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Anti-Cardiolipin and Anti-Phosphatidylglycerol Antibodies Prepared Against Bacterial Phospholipids

AUGUST J. DESIERVO

Department of Microbiology, University of Maine, Orono, Maine 04473

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Anti-phosphatidylglycerol and anti-cardiolipin antisera were prepared in rabbits by using phospholipids purified from *Micrococcus lysodeikticus*. Anti-phosphatidylglycerol antibodies were found in antisera when either phosphatidylglycerol or cardiolipin were used as immunogens, but adsorption studies indicated they were not similar. Antibodies which reacted with phosphatidylinositol and phosphatidic acid were also found in the anti-cardiolipin antiserum. Structures of the antigenic groups in phosphatidylglycerol and cardiolipin are suggested from cross-reaction and adsorption studies. Adsorption studies with pure phospholipid antigens indicated the importance of the spacial orientation of phospholipid haptens for immunological reactivity.

The immunological reactivity of cardiolipin (CL), an acidic phospholipid which reacts with the Wassermann antibody found in syphilitic serum, has been extensively studied (4-6). However, a closely related phospholipid, phosphatidylglycerol (PG), the sole precursor of CL in bacteria as shown by DeSiervo and Salton (1) and found in relatively large amounts in bacterial membranes, had not been studied immunologically. The nature of the determinant antigenic groups of CL and PG haptens, purified from lipid extracts of *Micrococcus lysodeikticus*, has been explored in this paper with cross-reaction and adsorption studies. Moreover, the phospholipids of *M. lysodeikticus* having over 90% saturated C_{15} branched-chain fatty acids make them considerably different in fatty acid composition from beef heart CL (4, 10) although this difference had no apparent effect on antibody production. The absence of any effect on immunological activity due to the fatty acid composition of CL had previously been observed with synthetic CL (4). The use of antisera against the major membrane components, protein and phospholipid, in taxonomic studies and in elucidating phospholipid arrangement in membranes has proved feasible (3, 10). The antisera produced in this study apparently have several classes of anti-phospholipid antibodies and with sufficient characterization may be employed as highly specific tools in probing phospholipid structure and orientation in biological membranes.

MATERIALS AND METHODS

Preparation of phospholipids. The phospholipids CL, PG, and phosphatidyl inositol (PI) were isolated and purified from *M. lysodeikticus* as described by DeSiervo and Salton (1). Phospholipid extracts were separated by silica gel impregnated paper chromatography and stored in chloroform at −80 C. Phosphatidic acid (PA) was obtained commercially (General Biochemicals) and purified further by paper chromatography before use. Lecithin and cholesterol were obtained commercially (Sigma Chemical Co.).

Antisera production. CL immunizing antigen was prepared as described by Inoue and Nojima (5). CL antigen emulsion, consisting of CL from *M. lysodeikticus*, lecithin, and cholesterol in the ratio of 1:10:30 (wt/wt/wt), was prepared according to the method of the Venereal Disease Research Laboratory (VDRL) (9). The antigen emulsion was centrifuged, and the pellet was resuspended in 1.0% methylated bovine serum albumin (Sigma Chemical Co.), stored overnight at 4 C, and used for rabbit immunization. PG from *M. lysodeikticus* was substituted for CL in the preparation of PG immunizing antigen with the subsequent procedure remaining identical. Freshly prepared suspensions of the immunogens were injected intravenously every other day for 21 days into rabbits. Two rabbits were used for each immunogen. Serum samples from each rabbit were obtained prior to immunization. Each rabbit received a total of 2 to 4 mg of CL or PG. One week after the last injection, the rabbits were bled by heart puncture. The sera were prepared and stored at −80 C.

Examination of control and immune sera. Quantitative VDRL microflocculation tests (8) were carried out in tubes by using 0.1-ml volumes of serum serially diluted in 0.9% saline. VDRL slide test emulsions were prepared with CL, PG, and PI purified from *M. lysodeikticus* and from commericially obtained PA. They were diluted 1:5 with 1.0% saline added to each tube. The tubes were manually shaken for 4 min, and samples from each tube were examined at ×100 magnification. The reactivities of the sera were graded from 0 to +4. The absence of aggregates was indicated as 0, granular appearance as +1, small

aggregates as +2, medium-sized aggregates as +3, and large aggregates or complete aggregation as +4. The titer of the sera was recorded as the highest dilution showing a +1 reaction.

Adsorption of antisera with phospholipid haptens. A 5-ml amount of the VDRL antigen emulsion, equivalent to 0.15 mg of phospholipid hapten, was prepared for each phospholipid tested. The emulsion was centrifuged at 12,000 × *g* for 30 min. The pellets were resuspended in 1.25 ml of serum, allowed to react for 10 min, and centrifuged for 30 min at 12,000 × *g* to remove the emulsion or hapten-antibody complexes, or both, from the serum. The sera were subsequently membrane filtered (Millipore Corp.) to remove any remaining particles. The antisera were then tested for reactivity as described above.

Preparation of pure PG and CL emulsions. Chloroform solutions of PG and CL were dried under a stream of nitrogen and suspended in buffered saline (7) by sonic treatment. The emulsion was centrifuged at 12,000 × *g* for 1 h, and the supernatant was discarded. The emulsion pellets were resuspended in their respective antiserum and left for 10 min. The emulsion and emulsion-antibody complexes were removed from the sera by centrifugation followed by filtration.

RESULTS

Production of antibody against cardiolipin and phosphatidylglycerol. The production of anti-CL and anti-PG sera in rabbits, employing the immunological procedures described by Inoue and Nojima (5), is shown in Table 1. The antisera of rabbits immunized with CL antigen had titers against CL similar to those previously reported (3, 5). In addition, the anti-CL antisera also cross-reacted with PG antigen at a considerably lower titer. The antisera of rabbits immunized with PG antigen also had anti-PG activity with titers similar to those found in the anti-CL antisera. However, there was no cross-reaction with CL antigen. Since this study appears to be the first report of anti-PG antibodies, the possibility of nonspecific reactions with PG antigen was tested. Pre-immunization serum samples from all rabbits were tested with CL and PG antigens as well as with PI and PA antigens. No antibody activity against these phospholipids was found.

TABLE 1. *Production of anti-CL and anti-PG antisera in rabbits*

Antigen	Antibody titer by the microflocculation test[a]			
	Anti-CL antisera		Anti-PG antisera	
	Rabbit		Rabbit	
	1	2	3	4
Cardiolipin	1:64	1:64	<1:2	<1:2
Phosphatidylglycerol	1:16	1:8	1:8	1:8

[a] Titer of each rabbit prior to immunization was <1:2.

Cross-reaction of anti-CL and anti-PG antisera with other phospholipids. The finding of anti-PG activity in the anti-CL antiserum led to examining these sera for additional antiphospholipid activity. Anti-CL and anti-PG antisera were tested with PI and PA. Results with untreated anti-CL antiserum are shown in Table 2. Titers were obtained with both PI and PA. However, there was no cross-reaction of PI and PA with anti-PG antiserum. The level of activity found with PI and PA antigens in the anti-CL antiserum was similar to that found with PG antigen.

Adsorption of anti-CL and anti-PG antisera with homologous and heterologous haptens. The observation that anti-CL antiserum contained other than anti-CL activity led to adsorption studies with homologous and heterologous antigens in order to determine whether more than one class of antibodies were present in the antiserum. Anti-CL and anti-PG antisera were adsorbed with CL, PG, PI, and PA antigens (Table 2). The anti-PG antiserum activity was adsorbed only by its homologous antigen. Its titer was unchanged by adsorption with CL, PI, or PA. The results with the anti-CL antiserum were more varied. Anti-CL activity was only adsorbed by its homologous antigen. The anti-PG activity was adsorbed by both CL and PG but not by PI or PA antigens. Both the PI and PA reactivity was removed by all of the antigens used. Some residual anti-PA activity remained after adsorption with PG and PI. The degree of flocculation was very low (+1) even at the 1:2 dilution. Although the anti-PI and anti-PA activity appears to be similar, there may be some specific anti-PA antibodies with which PG and PI antigens are unable to react.

These results indicate that the anti-CL serum contained at least three classes of antibodies, all of which were adsorbed from the serum with CL antigen. The data suggest that different parts as well as the whole CL molecule can function as the antigenic determinants for antibody production. These studies also indicate that the anti-PG antibodies found in the anti-PG antiserum are not identical to those found in the anti-CL serum.

Adsorption of antisera with pure homologous phospholipids. Pure CL and PG emulsions were prepared and used to adsorb their

TABLE 2. *Titers of untreated and adsorbed sera*

Antiserum	Antigens	Sera adsorbed with the following phospholipid antigens				
		Untreated	CL	PG	PI	PA
Anti-cardiolipin	CL	1:64	<1:2	1:64	1:64	1:64
	PG	1:16	<1:2	<1:2	1:8	1:8
	PI	1:16	<1:2	<1:2	<1:2	<1:2
	PA	1:16	<1:2	1:4	1:4	<1:2
Anti-phosphatidyl-glycerol	PG	1:8	1:8	<1:2	1:8	1:8

homologous antiserum. The concentration of phospholipid haptens (by weight) was over 16 times that found in the complex VDRL antigen preparations used in the preceding adsorption studies. There was a significant decrease in antibody titer from this treatment, although, under the same conditions of adsorption, the complex VDRL antigens adsorbed all the detectable antibody from the sera (Table 3).

TABLE 3. *Adsorption of anti-CL and anti-PG antisera with pure phospholipid haptens*

Dilution of antiserum	Quantitative VDRL microflocculation test[a]			
	Anti-CL antiserum		Anti-PG antiserum	
	Untreated	Adsorbed with CL	Untreated	Adsorbed with PG
1:2	+4	+4	+3	+2
1:4	+4	+3	+2	+1
1:8	+4	+3	+2	+1
1:16	+3	+2	+1	0
1:32	+3	+1	0	0
1:64	+2	0	0	0
1:128	+1	0		
1:256	0	0		

[a] Untreated and adsorbed antisera were tested only with their homologous antigens. 0 to +4 notation is described in Materials and Methods.

DISCUSSION

These studies have shown that it is possible to obtain anti-PG antiserum with PG hapten by using the same procedure employed to induce the formation of anti-CL antibodies. The fatty acid composition of the CL hapten from *M. lysodeikticus*, which primarily consists of C_{15} branched-chain fatty acids, had no apparent effect on the production of anti-CL antibodies. The anti-PG antibodies in the anti-PG sera were adsorbed only by their homologous antigen and not by CL, PI, or PA antigens. Cross-reactions with these phospholipid antigens were not observed. Further, these anti-PG antibodies were not the same as those produced when CL antigen was used as the immunogen as these antibodies were adsorbed by both CL and PG antigens. Additional cross-reaction and adsorption studies with PI and PA antigens suggested that anti-CL antiserum contained at least three classes of antibodies. One class apparently reacts with the entire CL molecule. A specific reactive site within CL cannot be defined since Inoue and Nojima (4, 6) have shown, in studies of Wassermann antibodies and antisera prepared against CL and CL analogues, that the diglyceride structure as well as the central hydroxyl group and two phosphate groups are important factors in the immunological reactivity of CL. A second class reacts with PG and cannot be adsorbed with PI and PA. A third class appears to react with PI and PA and can be adsorbed by CL and PG as well as PI and PA. The structures of the reactive sites to which these three classes of antibodies may be made are shown in Fig. 1 as A, B, and C, respectively. Structures B and C are both found as part of the CL structure (A) and would therefore explain the production of antibodies which react with similar structures found in other phospholipids when CL is used as the hapten.

A fourth structure is the PG molecule (Fig. 1D), which has two vicinal hydroxyl groups. This structure is not found as part of CL and might therefore illicit a fourth class of antibodies in which the vicinal hydroxyl groups play an important role. The PG molecule does contain structures B and C but no evidence of antibodies against these structures was found. This may result from the relatively low immunological response to PG antigen compared with CL antigen. However, it has been shown by Kataoka and Nojima (7) that antisera prepared against PI antigen did not cross-react with CL and PA, and by Fowler and Allen (2) that antisera prepared against PA did not cross-react

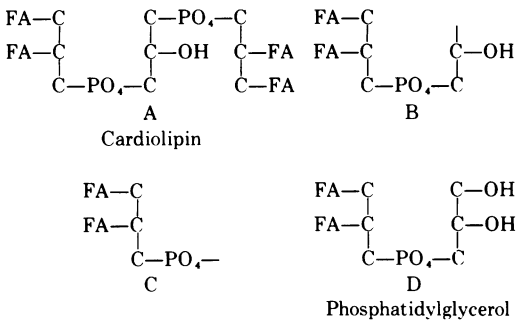

FIG. 1. *Diagramatic representation of four postulated structures of phospholipid antigenic groups. Structures A and D represent the entire molecules of CL and PG. Structure B is found as part of CL and PG. Structure C is found as part of CL, PG, PI, and PA. Hydrogen atoms are omitted from the structures for clarity. FA, Fatty acid residues.*

with CL even though structure C (Fig. 1) is common to all these phospholipids.

Adsorption studies using pure PG and CL emulsions were much less effective than VDRL antigen preparations in complexing with anti-PG and anti-CL antibodies even at considerably higher hapten concentrations. These results suggest the importance of the structural orientation of CL and PG hapten in complex lipid antigens.

These data suggest that antisera prepared against CL and PG may elicit several classes of antibodies which react with specific areas of phospholipid structure. Although this interpretation seems to fit best with the results of the adsorption experiments, other interpretations are possible. Further study is obviously necessary in this area to fully understand phospholipid determinants with the eventual possibility that these anti-phospholipid antisera could be used as tools in analyzing the accessibility and orientation of phospholipids on the surfaces of biological membranes.

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