Clin. exp. Immunol. (1984) **56**, 193–199.

Anti-phospholipid antibodies and biological false positive serological test for syphilis in patients with systemic lupus erythematosus

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(Accepted for publication 31 October 1983)

SUMMARY

Utilizing a newly developed solid phase enzyme immunoassay for the detection of anti-phospholipid antibodies, we found that 41·7% of sera from patients with active systemic lupus erythematosus (SLE) had the anti-cardiolipin antibody and 22·7% of sera were positive for the anti-phosphatidylinositol (PI) antibody. But antibodies to lecithin, cephalin and sphingomyelin were rarely found in this assay. We also examined antibodies to the VDRL (Venereal Disease Research Laboratory) antigen which consists of cardiolipin–cholesterol–lecithin mixtures. The incidence of the anti-VDRL antibody in the IgG class was 29·2% and 25% in the IgM class, and the magnitude of these antibodies correlated well with the incidence of biological false positive serological test for syphilis (BFP-STS), but no association between the anti-VDRL antibody titre and the anti-cardiolipin antibody titre was observed. Furthermore, the cardiolipin binding activity of sera from patients with SLE was inhibited by DNA and poly(I), both of which have polynucleotide backbones structurally similar to the phosphodiester groups of cardiolipin. On the contrary, these polynucleotides failed to inhibit the PI and the VDRL binding of SLE sera. These observations may support the concept that the anti-cardiolipin antibody in sera from patients with SLE might be a part of the anti-DNA antibodies, which are serologically polymorphic in that they can react with a variety of polynucleotides, but differ from the BFP reactors.

Keywords systemic lupus erythematosus BFP-STS anti-DNA antibodies anti-cardiolipin antibodies

INTRODUCTION

A biological false positive serological test for syphilis (BFP-STS) is observed frequently in patients with systemic lupus erythematosus (SLE), and is now a well recognized feature of this disease (Harvey & Shulman, 1974).

Previously, we have demonstrated that polyspecific anti-DNA antibodies in SLE sera that reacted with both single stranded DNA and double stranded DNA (anti-ss/dsDNA antibody) had the ability to cross-react with phospholipids, especially cardiolipin (Koike, Tomioka & Kumagai, 1982b). Hence, it would appear to us that the BFP-STS of SLE serum was the result of the cross-reactivity of these anti-DNA antibodies with cardiolipin.

The present study is an attempt to clarify the specificity of the anti-cardiolipin antibody and the

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other anti-phospholipid antibodies such as anti-phosphatidylinositol(PI) antibody and anti-VDRL (Venereal Disease Research Laboratory) antibody in sera from patients with SLE which could be detected by a newly developed solid phase enzyme immunoassay (EIA), and to elucidate the relationships of these anti-phospholipid antibodies and the anti-DNA antibody with BFP-STS in SLE sera. In the experiment reported below we concluded that the anti-cardiolipin antibody in sera from patients with SLE is to be regarded as a part of the anti-DNA antibodies but differs from the anti-PI antibody and the BFP reactor.

MATERIALS AND METHODS

Patients and sera. Sera were obtained from patients with SLE who satisfied the diagnostic criteria of the American Rheumatism Association for SLE. All sera were stored at −20°C in small aliquots until used.

Phospholipids. Cardiolipin (Sigma Chemical Company, St Louis, Missouri, USA) and phosphatidylinositol (Serdary Research Laboratories, London, Ontario, Canada) were supplied in ethanol solution and chloroform solution, respectively. DL-α-lecithin, cephalin and sphingomyelin were purchased from Miles Research Products, Elkhart, Indiana, USA. The VDRL (Venereal Disease Research Laboratory) antigen was made from an alcoholic solution containing 0·04% cardiolipin, 0·8% cholesterol and 0·24% lecithin. Miscelles were created in phosphate-buffered saline (PBS, pH7·4) by sonication.

Polynucleotides. Calf thymus DNA and synthetic RNA were purchased from PL Biochemicals, Milwaukee, Wisconsin, USA.

Enzyme immunoassay of anti-phospholipid antibodies. Antibodies to several phospholipids were measured by a solid phase enzyme immunoassay (EIA). Each well of polyvinylchloride plates (Dynatech Laboratories, Alexandria, Virginia, USA) was first coated with 50 μl of phospholipid solution, 1 mg/ml in 0·01 M PBS, pH 7·4, by incubation overnight at 4°C. After discarding the phospholipid solution, 200 μl of 10% fetal calf serum (FCS) solution was added to each well and incubated for 1 h at room temperature to block non-specific binding of immunoglobulins to the well surface. After five washes in PBS, 50 μl of serum to be tested (diluted with PBS containing 10% FCS) was added to each well, and the preparations were then incubated for 1 h at room temperature. After five washes in PBS, 50 μl of alkaline phosphatase (Sigma) labelled mouse monoclonal anti-human IgG (Wako Chemical Co., Tokyo, Japan) was added to each well, and the plate was incubated for 1 h, then 100 μl of *p*-nitrophenyl phosphate (Sigma) in 1 M diethanolamine buffer, pH 9·0, was added to each well. After 1 h incubation, the optical absorbance was measured at 405 nm by a Microelisa Auto Reader, MR 580 (Dynatech). The values of anti-phospholipid antibodies were expressed as units of the standard serum.

Inhibition study. The specificity of anti-phospholipid antibodies was determined by a competitive inhibition EIA. A mixture of 100 μl each of the inhibitors at varying dilutions and the samples to be tested was incubated for 1 h at 37°C. Prior to inhibition, the test samples were diluted to 50% of the maximum phospholipid binding. The results of inhibition were expressed as percent inhibition of phospholipid binding activity, calculated as follows:

$$\%\ \text{inhibition} = \left(1 - \frac{\text{OD 405 nm count in the presence of inhibitor} - \text{background}}{\text{OD 405 nm count in the absence of inhibitor} - \text{background}}\right) \times 100.$$

The background represents the count in wells without phospholipid as antigens.

Serological test for syphilis. A serological test for syphilis of SLE sera was carried out using a flocculation test kit (IATRON Laboratories Inc., Tokyo).

RESULTS

Anti-phospholipid antibodies in SLE sera

Fig. 1 illustrates the representative phospholipid binding of one SLE serum (SLE-TO) by means of a solid phase EIA. It is apparent that SLE-TO has the antibodies mainly reactive with cardiolipin and

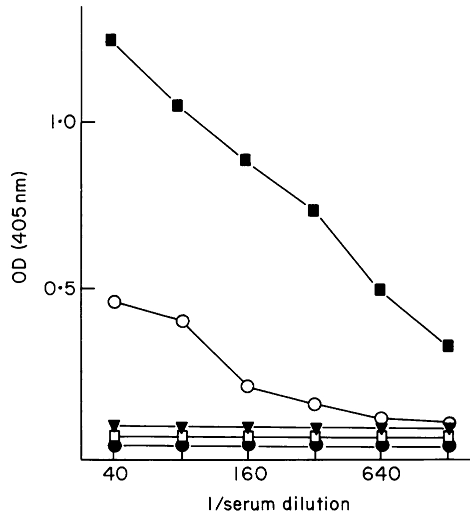

Fig. 1. Representative binding activity of SLE-TO serum to several phospholipids (cardiolipin, ■; phosphatidylinositol, ○; sphingomyelin, ▼; lecithin, □ and cephalin ●) by a solid phase EIA.

PI. No antibodies which bound to other phospholipids, such as lecithin, cephalin and sphingomyelin were observed.

Twenty-four sera from patients with active SLE were selected and the subsequent experiments were performed. As shown in Fig. 2, 41·7% of individual sera from patients with active SLE had the anti-cardiolipin antibody (Fig. 2a) and 22·7% of sera were found to be positive for the anti-PI antibody (Fig. 2b). In contrast to these results, antibodies to lecithin, cephalin and sphingomyelin were rarely found in this assay (Fig. 3).

In an attempt to clarify the relationship between the magnitude of anti-cardiolipin antibody and the biological false positive serological test for syphilis (BFP-STS), the antibody to VDRL antigen was measured by a modified flocculation test. Of all cases tested, three out of 24 (12·5%)

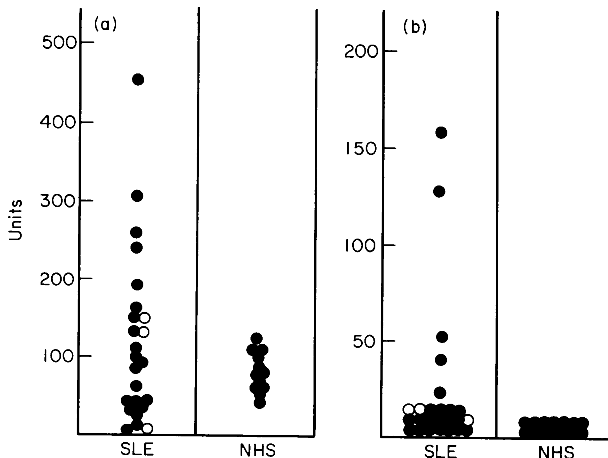

Fig. 2. Levels of anti-cardiolipin antibody (a) and anti-phosphatidylinositol antibody (b) in sera from 24 SLE patients and normal individuals. The results are expressed as units of the standard SLE-TO serum. Open circles represent the sera which were positive for the VDRL agglutination reaction.

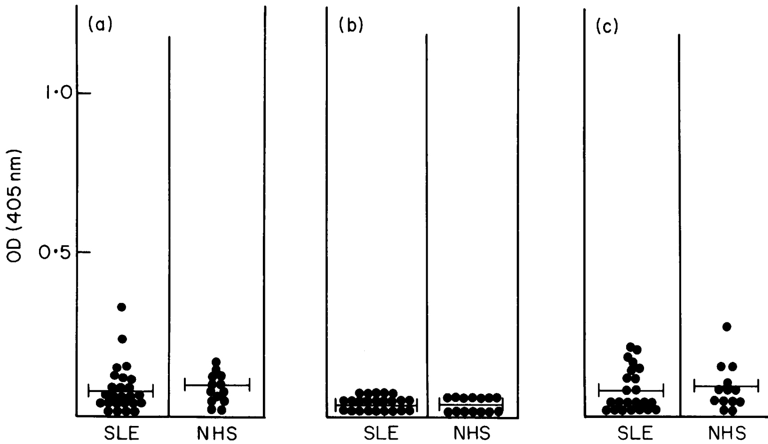

Fig. 3. Binding activity of 24 SLE sera and normal individuals to lecithin (a), cephalin (b) and spingomyelin (c).

were found to be positive for the VDRL agglutination reaction. However, as illustrated in Fig. 2a, there was no association between these BFP reactors (O) and the anti-cardiolipin antibody titre. Similar results were obtained in relation to the anti-PI antibody and BFP-STS (Fig. 2b).

Anti-VDRL antibody in SLE sera

The VDRL antigen is composed of cardiolipin, lecithin and cholesterol. We prepared this VDRL antigen and employed it for the detection of anti-VDRL antibody in SLE sera utilizing the same solid phase EIA. The incidence of anti-VDRL antibody in the IgG class was 29·2% and 25% in the IgM class (Fig. 4). However, there was no association between the titres of anti-cardiolipin antibody and anti-VDRL antibody (data not shown). In contrast to this result, a strong correlation between

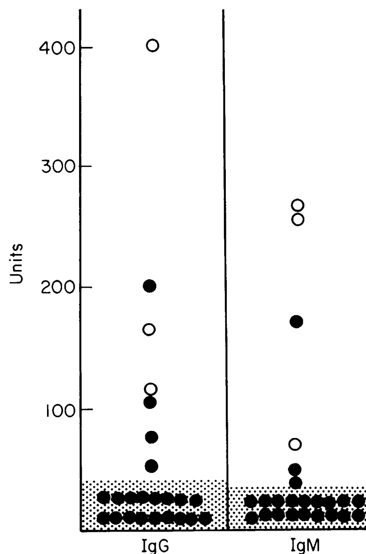

Fig. 4. Levels of anti-VDRL antibody in IgG and IgM class in sera from 24 SLE patients. The shaded area expresses a two-fold standard deviation of the mean value of normal controls. Open circles represent the sera which were positive for the VDRL agglutination reaction.

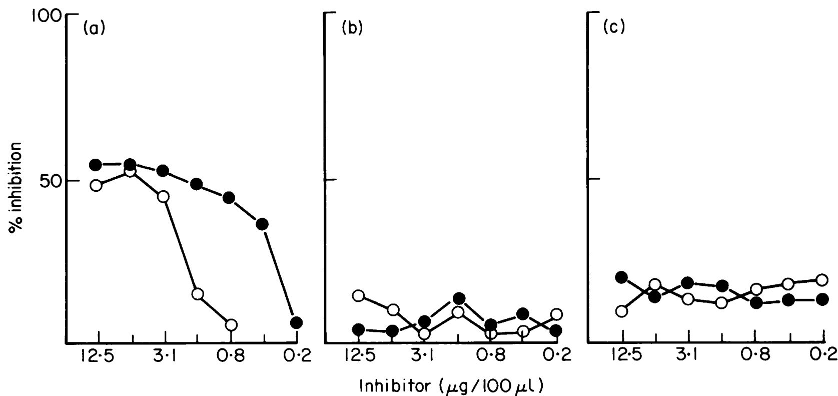

Fig. 5. Representative competitive inhibition EIA with SLE-TO serum. Values represent the mean percentage inhibition of cardiolipin binding (a), PI binding (b) and VDRL binding (c) for triplicate samples in the presence of indicated inhibitors (DNA, ● and poly(I), ○).

the anti-VDRL antibody detected by EIA and BFP-STS detected by the flocculation test was observed (Fig. 4, ○).

Reactivity of anti-phospholipid antibodies with DNA and RNA

We have previously reported that polyspecific anti-DNA antibodies in SLE sera had the ability to cross-react with cardiolipin. Next, we designed competitive inhibition studies to elucidate the relation between these anti-phospholipid antibodies and polynucleotide binding activity.

The representative results using SLE-TO serum are provided in Fig. 5. The cardiolipin binding of SLE-TO serum was partly inhibited by both DNA and poly(I) (Fig. 5a). In contrast to this result, these polynucleotides failed to inhibit the PI binding of the same SLE serum (Fig. 5b) and the binding to the VDRL antigen was not influenced by either DNA or RNA (Fig. 5c). Essentially similar results were obtained in other SLE sera which revealed positive reactions to the phospholipids (data not shown).

DISCUSSION

We designed a solid phase EIA for the detection of anti-phospholipid antibodies. By means of this EIA, we found that 41·7% of SLE patients had the antibody to cardiolipin and 22·7% to PI, repectively, but there were few antibodies to lecithin, cephalin and sphingomyelin.

It has been known that cardiolipin and PI are strong immunogens among the many phospholipids (Marcus & Schwarig, 1976). Guarnieri & Eisner (1974) have reported the cross-reactions of anti-cardiolipin antibodies which were raised in rabbits with PI and nucleic acids. However, according to our study, there is no association between the anti-cardiolipin antibody titre and anti-PI antibody titre in sera from patients with SLE (data not shown). Furthermore, the cardiolipin binding of SLE sera alone was inhibited by DNA and RNA, but the PI binding activity could not be influenced by these polynucleotides. Recently, using monoclonal mouse hybridoma antibodies, we also made the observation that the anti-DNA antibody which reacted with a wide range of polynucleotides could bind only to cardiolipin but failed to bind to PI and the other phospholipids. When these results are considered together, we may conclude that the anti-cardiolipin antibody and the anti-PI antibody in SLE sera are different antibodies.

A BFP-STS is a common feature of SLE (Harvey & Shulman, 1974), and it is assumed that the BFP reactors in sera from patients with SLE are due to the antibodies which react with cardiolipin. Antibody to DNA is also found frequently in SLE sera and is one of the major serological markers of this disease (Deicher, Holman & Kunkel, 1959; Stollar *et al.*, 1962; Arana & Seligmann, 1967; Koffler *et al.*, 1971; Koike *et al.*, 1982a, 1982b). Guarnieri & Eisner (1974) have made the observation that DNA and cardiolipin, the latter of which consisted of two phosphodiester groups

with interior glycerol moiety and a chemical structure of which resembling that of the polynucleotide backbone, reacted equally with anti-cardiolipin antibodies raised in rabbits, and that all of the antibodies to cardiolipin could be absorbed by DNA. Recently, using the monoclonal mouse hybridoma antibodies, Lafer *et al.* (1981) have reported that phospholipids, especially cardiolipin, can compete with the DNA binding activity of monoclonal anti-DNA antibodies. We have also demonstrated that polyspecific anti-DNA antibodies in sera from patients with SLE had the ability to cross-react with cardiolipin (Koike *et al.*, 1982b).

Considered together, it would appear to us that the BFP-STS of SLE was the result of the cross-reactivity of the polyspecific anti-DNA antibody with cardiolipin (Stollar, 1975). However, the incidence of SLE in BFP-STS is less frequent than that of anti-DNA antibodies (Harvey & Shulman, 1974), and, as previously described elsewhere, we found no association between the BFP-STS, detected by the agglutination test, and the anti-DNA antibody titre (Koike *et al.*, 1982b). Hence, the experiments were done to clarify the relationship between the anti-DNA antibody and the BFP-STS in sera from patients with SLE.

We made up the VDRL antigen which consists of cardiolipin–cholesterol–lecithin mixtures and employed it for the EIA. Consequently, we found that 29·2% of SLE patients had the antibodies to the VDRL antigen and that the magnitude of these antibodies correlated well with the incidence of BFP-STS which was detected by the agglutination test. Contrary to these findings, no association between the anti-VDRL antibody titre and the anti-cardiolipin antibody titre was observed.

Results from the competitive inhibition studies also indicated that polynucleotides had no effect on the VDRL antigen binding activity of anti-VDRL antibody in SLE sera, while the cardiolipin binding activity was inhibited by DNA and RNA. These observations may support the concept that the anti-cardiolipin antibody in sera from patients with SLE might be a part of the anti-DNA antibodies, which are serologically polymorphic in that they can react with a variety of polynucleotides, but differ from the BFP reactors.

The biological significance of these anti-phospholipid antibodies in SLE, especially anti-VDRL antibody and anti-PI antibody is still unclear. Shoenfeld *et al.* (1980) have reported that the incidence of BFP-STS in SLE patients with circulating anticoagulant is higher than in those without. Similarly, thrombocytopenia is more frequent in SLE patients with BFP-STS (Schleider *et al.*, 1976). On the basis of these observations, it is assumed that the association between circulating anticoagulant, BFP-STS and thrombocytopenia is due to the cross-reaction of the antibodies to the similar structures of the VDRL antigen, electron microscopic studies of which revealed that cardiolipin–lecithin–cholesterol liposomes consisted of lamellar structures surrounding a cholesterol core (Kanemasa, 1974).

Guarnieri (1974) has made the observation that rabbit antibodies to PI were adsorbed by myelin and synaptosomes. Though the precise specificity of the anti-PI antibodies in SLE sera is not yet clearly defined, these antibodies might be responsible for the neurological manifestations of this disease.

In conclusion, this work demonstrates that SLE patients possess the autoantibodies to several phospholipids, such as cardiolipin, PI and cardiolipin–cholesterol–lecithin mixtures (VDRL antigen) and that the anti-cardiolipin antibody is a part of the polyspecific anti-DNA antibodies but differs from anti-PI antibody and anti-VDRL antibody (BFP reactor).

This work was supported by grants from the Ministry of Health and Welfare, Science and Culture, Japan, and from the Japan Medical Research Foundation. We are grateful for the support and advice of Professor M. Taniguchi, Department of Immunology, School of Medicine, Chiba University, for the technical assistance of Ms T. Sato and the secretarial assistance of Ms H. Fukuda. We also wish to thank Mr A. Gerz for his proof reading.

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