Purified Protoplasmic Peptides of Mycobacteria: Isolation of Species-Specific Peptides from Protoplasm of Mycobacteria

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Infections with mycobacteria other than tubercle bacilli are responsible for a variable percentage of cross-reactions to tuberculin. Two major suggestions for circumventing this problem have been made: the first, development of a quantitative tuberculin test, is based on the fact that most cross-reactions are smaller than those caused by true tuberculous infections; the second, preparation of purified skin test antigens from other mycobacteria, is based on the hope that greater specificity will be displayed by homologous sensitin. Effort so far has been focused on the culture filtrates as the source of antigen. This article describes the preparation of low molecular weight purified protoplasmic peptides (PPP) of specificity and sensitivity superior to purified protein derivatives.

Magnusson (10) recently reviewed the world literature dealing with tuberculin and mycobacterial extracts (endotuberculins) and concluded that presently available endotuberculins offer no advantage over conventional tuberculins prepared from culture filtrates. Larson and co-workers (7) reported similar observations after comparing purified protein derivatives (PPD) with protoplasmic extracts in sensitized guinea pigs. On the other hand, Someya and co-workers (14), Morisawa and co-workers (11), and Counts and Kubica (3) demonstrated a greater specificity of peptide or protein sensitins isolated from mycobacterial protoplasm over conventional tuberculins prepared from culture filtrates. Difficulties in the identification of mycobacterial pathogens in situ by use of PPD have been reported by Edwards and Palmer (4), Chapman and co-workers (2), Worthington (19), Magnusson (10), and recently by Vakilzadeh and co-workers (16). Since decades of research have failed to greatly improve the specificity of the tuberculin skin test when culture filtrate antigens are used, a new approach has been taken to produce from protoplasm of mycobacteria an antigen of greater specificity and greater sensitivity than the PPD currently available.

MATERIALS AND METHODS

All cultures used in this study were identified by numerical taxonomy methods previously described, and each strain employed was shown to have a similarity of 93% or higher to the hypothetical median strain of its species (6). Organisms included in the investigation were *Mycobacterium tuberculosis* (H37Rv), *M. bovis* (strain BCG Tice), *M. kansasii* (P-8), *M. gastri* (ATCC-15754), *M. terrae* (T-75-5), *M. triviale* (T-319-3), *M. scrofulaceum* (T-72-5), and *M. intracellulare* (T-67-5).

Each culture was grown for 5 days in Middlebrook 7H-9 broth (Difco) and then plated onto Dubos-Middlebrook oleic acid albumin agar (DMA) to check culture purity and to pick single colonies of each strain for propagation. The single colonies were transferred to 250-ml screw-cap Erlenmeyer flasks containing 50 ml of modified Proskauer and Beck liquid medium (18). These cultures were incubated at 35 C in an atmosphere of 5 to 10% CO_2 until the organisms grew as a veil on the surface of the medium. Portions of the veil were transferred with a platinum spatula to the surface of several 2,500-ml low-form flasks, each containing 500 ml of modified Proskauer and Beck medium (18).

After incubation for 3 weeks at 35 C in a CO_2-enriched atmosphere, the floating veil usually covered the surface of the medium and contained from 0.5 to 1.0 g (dry weight) of mycobacteria. Any cultures in which the veil already had sunk, or which appeared

grossly contaminated, were discarded. Cultures were killed by adding crystalline phenol to a final concentration of 1%. Phenolized cultures were kept at 35 C for 1 week at which time multiple attempts to obtain growth from the washed, resuspended cells failed to yield any viable organisms.

The phenol-killed mycobacteria were separated from the liquid medium by centrifugation at 10,000 × g in a Sorvall RC-2 refrigerated centrifuge. The bacterial mass was washed three or more times in distilled water, lyophilized, and stored at −20 C until used.

The washed, dried bacteria (1 or 2 g) were resuspended in 100 ml of distilled water, and a homogeneous suspension of cells was obtained either by stirring the cells overnight at 4 C or by grinding them in a Ten Broeck tissue grinder. The mycobacterial suspension was then passed into a Sorvall Ribi cell fractionator (model RM-1) at a flow rate of 100 ml/20 min. The bacteria were disrupted under pressures varying from 15,000 to 30,000 psi, and the temperature at the point of disruption was maintained at 5 to 10 C by means of a flowing stream of chilled, gaseous nitrogen. Merthiolate in a final concentration of 1:10,000 was added to the disrupted cell mass which was then placed at 4 C overnight (to permit extraction of protoplasmic contents).

Disruption of the cells was checked by electron microscopy. A 1-ml amount of the disrupted suspension was washed four times with distilled water, and a negatively stained preparation was prepared by mixing 0.1 ml of the washed suspension with 0.1 ml of a 1% aqueous solution of phosphotungstic acid. This mixture was floated on Formvar-coated grids and stained for 3 min. Excess fluid was removed from the edge of the grid by absorption into filter paper. Electron micrographs were made on Kodak contrast plates using a Philips electron microscope 200 at 40 kv.

The disrupted cell mass was further processed by differential centrifugation in both a Sorvall Model RC-2 refrigerated centrifuge and a model L2-65B ultracentrifuge (Beckman Instruments Co., Fullerton, Calif.). The disrupted cell suspension was first centrifuged at 3,020 × g for 1 hr to remove any intact cells as well as most of the cell wall material. The sediment was discarded and the supernatant material was centrifuged at 12,100 × g for 30 min. The supernatant fluid was subjected to one more sedimentation at 41,000 × g for 15 min. The opalescent supernatant fluid, referred to as crude protoplasmic extract (CPE), was then transferred to 13.5-ml cellulose nitrate tubes and centrifuged for 3 hr at 144,700 × g in an ultracentrifuge with a type 65 fixed-angle rotor. The sediment was discarded. The supernatant fluid was passed through a membrane filter of 0.45 μm pore size and then lyophilized to a yellow-tinted powder. The powder was extracted for 12 hr with ethyl alcohol-diethyl ether (1:1) to remove lipids soluble in neutral solvents. The extracted powder was then dissolved in distilled water containing 1:10,000 merthiolate and was lyophilized and stored at −20 C until used. This lyophilized product was referred to as purified protoplasmic peptide (PPP).

The CPE was subjected to several tests before and after ultracentrifugation to establish the purity of the final product. Protein content was determined both by micro-Kjehldahl and biuret tests. Aqueous solutions of CPE and PPP (10 mg%) were assayed in a DU spectrophotometer (Beckman Instruments Co.) at 260 and 280 nm. The formula of Kalcker (5) was used to calculate the content of protein and desoxyribonucleic acid (DNA); the amount of DNA was confirmed by the diphenylamine test (13). Ribonucleic acid and pentose were detected by the orcinol method and other carbohydrates were detected by the anthrone reaction (13).

The ultracentrifuged, lipid-extracted PPP was assayed before and after hydrolysis in 2 N HCl for 4 hr at 110 C. Samples varying from 0.05 to 1.0 mg were fractionated by chromatography on Whatman no. 2 paper by using 1-butanol-acetic acid-distilled water (8:2:2). The presence of peptides and amino acids was ascertained by spraying chromatograms with ninhydrin reagent (17), dimethyl-amino-benzaldehyde (Ehrlich's) reagent (17), sodium nitroprusside, and sodium cyanate reagent (17). The Elson-Morgan test (17) was used to detect amino sugars. Other carbohydrates were observed by the aniline-diphenylamine-phosphate reagent and the 2,3,5-triphenyltetrazolium chloride test for reducing sugars (17). Carbohydrates were differentiated by the thin-layer method of Lato et al. (9). A 10-mg amount of PPP was separated by gel filtration on Sephadex G75; the effluent from the column was passed through a continuous-flow cell and scanned from 200 to 300 nm in a DU spectrophotometer for presence of nucleotides and aromatic amino acids.

Molecular weight determinations of PPP were attempted by ultracentrifugal analysis of the product. A solution (1 mg/ml) was centrifuged at 250,000 × g in a Spinco model E centrifuge according to the method of Morisawa et al. (11).

Preliminary fractionations and characterizations of PPP from different mycobacteria were attempted by paper chromatography according to methods described above.

Results of skin hypersensitivity tests in sensitized guinea pigs have been reported (15).

RESULTS

Separation and purification of protoplasmic extracts. To obtain specific skin test sensitins, the bacterial mass had to be disrupted with care. Pressure in the Ribi cell fractionator was regulated in such a way that acid-fast rods burst at only one end, leaving the remainder of the cell wall largely intact (Fig. 1–3). The required pressure varied with the smoothness and concentration of the suspension and the size of the cells, but once these parameters were established for any one species, preparation of large quantities of PPP was relatively easy. Pressures from 15,000 to 25,000 psi were satisfactory for *M. tuberculosis*, *M. bovis* (BCG), *M. triviale*, and *M. terrae*, whereas *M. intracellulare* and *M. scrofulaceum* required 25,000 to 30,000 psi.
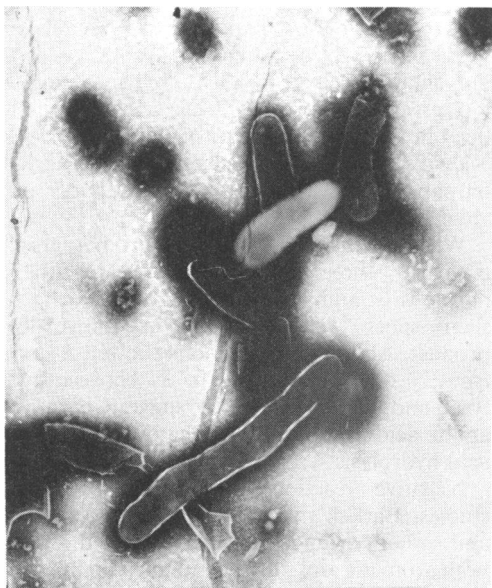
FIG. 1. *Cells of M. gastri disrupted under 30,000 psi. Note that most cell walls are intact and that protoplasmic contents have been released through an opening at one end of the cell. X 8,190.*

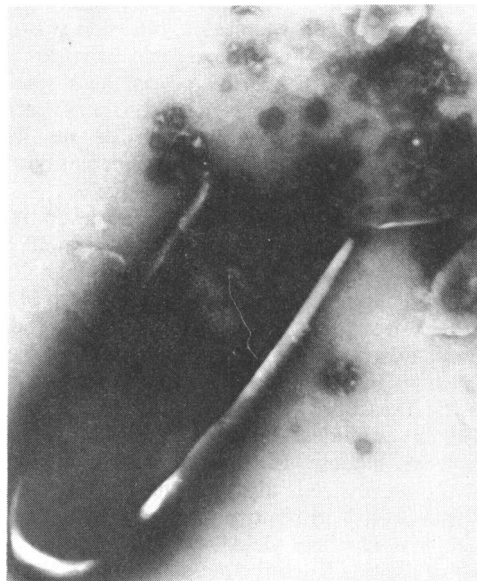FIG. 3. *Single cell of M. gastri disrupted under 30,000 psi. One end of the cell has been opened to release protoplasm. X 30,960.*
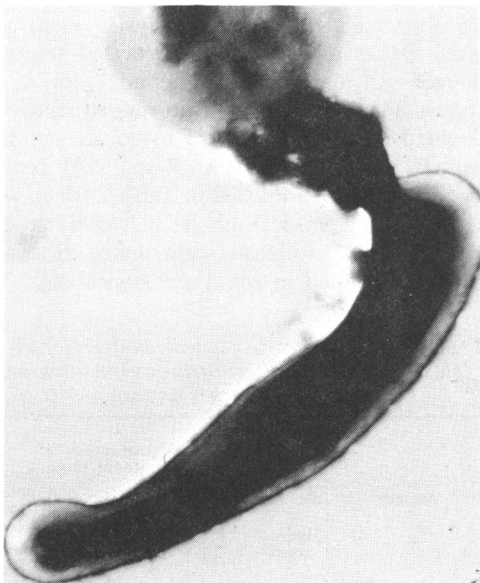

FIG. 2. *Single cell of M. bovis BCG disrupted under 25,000 psi. Protoplasmic contents are exuding through small "puncture" at one end of cell. X 30,960.*

FIG. 4. *Cells of M. gastri disrupted under 48,000 psi. Note that most of the cells have been "shattered" by the excessive pressure. Protoplasmic contents from cells disrupted in this manner yield highly cross-reactive PPP. X 8,190.*

Pressures in excess of 30,000 psi usually caused complete fragmentation of cell walls (Fig. 4) and resulted in high degrees of cross-reactivity in the PPP sensitin.

Differential centrifugation provided the simplest and most effective way to purify the CPE. Intact cells were removed at 3,000 X *g*, cell walls at 12,000 to 41,000 X *g*, and high molecular

weight proteins, nucleic acids, and ribosomes at 144,700 × g (20). Membrane filtration was used to remove most water-insoluble particles and some lipids which still floated on the surface of the ultracentrifuged material. All centrifugations were made in distilled water, since the lower specific gravity ensured better sedimentation than was possible in buffered salt solutions.

Table 1 reflects the purification achieved by the ultracentrifugation step. Protein content was reduced from 70 to 80% in CPE to less than 5% in PPP. Nucleic acids, which comprised 2 to 3% of CPE, were not detectable in the final product. Only two sensitins [from Bacillus Calmette-Guérin (BCG) and M. gastri] showed any residual carbohydrate after ultracentrifugation, and this was identified by thin-layer chromatography as glucose. Ethyl alcohol-ether extraction of the lyophilized, ultracentrifuged product was still necessary to remove the detectable amounts of lipids. The final yield of PPP represented 1 to 5% of the total bacterial mass.

Ultracentrifugal gradient analyses of the final PPP revealed the molecular weight of the product to be less than 10,000.

Preliminary chemical analyses of sensitins. The purified protoplasmic product consisted primarily of peptides which, in three instances (BCG, M. tuberculosis, and M. scrofulaceum), were contaminated with nucleotides. Fractionation of these hydrolyzed and unhydrolyzed PPP on Sephadex G75 and analyses of effluents in a DU spectrophotometer revealed only one absorption maximum at 255 nm, which was attributed to nucleotides. No extinction was observed at 280

nm, indicating an absence of tyrosine, tryptophane, and other aromatic amino acid-containing proteins or peptides. Lack of absorption at 260 nm, supported by a negative diphenylamine test, indicated the absence of nucleic acids. The nucleotides in M. tuberculosis, BCG, and M. scrofulaceum also revealed themselves as single blue spots on paper chromatograms examined under ultraviolet light.

When hydrolyzed PPP was chromatographed on paper, three to six fractions stained bluish-purple with ninhydrin reagent. Only two to four such spots were detected in unhydrolyzed product. Although all fractions showed R_F values from 20 to 25, the change in R_F between hydrolyzed and unhydrolyzed PPP suggested that some amino acid fragments had been dissociated by the acid hydrolysis.

Negative reactions with spectrophotometric studies, Ehrlich's reagent, sodium nitroprusside, and sodium cyanide reagents suggested a lack of both aromatic and sulfur-containing amino acids.

The PPP recovered from different species of mycobacteria were studied by paper chromatography. Twenty commercially available amino acids and 12 peptides consisting of two to three amino acids were used as controls on the supposition that acid hydrolysis of PPP might release individual amino acids or short peptides. None of the amino acids or peptides showed exactly the same R_F as any PPP or hydrolyzed fractions thereof. An alanine-glycine peptide with R_F 25 and a blue-purple ninhydrin-stained spot resembled the purple spots of mycobacterial PPP with R_F values ranging from 20 to 25. All peptide fractions of the PPP listed in Table 2 which were separated on paper (both from hydrolyzed and unhydrolyzed products) were eluted from the paper and tested in sensitized guinea pigs. The

TABLE 1. *Chemical analysis of intracellular mycobacterial products performed at two different stages of differential centrifugation*

Protoplasmic product	Protein (micro-Kjehldahl)	Desoxyribonucleic acid	Ribonucleic acid + pentoses	Lipids	Carbohydrates
	%	%	%	%	%
Mycobacterium tuberculosis					
41,000 × g	80	2	2	7	0.7
144,700 × g	<5	0	0	5	0
M. intracellulare					
41,000 × g	75	1.9	1.8	15	0.8
144,700 × g	<5	0	0	8.5	0
M. kansasii					
41,000 × g	71	2.2	2.8	12	1.6
144,700 × g	1	0	0	9	0

TABLE 2. *Preliminary chemical analysis of protoplasmic sensitins after ultracentrifugation and ethyl alcohol-ether extraction*

Antigen	No. of ninhydrin-stained spots		No. of carbohydrate fractions		No. of nucleotide fractions
	Hydrolyzed	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	
Mycobacterium tuberculosis..	4	3	0	0	1
BCG..........	6	4	1	1	1
M. scrofulaceum........	4	2	0	0	1
M. triviale....	3	2	0	0	0
M. gastri......	5	2	1	1	0

properties of the PPP sensitins in gel precipitation studies and as skin test antigens in sensitized guinea pigs will be described in the second paper in this series.

DISCUSSION

The need for more specific skin test antigens for mycobacteria prompted our earlier investigation of protoplasmic extracts of these organisms (3). Although this earlier study demonstrated an improved sensitin, the antigen, primarily protein, was still contaminated with nucleic acids and carbohydrates. Counts and Kubica (3) pointed out the need to prepare such antigens carefully to maintain the biologically active functional group(s). The present study was conducted in an effort to establish optimal conditions for disruption of cells and recovery of the active, specific sensitins. When appropriate pressures were used, the Ribi cell fractionator inflicted minimal damage on the mycobacterial cell, with subsequent retention of optimal differential properties of the final product.

Differential centrifugation and systematic investigation of all sediment and supernatant fractions were conducted in an effort to localize the active product with a minimum of initial chemical treatment. Chemical analyses of the protoplasmic extract after centrifugation at 41,000 $\times$ g revealed a protein content of 70 to 80%. Membrane filtration of the product reduced the protein to 20 to 30% without loss of skin test activity or specificity in gel diffusion tests (15). We then thought that the activity might reside in a fraction sedimenting with the ribosomes rather than in the large protein molecules; consequently, ultracentrifugal separations, as suggested by Youmans et al. (20), were attempted.

After sedimentation of the protoplasmic extract at 144,700 $\times$ g, we were surprised to find that not the ribosomal material in the sediment but the contents of the supernatant fluid reacted more specifically both in gel diffusion tests with homologous rabbit antiserum and in hypersensitized guinea pigs (15). Further fractionation and chemical analyses revealed the active product to be a PPP.

Subsequent investigations were conducted to determine the need for buffer salts in the extraction process. Since PPP had a molecular weight of less than 10,000, we were concerned that dialysis might result in loss not only of buffer salts but also of some active product. Then, too, lyophilization of PPP in buffer salts made it difficult to determine the true dry-weight yield of active material. Extraction of PPP in distilled water was shown to have no detrimental effect on

the product, and it permitted an accurate calculation of its yield and activity.

Seibert's analysis of the PPD skin test antigen (12) revealed the presence of two polysaccharides. Polysaccharide I, an antigenic heteropolysaccharide containing arabinose, galactose, and mannose, has a molecular weight of 7,000 to 9,000. Birnbaum and Affronti (1) recently showed that polysaccharide I was common to both *M. tuberculosis* and *M. kansasii*. Polysaccharide II was described (12) as a lipid-contaminated polyglucosan of molecular weight 100,000. Neither of these polysaccharides could be demonstrated in PPP.

Protein fractions having variable species specificity have been isolated from culture filtrates, cell walls, and protoplasm of mycobacteria (3, 7, 8, 12, 19; H. T. Fauser et al., Bacteriol. Proc., p. 81–82, 1969). Even though some of these products have a certain species specificity, most of them represent higher molecular weight materials than the PPP here described.

PPP has a molecular weight less than 10,000, and it contains relatively few components. One is encouraged, therefore, about a possible complete chemical analysis of the product in regard to amino acid content, sequence, and configuration. This prospect offers the advantage that cumbersome skin test standardizations in vivo may soon be unnecessary, since dosage of sensitin will be based upon actual weight of a chemically defined substance instead of on a poorly characterized unit whose activity must be reestablished in each new batch of PPD.

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