Phosphatide Antigens of Mycobacteria. (30456)

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Several years ago, Boquét and Nègre(1) and recently Weiss and Dubos(2) reported that methanol extract of *Mycobacterium tuberculosis*, "antigène méthylique," is antigenic in animals. Lederer(3) identified a phosphatidyl inositotrimannoside in an ether-insoluble phosphatide fraction of "antigène méthylique" which appeared to have antigenic activity. Takahashi and coworkers(4, 5) in an extensive study detected antibodies against phosphatides from strains H37 Rv and BCG of *M. tuberculosis* in sera from tuberculous animals and humans. Subrahmanyam *et al*(6) examined 125 samples of tuberculous sera for antiphosphatide antibody titer and reported that intact phosphatide moiety is essential for the serologic reaction, and the antigens are present in virulent as well as avirulent strains of mycobacteria. The present paper deals with the isolation and identification of phosphatide antigens from certain virulent, avirulent and saprophytic strains of mycobacteria.

Material and methods. Large quantities of *M. tuberculosis* strains H37 Rv, H37 Ra, and *M. avium* were grown for 4 weeks in a number of 4-liter Haffkine flasks containing modified Youmans and Karlson medium(7). M. 607 and *M. phlei* were grown for 4 days in the same medium. Methods for inoculation, culture of microorganisms and isolation of lipids were essentially as described previously(7,8). A minor modification in the procedure for isolation of lipids was the omission of the acetone wash step. Instead, the bacilli collected by centrifugation after washing with cold, deionized water were transferred to a quickfit round bottomed flask containing acetone and dried under reduced pressure below 50°C by a rotary evaporater. The dried bacilli were taken up with chloroform-methanol (2:1) for lipid extraction(8). Neutral lipids and pigments were separated from the lipid extracts by silicic acid column chromatography using chloroform, and chloroform-acetone (1:1) as eluants. Phosphatides were then eluted from the column with chloroform-methanol (1:1) and methanol. For separation of different phosphatides, the phosphatide fraction was rechromatographed on fresh silicic acid columns with suitable aliquots of chloroform-methanol (98:2, 19:1, 17:3, 3:1, 3:2 and 1:4) as eluants. The different phosphatides in the 6 fractions (fractions I to VI corresponding to the 6 different concentrations of methanol in chloroform eluants) were identified with marker lipids and other analytical methods detailed elsewhere(7,8). Deacylation of the lipid samples was carried out according to the method of Ballou *et al*(9). The method of Hanahan and Olley(10) was used to estimate α-linked glycerol. Mannose in the deacylated products was estimated according to the anthrone method of Johanson(11). The deacylated products were hydrolyzed under reflux for 10 hours with 1 N HCl and mannose in the hydrolysate was detected by paper chromatography with isopropanol-pyridine-water-acetic acid (8:8:4:1) as developing solvent and sodium periodate-benzidine as dip reagent(12) for detection. For estimation of inositol, the lipid samples were hydrolyzed for 48 hours with 6 N HCl at 120° in sealed tubes. The hydrolysate after a preliminary extraction with ether was evaporated to dryness free of HCl, dissolved in an aliquot of water and inositol in the aliquot was separated by paper chromatography with isopropanol-pyridine-water-acetic acid solvent. The inositol position on the chromatogram was detected by spraying marker inositol strip with alkaline-silver nitrate reagent and inositol was eluted from the paper with water. Inositol in the eluates was determined by the periodate oxidation method of Agranoff *et al*(13). Other analytical methods employed in the detection of lipids were as described elsewhere(7). Sera from patients with advanced tuberculosis were obtained from

TABLE I. Kaolin-Agglutination with Phosphatides of Different Species of Mycobacteria.

S. No.	Species	No. of tuberculous sera examined	Antibody titer 8	16	32	64	128	256	512
1.	H37 Rv	18				7	9	2	
2.	H37 Ra	18				7	9	2	
3.	*M. avium*	18				8	8	2	
4.	*M. phlei*	22			2	10	8	2	
5.	M. 607	22			2	10	8	2	
6.	Antigen of Takahashi	22			2	10	8	2	

Silver Jubilee Tuberculosis Hospital, Delhi. The kaolin-agglutination procedure of Takahashi(14,15) was employed for detection of antibodies in sera with purified phosphatides of mycobacteria as antigens. The procedure involved sensitization of kaolin particle suspension with test antigen and treatment of sensitized particles with tuberculous serum. The antigen consisted of a methanolic solution of purified phosphatides at a concentration of 14 μg of lipid phosphorus per ml. Ovolecithin was not included in antigen solutions. The results were interpreted as very strongly positive (+++), strongly positive (++), positive (+) and negative depending on the extent of agglutination. The lowest serum dilution giving a positive reaction was taken as the titer of the serum.

Sera from patients suffering from asthma, chronic bronchitis and bronchiectasis were obtained from the Vallabhbhai Patel Chest Institute Clinic, Delhi. The samples were examined by the kaolin-agglutination test for cross reactivity with purified phosphatide antigens.

Results. The total phosphatides isolated from *M. phlei* and M. 607 saprophytic strains were examined for the presence of phosphatide antigens by the kaolin-agglutination technique with tuberculous sera. The serum samples were simultaneously tested with phosphatide antigens from H37 Rv, H37 Ra, *M. avium* and the antigen of Takahashi for comparison. The antigen of Takahashi, supplied by Dr. Setsuo Sakai, Daiichi Seiyaku Co. Ltd., Tokyo, was essentially a total phosphatide fraction of H37 Rv or BCG extracted by a different procedure(17) than the one used in the present investigation. Results on the agglutination reaction, presented in Table I, reveal that the antigens are present in saprophyte strains as well as *M. tuberculosis*.

Phosphatides from H37 Rv, H37 Ra, *M. avium*, M. 607 and *M. phlei* were prepared as described and then each of the factions collected (fractions I to VI) was tested with serum from tuberculous patients by the kaolin-agglutination reaction. Major lipids of fractions I, II and III were identified using marker lipids or chemical analysis wherever necessary as phosphatidic acid (trace amounts), polyglycerolphosphatide(6) and phosphatidyl ethanolamine(16) respectively. These fractions did not agglutinate kaolin particles in presence of tuberculous sera. Fractions eluted with chloroform-methanol, 3:1, 3:2 and 1:4 (fraction IV, V, VI) gave strong agglutination reactions. The purity of the fractions was assessed on silicic acid impregnated papers using diisobutylketone-acetic acid-water, 40:25:5, as developing solvent. On rhodamine 6G staining it was found that fractions IV, V and VI contained mixture of lipids. Fraction IV comprised of lysophosphatidyl ethanolamine(8), phosphatidyl inositol and a third component (Rf 0.26). Fraction V contained 3 lipids (Rfs. 0.12, 0.21, 0.26) one of which had the same Rf as the third component of fraction IV (Rf 0.26). Fraction VI had 2 lipids which corresponded with the other 2 fractions of fraction V (Rfs. 0.12, 0.21). Rechromatography of the fractions on silicic acid columns did not result in any better separation. Therefore, each of the lipids in the 3 fractions was obtained in pure form by chromatography on a number of silicic acid impregnated papers. 0.5-0.8 mg of lipid was applied in streaks on the papers and developed with diisobutylketone-acetic acid-water. The position of the individual phosphatides on papers

was identified by developing a narrow marker strip with rhodamine 6G stain. The separated lipids were eluted from the paper with ethanol-chloroform-water (5:2:2) and the corresponding phosphatides were pooled together. The final pools were evaporated to dryness, dissolved in an aliquot of chloroform and tested for purity by rechromatography. Each of the purified lipids was then examined in the kaolin-agglutination reaction. Lysophosphatidyl ethanolamine and phosphatidyl inositol gave negative results when tested for agglutination while the 3 other lipids showed strong activity. Estimation of ester value, α-linked glycerol, phosphorus, mannose and inositol of the active lipid fractions revealed that they were phosphatidyl inosito-mono (Rf 0.26), di (Rf 0.21) and penta (Rf 0.12) mannosides having Rf values of 0.26, 0.21 and 0.12, respectively, as characterized by Ballou *et al*(9). Seventeen sera from patients with asthma, bronchitis, bronchiectasis did not show evidence of presence of antibodies when examined with the purified mannosides from mycobacteria.

Discussion. Anderson *et al*(18) found that polysaccharides from human tubercle bacilli contain firmly bound lipids which are strong antigens. Several other components of tubercle bacilli have been shown(19) to elicit antibody production in tuberculous hosts although none of the components so far identified is capable of producing immunity against tuberculosis. Three different types of antibodies(4,5,17) have been identified in sera from patients suffering from tuberculosis each being directed specifically against proteins, polysaccharides and phosphatides of the bacilli. Antiphosphatide antibody titer of sera, unlike that of antiprotein and antipolysaccharide antibodies, appears to reflect the progression of tuberculosis(4,5). The present investigation reveals that even saprophytic strains such as M. 607, *M. phlei* possess phosphatides capable of reacting readily with the corresponding antibodies in tuberculous sera. The phosphatides responsible for this serologic reaction are phosphatidyl inositomannosides characterized by Ballou *et al* (9). Further, the chemical nature of the major phosphatides of the 5 strains of mycobacteria investigated here does not differ significantly. However, it is to be noted that only the non-fatty acid portions of the phosphatides have been characterized in the present study. It would be of considerable interest to identify the nature of fatty acid moieties of the phosphatides of virulent and avirulent strains of *M. tuberculosis* in order to assess the role, if any, of phosphatides of the bacilli in the pathogenicity of tuberculosis.

It seems likely that these lipids act as haptens. Phosphatidyl inositol itself had no activity in the serologic reaction but its linkage with one or more mannose units appears to make it haptenic in character. This is analogous to the glycosphingolipid of human epidermoid carcinoma investigated by Rapport *et al*(20) where the glycolipid was hapten while the ceramide had no such activity. Evidence is accumulating of the immunogenic nature of glycolipids. Most lipid haptens so far investigated contain at least one molecule of a sugar covalently linked to the lipid portion of the molecule. It has been reported(21) that Forssman hapten is glycosphingolipid in nature. Brady *et al*(22) detected antiganglioside antibodies in serum of some cases of multiple sclerosis.

The specificity of the phosphatide-antiphosphatide reaction suggests that phosphatidyl inositomannosides of mycobacteria may be of considerable value in the serodiagnosis of tuberculosis.

Summary. The nature of phosphatide antigens of 5 species of mycobacteria has been investigated by the kaolin-agglutination technique of Takahashi using serum from tuberculous patients as antibody source. The phosphatide antigens are present in virulent (H37 Rv, *M. avium*), avirulent (H37 Ra) and saprophyte (M. 607, *M. phlei*) strains of mycobacteria. The phosphatide antigens have been purified by silicic acid column and paper chromatography and identified as phosphatidyl inositomannosides.

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Physiologic and Morphologic Studies on Atherosclerotic Pigeons.* (30457)

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White Carneau pigeons develop both aortic and coronary artery atherosclerosis in their natural environment on grain diets(1). The lesions begin developing as microscopic "fatty streaks" in the aortas of birds 2-3 weeks of age(2). By 3 years of age at least 95% of the birds have aortic lesions. Coronary artery atherosclerosis occurs in about 75% of mature White Carneau pigeons(3). Myocardial infarction has been reported in birds over 10 years of age(4). Distinct variations can be seen in the extent of both aortic and coronary artery atherosclerosis among mature birds. The purpose of this paper is to report the results of an experiment designed to determine the importance of blood pressure, serum cholesterol, and serum triglycerides in accounting for this variability seen in mature birds.

Materials and methods. One hundred and forty-six White Carneau pigeons were obtained from the Palmetto Pigeon Plant at Sumter, S. C. Their mean age was 8 years; 63 were male and 83 were female. The birds had been maintained by Palmetto Pigeon Plant from hatching until they were obtained for the experiment. During this period they were fed a free choice of corn, milo, peas, and wheat, along with a grit supplement. They were maintained in a similar fashion in our laboratory for periods up to one month.

White Carneau pigeons are quite docile and it was possible to obtain blood pressure measurements without general anesthesia. The birds were restrained on their backs by secur-

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