New Tuberculins[1] (34789)

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It is well known that preparations of tuberculin, old tuberculin (OT), "purified protein derivative" (PPD), and others, contain variable amounts of serologically active polysaccharide (polysaccharide I of Seibert). It will be shown here that they may also be contaminated with serologically active phosphoglycolipids. The purpose of this report is to describe a method for eliminating such substances from tuberculin preparations and to present preliminary results of studies of the comparative activities of such preparations in eliciting reactions of hypersensitivity in the skin of experimental animals and human subjects.

Materials and Methods. Treatment of tuberculins. The procedure employed in treatment of all tuberculin preparations, in general, involved oxidation with periodate in aqueous solution and, where indicated for preparations containing serologically detectable amounts of phosphoglycolipid, prior extraction of lyophilized material with chloroform:methanol:water (80:20:1). Crude OT[2] was always dialyzed and clarified by centrifugation and filtration to remove insoluble material, including any polysaccharide II, before being submitted to comparative testing as untreated, dialyzed old tuberculin (DOT). The following steps were carried out at room temperature (20–24°).

After dialysis against 0.01 *M* disodium ethylenediaminetetraacetic acid at pH 7 under toluene and then against several changes of deionized water, the material was freeze-dried, weighed, and dissolved at a concentration of 10 mg/ml of pyrogen-free physiological saline adjusted to neutrality with sodium bicarbonate. The solution was then centrifuged at 1500*g* for 30 min, and the supernatant fluid was filtered through a 0.22-μ Millipore filter in a "Filter-fuge" in 5-ml aliquots, with change of filter for each such sample. After dialysis of the filtrate against several changes of deionized water under toluene, the filtrate was freeze-dried in tared tubes and identified as DOT. Much of the serologically active phosphoglycolipid was extracted from the dry DOT by homogenizing in a Tri-R tissue grinder with repeated aliquots of chloroform:methanol:water (80:20:1).

For oxidation, DOT or PPD was dissolved in 0.2 *M* phosphate buffer at pH 7.6 at a concentration of 10 mg/ml, and 1/5 volume of freshly prepared 0.5 *M* sodium metaperiodate was added. The reaction was carried out in the dark during 48–72 hr.

Finally this solution was dialyzed against deionized water, freeze-dried, and dissolved in neutral phosphate-buffered pyrogen-free saline at 10 mg/ml and stored below —20° under toluene in a serum bottle as "NDOT" (from DOT) or "NPPD" (from PPD).

Serologic testing. The quantities of serologically active polysaccharide (PS) and phosphoglycolipid (PL) were determined by passive hemagglutination inhibition (PHAI) tests employing the microtiter technique. The antisera were prepared in New Zealand albino rabbits against living *M. bovis* BCG[3]

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[2] Kindly supplied by Lederle Laboratories, through the courtesy of Dr. Murray S. Cooper, as 4× concentrated OT.

[3] Kindly provided by the Tice Laboratories, University of Illinois, Chicago, through the courtesy of Dr. Sol Rosenthal, as "BCG vaccine."

introduced twice by intravenous route.

Sensitization of sheep red cells (RBC). RBC sensitized with PS: RBC were sensitized with phenol-soluble fraction of OT (Fraction PS, Lab No. 2725-28, Fr. II Lederle Laboratories) (1), by using 200 μg of PS per ml of 0.5% of RBC suspension in buffered saline and incubating at 37° for 1 hr.

RBC sensitized with PL: RBC were sensitized with a partially purified PL fraction from hot methanol extracts of BCG by using 40 μg of PL/ml of 0.5% RBC suspension and incubating at 37° for 1 hr. This fraction was eluted from a silicic acid:celite (2:1) chromatographic column with chloroform:methanol (1:1) and corresponds to a mixture of phosphoglycolipids described by Pangborn (2), and confirmed in these laboratories, as containing the serologically active phosphoglycolipids.

The buffer solution employed was one which has proved to be satisfactory for avoiding complement-dependent hemolysis (with ethylenediaminetetraacetate) and esterase-dependent deacylation of PL (with phospholine iodide[4]). The PHAI tests were carried out in the usual fashion, with 2-fold dilutions of material to be tested against a constant amount of antiserum being allowed to react at room temperature for 30 min before addition of sensitized RBC and standing at 4° overnight. PPD, NPPD, DOT, and NDOT were compared with purified PS and PL.

Skin tests. The tuberculin preparations employed in the animal studies reported here were anlayzed[5] for nitrogen content, with the following results: PPD, (Lederle) 10.0%, NPPD, 11.5%; DOT, 8.0%; NDOT, 11.1%. For the tests on human subjects PPD (Parke, Davis) was employed along with the same DOT and NDOT described above.

Tuberculin preparations were diluted for all skin-testing in pyrogen-free "Travenol" sterile isotonic saline containing 0.3% phenol and 0.0005% Tween 80.

The sites for multiple injections in animals or human subjects were randomized, and on human subjects, test sites on the same forearm were selected at least 5 cm apart. All skin tests were read blind or double-blind.

Albino Hartley strain guinea pigs weighing 500–900 g were used. Infection with virulent *M. tuberculosis* H37Rv was aerogenically induced with small numbers of infectious units (15–40 primary tubercles on sacrifice) and they were skin-tested on the flanks at 26 days after infection. Electric clippers were used to remove the hair from the sites for injections.

Results. Tuberculin preparations. Three different lots of 4× concentrated OT have been periodate-oxidized and extracted as described above, and quantitative PHAI titrations revealed that such treatment reduced the serologically active PS concentration at least 100-fold. The preparation (Lederle lot No. 2723-85) used in the skin-test studies described here contained approximately 15% of PS before treatment (DOT) and less than 0.1% after treatment (NDOT 102368). DOT proved to contain about 0.8% of PL and NDOT, less than 0.01% of such.

The concentration of PS in the preparation of PPD[6] used in the studies reported here, proved to be 2% before treatment and less than 0.1% (the minimum detectable) after periodate-oxidation. The same PPD contained about 0.8% of Pl, and the NPPD, less than 0.01% of PL.

Several experiments have shown that the phosphoglycolipids are more resistant to periodate-oxidation than the polysaccharide in tuberculin.

The total yield of oxidized or oxidized and extracted products (NPPD or NDOT) was 50–70% of the starting material. These products were always less amber than the original substrates and they required some electrolyte (NaCl with some sodium bicarbonate) for ready solubility in water. They always gave limpid solutions of low viscosity.

[4] Generously supplied by Ayerst Laboratories, through the courtesy of Mr. B. Mollov.

[5] Kindly performed by Dr. Percy Minden of the Scripps Clinic and Research Foundation, LaJolla, California.

[6] Provided as NIH Research Material (PH 43-68-1742-Lederle) and containing 30,000 "tuberculin units" per mg.

Skin tests on guinea pigs. In Table I are presented the results of some skin tests on guinea pigs. They reveal no significant differences in the reactions of erythema between DOT and NDOT at 24 hr. Observations at earlier and later times also revealed no important differences. Similarly, other characteristics of the reactions in these and other animals previously sensitized with *M. tuberculosis* or BCG vaccine, such as induration, central blanching, etc., were not seen to differ. Induration is not recorded in Table I because it is not precisely measurable in guinea pigs. Diluent controls were always negative.

Skin tests on tuberculosis patients. In Table II are presented some results of skin testing of 13 patients with bacteriologically proved active or recently active (within the previous year) pulmonary tuberculosis. It is clear that the diameter of the indurated area at 48 hr is slightly larger with DOT than with NDOT. On the basis of the evidence (3) that the diameters of intradermal tuberculin reactions are proportional, within limits, to the logarithms of the doses, then, on a weight basis, NDOT was calculated to have, in these patients, 65–75% of the induration-inducing activity of DOT. However, in a few of these patients NDOT was seen to elicit much less erythema than DOT. This is strikingly evident in patient W. F.: an area of intense and painful erythema, at least 50 mm in diameter, surrounded with indurated reaction site where DOT had been injected, while no trace of such a reaction was evident about the site of reaction to NDOT.

TABLE I. Skin Reactions to PPD, NPPD, DOT, and NDOT in Guinea Pigs after Aerogenic Infection with *M. tuberculosis* H37Rv.

Guinea pig no.	5 μg			
	PPD	NPPD	DOT	NDOT
19	18[a]	13	15	10.5
20	13	13.5	11	10.5
21	15.5	19	13	19
22	19	13.5	9.5	12
23	11.5	14.5	11	12.5
24	11	13	11	11.5
25	12	12	11	10
26	12.5	13.5	12	13.5
27	15.5	14	13	13.5
44	12.5	15.5	15	14.5
78	12.5	13	11	13.5
79	11.5	12	12	14
103	11	11	13	11
113	11	11	13.5	15.5
114	12	12	10.5	12.5
116	10.5	13.5	14	13.5
117	12	11.5	12.5	12.5
Averages	13.0	13.3	12.2	12.9

[a] Diameter of erythema in millimeters at 24 hr.

TABLE II. Skin Reactions to DOT and NDOT in Patients with Active or Recently Active Pulmonary Tuberculosis.

Patient init.	0.2 μg		Patient init.	0.02 μg	
	DOT	NDOT		DOT	NDOT
T.P.	27/27[a]	22/22	A.S.	25/20	18/15
E.M.	18/18	18/18	J.B.	0/25	0/23
L.W.	35/18	30/18	A.St.	17/17	12/12
A.S.	70/15	55/12	W.J.	17/17	16/16
M.S.	20/20	15/15	L.W.	16/12	15/13
W.F.	50/20	15/15	D.M.	14/14	10/10
			F.T.	20/14	17/15
Av (indur.)	19.7	16.7		17.0	14.9

[a] mm Erythema/mm Induration, at 48 hr.

The results of testing another group of 18 tuberculous patients with PPD and NPPD are shown in Table III.

Skin tests on healthy human subjects. In Table IV are shown the results of tests on seven healthy individuals who gave some reactions to the one or more of the reagents tested. None of these individuals had a history of tuberculosis and their chest roentgenograms had always been read as "normal." It will be noted that the first four individuals listed gave reactions of erythema with or without induration to either PPD or DOT or both, but no reactions of induration to NDOT. Two responded to NDOT with significant erythema, but the boggy induration which was observed at the sites of their reactions to PPD at 48 hr were absent from the sites of reaction to NDOT.

A striking observation was made regarding subjective sensations at the reaction sites:

TABLE III. Skin Reactions to PPD and NPPD in Patients with Active or Recently Active Pulmonary Tuberculosis.

	0.1 μg			
	PPD		NPPD	
Patient init.	24 hr	48 hr	24 hr	48 hr
J.J.	0/11[a]	0/13	0/12	0/13
M.G.	11/11	10/10	12/12	14/14
D.N.	14/14	13/13	17/17	12/16
J.B.	13/13[b]	13/13[b]	13/0	13/0
W.S.L.	?/17	0/14	?/17	0/13
J.M.	18/18	12/12	17/17	15/15
O.A.	0/18	0/16	0/19	0/15
C.W.M.	13/13	12/12	11/11	13/13
W.J.	9/9	9/9	10/10	10/10
E.W.	11/11	11/11	11/11	11/11
R.F.	14/14	11/11	12/12	12/12
J.H.	18/18	16/16	18/18	14/14
J.M.	11/10	10/10	14/14	12/12
J.L.S.	11/11	14/14	12/12	9/9
G.J.	0/17	0/18	0/17	0/15
H.R.	0/11	0/12	0/11	0/12
M.B.	14/14	25/14	15/15	11/11
J.M.[c]	3 mm (ecchymosis)		8/0	5/5 (ecchymosis)
Av (indur.)	12.8	12.1	12.5	11.7

[a] mm Erythema/mm Induration.
[b] Very slight induration.
[c] Under steroids.

sensations of pain or itching, or both, were reported by all seven of these subjects at the sites of injection of PPD and DOT, but only one subject (R. B.) reported such at the site of injection of NDOT, and it was said to be mild relative to the sensations of itching and pain at the other sites.

In previous tests, subjects B. S. and Z. R., had similarly given reactions of erythema, without induration, to PPD and no reactions to NDOT prepared from a different lot of DOT.

Discussion. Periodate oxidation of polysaccharides in polysaccharide–protein complexes or mixtures has previously been described (4). It has not been applied before to tuberculins. Results of the studies reported here show that the periodate oxidation of tuberculin can remove both polysaccharide and phosphoglycolipid, either before (DOT) or after (PPD) precipitation from Old Tuberculin. It is also shown that this modification does not significantly modify its activity in eliciting skin reactions in guinea pigs rendered hypersensitive by infection with living *M. tuberculosis*.

On the other hand, these preliminary studies show that some human beings, both those with active or recently active pulmonary tuberculosis and healthy individuals, may not give the same responses to these preparations. This is particularly striking with respect to erythematous reactions, with or without induration, which are much more frequently encountered with PPD or DOT than with the oxidized product. One would be tempted to conclude that the presence of serologically active polysaccharides and, or phosphoglycolipids, in the former and their absence in the latter may explain this difference. If this be so, however, the reactions of

TABLE IV. Skin Reactions to PPD, DOT, and NDOT in Healthy Human Subjects.

		0.2 μg					
		PPD		DOT		NDOT	
Subject	Sex, age	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
B.S.	F, 23	12/0[a]	11/0	12/0	14/0	0/0	0/0
Z.R.	F, 34	15/0	9/0	15/0	9/0	12/0	2/0
E.R.	F, 48	—	35/35	—	—	—	22/0
F.M.	M, 44	—	40/40	—	—	—	25/0
R.B.	M, 31	15/15	25/20	35/25	35/25	18/25	16/16
R.H.	M, 18	15/15	16/16	15/15	20/18	16/15	16/16
R.A.	M, 30	15/15	25/25	14/14	15/13	13/13	9/7

[a] mm Erythema/mm Induration.

erythema cannot be attributed to antigen determinant groups on these substances, *per se,* because it seems unlikely that they alone would elicit reactions in hypersensitive individuals in the very small amounts (0.001–0.03 μg) present in the applied doses of untreated tuberculins. Therefore, at least two other possibilities suggest themselves. Either the reactions elicited by PPD or DOT and not by NDOT are referable to immediate or delayed-type responses to determinant groupings of protein-polysaccharide or protein-phosphoglycolipid complexes which are not represented in the proteins alone; or, some of the protein (or polypeptide) molecules are also modified by periodate-oxidation, thus losing their activity in contributing to the erythematous response in some humans.

In any event, it is clear that the responses of guinea pigs and human beings to some of the antigenic groupings in tuberculin are distinctly different in a qualitative sense.

Finally, these studies show that crude tuberculoprotein preparations can easily be freed of immunologically active, nonprotein antigens without loss of thier ability to induce the responses characteristic of delayed-type hypersensitivity in tuberculous guinea pigs and human beings. Their possible uses in experimental laboratory investigations and diagnostic or epidemiologic studies remain to be explored.

Summary. Periodate oxidation of tuberculin preparations destroys the serologically active polysaccharide and phosphoglycolipid which they contain. This does not modify the biologic activity of such tuberculins as measured by skin responses in tuberculin-hypersensitive guinea pigs; but it does modify the characteristics of the skin response in some human beings, especially nontuberculous, tuberculin reactors.

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