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Monoclonal hybridoma anti-cardiolipin antibodies from SLE mice

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SUMMARY

To determine whether the anti-cardiolipin antibodies are identical with the lupus anticoagulant and other antibodies to phospholipids and DNA, we prepared monoclonal hybridoma autoantibodies to cardiolipin from SLE-prone MRL/lpr mice and characterized their specificity. Using a somatic cell hybridization technique, we established three hybridoma clones which produce antibodies to cardiolipin (CAL-1: IgG2b, k, CAL-2: IgM, k and CAL-3: IgM, k). These hybridoma antibodies preferentially reacted with cardiolipin and phosphatidylserine, weakly reacted with phosphatidylinositol, but not with other phospholipids such as phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and VDRL antigen. Two hybridoma anti-cardiolipin antibodies bound to ssDNA and were found to act as the lupus anticoagulant when mixing activated partial thromboplastin time with cephalin. These autoantibodies may prove to be good tools for elucidating mechanisms of thrombosis, thrombocytopenia, fetal loss and other related manifestations found in patients with systemic lupus erythematosus.

Keywords anti-cardiolipin antibody lupus anticoagulant anti-DNA antibody BFP-STS systemic lupus erythematosus

INTRODUCTION

Anti-phospholipid antibodies, including anti-cardiolipin antibodies, lupus anticoagulant and the biological false positive serological test for syphilis (BFP-STS) have often been detected in sera from patients with systemic lupus erythematosus (SLE) and related autoimmune disorders (Harris, Gharavi & Hughes, 1985a; Shulman, 1987). These patients with anti-phospholipid antibodies are prone to thromboembolic manifestations such as cerebral or myocardial infarctions, pulmonary embolism, deep venous thrombosis and intrauterine fetal death due to placental infarction (Mueh, Herbst & Rapaport, 1980; Carreras *et al.*, 1981; Boey *et al.*, 1983; Harris *et al.*, 1983; Lockshin *et al.*, 1985).

We reported that anti-DNA antibodies from SLE and SLE-prone mice cross-reacted with cardiolipin (Koike *et al.*, 1982a, 1984a,b). In contrast, Harris *et al.* (1985b) found that affinity purified anti-cardiolipin antibodies reacted with negatively charged phospholipids, but not with ssDNA. There is a report that monoclonal human hybridoma lupus anticoagulant antibodies react with cardiolipin and DNA (Rauch, Meng & Tannenbaum 1987). Hence, the relation between anti-phospholipid antibodies and anti-DNA antibodies remains controversial. To determine whether the anti-cardiolipin antibodies are identical with the lupus anticoagulant or other antibodies including anti-DNA antibodies, we prepared monoclonal hybridoma antibodies to cardiolipin from SLE-prone MRL/lpr mice. Attempts were made to characterize the precise specificity of the antibodies.

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MATERIALS AND METHODS

Mice and sera

MRL/lpr mice were obtained from colonies cared for in our laboratory. Blood was collected from the periorbital sinus. All the sera were kept at −70°C until use.

Cell fusion

Somatic cell hybridization was carried out as described by Oi & Herzenberg (1980). In brief, P3U1 cells were fused with spleen cells from MRL/lpr mice and the hybrid cell lines producing anti-cardiolipin antibodies were cloned by repeated procedures of limiting dilution.

Purification of mouse immunoglobulins from ascites

Ascites were obtained by giving an intraperitoneal injection of established mouse hybridoma cells to nude mice (ICR nu/nu). Euglobulin fractions of the ascites containing IgM monoclonal antibody were prepared. Ascites were dialysed against 0·01 M

sodium-phosphate buffer, pH 6·5 and the pellet obtained after centrifugation at 1,200 *g* 30 min at 4°C was suspended in 3% NaCl solution and extensively dialysed against 0·01 M phosphate-buffered saline (PBS, pH 7·4). Ascites containing IgG antibody were purified using Affi-Gel Protein A MAPS II Kit (Bio-Rad Laboratories, Richmond, California, USA).

Phospholipids

Cardiolipin (Sigma Chemical Company, St Louis, Missouri, USA) came supplied in ethanol solution. Phosphatidylserine, phosphatidylinositol and phosphatidylcholine in chloroform solution were purchased from Serdary Research Laboratories, London, Ontario, Canada. Sphingomyelin and phosphatidylethanolamine were purchased from Koch-Light Laboratories, Colonbrook Berks., UK and Fluka Chemie AG, Buchs, Switzerland, respectively. Micelles were obtained in PBS by sonication.

Polynucleotides

Calf thymus DNA was purchased from PL Biochemicals, Milwaukee, Wisconsin, USA. The double-stranded DNA (dsDNA) was obtained by digestion of calf thymus DNA with S1 nuclease (Seikagaku Kogyo, Tokyo, Japan), then dialysed and fractionated on a benzoyl-naphthoyl-DEAE-cellulose column (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany). The single-stranded DNA (ssDNA) was prepared by heating calf thymus DNA at 100°C for 10 min, then immediately cooling in an ice bath.

Enzyme immunoassay of anti-phospholipid antibodies

Antibodies to several phospholipids were measured by solid phase EIA, as described (Koike *et al.*, 1984a,b). Each well of the polyvinylcholride 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 the preparations were incubated for 1 h at room temperature to block the non-specific binding of immunoglobulins to the well surface. After five washes in PBS, 50 μl of serum or a monoclonal antibody 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 rabbit anti-mouse IgG or anti-mouse IgM (Cappel Laboratories, West Chester, Pennsylvania, USA) was added to each well, and the plate was incubated for 1 h. Next, 100 μl of p-nitrophenyl phosphate (Sigma) in 1 M diethanolamine buffer, pH 9·8, was added to each well. After 1 h of incubation, the optical absorbance was measured at 405 nm using a Microelisa Auto Reader, MR 580 (Dynatech).

Serological test for syphilis

Serological tests for syphilis using the hybridoma antibodies and mouse serum pools were measured by a modified flocculation test (IATRON Laboratories, Tokyo, Japan).

Enzyme immunoassay of anti-DNA antibodies

Antibodies to DNA were measured by a solid-phase enzyme-immunoassay (EIA) (Koike *et al.*, 1982a,b), but with some modification. Each well of the polyvinylchloride plates (Dynatech) was first coated with 50 μl of 0·1% methylated bovine serum albumin (M-BSA) solution by incubation for 1 h at room temperature. After discarding the M-BSA solution, 50 μl of DNA solution, 10 μg/ml in PBS, containing 1 mM EDTA, was added to each well and the preparations were then incubated for 1 h at room temperature. After three washes in PBS, 200 μl of 10% FCS solution was added to each well and the preparations were incubated for 1 h at room temperature to block the non-specific binding of immunoglobulins to the well surface. After five washes in PBS containing 0·05% Tween 20 (PBS-Tween), 50 μl of serum or a monoclonal antibody to be tested was added to each well, and the preparations were again incubated for 1 h at room temperature. After ten washes in PBS-Tween, 50 μl of alkaline phosphatase (Sigma) labelled rabbit anti-mouse IgG or anti-mouse IgM (Cappel Laboratories) 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 9·8, was added to each well. After 1 h of incubation, the optical absorbance was measured at 405 nm, using a Microelisa Auto Reader, MR 580 (Dynatech).

Immunoglobulin subclass of monoclonal antibodies

The immunoglobulin subclass of monoclonal antibodies was determined by the use of MonoAb-ID EIA Kit A (Zymed Laboratories, Inc., South San Francisco, California, USA).

Tests of anticoagulant activity

A mixing activated partial thromboplastin time (APTT) with cephalin by the method of Proctor and Rapaport (1961) was used with some modification. Platelet poor plasma (PPP) was obtained from nine parts of whole blood into one part of 3·8% sodium citrate and spun at 1,300 *g* for 15 min. Two-hundred microlitres of purified monoclonal hybridoma antibody and 200 μl of PPP were mixed and the preparations were incubated for 1 h at 37°C. After this procedure, 0·1 ml of samples was added to 0·1 ml of a diluted (1 in 5) Actin (activated cephaloplastin reagent, American Dade, Aguada, Puerto Rico) and incubated for 2 min at 37°C; 0·1 ml of 0·02 M calcium chloride (International Reagents Corp., Kobe, Japan) was then added. The proper time from the addition of calcium chloride for the formation of a clot was determined by the use of Coag Stat (Daiichi Kagaku, Kyoto, Japan).

RESULTS

Phospholipid binding of monoclonal anti-cardiolipin antibodies

Using a somatic cell hybridization technique, we established three hybridoma clones (CAL-1: IgG2b, k, CAL-2: IgM, k and CAL-3: IgM, k) from SLE prone MRL/lpr mice. These clones produce antibodies to cardiolipin. In an attempt to clarify the phospholipid binding activity of these monoclonal antibodies, we carried out a solid-phase EIA using phosphatidylserine, phosphatidylinositol, sphingomyeline, phosphatidylcholine and cardiolipin, as the antigens.

Figure 1 shows the representative phospholipid binding of one hybridoma monoclonal antibody, CAL-1. It is obvious that this monoclonal antibody preferentially bound to cardiolipin, phosphatidylserine and phosphatidylinositol, but not to other phosphalipids such as phosphatidylcholine and sphinogomyelin. Similar results were obtained from two other monoclonal anti-cardiolipin antibodies (CAL-2 and CAL-3) (Table 1).

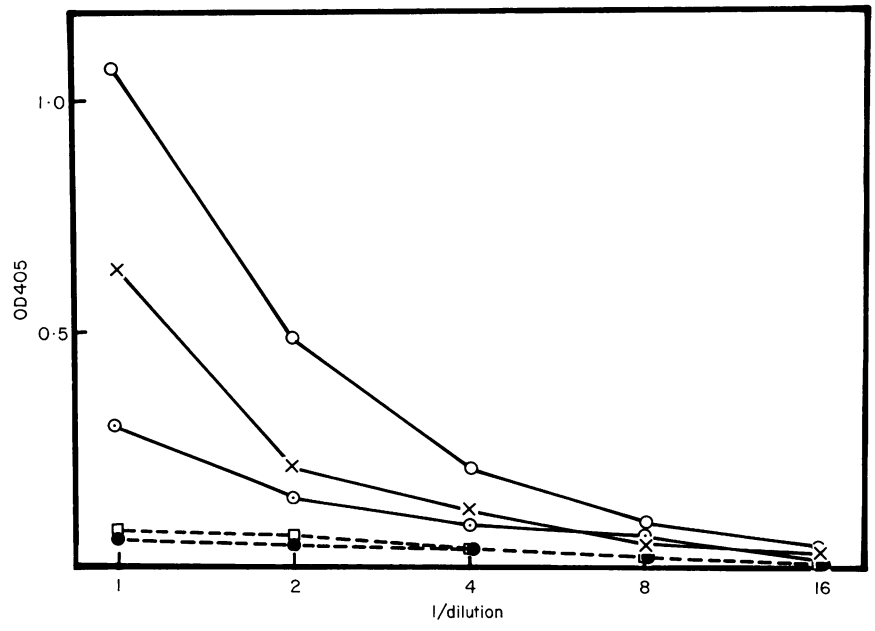

Fig. 1. Representative phospholipid binding of a monoclonal anti-cardiolipin antibody (CAL-1) derived from MRL/lpr mouse. ○ Cardiolipin × phosphatidylserine, ⊙ phosphatidylinositol, □ sphingomyelin, ● phosphatidylcholine.

Table 1. Binding activity of monoclonal anti-cardiolipin antibodies to phospholipids (OD 405 nm × 10^{-3})

		Binding to					
Clones	Isotype	CL	PS	PI	PC	SM	PE
CAL-1	IgG2b	872	537	229	< 50	< 50	< 50
CAL-2	IgM	446	589	75	< 50	< 50	< 50
CAL-3	IgM	619	375	144	145	< 50	< 50

CL cardiolipin, PS phosphatidylserine,
PI phosphatidylinositol, PC phosphatidylcholine,
SM sphingomyelin, PE phosphatidylethanolamine

Anticoagulant activity of monoclonal anti-cardiolipin antibodies

To determine whether the monoclonal anti-cardiolipin antibody had anticoagulant activity, we carried out a mixing APTT, using normal human platelet poor plasma and purified monoclonal antibodies from ascites at the indicated dilution. These monoclonal anti-cardiolipin antibodies have the potential to prolong clotting time in *in vitro* coagulation assays, in a dose dependent manner. A representative result using CAL-3 is shown in Fig. 2.

Anti-VDRL antibody and anti-cardiolipin antibody

To search for a relationship between the biological false-positive serological test for syphilis (BFP-STS) and anti-cardiolipin antibody, the reactivity to VDRL antigen was measured using a flocculation test. With all the tested hybridoma antibodies and serum pool of MRL/lpr mice that had the high titre of anti-cardiolipin antibodies, virtually no positive agglutination reaction was observed, other than serum from a positive control from syphilitics (Table 2).

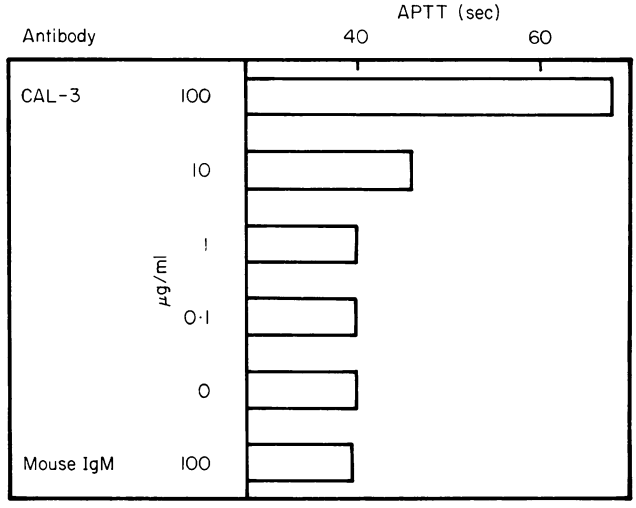

Fig. 2. Anticoagulant activity of a monoclonal anti-cardiolipin antibody (CAL-3). Mixing APTT was done using normal human PPP and purified CAL-3 antibody at the indicated dilution.

Table 2. VDRL antigen reactivity of monoclonal anti-cardiolipin antibodies by a flocculation test

Clones	Agglutination reaction
CAL-1	−
CAL-3	−
MRL serum	−
Syphilitics	+ + +

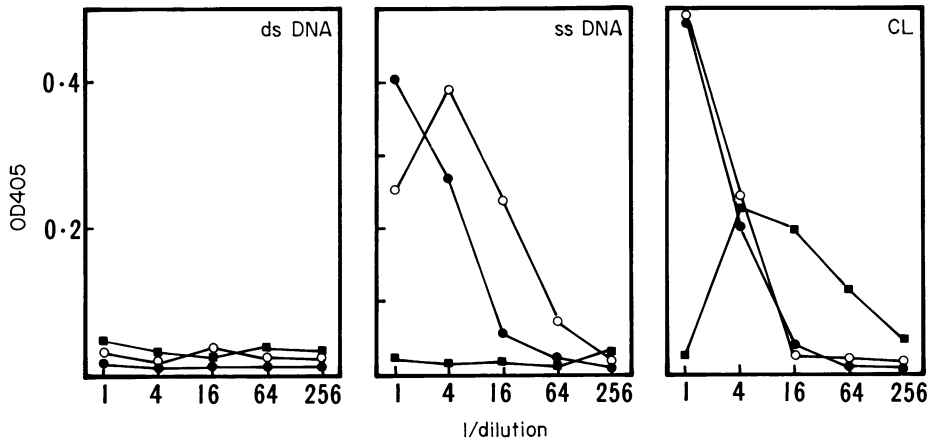

Fig. 3. Binding activity of monoclonal anti-cardiolipin antibodies to ssDNA, dsDNA and cardiolipin. ● CAL-1, ■ CAL-2, ○ CAL-3.

Table 3. Binding activity of mouse monoclonal antibodies to DNA and cardiolipin (OD 405 nm × 10^{-3})

Clones	Isotypes	Binding to ssDNA	dsDNA	CL
BWH13H1	IgG2a	914	769	216
M.3A3A3	IgG2b	996	994	268
M.8D10	IgG1	1107	1016	< 50
M.3A9D8	IgG2a	1051	1088	< 50
M.1D3D2	IgG2a	1110	1121	< 50
M.1H1D11	IgG2a	1074	1100	< 50
B.5A10E9	IgG2a	1067	1123	< 50
B.2H7B9	IgG2a	1173	1013	< 50

CL cardiolipin

DNA binding of monoclonal anti-cardiolipin antibodies

We reported that antibodies reacting with DNA can be basically classified into at least two categories (Koike *et al.*, 1982a,b). One includes those which react exclusively with ssDNA. The other includes antibodies which are serologically polymorphic, in that some react with a variety of polynucleotides including ssDNA, dsDNA and synthetic RNA and with cardiolipin.

Based on these observations, we examined the DNA binding activity of three monoclonal hybridoma antibodies to cardiolipin and representative results were shown in Fig. 3. Two clones (CAL-1 and CAL-3) preferentially reacted with ssDNA but not with dsDNA.

When we examined the binding activity of eight monoclonal antibodies which bound to both ssDNA and dsDNA, two out of eight reacted with cardiolipin. All the reaction profiles of monoclonal antibodies to DNA and cardiolipin are given in Table 3.

DISCUSSION

We prepared three monoclonal hybridoma clones which produce antibodies that preferentially reacted with cardiolipin and phosphatidylserine, weakly reacted with phosphatidylinositol, but not with other phospholipids such as phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and VDRL antigen. We also demonstrated that two monoclonal anti-cardiolipin autoantibodies bound to ssDNA and acted as a lupus anticoagulant.

There is a report of a patient with macroglobulinaemia from whom purified monoclonal IgM protein and its Fab tryptic fragment induced a prolongation of all phospholipid-dependent coagulation tests, using normal plasma (Thiagarajan, Shapiro & De Marco, 1980). It was suggested that the mechanism of coagulation inhibition was the result of a specific reaction of monoclonal IgM paraprotein with acidic phospholipids, including phosphatidylserine, phosphatidylinositol and phosphatidic acid. The binding to cardiolipin was apparently not examined. Shapiro & Thiagarajan (1982) noted that γ-globulin fractions of sera from 17 patients with the lupus anticoagulant cross-reacted with negatively charged phospholipids.

Harris *et al.* (1985b) reported that the affinity purified anti-cardiolipin antibodies showed high binding to negatively charged phospholipids such as phosphatidylserine, phosphatidic acid, phosphatidylinositol *etc.* and that these antibodies had lupus anticoagulant activity. Branch *et al.* (1987) demonstrated using the specific phospholipid EIA that sera containing lupus anticoagulant activity had antibodies against phosphatidylserine, cardiolipin, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol. They also observed that anticoagulant activity was always associated with the presence of antibodies against phosphatidylserine. Harris *et al.* (1985b) and our group (unpublished observations) found much the same evidence of a high correlation of binding activity to cardiolipin, with binding activity to phosphatidylserine.

All these results taken together suggest that the anti-cardiolipin autoantibodies found in SLE and related autoimmune disorders are, at least in part, identical to anti-phosphatidylserine antibodies and act as the lupus anticoagulant.

We reported that anti-DNA antibodies in SLE sera and monoclonal anti-DNA antibodies derived from SLE-prone mice, that reacted with both ssDNA and dsDNA, could cross-react with cardiolipin (Koike *et al.*, 1982a, 1984b). This is in keeping with the findings of Lafer *et al.* (1981) that the phospholipid groups, in particular cardiolipin have the potential to inhibit ssDNA binding of the anti-DNA monoclonal antibodies which react with a wide range of polynucleotides.

Contrary to these findings, Harris *et al.* (1985b) reported that the affinity purified anti-DNA antibodies did not bind to cardiolipin. Eilat, Zlotnick & Fischel (1986) concluded that anti-DNA antibodies and anti-cardiolipin, antibodies were a different antibody population.

For clarification, we extended experiments to examine the cardiolipin binding of our monoclonal anti-DNA antibodies which bound to both ssDNA and dsDNA (anti-ss/dsDNA antibody), using the EIA method. We found that 25% of the monoclonal anti-ss/dsDNA antibodies derived from SLE-prone mice showed a cross-reaction with cardiolipin. These results are basically in accord with observations by other investigators (Lafer *et al.*, 1981; Shoenfeld *et al.*, 1983). Smeenk, Lucassen & Swaak (1987) reported that the discrepant findings regarding the cross-reaction of anti-DNA antibodies to cardiolipin might be due to the procedures used to obtain sera or hybridomas with anti-DNA activity. They found a relationship between cross-reactivity and avidity, that is, anti-DNA antibody of high avidity to DNA showed little cross-reactivity to cardiolipin and anti-DNA antibody of low avidity was found to cross-react with cardiolipin. We found that hybridoma clones with a restricted specificity for binding to ssDNA virtually showed no cross-reactivity to all phospholipids, including cardiolipin (Koike *et al.*, 1984b, 1985).

The cross-reactivity of anti-cardiolipin antibody to ssDNA was also described by Colaco, Scadding & Lockhart (1987), and this may be explained by the presence of shared idiotypes on anti-cardiolipin antibodies and anti-ssDNA antibodies, as shown by Valesini *et al.* (1987).

It has become apparent that the presence of anti-cardiolipin antibody is strongly associated with thrombosis, thrombocytopenia, recurrent fetal losses and Coombs' positivity in SLE and related autoimmune disorders (Mueh, *et al.*, 1980; Carreras *et al.*, 1981; Boey *et al.*, 1983; Harris *et al.*, 1983; Lockshin *et al.*, 1985). Whether or not the anti-cardiolipin antibodies are responsible for the occurrence of these manifestations is now the subject of intensive study. Our monoclonal hybridoma anti-cardiolipin autoantibodies should prove to be useful for elucidating these mechanisms.

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