Comparative Study of $^{14}$C-Labeled Purified Protein Derivative from Various Mycobacteria

II. Skin Cross-Reactivity in Sensitized Guinea Pigs

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A study on skin cross-reactivity between stabilized $^{14}$C-labeled mycobacterial antigens, namely tuberculin purified protein derivative (PPD; from Mycobacterium tuberculosis), PPD-A (M. avium), PPD-Y (M. kansasii), PPD-G (M. scrofulaceum), PPD-B (M. intracellulare), and PPD-F (M. fortuitum), has been carried out in groups of guinea pigs sensitized with one of the following heat-killed mycobacteria: M. tuberculosis, M. avium, M. kansasii, M. scrofulaceum, M. intracellulare, or M. fortuitum. For each type of sensitization, the average response for the corresponding PPD antigen was higher than the average response for any of the other antigens. However, the responses to the heterologous PPD antigens were not necessarily significantly different among themselves, and the significant differences of the heterologous PPD antigens were distributed differently according to the type of sensitization. Therefore, $^{14}$C-PPD antigens skin cross-reacted in guinea pigs essentially in the same manner as reported by others for nonradioactive PPD antigens.

Previous studies on the specificity of purified protein derivatives (PPD antigens) prepared from various mycobacteria have shown that their PPD antigens possess a certain species specificity. This was revealed by the fact that animals infected with a given mycobacterium usually showed more extensive reactions to the PPD antigen produced from homologous mycobacteria (the mycobacterium with which the animals were infected) than to the PPD antigen produced from other species of mycobacteria (2, 3, 6, 8).

The increasing interest in the pathogenic significance of some mycobacteria other than Mycobacterium tuberculosis, such as M. kansasii, M. scrofulaceum, M. intracellulare, and M. fortuitum, and the skin cross-reactivity which exists between the PPD antigen produced from the culture filtrates of these mycobacteria and tuberculin PPD produced from M. tuberculosis made it worthwhile to compare the extent of their cross-reactivity in guinea pigs sensitized selectively with these different species of mycobacteria and to find out the significance of their cross-reactivity. However, the extent and the significance of the PPD antigen cross-reactivities can be meaningful only if the PPD antigens used are known to be stable. Since stabilized PPD antigens are now available (4-6), we felt that this study should reveal with some accuracy the true degree of skin cross-reactivity of the PPD antigens being tested and should show if cross-reactivity between various $^{14}$C-labeled PPD antigens is of the same order as the cross-reactivity reported by others (3, 6, 8) with nonradioactive PPD antigens.

MATERIALS AND METHODS

Reagents. Phenol (Analar) was supplied by the British Drug Houses Ltd., Poole, England; Tween 80 [polyoxyethylene (20) sorbitan mono-oleate] was supplied by Atlas Chemical Industries, Wilmington, Del.

$^{14}$C-labeled PPD antigens. The preparation and purification of $^{14}$C-tuberculin PPD antigen from the culture filtrate of M. tuberculosis Johnston strain and of $^{14}$C-PPD antigens from the culture filtrates of M. avium ($^{14}$C-PPD-A), M. kansasii Chadwick strain ($^{14}$C-PPD-Y; group I of Runyon (9, 10)), M. scrofulaceum Gause strain ($^{14}$C-PPD-G; group II of Runyon), M. intracellulare Battey bacillus, Boone strain ($^{14}$C-PPD-B; group III of Runyon), and M. fortuitum ($^{14}$C-PPD-F; group IV of Runyon) have been described previously (5). The average specific radioactivity of the various $^{14}$C-PPD antigens used was 0.26 μCi per mg of dry powder $^{14}$C-PPD (0.18 to 0.48 μCi/mg). The potency of the various $^{14}$C-PPD antigens relative to the potency of the corresponding nonradioactive PPD antigens was of the same order (5).

Adsorption of PPD antigens. Previous adsorption studies have shown that all PPD antigens adsorb
quite readily to glass and that their adsorption can be effectively prevented by the addition of 0.0005% Tween 80 to the diluent (4, 5).

**Diluent.** The following buffer solution was used to prepare the diluent: isotonic phosphate-buffered saline (pH 7.38) containing 1.45 g of KH₂PO₄, 7.60 g of Na₂HPO₄·2H₂O, and 4.80 g of NaCl per liter after autoclaving. To the diluent 0.0005% Tween 80 was added as a stabilizer and 0.3% phenol was added as a preservative (5).

**Dilution of PPD preparations.** Dilutions containing 0.001 mg of ¹⁴C-PPD antigen per ml were prepared for biological assays.

**Syringes.** One-milliliter B-D tuberculin plastic disposable syringes obtained from Becton, Dickinson and Co., Rutherford, N.J., were used throughout this work.

**Guinea pigs.** The assays were performed on three groups of white, female guinea pigs. Each animal weighed between 450 and 550 g in groups 1 and 2 and between 650 and 750 g in group 3.

**Sensitization.** The sensitizing injection was given intramuscularly in the inner part of the thigh. The dose injected in each animal was 0.1 ml of 10 mg/ml.

### Table 1. Combinations of four ¹⁴C-PPD antigens to be tested in guinea pigs sensitized with either M. avium, M. kansasii, M. scrofulaceum, or M. intracellulare

| Sensitizationa of animals in group 1 | ¹⁴C-PPD antigens on test injected in pairsb |
|--------------------------------------|------------------------------------------|
| M. avium                            | PPD-A-PPD-Y                              |
|                                     | PPD-A-PPD-G                              |
|                                     | PPD-A-PPD-B                              |
|                                     | PPD-Y-PPD-G                              |
|                                     | PPD-Y-PPD-B                              |
|                                     | PPD-G-PPD-B                              |
| M. kansasii                         | PPD-A-PPD-Y                              |
|                                     | PPD-A-PPD-G                              |
|                                     | PPD-A-PPD-B                              |
|                                     | PPD-Y-PPD-G                              |
|                                     | PPD-Y-PPD-B                              |
|                                     | PPD-G-PPD-B                              |
| M. scrofulaceum                     | PPD-A-PPD-Y                              |
|                                     | PPD-A-PPD-G                              |
|                                     | PPD-A-PPD-B                              |
|                                     | PPD-Y-PPD-G                              |
|                                     | PPD-Y-PPD-B                              |
|                                     | PPD-G-PPD-B                              |
| M. intracellulare                   | PPD-A-PPD-Y                              |
|                                     | PPD-A-PPD-G                              |
|                                     | PPD-A-PPD-B                              |
|                                     | PPD-Y-PPD-G                              |
|                                     | PPD-Y-PPD-B                              |
|                                     | PPD-G-PPD-B                              |

- Thirty-six guinea pigs were used for each sensitization.
- Six guinea pigs were used for each pair of ¹⁴C-PPD antigens on test.
of the appropriate heat-killed bacilli suspension in Freund’s adjuvant and saline mixed in equal proportions.

In group 1, 144 guinea pigs were subdivided into four subgroups each comprising 36 animals. Each subgroup was sensitized with either M. avium, M. kansasii, M. scrofulaceum, or M. intracellulare. The animals were used for skin tests 8 weeks after vaccination.

In group 2, 90 guinea pigs were subdivided into six subgroups each comprising 15 animals, and each subgroup was sensitized with M. tuberculosis, M. avium, M. kansasii, M. scrofulaceum, M. intracellulare, or M. fortuitum. The animals were used for skin tests 8 weeks after vaccination.

Group 3 was made up of the same animals used in group 2, and the animals were skin-tested for the second time 14 weeks after vaccination.

Skin test. Skin tests were carried out on group 1 following the design outlined in Table 1 to study the cross-reactivities of four 14C-PPD antigens obtained from the culture filtrates of M. avium (PPD-A), M. kansasii (PPD-Y), M. scrofulaceum (PPD-G), and M. intracellulare (PPD-B). Table 1 shows that only two injections were performed in each animal, that is, one injection of each of two of the four PPD antigens under investigation. For each of the four types of sensitization (M. avium, M. kansasii, M. scrofulaceum, and M. intracellulare), we have six combinations or six sets of test doses of the four 14C-PPD antigens (PPD-A, PPD-Y, PPD-G, and PPD-B) taken two at a time. For one type of sensitization, it was necessary for statistical analysis that the same number of animals should be used for each of the six sets of test doses. Thus, for a particular sensitization, the total number of animals used was a multiple of six. In our case, 36 animals were used for each type of sensitization, and the six sets of test doses were injected in a random manner.

Skin tests were carried out in groups 2 and 3 according to the design outlined in Table 2 for the study of the following 14C-PPD antigens: tuberculin PPD, PPD-A, PPD-Y, PPD-G, PPD-B, and PPD-F. The scheme for this study is similar to the scheme described for group 1, except that there were now six different 14C-PPD antigens instead of four and in this design each combination of two PPD antigens was used in only a single animal for each sensitization. Thus, for each of the six types of sensitization with M. tuberculosis, M. avium, M. kansasii, M. scrofulaceum, M. intracellulare, or M. fortuitum, 15 animals were required, that is to say one guinea pig for each of the 15 pairs of challenge doses. This test therefore required 90 animals. The 15 pairs of challenge doses were injected in a random manner.

Each skin test dose consisted of 0.1 ml (0.0001 mg of 14C-PPD) of the PPD antigens used and was administered intracutaneously. All injections were given and read by the same person.

Reading of test. The reactions were read after about 24 hr. For each reaction site, the longitudinal and transverse diameters of erythema were measured in millimeters and recorded as the sum of these two diameters and used for statistical evaluation.

RESULTS AND DISCUSSION

Tables 3, 4, and 6 show that, for each type of sensitization, the mean response for the

| Sensitization of animals in group 1 with | Skin reaction (mm) |
|-----------------------------------------|-------------------|
|                                        | PPD-A | PPD-Y | PPD-G | PPD-B |
| M. avium                               | 22.65 | 17.57 | 19.44 | 19.94 |
| M. kansasii                             | 13.21 | 27.62 | 16.54 | 17.96 |
| M. scrofulaceum                         | 16.79 | 19.33 | 27.92 | 19.95 |
| M. intracellulare                       | 15.97 | 19.10 | 17.64 | 22.68 |

* Each mean is based on eighteen values of the sum of two diameters. Two injections, one per flank, in random combinations as described in Table 1, were given to each animal.

* Boldface numbers indicate the homologous skin reaction.

| Sensitization of animals in group 2 with | Skin reaction (mm) |
|-----------------------------------------|-------------------|
|                                        | Tuberculin PPD  | PPD-A   | PPD-Y   | PPD-G   | PPD-B   | PPD-F   |
| M. tuberculosis                         | 27.13            | 13.13   | 24.73   | 17.13   | 22.53   | 10.13   |
| M. avium                                | 25.76            | 34.16   | 21.16   | 20.76   | 22.56   | 12.16   |
| M. kansasii                             | 20.20            | 15.20   | 23.60   | 16.00   | 15.20   | 13.00   |
| M. scrofulaceum                         | 22.20            | 21.60   | 23.80   | 22.00   | 22.60   | 16.60   |
| M. intracellulare                       | 20.00            | 21.60   | 24.20   | 24.20   | 31.80   | 17.20   |
| M. fortuitum                            | 14.36            | 10.36   | 15.56   | 9.76    | 14.16   | 19.56   |

* Each mean is based on five values of the sum of two diameters. Two injections, one per flank, in random combinations as described in Table 2, were given to each animal.

* Boldface numbers indicate the homologous skin reaction.
Table 5. Mean responses* of cross-reactivity to \(^{14}\text{C}-\text{PPD}\) antigens in guinea pigs sensitized (14 weeks) with various mycobacteria

| Sensitization of animals in group 3 with | Skin reactions (mm) |
|----------------------------------------|---------------------|
|                                        | Tuberculin PPD      |
|                                        | PPD-A               |
|                                        | PPD-Y               |
|                                        | PPD-G               |
|                                        | PPD-B               |
|                                        | PPD-F               |
| \(M.\text{ tuberculosis}\)              | 22.80\(^b\)         |
| \(M.\text{ avium}\)                    | 19.40               |
| \(M.\text{ kansasii}\)                 | 20.33               |
| \(M.\text{ scrofulaceum}\)             | 21.13               |
| \(M.\text{ intracellular}\)            | 20.13               |
| \(M.\text{ fortuitum}\)                | 11.50               |

* Each mean is based on five values of the sum of two diameters. Two injections, one per flank, in random combinations as described in Table 2, were given to each animal.

* Boldface numbers indicate the homologous skin reaction.

Table 6. Combined mean responses* of cross-reactivity to \(^{14}\text{C}-\text{PPD}\) antigens in guinea pigs sensitized with various mycobacteria

| Sensitization of animals in groups 2 and 3 with | Skin reactions (mm) |
|-----------------------------------------------|---------------------|
|                                              | Tuberculin PPD      |
|                                              | PPD-A               |
|                                              | PPD-Y               |
|                                              | PPD-G               |
|                                              | PPD-B               |
|                                              | PPD-F               |
| \(M.\text{ tuberculosis}\)                  | 24.30\(^b\)         |
| \(M.\text{ avium}\)                        | 22.33               |
| \(M.\text{ kansasii}\)                     | 20.01               |
| \(M.\text{ scrofulaceum}\)                 | 21.80               |
| \(M.\text{ intracellular}\)                | 20.10               |
| \(M.\text{ fortuitum}\)                    | 12.90               |

* Mean responses from Tables 4 and 5.

* Boldface numbers indicate the homologous skin reaction.

Table 7. Significant differences among the mean responses to \(^{14}\text{C}-\text{PPD}\) antigens PPD-A, PPD-Y, PPD-G, and PPD-B) in guinea pigs sensitized with either \(M.\text{ avium}\), \(M.\text{ kansasii}\), \(M.\text{ scrofulaceum}\), or \(M.\text{ intracellular}\)

| Sensitization of animals in group 1 with | Skin reaction (mm) | Significant difference minimum\(^a\) (mm) |
|----------------------------------------|---------------------|----------------------------------------|
|                                        | PPD-A               | PPD-B               | PPD-G               | PPD-Y               |                              |
| \(M.\text{ avium}\)                   | 22.65               | 19.94               | 19.44               | 17.57               | 2.54                        |
| \(M.\text{ kansasii}\)                | 27.62               | 17.96               | 16.54               | 13.21               | 2.25                        |
| \(M.\text{ scrofulaceum}\)            | 27.42               | 19.96               | 19.33               | 16.79               | 2.56                        |
| \(M.\text{ intracellular}\)           | 22.68               | 19.10               | 17.63               | 15.97               | 1.99                        |

* For \(M.\text{ avium}\), there is no significant difference between PPD-B, PPD-G, and PPD-Y. For \(M.\text{ kansasii}\), there is no significant difference between PPD-B and PPD-G. For \(M.\text{ scrofulaceum}\), there is no significant difference between PPD-B and PPD-Y; and PPD-Y and PPD-A. For \(M.\text{ intracellular}\), there is no significant difference between PPD-Y and PPD-G; PPD-G and PPD-A.

\(^b\) For calculation, see Cochran and Cox (1).
| Sensitization of animals in groups 2 and 3 with | Skin reaction (mm) | Significant difference minimum<sup>b</sup> (mm) |
|---------------------------------------------|-------------------|------------------------------------------|
| **M. tuberculosis**                          |                   |                                          |
| Tuberculin PPD                              | 24.30             |                                          |
| PPD-Y                                       | 21.05             |                                          |
| PPD-B                                       | 18.97             |                                          |
| PPD-G                                       | 16.55             |                                          |
| PPD-A                                       | 11.55             |                                          |
| PPD-F                                       | 10.88             |                                          |
| **M. avium**                                |                   |                                          |
| PPD-A                                       | 27.59             |                                          |
| Tuberculin PPD                              | 22.33             |                                          |
| PPD-B                                       | 20.25             |                                          |
| PPD-G                                       | 20.25             |                                          |
| PPD-Y                                       | 19.58             |                                          |
| PPD-F                                       | 14.00             |                                          |
| **M. kansasii**                             |                   |                                          |
| PPD-Y                                       | 21.10             |                                          |
| Tuberculin PPD                              | 20.01             |                                          |
| PPD-G                                       | 15.01             |                                          |
| PPD-B                                       | 14.85             |                                          |
| PPD-A                                       | 13.43             |                                          |
| PPD-F                                       | 12.69             |                                          |
| **M. scrofulaceum**                         |                   |                                          |
| PPD-G                                       | 26.80             |                                          |
| PPD-B                                       | 23.38             |                                          |
| PPD-Y                                       | 22.05             |                                          |
| Tuberculin PPD                              | 21.80             |                                          |
| PPD-A                                       | 21.14             |                                          |
| PPD-F                                       | 13.14             |                                          |
| **M. intracellulare**                       |                   |                                          |
| PPD-B                                       | 28.75             |                                          |
| PPD-Y                                       | 26.17             |                                          |
| PPD-G                                       | 22.75             |                                          |
| PPD-A                                       | 22.00             |                                          |
| Tuberculin PPD                              | 20.10             |                                          |
| PPD-F                                       | 18.92             |                                          |
| **M. fortuitum**                            |                   |                                          |
| PPD-F                                       | 14.82             |                                          |
| PPD-Y                                       | 13.23             |                                          |
| Tuberculin PPD                              | 12.90             |                                          |
| PPD-B                                       | 11.48             |                                          |
| PPD-A                                       | 9.74              |                                          |
| PPD-G                                       | 9.24              |                                          |

<sup>a</sup> For *M. tuberculosis*, there is no significant difference between PPD-Y and PPD-B; PPD-B and PPD-G; and PPD-A and PPD-F. For *M. avium*, there is no significant difference between tuberculin PPD, PPD-B, PPD-G, PPD-Y. For *M. kansasii*, there is no significant difference between PPD-Y and tuberculin PPD; and PPD-B, PPD-G, PPD-A, PPD-F. For *M. scrofulaceum*, there is no significant difference between PPD-G and PPD-B, and PPD-B, PPD-Y, tuberculin PPD, and PPD-A. For *M. intracellulare*, there is no significant difference between PPD-G and PPD-A; PPD-A and tuberculin PPD; and tuberculin PPD and PPD-F. For *M. fortuitum*, there is no significant difference between PPD-F and PPD-B; PPD-B and PPD-A; and PPD-A and PPD-G.

<sup>b</sup> For calculation, see Cochran and Cox (1).
corresponding ¹⁴C-PPD antigen was significantly higher than the mean response for any of the other ¹⁴C-PPD antigens.

Table 4 gives the mean response to tuberculin PPD, PPD-A, PPD-Y, PPD-G, PPD-B, and PPD-F obtained in guinea pigs (group 2) sensitized with *M. tuberculosis*, *M. avium*, *M. kansasii*, *M. scrofulaceum*, *M. intracellulare*, or *M. fortuitum*. As expected, for each sensitization, the corresponding PPD gave the highest mean response. These results are in agreement with the findings for nonradioactive PPD antigens, which were reported to give more extensive reactions in humans or animals infected with the homologous mycobacteria than to antigens produced from other species (3, 6, 8).

Table 5 gives the mean response to ¹⁴C-PPD antigens (tuberculin PPD, PPD-A, PPD-Y, PPD-G, PPD-B, and PPD-F) obtained with 3 guinea pigs (same animals as used in group 2 and now being tested for the second time at 14 weeks after vaccination). For each sensitization, except for *M. fortuitum*, the corresponding PPD antigen gave the highest mean response. However, in group 3 the pattern of response to the various PPD antigens in the animals sensitized with *M. intracellulare* and *M. fortuitum* showed a detectable change from the response obtained in the animals in group 2. Although the differences were significant in these two cases, they were not very large. Furthermore, in the avian-sensitized animals of group 3 (Table 5), both tuberculin PPD and PPD-G produced higher mean responses than PPD-B, whereas in avian-sensitized animals of group 2 (Table 4) both tuberculin PPD and PPD-B produced higher mean responses than PPD-Y. Finally, for the animals of group 3 sensitized with *M. fortuitum*, both tuberculin PPD and PPD-Y gave somewhat higher mean responses than the corresponding PPD-F (Table 5).

Table 6 gives the combined means of the skin test results of Tables 4 and 5. When the results reported in Tables 3 and 6 were examined for significant differences among the mean responses to the PPD antigens for the different types of sensitization, we found that for the animals in group 1 (Table 3) and for each sensitization the corresponding PPD antigen gave the highest mean response. However, the responses to the heterologous PPD antigens although significantly different from the homologous PPD antigen were not necessarily significantly different among themselves, and the significant differences of the heterologous PPD antigens were distributed differently according to the type of sensitization (Table 7). In the animals in groups 2 and 3 (Table 6), we also found that, on the average, for each sensitization the corresponding PPD antigen gave the highest mean response, but in animals sensitized with *M. kansasii*, *M. scrofulaceum*, and *M. fortuitum* no significant difference could be found with the heterologous PPD antigens such as tuberculin PPD, PPD-B, and PPD-Y, respectively (Table 8). The results of this experiment were very similar to those reported in Table 7, and the order of the responses was the same in both instances, except for the response to PPD-G and PPD-B in the animals sensitized with *M. kansasii*. However, this difference was not serious since the mean responses to the two PPD antigens were very similar.

These results were not essentially different from those reported by others (3, 6, 8) with nonradioactive PPD antigens. It can therefore be concluded that ¹⁴C-PPD antigens which were used throughout this work gave skin cross-reactivities comparable to those reported for nonradioactive PPD antigens.

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