BE and CF of M. tuberculosis H37Rv (Fig. 3A, B, C) were similar graphically and showed a maximum of ten major fractions. The chromatography of Sephadex fraction 1 from the two lots of commercial PPD (Fig. 3D, E) differed in the number of peaks eluted (six in E and ten in D).

Skin testing. Figure 4 demonstrates the results of skin tests with Sephadex fractions on M. tuberculosis H37Rv-infected guinea pigs, 6 weeks postinfection. The maximum delayed hypersensitivity activity, as judged by the 48-hr readings, resided in Sephadex fraction 1. Lesser reactions were elicited with other Sephadex fractions; however, the average diameters of induration never exceeded 8 mm.

Measurement of induration within 4 to 24 hr on negative control animals indicated a degree of nonspecific inflammation or irritation elicited by all Sephadex fractions. These early reactions were also present in infected animals in approximately the same degree. OT elicited the same specific and nonspecific reactions.

Intradermal injection of 100 µg of DEAE-cellulose fractions obtained from BE (Fig. 3A) demonstrated that, at 48 hr, all except the first fraction were capable of eliciting delayed hypersensitivity at some time during the course of a progressive tuberculous disease (Fig. 5). DEAE-cellulose fraction 1, even in doses of 200 µg, caused only erythema. There was no uniformity in the capability of the fractions to
induce delayed reactions. Early and more marked delayed reactions were induced by DEAE-cellulose fractions 4 and 5, particularly 4. These fractions produced noticeable reactions 5 days postinfection when the OT skin test was still negative. DEAE-cellulose fractions 2 through 10 gave significant delayed skin reactions at 2 weeks postinfection. Fractions 2, 3, 5, 7, and 10 induced weaker skin reactions which reverted to negative as the infection progressed to the 84th day. Fractions 4, 6, and 8 induced the most sustained and significant reactions, as did OT.

**DISCUSSION**

Comparison of commercial tuberculins (PPD and OT) with Sephadex G-25 fraction 1 of *M. tuberculosis* H37Ra has shown the superiority of the latter as a tuberculin. Unlike PPD and OT, Sephadex fraction 1 contains none of the low-molecular-weight, nonspecifically irritating materials. By its simple manner of production, its composition is constant and includes the elements of the tubercle bacillus able to elicit tuberculin reactions. It does, however, contain DEAE-cellulose fraction 1 which does not elicit delayed dermal hypersensitivity.

The skin test results with Sephadex G-25 fractions support the premise that tuberculin activity is found within the high-molecular-weight portion eluted in the "void" volume of Sephadex G-25 columns. Activity found in subsequent fractions is due to trailing from the first fraction or nonspecific reactions. Other investigators have demonstrated tuberculin activity by Sephadex chromatography. Chaparas and Baer (1) showed that immunologic activity of BCG was associated mostly with the larger...
molecules in the first peak eluted off G-25 and G-50 columns with lesser antigenic activity in subsequent fractions. Norlin and Ernevald (5) found on Sephadex chromatography of concentrated M. microti culture filtrates that antigenicity was mostly localized in the first peak. Again, some was also present in the second peak. Stöckl, Krocza, and Mathois (8) also reported that material of optimum tuberculin specificity was present in the first peak eluted from Sephadex G-25 or G-50 columns. There is, therefore, ample evidence of the localization of tuberculin activity in the first fraction eluted from Sephadex G-25 or G-50 columns.

Skin test results with all Sephadex fractions caused early nonspecific induration in normal animals. This may be a factor in the puzzling early skin reactions seen occasionally in tuberculin skin testing. Although chemically irritating reactions in normal guinea pigs have been denied by Smith (7), Van Waveren (9) has reported nonspecific irritation in the same species injected with the tuberculin of heat-concentrated synthetic medium. Pepys (6) has mentioned nonspecific inflammation modifying the vascularity and thus the persistence of antigens in the tuberculin reaction.

Comparable Sephadex chromatography of seven commercial lots of PPD from two firms clearly demonstrated that PPD is neither homogeneous nor uniform. In addition to the essential Sephadex fraction 1, they also contained the smaller molecular, nonessential, and nonspecifically inflammatory materials in variable quantities. Either 50% ammonium sulfate precipitation is not uniform in its effects from batch to batch of PPD even when processed by the same company, or the drop in pH of the culture medium with bacterial growth leads to a variable precipitation of tuberculoproteins. Corper and Cohn (2) have shown that incubation of M. tuberculosis cultures will lower the medium pH to below six. There is also definite loss of protein on heating a tuberculin solution when the pH is 6.0 or less. The variability of tuberculins has been the subject of a report by Landi and Held (3). PPD prepared by seven precipitation methods from the culture filtrates of M. tuberculosis H37Rv were found to contain anywhere from 48 to 98% tuberculoprotein. In addition, the biological activity of PPD varied from method to method and from lot to lot.

Further evidence of the superiority of Se-
Sephadex fraction 1 as a tuberculin is demonstrated by DEAE-cellulose chromatography (with DEAE-cellulose fractions) and skin testing of H37Rv-infected guinea pigs. Firstly, 9 of the 10 fractions from DEAE-cellulose chromatography Sephadex fraction 1 can elicit delayed hypersensitivity. Only DEAE-cellulose fraction 1 failed in this respect, suggesting its possible nonprotein nature. On the other hand, DEAE-cellulose chromatography of Sephadex fraction 1 from two commercial lots of PPD showed a variable content of potentially essential tuberculin fractions.

Secondly, serial skin tests on tuberculous guinea pigs showed that the onset and intensity of delayed hypersensitivity reactions to DEAE-cellulose fractions were not uniform. Tests with fractions 4 and 6 became positive first, even before OT. Furthermore, these two fractions and OT were consistently able to elicit delayed skin reactions over the 84 days of progressive tuberculous infection. Other fractions elicited less intense reactions and then only transiently, giving negative skin test results at the end of the 12 weeks of observation. The results suggest that some fractions (e.g., 4) are better than either crude tuberculin or other DEAE-cellulose fractions and can detect mycobacterial sensitization earlier. Conversely, loss of reactivity to other fractions (e.g., to DEAE-cellulose fractions 3 and 7) may indicate far advanced infection.

Sephadex G-25 chromatography is a better method for producing a standardized tuberculin based on weight. Either CF or BE may be used as the starting material. The latter is preferred because of greater yield and the easier handling of small volumes. In addition to containing the necessary components for the tuberculin reaction, Sephadex fraction 1 represents a clean separation of the nonessential and nonspecific inflammatory low-molecular-weight from the high-molecular-weight substances, wherein tuberculin activity resides.

Commercial PPD, OT, the BE, and the CF of *M. tuberculosis* H37Ra were studied by Sephadex G-25 and DEAE-cellulose chromatography. Seven lots of commercial PPD were variable in composition and content. They contained both essential and nonessential materials for the tuberculin reaction. Skin tests with Sephadex fractions in *M. tuberculosis* H37Rv-infected guinea pigs showed that tuberculin activity was localized in the high-molecular-weight fraction. All other Sephadex fractions were nonessential and nonspecifically irritating. Fractions from chromatography of Sephadex fraction 1 on DEAE-cellulose columns showed that all but the first were able to elicit the tuberculin reaction. There was a variability in the capacity to elicit delayed hypersensitivity according to the fraction injected and the stage of tuberculous infection in guinea pigs. Sephadex G-25 fraction 1 would appear to be a better tuberculin as it excludes nonessential, nonspecific irritating elements and contains the complement able to elicit the tuberculin reaction. The simplicity of its production would be economically advantageous.

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