

Reactivity Patterns of Human Anticardiolipin and Other Antiphospholipid Antibodies in Syphilitic Sera

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Sera from patients with proven cases of syphilis were tested for the presence of antibodies to structurally important phospholipids by using qualitative and quantitative assays. All 47 sera examined qualitatively contained antibodies to cardiolipin, phosphatidic acid, and phosphatidylserine, but not antibodies to other selected phospholipids. Such reactivity was not found in normal (Red Cross) sera. Although the degree of antibody binding to phospholipids varied in individual sera, reactivity was almost always greater with cardiolipin than with phosphatidic acid or phosphatidylserine. Binding saturability was found in sera as the cardiolipin concentration was increased over a constant area of nitrocellulose paper. Anti-cardiolipin binding measured by the protein A method gave results similar to the results measured by using anti-immunoglobulin G, which supports the conclusion that binding was to the F_{ab} portion of the immunoglobulin molecule. When measured as a function of serum concentration and plotted in double-reciprocal fashion, the anti-cardiolipin binding data for two syphilitic sera had similar K_d values but different B_{max} values. Stoichiometric calculations indicated that approximately 11,000 to 16,000 mol of cardiolipin appeared to be bound per mole of labeled second antibody. These observations may mean that the anti-cardiolipin antibody does not recognize the individual cardiolipin molecule as the antigenic site but recognizes some structural form of the phospholipid or that steric hindrance related to the interaction of the phospholipid with nitrocellulose paper prevented the bulk of cardiolipin molecules from reacting. The structural specificity of the antibodies identified excludes the possibility that these antibodies are directed against the phosphodiester linkage. These findings should give impetus to future study of a potential pathogenic or marker role for these antibodies in syphilis and in other syndromes in which membrane damage may be a primary event.

Cardiolipin (CL) was discovered in 1942 (14), and final identification of the structure of this compound was provided when diphosphatidylglycerol was synthesized and found to be identical to ox heart CL with respect to chromatographic behavior, melting point, optical rotation, and infrared absorption. The synthetic compound was also shown to substitute for CL in the serological tests for syphilis (2).

Although still used for screening purposes, flocculation tests have been replaced by more specific tests for treponemal antigens to confirm diagnoses of syphilis. In connection with false-positive tests for syphilis, there has been recent interest in anti-CL antibodies, which have been observed with increasing frequency in patients with central nervous system manifestations of systemic lupus erythematosus (SLE) and other clinical situations in which vascular thrombosis occurs (4, 5, 18). This new interest brings up the question of whether anti-CL antibodies in patients with syphilis are distinguishable from anti-CL antibodies in patients with other conditions. However, impeding investigations into this question is the fact that little is known about the reactivity of anti-CL antibodies in patients with syphilis at the molecular level, including the specificity of various portions of the phospholipid (PL) molecule, the physical form of the PL, the stoichiometry, whether the antibodies are directed against host or spirochetal CL, and whether the antibodies could play any pathogenic role, particularly against cell membranes.

In this paper we describe a method for examining anti-PL antibodies, both qualitatively and quantitatively in parallel

and in such a way as to study the headgroup specificity of the major PL classes. Binding curves were studied in detail, with both PL and serum concentrations as variables.

MATERIALS AND METHODS

The sera which were tested were obtained from normal blood donors (Red Cross, Buffalo, N.Y.) and from individuals with documented cases of syphilis (Erie County Health Department, Division of Public Health, Buffalo, N.Y.). The fluorescent treponemal antibody absorption test for syphilis was positive in each patient, and all sera were reactive in the Venereal Disease Research Laboratory flocculation test. Goat anti-human immunoglobulin G (IgG) and nitrocellulose membranes were purchased from Bio-Rad Laboratories, Rockville Center, N.Y. Goat anti-human IgG was purchased from Jackson ImmunoResearch Laboratories, Avondale, Pa. [^{14}C]acetic anhydride (30 to 40 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass. Anti-human IgA-, IgM-, and IgG-peroxidase conjugates, diaminobenzidine, palmitic acid, and the following PLs were purchased from Sigma Chemical Co., St. Louis, Mo., and from Avanti Polar Lipids, Birmingham, Ala.: CL (diphosphatidylglycerol), dicaproylphosphatidylcholine, dilauroylphosphatidylcholine, egg phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. Hydrofluor was supplied by National Diagnostics, Somerville, N.J.

Qualitative anti-PL antibody identification. A 40- μ g portion of each dried (N_2) lipid was dissolved in 5 μ l of chloroform and spotted onto nitrocellulose papers (3.5 by 8 cm). The papers were agitated in 3% gelatin blocking buffer (50 mM Tris hydroxide, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3 ,

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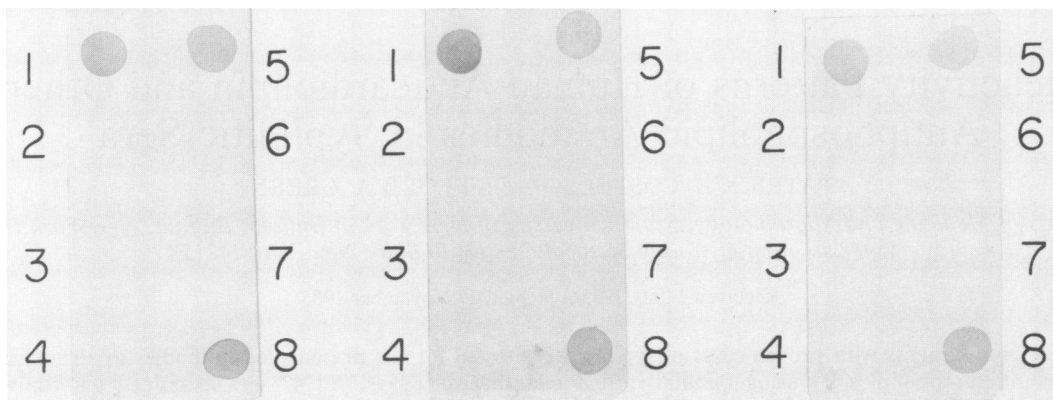


FIG. 1. Qualitative anti-PL antibody staining on nitrocellulose papers. Each paper contained the antibody reactivity in the sera (1:100) from three different subjects with syphilis. Portions (40 μ g) of PLs were spotted in 5- μ l volumes onto each paper as follows: spot 1, egg phosphatidic acid; spot 2, palmitic acid; spot 3, dicaproylphosphatidylcholine; spot 4, phosphatidylethanolamine; spot 5, beef heart CL; spot 6, dilauroylphosphatidylcholine; spot 7, egg phosphatidylcholine; and spot 8, bovine brain phosphatidylserine.

0.1% Tween 20, pH 7.4) for 1 h at 25°C. The papers were then incubated for 16 h in blocking buffer containing serum (1:100) or as indicated below. The strips were then washed three times in blocking buffer for 10 min and were incubated with goat anti-human IgG-peroxidase conjugate diluted 1:1,000 in phosphate-buffered saline at room temperature for 90 min. The papers were washed three times with phosphate-buffered saline, and color development was visualized after the papers were placed in a solution containing 50 mg of diaminobenzidine per 100 mg of phosphate-buffered saline and 0.04% H_2O_2 .

Quantitative anti-PL antibody identification. For experiments in which the serum (antibody) concentration was varied, nitrocellulose papers were cut into circles (diameter, 1 cm) and spotted with 65 nmol of PL. The circles and a blank were placed in 50-ml conical centrifuge tubes containing 4 ml of 3% gelatin blocking buffer and were incubated for 1 h in a 33° angle centrifuge rack. Syphilitic serum was added in amounts of 5, 10, 20, 30, 50, 70, and 100 μ l. For experiments in which the serum concentration was held constant, 3 to 136 nmol of CL was spotted onto the circles. After incubation for 60 min, normal or syphilitic serum diluted 1:50 in blocking buffer was added. The tubes were agitated for 16 h at 25°C in the angled racks. The papers were removed and washed three times for 15 min in 50-ml portions of blocking buffer containing decreasing concentrations of gelatin (3, 1, and 0.5%). The paper circles were reacted for 1 h with ^{14}C -labeled anti-human IgG diluted 1:200 in phosphate-buffered saline (pH 7.4) or for 2 h with ^{14}C -labeled protein A diluted 1:200 in 0.1 M barbital buffer (pH 8.6). The circles were washed three times and counted with a Tri-Carb model 460CD scintillation counter (Packard Instrument Co., Inc., Rockville, Md.) after addition of 10 ml of Hydrofluor. ^{14}C -labeled protein A was prepared as previously described (13). Goat-derived affinity-purified anti-human IgG was prepared from ^{14}C -labeled acetic anhydride in the same manner. The specific activity of the labeled antibody protein was measured and used to convert the number of disintegrations per minute to the number of moles of second antibody.

For most experiments the amount of antibody binding was plotted after subtraction of the number of background counts (nitrocellulose without PLs) for each experimental point. The binding of the labeled second antibody or labeled protein A to the nitrocellulose paper and the binding to the paper with PL but without human serum were negligible.

RESULTS

The qualitative assay for bound antibody to PLs in which an enzyme-labeled assay (peroxidase) was used to rapidly compare the reactivities of many lipids with the same serum. In contrast to sera from normal subjects, which in this system showed little or no reactivity, all of the sera from patients with syphilis ($n = 47$) contained antibodies to CL, phosphatidic acid, and phosphatidylserine but not antibodies to phosphatidylcholine (egg phosphatidylcholine or phosphatidylcholine with synthetic short-chain fatty acids), phosphatidylethanolamine, or a free fatty acid (palmitic acid) (Fig. 1). Therefore, CL, phosphatidic acid, and phosphatidylserine were used for quantitative measurements of antibody binding.

All of the binding study data below refer to individual sera from subjects with syphilis or normal subjects. No pooled sera were used. Figure 2 shows binding curves in which the amount of ^{14}C -labeled anti-IgG was measured as a function

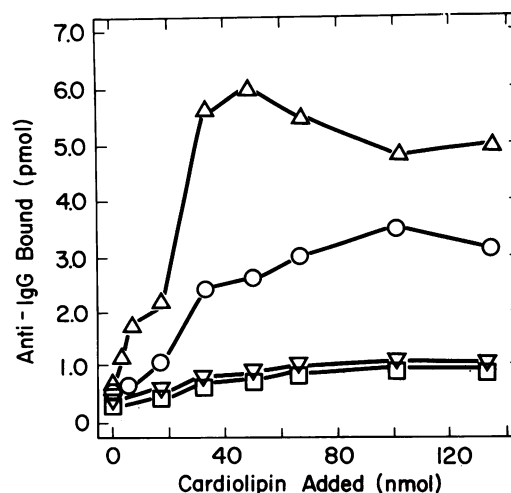


FIG. 2. Quantitative anti-CL binding in sera from two individuals with syphilis (Δ and \circ) and in sera from two normal individuals (∇ and \square). The labeled second antibody was added as described in Materials and Methods. The serum concentration was 1:50 in blocking buffer. CL was added in increasing amounts in a constant volume of 5 μ l.

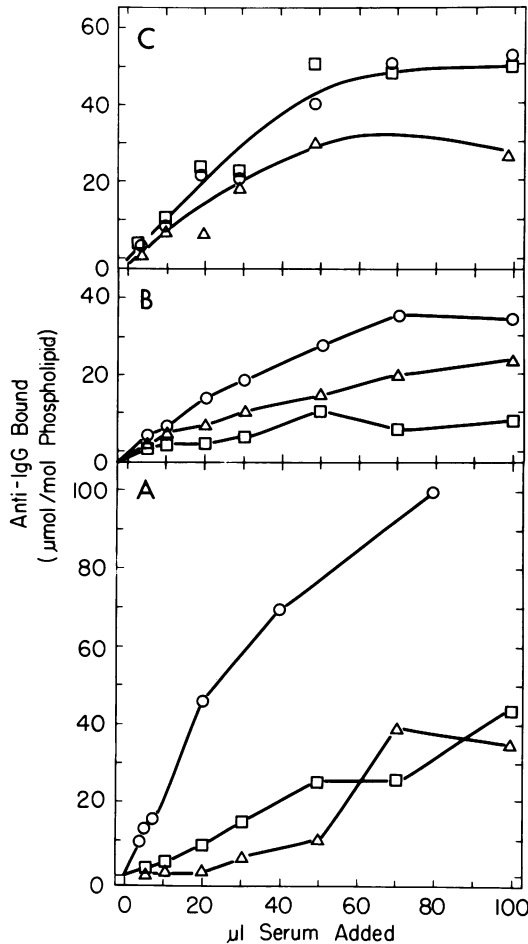


FIG. 3. (A) Antibody binding to different PLs in serum from a single syphilitic subject as a function of increasing serum concentration (increasing amounts of serum in a constant volume of 4 ml). Symbols: ○, CL; □, phosphatidylserine; △, phosphatidic acid. The PLs were placed on nitrocellulose papers in equimolar amounts. (B) Antibody binding to PLs in serum from a second syphilitic subject. The conditions used are described in the legend to Fig. 2. Symbols: ○, CL; △, phosphatidylserine; □, phosphatidic acid. (C) Antibody binding to PLs in serum from a third syphilitic subject. The conditions used are described in the legend to Fig. 2. Symbols: ○, CL; □, phosphatidylserine; △, phosphatidic acid.

of the amount of CL placed on the nitrocellulose papers (constant volume and constant area). With two syphilitic sera saturation was reached, but with the normal sera there was only a slight increase above the nonspecific binding level which was represented by the amount of antibody found when no CL was present. There was a slight tendency for some of the binding curves to decrease at higher CL levels; the significance of this observation is not clear.

Figure 3A shows the binding curves when another serum from a subject with syphilis was used. In this case the binding to CL was compared with the binding to phosphatidylserine and phosphatidic acid, which were placed in equimolar amounts on the nitrocellulose papers. The data were derived by using constant amounts of PLs, increasing amounts of serum, and a constant volume. The amount of binding was much less with phosphatidylserine and phosphatidic acid than with CL, with no clear tendency to plateau in any case. Figure 3B shows the results of an

experiment in which we studied the binding curves for another serum from a syphilitic patient in relation to CL, phosphatidylserine, and phosphatidic acid. Again the binding to CL was greater and appeared to plateau, whereas with the other two PLs such a trend was not obvious.

The data for serum from another syphilis patient are shown in Fig. 3C. In this case, the binding to CL was indistinguishable from the binding to phosphatidylserine, and the binding to both of these compounds was considerably greater than the binding to phosphatidic acid. This situation was quite unusual in our experience, but does illustrate a variation that could be significant. This finding could not be pursued because additional samples of sera from the same individuals were unobtainable. In most cases the CL binding far exceeded the binding to any other PL studied.

To further examine anti-CL reactivity, the levels of binding of two different syphilitic sera were studied by using two different compounds to measure binding, namely ¹⁴C-labeled IgG and ¹⁴C-labeled protein A. As Fig. 4 shows, peak binding levels varied from case to case, but there were consistent points of binding saturation around 60 nmol of added CL, with no substantial change thereafter. It is also apparent that the level of binding measured by using anti-IgG was greater. However, the plateau levels which were reached by each method differed by a factor of two or less.

Figure 5 shows the anti-CL binding curves for two additional sera from patients with syphilis. When antibody binding was plotted as a function of increasing serum concentration, the typical hyperbolic binding curve was evident. When the binding data were then linearized by using a double-reciprocal plot (Fig. 5, inset), the intercepts on the abscissa (apparent K_d) were about the same. However, the ordinate intercepts differed, indicating that there were differences in the B_{max} (reciprocal of moles of IgG bound per mole of antigen at saturation). Calculation of the apparent stoichiometry based on these intercepts gave values of approximately 11,000 to 16,000 mol of CL bound per mol of labeled anti-IgG antibody.

DISCUSSION

All 47 sera from patients with syphilis studied had demonstrable antibodies to CL in a system with no other lipids present. The majority also demonstrated antibody binding to

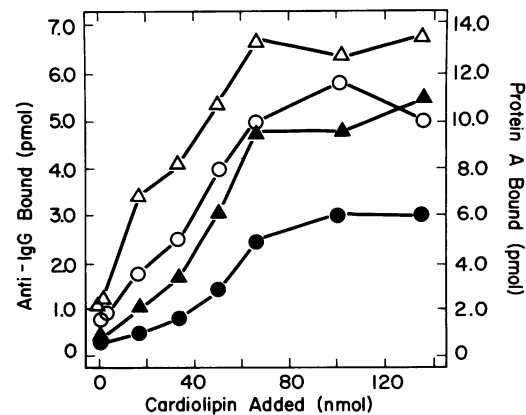


FIG. 4. Anti-CL binding in two syphilitic sera, as measured by the amount of bound goat ¹⁴C-labeled anti-IgG (○ and △) and by the amount of bound ¹⁴C-labeled protein A (● and ▲). The serum concentrations used were constant (1:50).

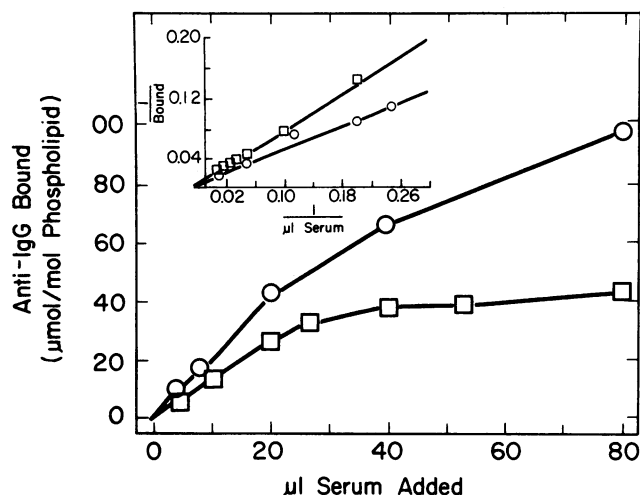


FIG. 5. Anti-CL binding in sera from two syphilitic subjects as a function of increasing serum concentrations. CL (65 nmol) was applied to nitrocellulose papers. The inset shows a double-reciprocal plot derived from the same data.

phosphatidic acid and to phosphatidylserine to lesser extents. No reactivity was found to egg or short-chain synthetic phosphatidylcholine, phosphatidylethanolamine, cholesterol, or fatty acids. We also found (data not shown) very little antibody activity toward phosphatidylglycerol. These results indicate that there was a high degree of structural specificity for the anti-CL and other PL antibodies which were found in the syphilitic sera which we studied. Antibodies directed against CL have been classically studied in animals in a complex system which also contains phosphatidylcholine and cholesterol (8). We concluded that CL is a hapten and requires the presence of phosphatidylcholine for full reactivity. However, the enhancement of CL antigenicity by phosphatidylcholine and cholesterol may be subject to change by factors other than those which modify antigen-antibody inhibition.

Our qualitative studies of sera from Red Cross donors revealed no anti-PL reactivity. In other experiments (data not shown), lowering the concentration of gelatin and Tween 20 in the blocking buffer did result in some visualization of anti-CL antibodies in normal sera, as well as higher levels of background binding (paper without PLs). This difference most probably resulted from selective factors based on affinity.

Although the stoichiometry between goat anti-IgG and human IgG bound on nitrocellulose paper is not definitely known, it is known that the protein A stoichiometry is 2 to 1 (12), and results with the same sera (Fig. 4) showed somewhat similar extents of reactivity compared with labeled anti-IgG. That we were able to regularly measure immunoglobulin binding to CL by using the protein A method implies that the F_c portion of the immunoglobulin molecule was free and available for interaction with protein A. This in turn implies that the binding of antibody to the CL molecules on the paper was to the Fab portion of the antibody molecule.

The stoichiometry between PLs and IgG could mean that individual molecules are not recognized as such at the antigen combining site, but rather are recognized as some multiple of molecules, perhaps in the form of a bilayer structure, which hydrated CL is known to assume. The preliminary observation that divalent cations affect antibody

binding supports this hypothesis. These cations are known to induce phase changes in CL, specifically a change from a bilayer structure to a hexagonal structure (H_{II}) (15). Alternative explanations for the observed stoichiometric relationship cannot be excluded by our results. It is possible that some CL impregnated in the nitrocellulose paper is physically excluded from interaction in toto with antibody or that CL in the paper resulted in steric hindrance of antibody binding.

Renewed interest in syphilis serology is becoming apparent, perhaps partly as a result of the identification of anti-PL antibodies in sera from SLE patients. Colaco and Male measured the relative affinity of anti-PL binding in syphilitic sera with a solid-phase radioimmunoassay (1). Compared with SLE sera, modest elevations of anti-CL binding were found in 15 of 23 syphilitic sera. The IgM anti-CL binding levels were somewhat greater than the levels for IgG. Anti-phosphatidic acid binding was found to a lesser extent. In contrast to our results with IgG antibody, these workers also found IgM antibody activity toward phosphatidylethanolamine, but binding to phosphatidylserine was minimal in the few sera which were tested.

Although their pathogenic role remains unestablished, human antibodies to PLs (especially to CL) have been described in association with a variety of other diseases in which membrane damage may be a predominant event, including multiple sclerosis (3) and autoimmune thrombocytopenic purpura (6). Special attention has recently been directed toward a subset of patients with SLE who as a group demonstrate an association between the presence of anti-CL antibodies (measured by radioimmunoassay) and the binding of the lupus anticoagulant, thrombocytopenia, and venous and arterial thrombotic events (4, 5, 18). There is conflicting evidence regarding the specificity of anti-CL activity in SLE sera, especially concerning the cross-reactivity of CL (and possibly other PL) antibodies with native and denatured DNAs (7, 9-11). In SLE a case has been made for a common reactive antigenic site located in the phosphate diester linkage area which is common to the nucleic acid and PL molecular structures (9, 16). Our results argue strongly against the possibility that anti-PL antibodies, at least in syphilis, are directed at this epitope since this site is found in all classes of PL. If this were the reactive site, we would have observed similar binding to all of the PLs tested.

The results of the studies described above indicate that PL antibodies with specific reactivity patterns exist in syphilitic sera. The question of the disease specificity of these and similar antibodies will require further study. Some evidence that such specificity may exist has been reported by Shoenfeld et al. (17), who found discordancy in terms of the frequency of an anti-CL-related antibody (the lupus anticoagulant) in syphilitic sera on the one hand and in sera from SLE patients and false-positive serologic syphilis test reactors without connective tissue disease on the other.

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