ORIGINAL ARTICLE

Specificity of Antibodies to Single-stranded (ss) DNA in SLE Patients with Anti-phospholipid Syndrome

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Abstract. Although the concept of anti-phospholipid syndrome has been proposed in patients with SLE and other rheumatic diseases, the immunological mechanism is still controversial. Recently, the crossreactivity between anti-cardiolipin antibody and anti-ssDNA antibodies has been discussed in relation to this syndrome. In the present study, the specificity such as the avidity and the crossreactivity of anti-ssDNA antibodies was examined to find a clue to clarify the question why all of anti-cardiolipin antibody positive patients do not have any specific clinical features, thrombosis and spontaneous abortion. The avidity of IgG anti-ssDNA antibodies was examined by salt elution studies in solid phase ELISA. The avidity of anti-ssDNA antibodies tended to be lower in 10 patients with specific features than in other 10 patients without those features. The crossreactivity of affinity purified IgG anti-ssDNA antibodies was investigated by competitive ELISA. Purified anti-ssDNA antibodies from 4 patients without specific features were slightly inhibited by negatively charged phospholipids, cardiolipin and phosphatidylserine, whereas purified anti-ssDNA antibodies from 2 patients with specific features, who were considered to have anti-phospholipid syndrome, were little inhibited by these phospholipids. The above results suggest that the specificity of anti-ssDNA antibodies appears, at least partly, in different manners whether specific features are present or absent in anti-cardiolipin antibody positive patients. Moreover, anti-ssDNA antibodies and anti-phospholipid antibodies may form separate groups of antibodies in patients with anti-phospholipid syndrome. (Keio J Med 41 (1): 10–15, March 1992)

Key words: anti-ssDNA antibodies, anti-cardiolipin antibody, anti-phospholipid antibodies, systemic lupus erythematosus

Introduction

Many autoantibodies can be detected in sera from patients with systemic lupus erythematosus (SLE), and the association between each autoantibody and various clinical manifestations has been found.¹ Most of these autoantibodies are directed to nuclear proteins or protein-nucleic acid complexes.¹ Moreover, antibodies to phospholipids have been tested as biologically false positive serologic tests for syphilis (BFP) or lupus anticoagulant (LAC).² Recently, antibodies to cardiolipin or other negatively charged phospholipids have been specifically examined by sensitive solid phase radioimmunoassay (RIA) and enzyme linked immunoassay (ELISA).^{3,4}

Anti-cardiolipin antibody is detected in about 40% of patients with SLE.^{2,5} Clinical characteristics such as thrombosis, recurrent fetal loss and thrombocytopenia are found in these antibody positive patients.^{2,3,5} Thus, the concept of “Anti-cardiolipin syndrome” or “Anti-phospholipid syndrome” has been proposed.^{6–8} However, some papers which are opposed to this syndrome have been published,^{9–12} because several patients with anti-cardiolipin antibody do not have these characteristic symptoms.

In the present study, we focused on the specificity of anti-ssDNA antibodies in anti-cardiolipin antibody positive patients. Because anti-ssDNA antibodies are detected in all sera from patients with SLE,¹ and the crossreactivity between anti-ssDNA antibodies and anti-

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cardiolipin antibody has been discussed.^{4,13–15} And, it has been reported that low avidity of anti-ssDNA antibodies may determine the property for the cross-reactivity of anti-ssDNA antibodies.^{16,17} We examined the avidity and the crossreactivity of anti-ssDNA antibodies in order to find a clue to clarify the question why all of anti-cardiolipin antibody positive SLE patients do not have the same clinical features.

Patients and Methods

Patients and sera

Sera were obtained from 20 patients with SLE who satisfied the revised criteria of the American Rheumatism Association for the classification of SLE.¹⁸ All sera were stored at -20°C until use, and were confirmed to contain antibodies to cardiolipin, ssDNA and double-stranded (ds) DNA by solid phase ELISA.

Assay of IgG autoantibodies by ELISA

IgG antibody to cardiolipin was screened by solid phase ELISA¹⁹ using triplicate of the same sample. One hundred μl and 100 $\mu\text{g}/\text{ml}$ cardiolipin from bovine heart (Sigma Chemical Company, USA) was coated onto 96 wells of polystyrene microtiter plates (Immulon I: Dinattech Laboratories, USA) and were evaporated to dryness. After the plates were blocked for 2 hours at 22°C with PBS containing 10% fetal calf serum (FCS), 100 μl of 1:50 dilution of each serum sample was added to each well and incubated for 2 hours at 22°C . Then the plates were washed three times with PBS. After incubation with goat anti-human IgG conjugated to alkaline phosphatase (Sigma Chemical Company, USA) and additional washes, bound antibodies were measured by reaction of 1 mg/ml p-nitrophenyl phosphate in 10% diethanol buffer. If the absorbance at 405 nm was equal or superior to the addition of the mean value calculated in 20 healthy controls plus 3 times of the standard deviation, the tested serum was judged to be positive for antibody to cardiolipin. Moreover, antibody titer was determined according to the standard curve by Walker Laboratories (USA).

IgG antibodies to ssDNA and dsDNA were examined by solid phase ELISA using UV-pretreated polystyrene microtiter plates (Immulon I).²⁰ Calf thymus dsDNA (Sigma Chemical Company, USA) was further purified by the combination of S1 nuclease treatment and BNDC (Benzoylated Naphthoylated DEAE Cellulose) column chromatography.²¹ ssDNA was obtained by heating the native form at 100°C for 10 minutes and then quickly chilling it in an ice-water bath.²⁰ One hundred μl of 10 $\mu\text{g}/\text{ml}$ DNA was coated onto 96 wells of polystyrene microtiter plates (Immulon I) which were pretreated

with UV for 12 hours at 22°C . After incubation for 12 hours at 4°C , the plates were washed three times with PBS. The plates were blocked for 2 hours at 22°C with PBS containing 3% bovine serum albumin (BSA), and then the plates were washed three times with PBS containing 0.1% Nonidet P-40 (NP40) (Sigma Chemical Company, USA) and more three times with PBS. After incubation with diluted sera, the plates were washed again. Bound antibodies to DNA were detected with alkaline phosphatase-labeled goat anti-human IgG. Sera were diluted to 1:200 for the detection of anti-ssDNA antibodies and to 1:1000 for the detection of anti-ssDNA antibodies. PBS containing 1% BSA and 0.1% NP-40 was used as a diluent.

Avidity of serum IgG antibodies to ssDNA

Elution studies in solid phase ELISA were performed to examine the avidity of serum IgG antibodies to ssDNA.¹⁷ In the preliminary experiment, ssDNA-coated plates were incubated with PBS or with 0.01 M phosphate buffer, pH7.4, containing 1.0 M NaCl for 30 minutes at 22°C . After the plates were washed five times with PBS, standard serum was added to each well and incubated. The following assay was performed in the same way as solid phase ELISA to detect anti-DNA antibodies.

In salt elution studies, after incubation of sera with ssDNA coated on wells of UV pretreated polystyrene microtiter plates, 100 μl of 0.01 M phosphate buffer, pH7.4, containing 1.0 M NaCl as elution buffer was added to each well. PBS was used as control in this step. The plates were incubated for 30 minutes at 22°C , and then washed five times with PBS. Residual activity of anti-ssDNA antibodies was detected with alkaline phosphatase-labeled goat anti-human IgG. Avidity of serum IgG anti-ssDNA antibodies was expressed as residual DNA binding activity, calculated as follows:

$$\text{Residual DNA binding (\%)} = \frac{\text{Absorbance at 405 nm of 1.0 M NaCl eluted wells}}{\text{Absorbance at 405 nm of control (PBS) added wells}} \times 100$$

Purification of serum IgG antibodies to ssDNA

IgG fraction was isolated by DEAE cellulose ion exchange column chromatography.²² Then, IgG antibodies to ssDNA was purified by the following affinity column chromatography.

The immunoadsorbent columns used for purification of serum IgG antibodies to ssDNA were prepared by coupling ssDNA to poly-L-lysine Sepharose 4B column according to the method described by Kubota, *et al.*²³ IgG fraction was applied to poly-L-lysine Sepharose 4B

column without ssDNA first to remove the nonspecific binding to Sepharose. Then, the flow-through fraction was applied to a column of ssDNA coupled to poly-L-lysine Sepharose 4B. The column was washed extensively with PBS until the absorbance at 280 nm of the out-flow was 0.001, and then bound antibodies were eluted with 20 mM sodium carbonate, pH10.5, containing 5% volume of dimethyl sulfoxide. The eluates were immediately mixed with 1M Tris-HCl, pH7.0, and dialysed against PBS. Immunoglobulin concentration in the eluates was determined with competitive ELISA.²⁴

Crossreactivity of purified IgG antibodies to ssDNA

The crossreactivity of purified IgG anti-ssDNA antibodies was determined by competitive ELISA.²⁴ Purified antibodies were used to avoid the effects of phospholipids in sera.

Prior to the competitive inhibition studies, sera were diluted to give 50% binding to ssDNA coated on plates under the previously described solid phase ELISA in the absence of any inhibitors. A mixture of 200 μ l each of the serum to be tested and the inhibitors at varying dilutions in PBS was incubated for 90 minutes at 37°C. ssDNA and micelles of cardiolipin or phosphatidylserine (from bovine brain, Sigma Chemical Company, USA) which were created in PBS by sonication²⁵ were used as inhibitors. PBS was used as control in this step. Then, the centrifugated mixture was added to each ssDNA coated well, and bound antibodies were detected with alkaline phosphatase-labeled goat anti-human IgG. The results of the competitive inhibition were expressed as percent inhibition of DNA binding activity, calculated as follows:

$$\text{Percent inhibition} = \left(1 - \frac{\text{Absorbance at 405 nm in the presence of inhibitor}}{\text{Absorbance at 405 nm in the absence of inhibitor}} \right) \times 100$$

Results

Classification of patients

Anti-cardiolipin antibody and anti-ssDNA/dsDNA antibodies were detected in all sera from 20 patients with SLE. However, arterial or venous thrombosis and/or spontaneous abortion, which were regarded as specific features in patients with anti-phospholipid syndrome,²⁶ were not found in 10 patients, but found in other 10 patients. Therefore, the patients could be divided into two groups; patients without specific features, and patients with specific features who could be diagnosed as anti-phospholipid syndrome.

Avidity of serum IgG antibodies to ssDNA

In the preliminary experiment for salt elution studies, the absorbance of the standard serum gave 0.342 against ssDNA coated on plates when phosphate buffer, containing 1.0M NaCl, was used as elution buffer. On the other hand, the absorbance of the standard serum gave 0.345 against ssDNA when only PBS was used. Therefore, this result indicated that high concentration of salt did not alter the antigenicity of the coated ssDNA.

Residual DNA binding was $66.7 \pm 21.1\%$ in patients with specific features and $80.5 \pm 13.5\%$ in patients without them (Fig 1). Although the difference was not statistically significant between patients with specific features and those without specific features, the avidity of anti-ssDNA antibodies tended to be lower in the formers than in the latters.

Crossreactivity of purified IgG antibodies to ssDNA

Purified IgG antibodies to ssDNA from 2 patients with specific features and 4 patients without these features were analysed. The representative results of competitive ELISA were shown in Fig 2 and Fig 3. Purified IgG anti-ssDNA antibodies from patient KC who had a specific feature were not inhibited by both cardiolipin and phosphatidylserine, but strongly inhibited by ssDNA (Fig 2). This result suggested that anti-ssDNA antibodies

Fig 1 The avidity of anti-ssDNA antibodies in patients with specific features and those without specific features. The avidity of anti-ssDNA antibodies was examined by salt elution studies in solid phase ELISA. Residual DNA binding activity after elution of phosphate buffer containing 1.0M NaCl was expressed. Either thrombosis or spontaneous abortion was defined as specific feature. The avidity of anti-ssDNA antibodies tended to be lower in patients with specific features than in those without specific features.

Fig 2 Representative result of competitive ELISA (Case KC with thrombosis as a specific feature). The crossreactivity of affinity purified IgG anti-ssDNA antibodies was examined by competitive ELISA. The results of the competitive inhibition were expressed as percent inhibition of DNA binding activity by the inhibitors, ssDNA or micelles of phospholipids. Affinity purified IgG anti-ssDNA antibodies from case KC were not inhibited by both cardiolipin (○—○) and phosphatidylserine (▲...▲), but strongly inhibited by ssDNA (●—●).

Fig 3 Representative result of competitive ELISA (Case MM without thrombosis or spontaneous abortion as specific features). The crossreactivity of affinity purified IgG anti-ssDNA antibodies was examined by competitive ELISA. The results of the competitive inhibition were expressed as percent inhibition of DNA binding activity by the inhibitors, ssDNA or micelles of phospholipids. Affinity purified IgG anti-ssDNA antibodies from case MM were strongly inhibited by ssDNA (●—●), and also inhibited by both cardiolipin (○—○) and phosphatidylserine (▲...▲).

from patient KC did not recognize negatively charged phospholipids, cardiolipin and phosphatidylserine, but ssDNA. On the other hand, purified IgG anti-ssDNA antibodies from patient MM who did not have these

Table 1 Percentage of Inhibition of Affinity Purified IgG anti-ssDNA Antibodies by Cardiolipin (CL) and Phosphatidylserine (PS)

Case	Specific Features	% Inhibition	
		CL	PS
1	(+)	0	0
2	(+)	5	3
3	(-)	16	18
4	(-)	25	19
5	(-)	16	17
6	(-)	14	15

features were strongly inhibited by ssDNA, and also inhibited by both cardiolipin and phosphatidylserine (Fig 3). This result suggested that anti-ssDNA antibodies from patients MM could react with both ssDNA and negatively charged phospholipids.

Table 1 depicts the results of percent inhibition at 100 µg/ml of inhibitors in competitive ELISA. Purified IgG anti-ssDNA antibodies from 2 patients with specific features were not adequately inhibited by both cardiolipin and phosphatidylserine, whereas purified IgG anti-ssDNA antibodies from 4 patients without these features were inhibited by cardiolipin and phosphatidylserine.

Discussion

Recent studies have revealed that anti-phospholipid antibody positive patients with SLE tend to have the characteristic clinical symptoms such as thrombosis, recurrent fetal loss and thrombocytopenia.^{2,3,5-8} Moreover, the concept of anti-phospholipid syndrome has been proposed.⁶⁻⁸ Although the diagnostic criteria for anti-phospholipid syndrome has been advocated,⁸ there are still some arguments as to the definition of this syndrome.⁹⁻¹² One of the reasons why the concept of this syndrome is unclear is the question whether all of anti-phospholipid antibody positive patients with SLE have these specific clinical features.

The titer and the isotype of anti-cardiolipin antibody have been studied to distinguish the antibody positive patients with specific features from those without specific features.^{7,8,27-29} Twenty-one percent of patients with SLE were found to show a high titer of anti-cardiolipin antibody,³⁰ and it was reported that there was a strong statistical correlation between elevated IgG anti-cardiolipin antibody levels and these specific clinical features.^{27,29} However, a high titer of anti-cardiolipin antibody could be observed in several patients with SLE who had no history of thrombosis, fetal death and thrombocytopenia.^{10,12}

On the other hand, antibodies to ssDNA are detected in all patients with SLE, and the polyspecificity has been found in mouse and human anti-DNA antibodies.^{4,13,15}

Thus, we investigated the specificity of anti-ssDNA antibodies in sera from anti-phospholipid antibody positive patients with SLE.

Anti-cardiolipin antibody and anti-ssDNA antibodies were detected in all sera from our 20 patients with SLE. Arterial or venous thrombosis and spontaneous abortion were defined as specific features in anti-phospholipid antibody positive patients with SLE according to the previous study.²⁶ Consequently, our patients could be divided into two groups whether these specific clinical features were present or absent.

First, we examined the avidity of antibodies to ssDNA to get a clue to the solution of this problem. Because Smeenk, *et al.* suggested that low avidity of anti-ssDNA antibodies determined the property for the crossreactivity of anti-ssDNA antibodies.^{16,17} The preliminary experiment showed that high concentration of salt did not have any effects on coated ssDNA. Residual DNA binding activity after salt elution tended to be lower in patients with specific features than in those without these specific features. These results suggested the importance of the avidity of anti-ssDNA antibodies in the immunological view in anti-phospholipid antibody positive patients, although more serum samples should be examined.

The crossreactivity between anti-cardiolipin antibody and anti-ssDNA antibodies has been demonstrated by several investigators, who studies mouse or human monoclonal antibodies to ssDNA,^{13,14,31} anti-cardiolipin antibody and anti-ssDNA antibodies in immunized mice,³² and even polyclonal antibodies to ssDNA in sera from human SLE patients.³³ However, Harris, *et al.* found that anti-cardiolipin antibody bound negatively charged phospholipids and had little crossreactivity with DNA or unrelated negatively charged polymers, and therefore that anti-phospholipid antibodies and anti-DNA antibodies are largely separate groups of antibodies.^{34,35} This question whether these antibodies have cross-reactivity is still controversial.

Therefore, at the next step, we examined the cross-reactivity of anti-ssDNA antibodies in the two groups; those with specific features and those without specific features. A few serum samples could be used because of enough serum volume to be tested.

Purified IgG antibodies to ssDNA were used to avoid the influence of any phospholipids in sera in competitive ELISA. Antibodies to ssDNA from 4 patients without specific features showed mild inhibition by negatively charged phospholipids, cardiolipin and phosphatidylserine. These results suggested that antibodies to ssDNA from these patients have the crossreactivity with negatively charged phospholipids. These results are explained by the idea that these antibodies to ssDNA recognize the phosphodiester group as an epitope, which both DNA and cardiolipin commonly possess.¹³

On the other hand, antibodies to ssDNA from 2 patients with specific features had little inhibition by negatively charged phospholipids. These results indicated that antibodies to ssDNA from these patients do not have the crossreactivity against phospholipids. Therefore, two kinds of antibodies, anti-cardiolipin antibody and anti-ssDNA antibodies, recognize the separate epitopes. These results confirm the previous studies that a group of crossreactive anti-DNA antibodies represent very small antibody population in sera from patients with SLE.^{36,37} Thus, anti-cardiolipin antibody in these patients, who are regarded to have anti-phospholipid syndrome,²⁶ may react only with cardiolipin or other negatively charged phospholipids. Because phospholipids form micelles in aqueous solution with the glyceride portion directed inward to the core of the micelle and with the phosphodiester groups directed outward,⁸ anti-cardiolipin antibody in sera from these patients may bind to the complexed form of cardiolipin and serum cofactor, β_2 -glycoprotein I.^{38,39} Further studies are necessary to examine the cofactor dependency in this group of patients.

A recent study has demonstrated the same cross-reactive idiotype on both anti-cardiolipin antibody and anti-ssDNA antibodies in sera from patients with SLE.⁴⁰ This study suggested that two kinds of autoantibodies originate from the same genetic origin. However, different IgG subclass and light chain distributions of anti-cardiolipin antibody and anti-ssDNA antibodies were found in sera from patients with SLE.⁴¹ All subclasses of anti-cardiolipin antibody were detected. This study suggested that extensive class switching may occur at the B cell by the antigen driven.⁴² Therefore further studies are necessary to determine the diversity about anti-cardiolipin antibody and anti-ssDNA antibodies in patients with anti-phospholipid syndrome.

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