Clin. exp. Immunol. (1990) **80**, 171–176

Heterogeneity of binding reactivity to different phospholipids of antibodies from patients with systemic lupus erythematosus (SLE) and with syphilis

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(Accepted for publication 29 November 1989)

SUMMARY

The binding specificities were investigated of anti-phospholipid antibodies derived from sera from 55 patients with SLE and related diseases, and from 33 patients with syphilis. Antibodies from both these groups of patients bind strongly to cardiolipin in solid-phase immunoassays, but only anti-phospholipid antibodies from patients with autoimmune diseases are associated with thrombotic complications and recurrent spontaneous abortions. IgG anti-phospholipid antibodies from both groups of patients cross-reacted with a range of negatively charged phospholipids, but binding to neutral phospholipids was largely restricted to sera from patients with syphilis. A monoclonal IgMλ anti-cardiolipin antibody, derived from a patient with autoimmunity, was used to inhibit binding of anti-phospholipid antibodies to cardiolipin and to phosphatidic acid. This antibody inhibited the binding of autoimmune sera to cardiolipin more strongly than sera from syphilis patients, but the converse pattern of inhibition of binding to phosphatidic acid was observed. The VDRL titre correlated with anti-phospholipid antibody activity in sera from syphilis patients, but not from those with autoimmunity. Lupus anti-coagulant activity correlated weakly with IgG antibody levels to each of the negatively charged phospholipids among the patients with autoimmunity. Lupus anti-coagulant activity did not correlate uniquely with any anti-phospholipid antibody specificity. These results provide further documentation of the great heterogeneity of anti-phospholipid antibodies associated with autoimmune disease and syphilis.

Keywords systemic lupus erythematosus anti-cardiolipin antibodies lupus anti-coagulant VDRL

INTRODUCTION

Solid-phase immunoassays for antibodies to cardiolipin have allowed the identification of anti-phospholipid antibodies in patients with many diseases, especially chronic infections and autoimmunity. Among the latter patients, anti-phospholipid antibodies have been associated with disease, including arterial and venous thrombosis, recurrent abortions, thrombocytopenia and the rash of livedo reticularis (Harris, Asherson & Hughes, 1988a). If there is any causal relationship between anti-phospholipid antibodies and these clinical manifestations, this implies that there must be heterogeneity between the antibodies associated with infection and those associated with autoimmunity.

Other assays of anti-phospholipid activity do show such heterogeneity. Anti-phospholipid antibodies in patients with syphilis are readily detected using VDRL test reagent, containing a mixture of cardiolipin, phosphatidylcholine and cholesterol, but this test is only infrequently positive in patients with autoimmunity. The converse of this observation is that the lupus anti-coagulant has been associated with anti-cardiolipin binding activity in sera from patients with autoimmune disease, but not from those with syphilis (Johansson & Lassus, 1974).

We set out to examine the binding specificities of antibodies to a range of negatively charged and neutral phospholipids. Sera from cohorts of patients with autoimmune disease or with syphilis were analysed. The results demonstrate great heterogeneity among antibodies to phospholipids, and raise further questions about the nature of the relevant pathogenetic specificities.

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PATIENTS AND METHODS

Patients

Sera from three groups of patients were studied. Fifty-five patients (46 women and nine men) had autoimmune disease; 42

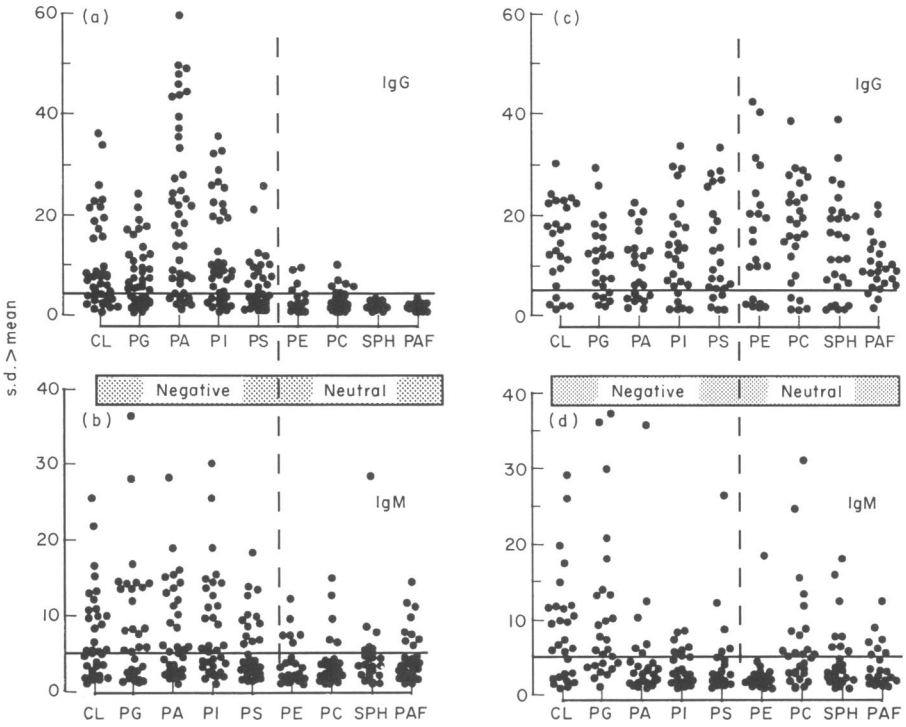

Fig. 1. Binding of IgG and IgM antiphospholipid antibodies to a range of antigens: CL, cardiolipin; PG, phosphatidylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; PAF, platelet-activating factor. Results are expressed in s.d. above a normal mean derived from 17 healthy controls. Samples from patients with SLE (a,b) and from patients with syphilis (c,d).

of these fulfilled the ARA revised criteria for the classification of SLE (Tan *et al.*, 1982). Thirteen patients had other autoimmune diseases: vasculitis (seven), mixed connective tissue disease (four) and undifferentiated connective tissue disease (two patients. These patients were included because they had raised titres of serum anti-phospholipid antibodies as detected by an ELISA for antibodies to cardiolipin. Sera from 33 patients (20 men and 13 women) with active syphilis were studied. Sera from 17 healthy laboratory personnel (14 women and three men) were studied as normal controls. All sera were stored at −20°C before use.

Anti-phospholipid antibody detection

ELISA. Antibodies to cardiolipin were detected by an ELISA as described (Loizou *et al.*, 1985). Antibodies to other phospholipids were detected using a modification of this assay system; in each case the particular phospholipid was coated at 1 μg in 40 μl/well onto half of the wells of a microtitre plate, the other half being coated with cardiolipin at the same concentration for comparison. The plates were blocked by incubation overnight at 4°C with phosphate-buffered saline (PBS)/10% fetal calf serum (FCS).

The following phospholipids were used (all from Sigma Chemical Company, Poole, UK): cardiolipin (in ethanol); phosphatidyl inositol, phosphatidyl choline (lecithin), phosphatidyl ethanolamine (cephalin) and L-α-phosphatidyl choline, β-acetyl-γ-*O*-alkyl (platelet-activating factor, PAF) (all in chloroform); phosphatidyl glycerol and phosphatidyl serine (both in 95:5 chloroform/methanol); and phosphatidic acid and sphingomyelin, as solids which were dissolved in one part chloroform and then nine parts ethanol before use.

Patient and control sera were tested in each assay at 1/100 dilution in PBS containing 10% FCS. In each assay four positive controls, with raised levels of anti-cardiolipin antibodies (ACA), were included in addition to the normal and patient sera. Anti-phospholipid levels were expressed as s.d. above the mean of the normal controls; levels >5 s.d. above this mean were considered positive.

Competitive binding assay. A human IgMλ monoclonal antibody derived from a patient with primary anti-phospholipid syndrome (Mackworth-Young *et al.*, in preparation) was used to inhibit the binding of IgG in patient sera to cardiolipin. A human monoclonal IgM antibody without binding reactivity to phospholipids (Serotec, Oxford, UK) was used as a control in these experiments. Microtitre plates were coated with cardiolipin (Loizou *et al.*, 1985), blocked and then incubated overnight at 4°C with 100 μl of PBS/FCS containing a range of concentrations of monoclonal antibody from 8 to 500 μg/ml. The assay was performed by incubating sera known to contain elevated levels of IgG anti-cardiolipin antibodies in the microtitre plates and probing with affinity-purified, goat anti-human IgG, known not to contain any anti-IgM reactivity (Tissue Culture Services, Slough, UK), followed by an alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma). All assays were performed in triplicate.

Lupus anti-coagulant. The presence of the lupus anti-coagulant was detected as previously described (Boey *et al.*, 1983). Prolongation of the kaolin cephalin clotting time (KCCT) of more than 25 sec in the mixing test was considered positive.

VDRL. The VDRL slid flocculation test was performed using a VDRL carbon antigen (Oxoid, Basingstoke, UK), the

Table 1. Spearman rank correlations between levels of antibodies to different phospholipids in sera from SLE and syphilis patients

	Cardiolipin	Phosphatidic acid	Phosphatidyl-glycerol	Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidyl-choline	Phosphatidyl-ethanolamine	PAF	Sphingomyelin
Correlation between IgG ACA level and IgG antibodies to									
SLE sera		0·93*	0·89*	0·89*	0·87*	0·07	0·08	0·28†	0·13
Syphilis sera		0·75*	0·72*	0·73*	0·77*	0·88*	0·68*	0·77*	0·72*
Correlation between IgM ACA level and IgM antibodies to									
SLE sera		0·90*	0·90*	0·88*	0·79*	0·47‡	0·33†	0·51*	0·43‡
Syphilis sera		0·74*	0·82*	0·68*	0·78*	0·82*	0·26	0·50‡	0·59*
Correlation between VDRL titre and levels of IgG antibodies to									
SLE sera	0·14	0·14	0·18	0·18	0·27†	0·17	0·02	0·02	0·17
Syphilis sera	0·70*	0·50‡	0·49‡	0·71*	0·59*	0·71*	0·49‡	0·63*	0·58‡
Correlation between VDRL titre and levels of IgM antibodies to									
SLE sera	0·22	0·24	0·21	0·25	0·26	0·31‡	0·27†	0·40‡	0·35‡
Syphilis sera	0·74*	0·60*	0·58‡	0·44†	0·46‡	0·68*	0·30	0·19	0·46‡
Correlation between level of lupus anti-coagulant and levels of anti-phospholipid antibodies in SLE patients									
IgG antibodies	0·40‡	0·36‡	0·27†	0·37‡	0·31†	0·03	0·34†	0·22	0·01
IgM antibodies	0·33†	0·23	0·28†	0·34†	0·19	0·12	0·13	0·17	0·23

* $P < 0·001$; † $P < 0·05$; ‡ $P < 0·01$

composition of which was 0·03% cardiolipin, 0·02% phosphatidylcholine (lecithin) and 0·09% cholesterol.

Statistical analysis

Non-parametric tests were used throughout. Correlations were performed using Spearman's rank correlation.

RESULTS

Spectrum of phospholipid binding activity

The binding of IgG and IgM to each phospholipid is shown in Fig. 1. The phospholipids were categorized as negatively charged or neutral. IgG and IgM antibodies in sera from patients with autoimmune diseases bound predominantly to the negatively charged phospholipids, whereas sera from the patients with syphilis showed an equal prevalence of binding to neutral and negatively charged phospholipids. These distinctions were more apparent for IgG binding than for IgM. The highest levels of IgG anti-phospholipid binding were to phosphatidic acid among the SLE sera and to phosphatidylethanolamine among the syphilis sera.

Correlations were performed between the level of binding of antibodies to cardiolipin and each of the other phospholipids (Table 1). Among the SLE patients there were strong correlations between the level of IgG reactivity to cardiolipin and each of the negatively charged phospholipids ($P < 0·001$ for each of these correlations). No significant correlations, with the exception of a weakly significant result for cardiolipin compared with PAF, were seen for IgG binding to cardiolipin and the neutral group of phospholipids among the sera from patients with autoimmunity.

These results contrasted with the findings in the sera from the patients with syphilis, where there were strong correlations between binding to cardiolipin and all of the negatively charged and neutral phospholipids ($P < 0·001$ for each of these correlations). The contrast between the binding properties of anti-

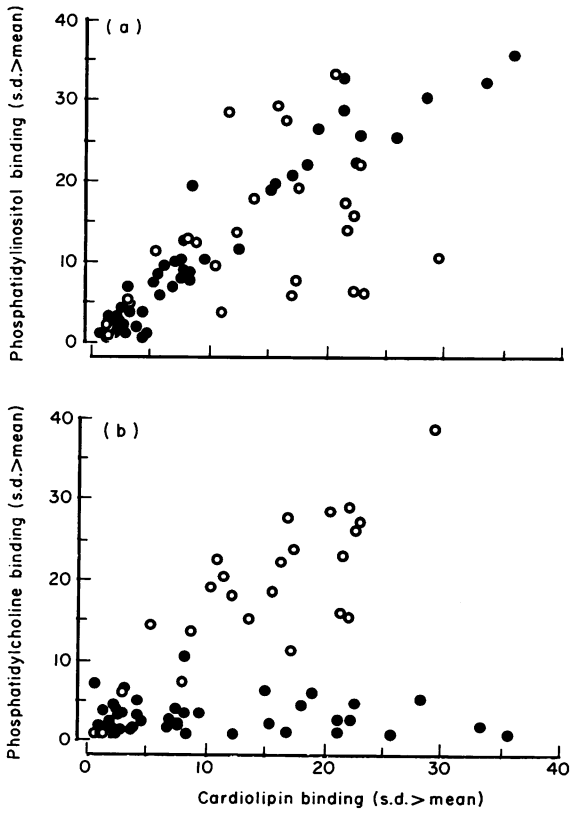

Fig. 2. Correlations between levels of IgG anti-phospholipid antibody binding to cardiolipin and to phosphatidylinositol, a negatively charged phospholipid (a), and phosphatidylcholine, a neutral phospholipid (b). Autoimmune sera, ●; syphilis sera, ○. Correlation coefficients are given in Table 1.

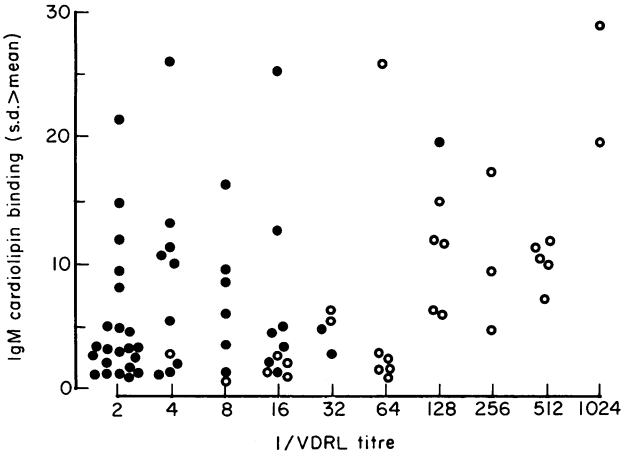

Fig. 3. Relationship between VDRL titre and levels of IgM binding to cardiolipin. Autoimmune sera, ●; syphilis sera, ○. Correlation coefficients are given in Table 1.

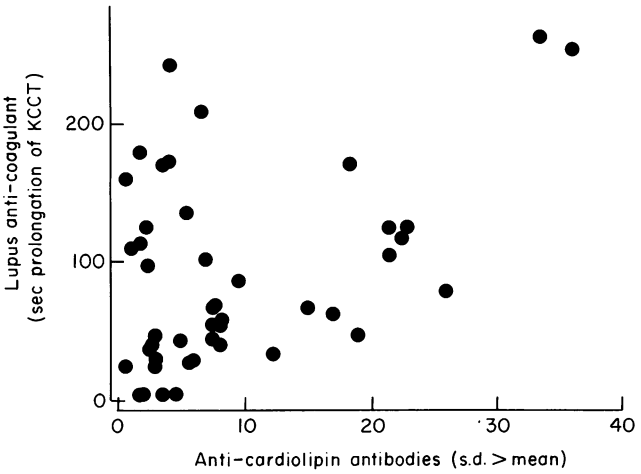

Fig. 4. Relationship between lupus anti-coagulant and levels of IgG binding to cardiolipin among the autoimmune sera.

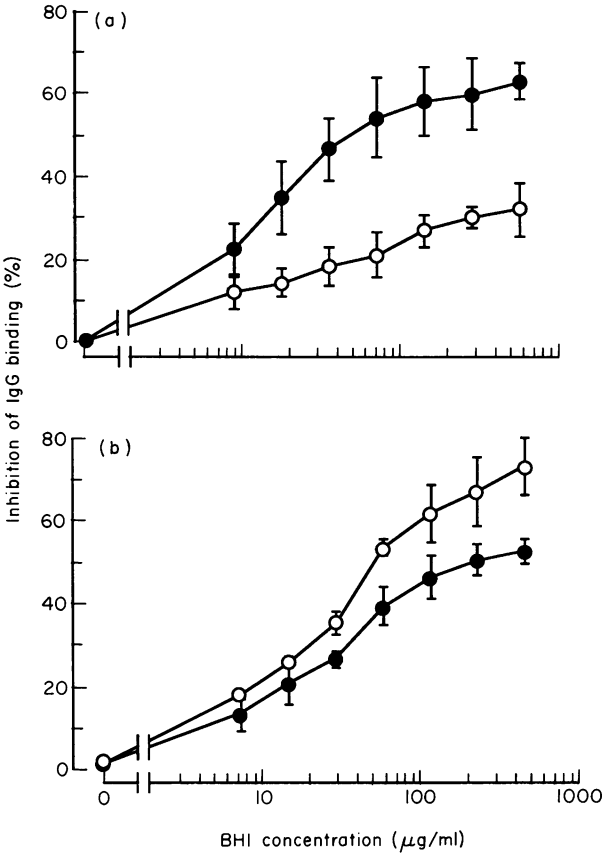

Fig. 5. Inhibition of binding of IgG anti-phospholipid antibodies to cardiolipin (a) and to phosphatidic acid (b) by varying concentrations of an IgM human monoclonal anti-phospholipid antibody (BH1). Results are expressed as the mean inhibition of six SLE sera (●) and of six syphilis sera (○) ± 1 s.d.

bodies to phospholipids, derived from autoimmune and syphilis sera, is shown in Fig. 2, correlating the binding of anti-phospholipid antibodies to cardiolipin compared with phosphatidylinositol (negatively charged) and phosphatidylcholine (neutral).

Anti-phospholipid binding activity and VDRL titre

The correlation between VDRL titre and anti-phospholipid binding is shown in Table 1. Again there was a discordance between the autoimmune and syphilis sera. This is illustrated for the relationship between VDRL titre and IgM anti-cardiolipin activity in Fig. 3. There was no correlation between these two activities amongst the autoimmune sera ($r_s = 0{\cdot}22$, $P > 0{\cdot}05$), but a strong correlation amongst the sera from patients with syphilis ($r_s = 0{\cdot}74$, $P < 0{\cdot}001$). A similar discordance between sera from autoimmune patients and those from syphilis patients existed for each phospholipid examined (Table 1). Among the syphilis sera the strongest correlations between anti-phospholipid binding activity and VDRL titre were for IgG and IgM anti-cardiolipin and IgG and IgM anti-phosphatidylcholine antibodies. These are the two phospholipids contained within the VDRL reagent. The only weak correlations between binding activity in the SLE sera and VDRL titre were in the case of IgM binding to each of the neutral phospholipids (Table 1).

Anti-phospholipid binding activity and lupus anti-coagulant

Lupus anti-coagulant activity was measured in plasma samples derived from each of the patients with autoimmune disease. There were weak correlations between the prolongation of the KCCT and the titre of antibodies to each of the negatively charged phospholipids, but none to the neutral phospholipids (Table 1). The correlation between the prolongation of the KCCT and IgG ACA is shown in Fig. 4. A group of subjects may be identified from this figure with prolongation of their KCCT but no elevation of the titre of antibodies to cardiolipin.

Inhibition of IgG anti-cardiolipin and anti-phosphatidic acid binding by a monoclonal IgM anti-cardiolipin antibody

The hypothesis was tested that IgG antibodies to phospholipids in sera from autoimmune patients bound to a different epitope to similar antibodies in sera from syphilitic patients. An IgM human monoclonal antibody with anti-cardiolipin reactivity, derived from a patient with the primary anti-phospholipid syndrome, was used to inhibit the binding of polyclonal IgG

from patient sera. The results are shown in Fig. 5. Sera from six SLE patients were compared with sera from six patients with syphilis. It may be seen that the inhibition of binding to cardiolipin of IgG antibodies from SLE sera was much greater than inhibition of binding of IgG antibodies from patients with syphilis. In contrast, the inhibition of binding to phosphatidic acid of IgG antibodies from SLE sera was less than inhibition of binding of IgG antibodies from patients with syphilis. No inhibition of binding to antibodies for either group of sera was seen when an irrelevant IgM monoclonal antibody was used as inhibitor (data not shown). This experiment suggests that different epitopes (although possibly partially overlapping) on cardiolipin and phosphatidic acid are recognized by IgG antibodies from patients with syphilis and SLE.

DISCUSSION

There is increasing evidence for heterogeneity of anti-phospholipid binding reactivity among antibodies derived from patients with syphilis and with autoimmune disease, mainly SLE and the primary anti-phospholipid syndrome. Anti-phospholipid antibodies from subjects with autoimmunity are associated with the presence of the lupus anti-coagulant; those from syphilis patients are not (Johansson & Lassus, 1974). In contrast, positive VDRL results are weak and infrequent in patients with autoimmunity, but common and of higher titre in patients with syphilis. The ACA assay provides a common denominator as a test for anti-phospholipid reactivity in the two diseases. In the present study we have found a difference in the recognition of cardiolipin by anti-phospholipid antibodies from patients with autoimmunity and syphilis. A human IgM monoclonal anti-phospholipid antibody, derived from a patient with primary anti-phospholipid syndrome, inhibited the binding of IgG ACA from sera from autoimmune patients more strongly than the binding of ACA from sera from patients with syphilis. The opposite pattern of inhibition was seen in the case of anti-phosphatidic acid binding; antibodies from syphilis sera were inhibited by the same monoclonal antibody more strongly than their counterparts from autoimmune sera. The two most likely explanations for these findings are either (i) that the binding constants of anti-phospholipid antibodies for cardiolipin are higher in syphilis than in autoimmunity and *vice versa* for the binding to phosphatidic acid; or (ii) that different epitopes are recognized on cardiolipin and phosphatidic acid by anti-phospholipid antibodies derived from the two groups of patients.

The explanation for the different correlations between the levels of anti-phospholipid antibodies measured by ACA, VDRL and lupus anti-coagulant assays is complex. It has been a reproducible observation that ACA assays do not correlate with VDRL titre among patients with SLE (Koike *et al.*, 1984; Harris *et al.*, 1985a; Eilat, Zlotnick & Fischel, 1986), although cardiolipin is the main phospholipid antigen in the VDRL test reagent. This lack of correlation broadly held true in the present study, although there were significant but weak correlations between the binding of anti-phospholipid antibodies from autoimmune sera to neutral phospholipids and the VDRL titre of these samples (Table 1). Similarly, previous studies have reported poor correlations between ACA and VDRL levels amongst sera fom patients with syphilis (Colaco & Male, 1985; Harris *et al.*, 1985a; Eilat *et al.*, 1986) although only very few subjects were studied. In the present study, highly significant correlations were found between the level of anti-phospholipid antibodies and VDRL titre (Table 1), although the correlations were relatively weak (Table 1, Fig 3).

We measured the specificity of anti-phospholipid antibodies by studying their reactions with a variety of negatively charged and neutral phospholipids in order to try to explain the different behaviour of anti-phospholipid antibodies from patients with different diseases in VDRL and lupus anti-coagulant assays. By ELISA there was a similar pattern of reactivity of both autoimmune and syphilis sera to the panel of negatively charged phospholipids, although autoimmune sera bound most strongly to phosphatidic acid. Similar spectra of binding have been reported previously to negatively charged phospholipids in autoimmune (Harris *et al.*, 1985a, 1985b; Cowchock, Smith & Gocial, 1986; Meyer *et al.*, 1987) and syphilis patients (Costello & Green, 1986; Meyer *et al.*, 1987).

The pattern of reactivity to neutral phospholipids differed between the two groups of patients. Sera from patients with SLE and the primary anti-phospholipid syndrome showed very little binding to neutral phospholipids in comparison with the syphilis samples, which bound to each of the neutral phospholipids tested. These findings differ from those of other workers (Costello & Green, 1986; Pedersen, Orum & Mouritsen, 1987; Harris *et al.*, 1988b) who did not find significant binding of syphilis sera to phosphatidylcholine (lecithin). Similarly cholesterol and lecithin did not inhibit the VDRL reaction (Pedersen *et al.*, 1987; Harris *et al.*, 1988b), in comparison with cardiolipin which inhibited moderately strongly.

Most assays for binding to phospholipids have been performed using antigen which is in micellar form, the most ubiquitous phase *in vivo*, or coated on microtitre plates. Such assays are sensitive and give results in patients with SLE and the primary antiphospholipid syndrome which correlate with the presence of clinical manifestations (Harris *et al.*, 1988a). However, the relevance of these results to the binding of these autoantibodies *in vivo* is less certain. Rauch *et al.*, (1986) showed that phosphatidylethanolamine, a neutral phospholipid, in lamellar form would not inhibit lupus anti-coagulant, but that the same phospholipid in hexagonal phase was a strong inhibitor.

There is also evidence for the importance of steric factors in the binding of antiphospholipid antibodies to the VDRL reagent, which comprises cardiolipin, lecithin and cholesterol. Lecithin plays an important role in determining the immunogenicity of several phospholipids including cardiolipin and phosphatidylinositol (Kataoka & Nojima, 1970). Although we found specific binding of syphilis sera to mictrotitre plates coated with lecithin, this differs from previous findings (Costello & Green, 1986; Pedersen *et al.*, 1987; Harris *et al.*, 1988b) and the observations that lecithin and cholesterol did not inhibit the reaction of syphilitic sera with the VDRL reagent (Pedersen *et al.*, 1987; Harris *et al.*, 1988b) suggests that the main antigenic moiety of this reagent is cardiolipin, and that the role of cholesterol and lecithin is to modify the antigenicity of the cardiolipin. Further evidence in support of this hypothesis comes from recent observations that VDRL antigen strongly inhibited ACA activity in sera from patients with syphilis, but only weakly inhibited similar activity from patients with SLE (Mouritsen *et al.*, 1989).

Our data provide further examples of the heterogeneity of

antiphospholipid reactivity amongst patients with SLE and the primary antiphospholipid syndrome. Although there were very strong correlations between the binding of these antibodies to cardiolipin and each of the other negatively charged phospholipids the correlations between the lupus anti-coagulant and binding to the panel of phospholipids were all comparatively weak (Table 1). A discrepancy between lupus anti-coagulant and anti-cardiolipin levels has been reported previously (Derksen *et al.*, 1986; Branch *et al.*, 1987). It was found that lupus anti-coagulant activity showed 100% correlation with the presence of antibodies binding to phosphatidyserine (Branch *et al.*, 1987). However, in the present study the correlation between the levels of the lupus anti-coagulant and binding to phosphatidylserine was no higher than in the cases of other negatively charged phospholipids (Table 1). Lupus anti-coagulant activity may not be uniquely associated with any single anti-phospholipid specificity. Anti-phospholipid pathogenicity seems more likely to reside in an epitope present on several phospholipids sharing structural similarity, rather than in the binding to a single species. The conformation of the phospholipid may be as important as its charge and primary structure.

ACKNOWLEDGMENTS

These studies were supported by the Arthritis and Rheumatism Council. We would like to thank Ms H. Wong (S.T.D. Laboratory, P.H.L.S., St Georges Hospital, Tooting, London) for supplying the sera from patients with syphilis and Mr P. Mellow (Department of Bacteriology, R.P.M.S.) for performing the VDRL assays.

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