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Purified Protoplasmic Peptides of Mycobacteria: In Vivo and In Vitro Comparison of the Species Specificity of Purified Protoplasmic Peptides and Purified Protein Derivatives of Mycobacterial Culture Filtrates

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The specificity of purified protein derivatives (PPD) prepared from the culture filtrates of *Mycobacterium tuberculosis* (PPD), *M. kansasii* (PPD-Y), *M. intracellulare* (PPD-B), and *M. scrofulaceum* (PPD-G) were compared to comparable protoplasmic extracts (PPP) of the same organisms by gel diffusion and delayed hypersensitivity reactions in sensitized guinea pigs. PPD and, to a lesser degree, PPD-Y demonstrated specificities sufficient to enable identification of homologously sensitized guinea pigs in the above group of four mycobacteria. PPD-B and PPD-G did not always elicit the largest reaction in homologously sensitized animals. The PPP sensitins from *M. tuberculosis* and *M. kansasii* produced as good skin reactions at 24 and at 48 hr as did their PPD counterparts. The PPP from *M. scrofulaceum* and *M. intracellulare* were more specific and more reactive than corresponding PPD, regardless of the time of comparison. Although based on different immunological mechanisms, the specificity of these two groups of sensitins, as demonstrated by delayed hypersensitivity, correlated well with serological comparisons in the gel diffusion test. The low degree of specificity of PPD-B and PPD-G in contrast to that of corresponding PPP was reflected in the precipitin bands in agar gel.

The in situ identification of mycobacterial infections in man and in lower animals is conventionally attempted by the intradermal injection of purified protein derivatives (PPD) derived from mycobacterial culture filtrates. It is an accepted, although by no means infallible, observation that these PPD generally elicit the largest reaction in homologously sensitized animals (4, 9, 15). Reports by Larson et al. (6, 7, 8) and Counts and Kubica (3) indicated that more specific skin reactions might be elicited in sensitized experimental animals by protoplasmic extracts of homologous mycobacteria. If such specific sensitins could be found, then the long-sought, accurate in situ identification of mycobacterial disease could become a reality. Beam et al. (1) recently described intracellular products obtained from young cultures of mycobacteria and referred to as purified protoplasmic peptides (PPP).

In this investigation, four of these PPP are compared to the corresponding PPD antigens by using both in vivo and in vitro tests. It was hoped the more rapid in vitro test might serve as a control on the purification and specificity of the final PPP sensitin.

MATERIALS AND METHODS

The species specificities of four PPP isolated from *Mycobacterium tuberculosis* (H37Rv), *M. kansasii* (P8), *M. scrofulaceum* (T-72-5), and *M. intracellulare* (T-67-5) were compared in vitro and in vivo with those of four corresponding purified protein derivatives (PPD, PPD-Y, PPD-G, and PPD-B). (These were kindly supplied by Lydia Edwards, Operational Research Section, Research Coordination Unit, TB Branch, National Communicable Disease Center,

Bethesda, Md.) The PPDs were used in concentrations of 1 mg/ml and 0.0005 mg/0.1 ml (25 TU). Methods for isolation and purification of PPP were described previously (1). All PPP were suspended in 0.066 M phosphate buffer (*p*H 7.2) containing 0.005% Tween 80 (3), and were used in concentrations ranging from 0.01 μg/ml to 1.0 mg/ml.

From 6 to 12 weeks prior to skin testing, groups of eight randomly bred albino guinea pigs of both sexes, each weighing 400 to 800 g, were sensitized by intramuscular injection of 1 mg (dry weight) of phenol-killed bacteria emulsified in Freund's adjuvant. The five species of mycobacteria used for sensitization included the four species above plus *M. bovis* (BCG Tice). A sixth group of eight uninoculated guinea pigs were retained as unsensitized controls.

Skin tests in guinea pigs were performed by the method of Magnusson (9, 10). Hair was removed from both sides of the animals by means of hair clippers, and a commercial depilatory cream was then applied to provide a smooth surface on both sides of the animal separated by a strip of hair (1 to 2 cm) directly over the spine. In this manner, 12 to 20 intradermal injections could be placed on the sides of each animal. To determine the quantity of protoplasmic extract needed to elicit a skin reaction comparable to that of PPD, the PPP of *M. tuberculosis* was titrated in 12 guinea pigs, half of which were sensitized with *M. tuberculosis* and the other half with *M. bovis* (BCG Tice). The quantity of PPP injected ranged from 0.001 to 100.0 μg per 0.1 ml.

All subsequent skin tests in both homologously and heterologously sensitized guinea pigs were done with 25 units (0.5 μg) of each PPD employed and both 0.1 and 1.0 μg of the respective PPP sensitins. To avoid bias in reading skin reactions, all sensitins were coded and injection sites were rotated on each guinea pig. The code was broken only after the skin reactions had been measured and recorded at 24 and 48 hr.

Skin test reactions were evaluated by calculating specificity differences by Magnusson's formula $SPD = (Aa + Bb) - (Ab + Ba)$, in which *Aa* was the homologous reaction to sensitin *A* in animals sensitized with organism *a*, and *Ab* represented heterologous reactions to sensitin *A* in animals sensitized with organism *b* (9). In a similar manner, *Bb* and *Ba* represented homologous and heterologous reactions to sensitin *B*.

Antisera for use in serological studies were prepared by subcutaneous, intramuscular, or intraperitoneal injection of albino rabbits weighing 3 to 5 kg each. A 100-mg amount of crude protoplasmic extract [CPE (1)] was injected along with 10 mg of homologous phenol-killed mycobacteria emulsified in 10 ml of hexadecane adjuvant (16). At 14 days after injection, the sensitized and uninoculated control rabbits were bled by cardiac puncture. Serum was collected and γ-globulins were separated by 2 or 3 precipitations with half-saturated ammonium sulfate. Globulins, redissolved and diluted from 1:2 to 1:64 in sterile 0.85% saline, were tested by gel diffusion against PPP or PPD, or both (200 μg/0.2 ml), as described by Gussoni (5). Gel diffusion plates were prepared by aseptically pouring 10 ml of autoclaved agar (0.9% Oxoid Ion Agar no. 2, 0.9% NaCl, and 0.4% sodium citrate) into plain plastic petri plates (15 by 100 mm). Agar was allowed to solidify on a level table to insure uniform depth throughout the plate.

For the gel diffusion test, each agar plate was set on a template and 13 penicillin assay cylinders were placed on the agar; one central cylinder was surrounded by two concentric rings of six cylinders each. The edges of the cylinders in the two rings were located 12.7 and 31.4 mm, respectively, from the edge of the central cylinder. Edges of the interacting cylinder wells were exactly 12.7 mm distant from one another (*see* Fig. 2–6). The center cylinder was filled with 0.2 ml of antiserum. The six cylinders in the surrounding inner ring were alternately filled with antigen and antiserum, and the cylinders in the outer ring were filled with antigen or antiserum in a sequence complementary to that of the inner ring; the cylinders in the outer ring served as a control check on the activity of reactants in the inner ring.

Antigen and antiserum were placed in the cylinders so that precipitin bands of homologous systems occurred in circular fashion either between the central cylinder and the inner ring cylinders or between the latter and the outer ring cylinders. Precipitin bands of heterologous nature appeared as radial bands between cylinders of the inner ring (*see* Fig. 2–6).

RESULTS

In vivo. The skin reactivity of sensitized guinea pigs to the homologous protoplasmic extract from *M. tuberculosis* (PPP-TB) is summarized in Fig. 1. No reaction was elicited by 0.001 μg of PPP-TB. Indurations ranging from <5 to 6 and 10 to 14 mm were elicited by 0.01 and 0.1 μg of PPP-TB, respectively. Dosages of the PPP ranging from 10 to 100 μg caused induration from 17 to >25 mm, often with severe central necrosis.

Results of skin tests performed with 25 units of various PPD, and 0.1 and 1.0 μg of the corresponding PPP in homologously and heterologously sensitized guinea pigs, were evaluated by the method of Magnusson (9); the calculated specificity differences (SPD) are recorded in Tables 1*a*, 1*b*, 2*a*, and 2*b*. The diameters of the 24- and 48-hr mean reactions from which the SPD

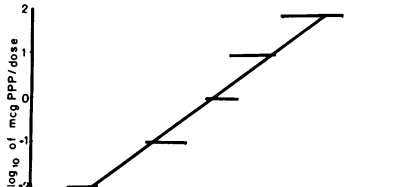

FIG. 1. *Relationship of PPP dosage to size of skin reaction (24 hr) in sensitized guinea pigs.*

TABLE 1a. *Specificity differences (SPD) of the 24-hr skin reactions in sensitized guinea pigs: purified protein derivatives*[a]

Mycobacterium sensitizing strain	Culture filtrates[b]			
	M. tuberculosis	*M. kansasii*	*M. scrofulaceum*	*M. intracellulare*
M. tuberculosis	0	7.2	13.1	17.2
M. kansasii	7.2	0	11.6	19.3
M. scrofulaceum	13.1	11.6	0	5.9
M. intracellulare	17.2	19.3	5.9	0

[a] SPD values and their standard deviations are determined by the method of Magnusson (9). The larger the SPD, the more reliable the separation of the paired mycobacterial strains by skin sensitivity. Standard deviation of the SPD ranged from 3.5 to 7.0.

[b] Twenty-five units per dose.

PPP were larger than the corresponding values for the PPD. At 24 hr, 1.0 μg of PPP was inferior to 0.1 μg in eliciting specific reactions; e.g., the SPD of *M. tuberculosis-M. kansasii* was only 9.3 mm.

The 48-hr observations (Tables 2a and 2b) revealed the opposite results; the 1.0-μg PPP dosages showed greater SPD values, all being 10 mm or more. At 48 hr, the SPD values of PPD, particularly combinations including *M. scrofulaceum*, *M. kansasii*, and *M. intracellulare*, were reduced to the 5-mm range. The SPD calculated for the 0.1-μg dosages of PPP at 48 hr also decreased to a range of 5.3 to 7.4 mm, mainly because of several negative homologous reactions at that time.

In general, these observations showed the 0.1-μg PPP to yield higher degrees of species specificity at 24 hr than did 25 units of comparable PPD. The specificity elicited at 48 hr by 1.0-μg

TABLE 1b. *Specificity differences (SPD) of the 24-hr skin reactions in sensitized guinea pigs: protoplasmic peptides*[a]

Mycobacterium sensitizing strain	Protoplasmic peptides							
	M. tuberculosis		*M. kansasii*		*M. scrofulaceum*		*M. intracellulare*	
	0.1 μg	1.0 μg	0.1 μg	1.0 μg	0.1 μg	1.0 μg	0.1 μg	1.0 μg
M. tuberculosis	0	0	15.4	9.3	16.5	12.0	15.0	16.0
M. kansasii	15.4	9.3	0	0	12.7	13.0	11.4	10.5
M. scrofulaceum	16.5	12.0	12.7	13.0	0	0	12.25	11.0
M. intracellulare	15.0	16.0	11.4	10.5	12.25	11.0	0	0

[a] SPD values and their standard deviations are determined by the method of Magnusson (9). The larger the SPD, the more reliable the separation of the paired mycobacterial strains by skin sensitivity. Standard deviation of the SPD ranged from 3.5 to 5.9.

were calculated are given in Tables 3 and 4. An SPD of 5 mm or more indicates a difference between two antigens, whereas SPD values in excess of 10.0 mm indicate that definitive identification of the sensitizing mycobacterium is possible by the skin test. In Table 1a the PPD identified those guinea pigs sensitized with homologous organisms. The SPD in animals sensitized with heterologous bacteria ranged from 5.9 to 19.3. Only the use of PPD to distinguish *M. intracellulare* from *M. scrofulaceum* sensitizations and *M. kansasii* from *M. tuberculosis* was somewhat less reliable, yielding SPD values of 5.9 and 7.2, respectively. Table 1b shows SPD values obtained with two dosages of PPP, at the 24-hr reading. No SPD for any heterologous reaction elicited by 0.1 μg of PPP was smaller than 11.4 mm. With the exceptions of the *M. tuberculosis-M. intracellulare* and *M. kansasii-M. intracellulare* comparisons, the SPD values for 0.1 μg of

TABLE 2a. *Specificity differences (SPD) of the 48-hr skin reactions in sensitized guinea pigs: purified protein derivatives*[a]

Mycobacterium sensitizing strain	Culture filtrates[b]			
	M. tuberculosis	*M. kansasii*	*M. scrofulaceum*	*M. intracellulare*
M. tuberculosis	0	8.0	12.0	18.0
M. kansasii	8.0	0	4.6	10.25
M. scrofulaceum	12.0	4.6	0	6.1
M. intracellulare	18.0	10.25	6.1	0

[a] SPD values and their standard deviations are determined by the method of Magnusson (9). The larger the SPD, the more reliable the separation of the paired mycobacterial strains by skin sensitivity. The standard deviation of the SPD ranged from 2.4 to 8.2.

[b] Twenty-five units per dose.

TABLE 2b. *Specificity differences (SPD) of the 48-hr skin reactions in sensitized guinea pigs: protoplasmic peptides*[a]

Mycobacterium sensitizing strain	Protoplasmic peptides							
	M. tuberculosis		*M. kansasii*		*M. scrofulaceum*		*M. intracellulare*	
	0.1 μg	1.0 μg	0.1 μg	1.0 μg	0.1 μg	1.0 μg	0.1 μg	1.0 μg
M. tuberculosis	0	0	7.4	10.3	6.8	24.0	7.0	16.5
M. kansasii	7.4	10.3	0	0	5.7	17.1	6.0	14.0
M. scrofulaceum	6.8	24.0	5.7	17.1	0	0	5.3	21.8
M. intracellulare	7.0	16.5	6.0	14.0	5.3	21.8	0	0

[a] SPD values and their standard deviations are determined by the method of Magnusson (9). The larger the SPD, the more reliable the separation of the paired mycobacterial strains by skin sensitivity. Standard deviation of the SPD ranged from 2.2 to 4.1.

TABLE 3. *Mean reactions to purified protein derivatives (PPD) and protoplasmic peptides (PPP) after 24 hr in 32 sensitized guinea pigs*

Mycobacterium skin test antigen	Sensitizing strains			
	M. tuberculosis	*M. kansasii*	*M. scrofulaceum*	*M. intracellulare*
	mm	mm	mm	mm
PPD of				
M. tuberculosis	12.8	11.4	0.6	0.6
M. kansasii	4.0	9.7	0	0.6
M. scrofulaceum	5.0	4.1	6.0	4.8
M. intracellulare	7.3	2.0	7.4	12.2
PPP of				
M. tuberculosis	9.6	0.9	0	0
M. kansasii	0	6.6	0.75	0.6
M. scrofulaceum	0	0	6.8	0
M. intracellulare	0	0	0	5.4

dosages of PPP was usually greater than the SPD of PPD at that time.

In an attempt to determine whether the protoplasmic extract from *M. tuberculosis* (PPP-TB) and *M. bovis* BCG (PPP-BCG) could be used to distinguish these two organisms from one another, two groups of guinea pigs, sensitized with either tubercle bacilli or BCG, were cross-skin tested. The resultant SPD value was 5.0 mm, indicating continued difficulties in separating naturally infected from BCG-immunized individuals. Further purification of the PPP-BCG may be necessary if an in situ differentiation of BCG from *M. tuberculosis* is desired.

In the first paper of this series (1), we indicated that the PPP from various organisms had been paper chromatographed, resulting in separation of several ninhydrin-staining spots. These spots were carefully eluted in phosphate buffer and injected into homologously sensitized guinea pigs. In the case of the PPP from *M. tuberculosis*, *M. scrofulaceum*, and *M. intracellulare*, only one of the ninhydrin-staining spots elicited a skin reaction in animals, and in all cases the R_F ranged from 20 to 25. Two of the ninhydrin-staining spots from PPP-BCG (R_F values of 20 and 25) were capable of eliciting skin reactions in BCG-sensitized animals.

In vitro. The specificities of the various PPD and PPP were compared in gel diffusion tests by using antimycobacterial γ-globulins. Figures 2–4 depict the stepwise purification of PPP. In Fig. 2, the crude protoplasmic extracts (CPE) of *M. tuberculosis* (t) and *M. bovis*, BCG(C) were tested against globulins for *M. tuberculosis* (T), *M. terrae* (R), *M. triviale* (V), and BCG(C). The marked cross-reactivity between *M. tuber-*

TABLE 4. *Mean reactions to purified protein derivatives (PPD) and protoplasmic peptides (PPP) after 48 hr in 32 sensitized guinea pigs*

Mycobacterium skin test antigen	Sensitizing strains			
	M. tuberculosis	*M. kansasii*	*M. scrofulaceum*	*M. intracellulare*
PPD of				
M. tuberculosis	10.8	3.4	0	0
M. kansasii	2.0	2.6	0	0.6
M. scrofulaceum	3.1	2.3	4.3	2.4
M. intracellulare	2.3	1.25	5.25	9.5
PPP of				
M. tuberculosis	12.2	6.4	0	2.25
M. kansasii	3.7	8.25	0.6	1.2
M. scrofulaceum	0.6	2.9	12.5	0
M. intracellulare	4.8	4.6	2.0	11.4

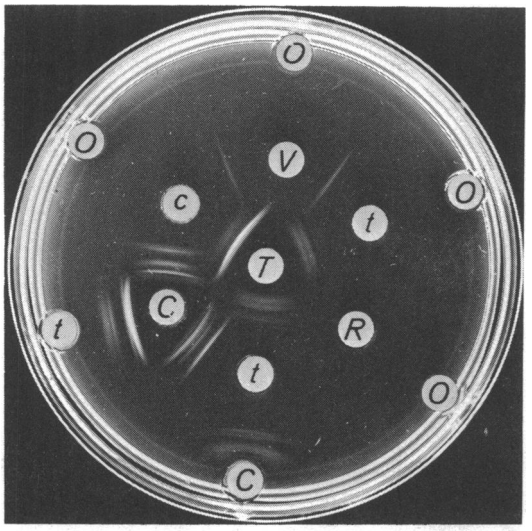

FIG. 2. *Gel diffusion reactions of crude protoplasmic extracts (CPE) of M. tuberculosis and M. bovis (BCG) with various globulins prepared against killed, whole cells of mycobacteria. Cells were disrupted and centrifuged at 40,000 × g. No lipid extraction of product. Control wells consisted of either sterile saline or normal rabbit globulin. Key to symbols for this and Fig. 3–6:*

Organism	Globulin	Protoplasmic extract	PPD
Control	*O*	*O*	
M. tuberculosis	*T*	*t*	*S*
M. bovis BCG	*C*	*c*	
M. kansasii	*K*	*k*	*Y*
M. intracellulare	*I*	*i*	*B*
M. triviale	*V*	*v*	
M. terrae	*R*	*r*	
M. gastri	*J*	*j*	
M. scrofulaceum	*M*	*m*	*G*

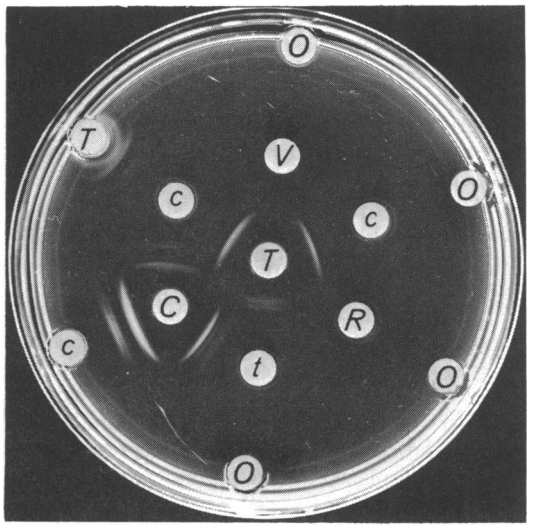

FIG. 3. *Gel diffusion reactions of protoplasmic extracts of M. tuberculosis and M. bovis (BCG) after ultracentrifugation. Cells were disrupted at 30,000 psi and centrifuged at 144,700 × g. No lipid extraction of product. See Fig. 2 for key to well designations.*

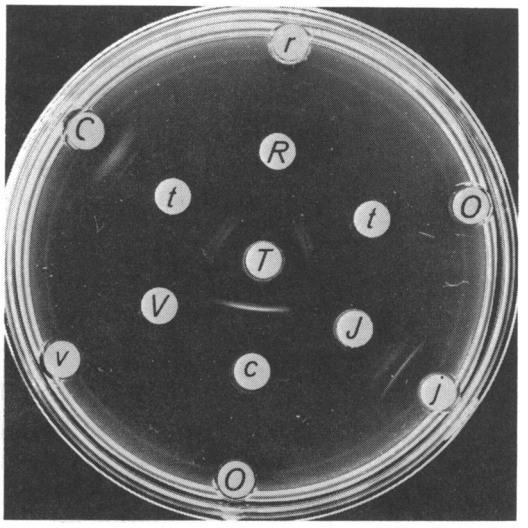

FIG. 4. *Gel diffusion reactions of purified protoplasmic products (PPP). PPP was prepared by disrupting cells at 30,000 psi and subjecting final product to ultracentrifugation at 144,700 × g and lipid extraction. See Fig. 2 for key to well designations.*

culosis and BCG is readily apparent in those antigen-antibody reaction zones showing four or more bands of precipitation. Cross-reactivity of globulins against *M. triviale* and *M. terrae* with CPE-t and CPE-c was minimal, showing no more than one radial line between the wells located in the inner circle.

Ultracentrifugation of the CPE eliminated precipitin cross-reactivity of mammalian mycobacterial extracts with globulins against *M. terrae* and *M. triviale;* however, cross-reactions within the *M. tuberculosis*-BCG system still persisted (Fig. 3). Figure 4 shows the precipitation bands which appear after the PPP have been ultracentrifuged and extracted with neutral organic solvents. Although PPP-TB and PPP-BCG cross-reacted in sensitized guinea pigs, they could be distinguished by gel diffusion (Fig. 4). The PPP-TB(t)-anti-*M. tuberculosis* globulin (T) system revealed a double band, whereas the cross-reacting BCG(C) showed only a single band. In the reciprocal reaction [i.e., anti-BCG globulin (C) against PPP-TB(t)] three bands were observed, but the homologous C-c system (not shown in Fig. 4) yielded four bands. This was not unexpected in view of the greater number of com-

ponents detected in PPP-BCG. Only a single band of precipitation was observed in the *M. terrae* (R-r) and *M. triviale* (V-v) systems, but three precipitin bands appeared in the *M. gastri* (J-j) system (*see* Fig. 4). The cross-reacting radial bands between cylinders of the inner ring were virtually eliminated by the ultracentrifugation-lipid extraction procedure. From these results, it appears that the gel diffusion test might serve as an accurate monitor of the extent of purification achieved with each PPP.

The reactivity of PPD-Y (from *M. kansasii*) is shown in Fig. 5. Globulins against *M. tuberculosis* (T), *M. scrofulaceum* (M), and *M. intracellulare* (I) were placed in the inner ring of cylinders, whereas anti-*M. kansasii* globulin (K) was in the central well. PPD-Y (represented by Y) was also placed in three cylinders of the inner circle. One band of identity was common to all these systems. Although the difference is not easily discernible in this photograph, the homologous *M. kansasii* system had two bands; the *M. tuberculosis* system had four.

In Figure 6, both PPD and PPP are reacted with globulins prepared against homologous and heterologous whole cells. The homologous reactivity of the PPP products is seen in the T-t (*M. tuberculosis*), I-i (*M. intracellulare*), and M-m (*M. scrofulaceum*) systems. It was interesting that among the PPD, only that for *M. tuberculosis* reacted with globulin against whole cells (the S-T system in Fig. 6). Neither the PPD from *M. intracellulare* (the B-I system) nor *M. scrofulaceum* (G-M system) showed homologous

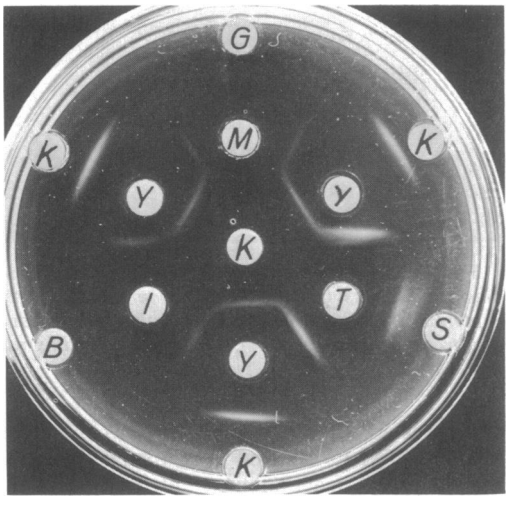

FIG. 5. *Gel diffusion reactions of PPD, PPD-Y, PPD-B, and PPD-G with various antimycobacterial globulins. See Fig. 2 for key to well designations.*

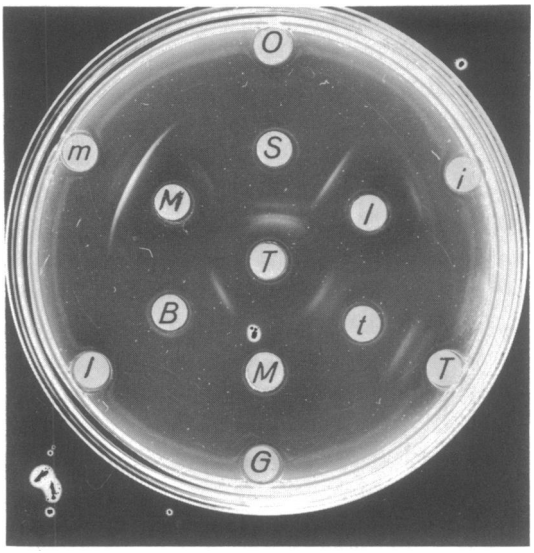

FIG. 6. *Gel diffusion comparison of PPD, PPD-B, and PPD-G with their corresponding purified protoplasmic extracts. See Fig. 2 for key to well designations.*

precipitin bands, yet PPD-B produced one band of precipitation with globulin against whole cells of *M. tuberculosis* (*note* B-T system, Fig. 6). By the same token, the PPD from *M. tuberculosis* also yielded one precipitin band with globulins against *M. intracellulare* (S-I wells, Fig. 6) and *M. scrofulaceum* (S-M wells). Although PPD from *M. intracellulare* (B) and *M. scrofulaceum* (G) did not react with globulins I and M prepared against different strains of these two species, they did react homologously with antisera against the specific strains of *intracellulare* and *scrofulaceum* used to produce PPD-B and PPD-G. In contrast, the PPP prepared from these two species reacted homologously with globulins against the several strains of *M. intracellulare* and *M. scrofulaceum* tested. In the *M. tuberculosis* system, on the other hand, PPP-TB(t) produces a double band with anti-*M. tuberculosis* globulin (T), whereas the comparable PPD (S in Fig. 6) shows a third band which reacts the *M. intracellulare* (I) globulin.

DISCUSSION

Edwards et al. pointed out that guinea pigs infected with different mycobacteria often expressed cross-reactivity to heterologous PPD antigens, but the average size of cross-reactions generally was smaller than the homologous reaction (4). To decrease or eliminate these cross-reactions, investigations were initiated in this laboratory (3) to isolate a more specific protein from the protoplasm of tubercle bacilli. In

dosages of 0.1 to 1.0 μg, this extract yielded skin reactions only in homologously sensitized guinea pigs. This preliminary success and three reports by Larson and co-workers (6–8) which showed species specificity of protoplasmic products stimulated our attempts to purify protoplasmic extracts in search of the species-specific protoplasmic products now labeled PPP (1). Tuberculin active peptides were extracted from intact mycobacteria by Morisawa and co-workers (11) and tested in vivo by Someya and co-workers (13). In spite of the similar molecular weight (<10,000), the product of Morisawa (11) was not identical with the PPP reported here because serological identity of the two sensitins could not be established by gel diffusion studies. The gel diffusion test proved to be a valuable tool for in vitro comparison of our PPP not only with PPD but also with the protoplasmic peptide of Morisawa prepared according to their description (11). The greater potency in vitro of PPP as compared to PPD was apparent when PPP from *M. scrofulaceum* and *M. intracellulare*, in concentrations of 1 mg/ml, produced precipitin bands in gel diffusion with 1:16 dilutions of antisera against several strains of these two species. On the other hand, the corresponding PPD (G and B) precipitated only their strain-specific antisera, and then only in the 1:4 dilution. In a study of culture filtrate antigens made from several strains of *M. intracellulare*, Chapman et al. (2) also noted that some strains of this species were more reactive than others in gel diffusion studies.

The high degree of reactivity of PPP sensitins in homologously sensitized guinea pigs is shown in Fig. 1. Counts and Kubica (3) could elicit skin reactions of 5 to 6 mm in diameter with 0.1 to 1.0 μg of their crude protein-containing antigen. Larson et al. obtained similar specific skin reactions with 0.015 to 0.28 μg of their product (7). The PPP described here elicited reactions of 5-mm induration with dosages of 0.01 μg (Fig. 1); however, 0.1 μg was more commonly used to elicit reactions in the range of 10 to 15 mm.

The peptide of Morisawa et al. and Someya et al. was less active than PPD (11, 13). The PPP from *M. tuberculosis* was as active as the corresponding PPD, whereas PPP-K (*M. kansasii*) appeared more specific than did PPD-Y from the same organism. The PPP of *M. intracellulare* and *M. scrofulaceum* were much more active than their PPD counterparts (Tables 1*a*, 1*b*, 2*a*, and 2*b*). It was observed that 0.1 μg of the appropriate PPP elicited a 24-hr induration usually with greater specificity differences (SPD values) than did the corresponding PPD which were used in dosages of 25 units (0.5 μg). However, possibly because of smaller molecular size and presumably more rapid diffusion away from the inoculation site by the time of mononuclear cell mobilization, the reaction to 0.1 μg of PPP faded faster than did the PPD reaction (14). On the other hand, when 1.0 μg of PPP was used, all reactions but one showed greater SPD values than did the corresponding 25 units of PPD. In this study, PPP was not used in 0.5-μg dosage, but this additional amount of protoplasmic material might be sufficient to provide a persistence of reaction comparable to that observed with PPD.

When using PPD, we encountered particular difficulties in identifying guinea pigs sensitized by *M. intracellulare* and *M. scrofulaceum*. The SPD values at both 24 and 48 hr were only 5.9 to 6.1 mm, thus confirming the studies of Smith and Johnston (12). If we disregard the 48-hr reading for the rapidly fading reaction to low-dosage PPP, the SPD values observed for PPP with these same two species were 11 to 21.8 mm.

The PPP from *M. bovis* BCG is not yet purified to a degree which permits distinctions of animals sensitized with *M. tuberculosis* from those vaccinated with BCG. Too, further purification of PPP-TB might enable complete removal of some of the nonskin-reactive, ninhydrin-staining spots with resultant greater specificity and sensitivity of the final product.

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