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Relationship between anti-cardiolipin and anti-endothelial cell antibodies in systemic lupus erythematosus

A. VISMARA, P. L. MERONI, ANGELA TINCANI*, E. N. HARRIS†, WILMA BARCELLINI, A. BRUCATO, M. KHAMASHTA,‡ G. R. V. HUGHES,‡ C. ZANUSSI & G. BALESTRIERI *Istituto di Medicina Interna, Mallattie Infettive & Immunopatologia University of Milan, *Servizio di Immunologia Clinica, Spedali Civili, Brescia; †Division of Rheumatology, Department of Medicine, University of Louisville, Louisville, Kentucky, and ‡The Lupus Research Laboratory, St. Thomas, Hospital, London*

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SUMMARY

Anti-endothelial cell antibodies (AECA) have been detected in 51 lupus sera by cell surface radioimmunoassay with a prevalence of 39·2% for IgG and 45·1% for IgM-AECA. No correlations were found between AECA and different clinical or laboratory parameters, including the presence of anti-cardiolipin antibodies and signs associated with the presence of anti-phospholipid antibodies. However, absorption with cardiolipin liposomes partially inhibited endothelial cell binding, and affinity purified anti-cardiolipin antibodies were able to react with intact human endothelial cells. The binding did not occur via Fc receptors since blocking of Fc receptors with rabbit IgG did not affect the endothelial cell reactivity. Taken together our findings support the hypothesis that antibodies directed against negatively charged phospholipids can be part of the anti-endothelial cell antibodies in certain lupus sera. The possible role of these cross-reacting antibodies in the pathogenesis of vascular thrombosis in lupus patients is discussed.

Keywords systemic lupus erythematosus anti-cardiolipin antibodies anti-endothelial cell antibodies thrombosis

INTRODUCTION

Anti-endothelial cell antibodies (AECA) have been demonstrated in sera from patients affected by systemic lupus erythematosus (SLE) and their presence has been related to vascular injury (Cines *et al.*, 1984; Le Roux *et al.*, 1986; Hashemi, Douglas-Smith & Izaguirre, 1987).

In addition a significant association between IgG AECA and lupus-like anticoagulant (LLAC) has been shown recently in a small series of SLE patients (Le Roux *et al.*, 1986). Carreras and Vermylen (1982) first showed that immunoglobulin fractions from sera of patients with LLAC activity inhibited prostacyclin release from endothelial cells, suggesting a possible interference of anti-phospholipid antibodies and endothelial cells (Carreras *et al.*, 1981; Carreras & Vermylen, 1982). However, further studies reported conflicting results (Izaguirre *et al.*, 1987; Rustin *et al.*, 1987; Petraiuolo, Bovill & Hoak, 1987; Hasselaar *et al.*, 1987).

Correspondence: Pier Luigi Meroni M.D., Istituto di Medicina Interna, Malattie Infettive & Immunopatologia, Padiglione Granelli-Ospedale Policlinico, University of Milan, Via F. Sforza 35, 20122-Milan, ITALY

It is from this standpoint that we proceeded to investigate the possible relationship between AECA and other anti-phospholipid antibodies associated with LLAC, namely the anti-cardiolipin antibodies (Harris *et al.*, 1983). For this purpose we studied 51 SLE sera for their binding activity to cardiolipin and endothelial cells using both whole sera and affinity purified anti-cardiolipin fractions.

Our results indicate that anti-endothelial cell antibodies are a heterogenous population which may contain subsets of antibodies cross-reacting with cardiolipin and phospholipid structures present on the surface of intact human endothelial cells.

MATERIALS AND METHODS

Patients

We studied 51 sera from SLE patients, who satisfied the revised American Rheumatism Association classification for SLE (Tan et al., 1982). The following information was recorded for all patients: age, sex, duration of the disease, prednisone and cytotoxic therapy, organ involvement (cutaneous, musculoskeletal, hematologic, renal, neurologic, serositis and Raynaud phenomenon), current or past clinical events reported to be associated with anti-phospholipid antibodies and laboratory

data (anti-nuclear and anti-dsDNA antibodies, complement, lupus-like anti-coagulant, venereal disease reference laboratory test).

Endothelial cell culture

Endothelial cells were harvested from human umbilical cord veins by collagenase digestion using established methods (Jaffe *et al.*, 1973). Endothelial cells were grown onto 0·1% gelatine coated tissue culture flasks (Costar, Cambridge, MA.), in medium E199 (Flow Labs., Irvine, Scotland, UK) supplemented with 20% heat-inactivated newborn calf serum (NCS, Gibco, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin (Eurobio, Paris, France) and 1% L-glutamine (Flow Labs., Irvine, Scotland, UK). Endothelial cell growth factor (50 μg/ml) prepared according to the method of Maciag *et al.* (1979) and heparin (90 μg/ml) were also added to cultures. Cells were fed every three days, and, when confluent, subcultured by exposure to 0·05% trypsin-0·02% EDTA (trypsin-EDTA, Flow Labs., Irvine, Scotland, UK). These cells met morphological criteria for endothelial cells and were positive for Factor VIII antigen (Jaffe, Hoyer & Nachman, 1973).

Anti-endothelial cell antibody assay

Anti-endothelial cell antibodies were detected using a solid-phase radioimmunoassay with modifications of the method of Cines *et al.* (1984). Primary cultures of endothelial cells from at least two donors were detached by exposure to trypsin-EDTA and placed onto gelatin-coated 96-well microtitre plates (Costar, Cambridge, MA.) at a concentration of 2×10^4 cells/well in complete medium. Cells reached a confluent monolayer in about 24 h. Plates were washed twice with Hank's Balanced Salt Solution (HBSS, Flow Labs., Irvine, Scotland, UK) 200 μl/well. Free protein binding sites were then blocked by adding 200 μl/well of blocking buffer (HBSS-1% Bovine Serum Albumin, Sigma Chemicals Co., St. Louis, Mo.) and incubated for 1 h at room temperature. After two washes with HBSS, 100 μl of the test serum (diluted 1/25 with HBSS) or 100 μl of affinity purified anti-cardiolipin antibodies (diluted with HBSS at a concentration of 250 μg/ml) were added to each well in triplicate. Plates were incubated for 2 h at room temperature and then washed three times with HBSS. One hundred microlitres of ^{125}I-labelled affinity purified goat anti-human IgG or IgM (diluted with HBSS at a concentration of 5 μg/ml) were added to each well and incubated for 2 h at room temperature. Four washes were then performed with HBSS (200 μl/well) and 100 μl of lysis buffer (0·1% SDS-0·025 M NaOH) were added to each well. Solubilized endothelial cells were collected using Q-tip swabs and associated radioactivity was measured in a gamma-counter.

The amount (ng) bound of anti-human IgG or IgM of the positive control sera at the standard dilution of 1/25 was arbitrarily chosen as 100% endothelial cell binding activity. Positive control sera were run on each plate and test sample binding activity was expressed as percentage of positive reference sera. This was accomplished by the following formula: (ng bound of sample/ng bound of reference serum) × 100. Thus, the conversion of bound values to a percentage of endothelial cell binding activity of the reference sera eliminated the between day variation and ensured accuracy in comparison of results. Values greater than three standard deviations above the mean of 16 normal subjects were considered raised, namely 33·30% for IgG-AECA and 33·28% for IgM-AECA. In absorption experiments and in experiments carried out with affinity purified fractions, the values were expressed as ng bound of the anti-IgG or anti-IgM antisera.

The following controls were included in each plate as references: a sample from a pool of 16 healthy subjects (negative sample), a positive (IgG or IgM) AECA reference serum, blocking buffer (blank) and Cohn Fraction II (Sigma Chemicals Co., St. Louis, Mo.) or normal human IgM (Behring Inst., Italy) diluted with HBSS at the concentration of 250 μg/ml when affinity purified preparations were tested.

The endothelial cells remained attached and confluent during every passage of the assay, without any significant morphological change as documented by examination under phase contrast light microscopy and Giemsa staining.

Anti-phospholipid antibodies

Anti-cardiolipin antibodies (ACA) were detected using a solid-phase radioimmunoassay as previously described (Tincani *et al.*, 1985; Meroni *et al.*, 1987). The test was performed according to recommendations of the recent workshop on standardization of the anti-cardiolipin antibody test (Harris *et al.*, 1987). Results were expressed as ng bound of ^{125}I-labelled affinity purified anti-human IgG or IgM. Values higher than three standard deviations above the mean of 40 normal sera were considered raised, namely 3·55 ng for IgG-ACA and 7·40 ng for IgM-ACA.

Anti-phosphatidylserine antibodies (APSA) were measured by a solid phase radioimmunoassay. Briefly, phosphatidylserine (Sigma Chemicals Co., St. Louis, Mo.; 25 μl, 50 μg/ml in methanol: chloroform 4:1) was coated on polystyrene microtitre wells (Indywell, Greiner, Germany) by evaporation under nitrogen as previously described (Harris *et al.*, 1985). The plates were blocked for 2 h and washed three times with 10% Fetal Calf Serum (FCS; Flow Labs., Irvine, Scotland, UK) in phosphate-buffered saline (PBS). The sera diluted 1/50 in FCS-PBS were added to plates and incubated for 4 h at room temperature. After three washes with FCS-PBS, affinity purified ^{125}I-labelled goat antibodies against human IgG or IgM were added. The plates were incubated overnight, washed three times and then the wells were counted.

Absorption experiments

Absorption experiments were performed as previously described (Meroni *et al.*, 1987). Briefly, the sera were mixed with cardiolipin liposomes, incubated for 1 h at 37°C and overnight at 4°C. The mixtures were then centrifuged at 30,000 *g* for 15 min at 4°C and the supernatants kept as absorbed sera.

The results of inhibition studies were expressed as percentage inhibition of anti-cardiolipin, anti-phosphatidylserine and anti-endothelial cells antibody binding activity. Percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \left(1 - \frac{\text{ct/min after cardiolipin absorption} - \text{background}}{\text{ct/min before cardiolipin absorption} - \text{background}}\right) \times 100$$

Affinity purified anti-cardiolipin antibodies

Affinity purified ACA were prepared from patients' sera using cardiolipin liposomes as previously described (Meroni *et al.*, 1987; Harris *et al.*, 1985).

Lupus like anticoagulant (LLAC)
LLAC was detected as previously described (Tincani *et al.*, 1985).

Antibodies to double stranded DNA (ds-DNA)
Anti-dsDNA were detected by the Farr assay using ^{14}C ds-DNA (Amersham International) as previously described (Tincani *et al.*, 1985). Results were given in percentage of antigen bound. The upper limit of normal (mean + 3 s.d.) was 25%.

Venereal disease reference laboratory test
The venereal disease laboratory reference test was performed by commercial slide flocculation test (Wellcome Foundation Limited, UK).

Statistical analysis
Statistical analysis was performed by chi-square tests with Yates' correction.

RESULTS

Anti-endothelial cell antibodies
Serial titration experiments were performed to determine the serum dilution that provided optimal experimental conditions. The 1/25 dilution, which displayed the highest binding activity in the reference serum and the lowest background values in normal sera, was selected (Fig. 1).

There was negligible binding of affinity purified ^{125}I-labelled goat anti-human IgG or IgM to endothelial cells or to gelatin coated plates. Experiments were also carried out with cells fixed by 0.0625% paraformaldehyde for 10 min at 22°C, as described by Cines *et al.* (1984). Six positive and six negative sera did not display significant variations in binding activity on fixed and unfixed endothelial cells (data not shown). However, to exclude any possible alterations in the endothelial cell surfaces, the assays were performed without paraformaldehyde fixation. Fifty-one SLE sera were tested for anti-endothelial cell antibodies (AECA); 20 were positive for IgG-AECA (39·2%) and 23 for IgM-AECA (45·1%); eight were positive for both isotypes (15·6%).

We were unable to find significant correlations between AECA and any clinical or laboratory parameters investigated and in particular with signs and symptoms reported to be associated with anti-phospholipid antibodies, such as venous or arterial thrombosis, fetal loss and thrombocytopenia. In contrast the positivity of IgG-ACA was significantly associated with history of thrombosis ($P < 0.05$), fetal loss ($P < 0.01$) and the presence of LLAC ($P < 0.05$).

Prevalence of anti-endothelial cell antibodies in ACA positive and ACA negative SLE sera
Figure 2 shows the distribution of IgG and IgM AECA in ACA positive and ACA negative SLE patients. Positivities for anti-endothelial cells were present either in anti-cardiolipin positive or negative sera and no correlation was found between the two antibodies.

Absorption experiments with cardiolipin liposomes
In order to assess whether anti-cardiolipin antibodies can be responsible, at least in part, for the endothelial cell binding, 18 sera positive both for ACA and AECA were tested for anti-endothelial cell activity after absorption with cardiolipin liposome suspensions. As shown in Table 1, using high concentrations of cardiolipin liposomes (12 mg/ml) we completely inhibited the anti-phospholipid activity detected either as anti-cardiolipin or anti-phosphatidylserine binding activity. In contrast, all the sera retained their activity against unfixed endothelial cells. However, partial inhibition, ranging from 10·1 to 50·1%, was found in seven out of 18 ACA and AECA positive SLE sera (Table 1).

The specificity of the absorption test was supported by the fact that antibody activities directed against different antigens, namely anti-nuclear or anti-mitochondrial type M2 antibodies, were not affected by the absorption procedures, as previously reported (Meroni *et al.*, 1987).

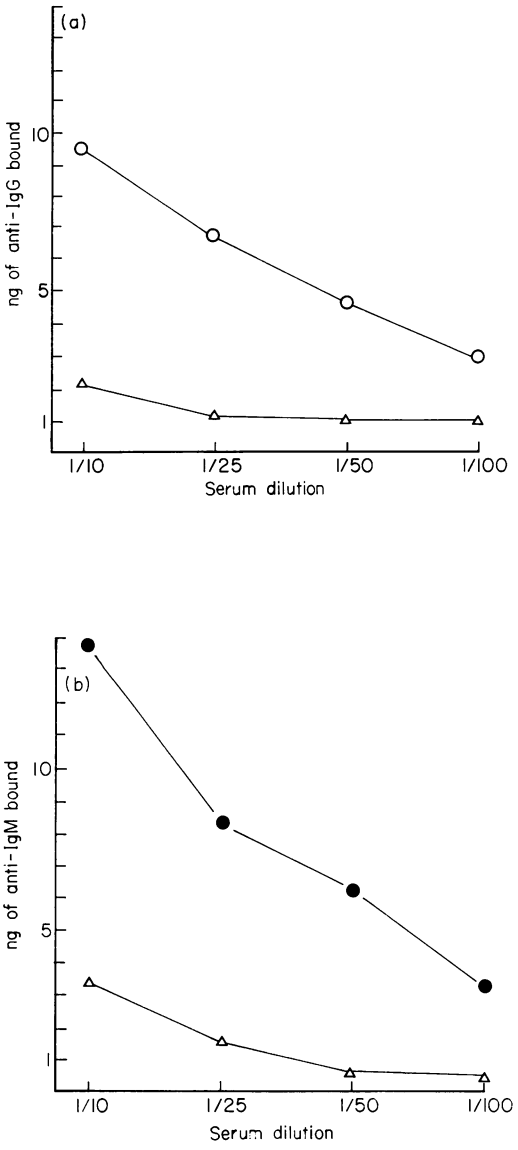

Fig. 1. Titration experiments with a reference serum positive for IgG anti-endothelial cell antibodies (Fig. 1a) and for IgM anti-endothelial cells antibodies (Fig. 1b). The control was a pool from 16 normal sera. The endothelial cell reactivity is expressed as ng of anti-IgG or anti-IgM bound. (○——○) IgG positive serum, (△——△) Normal human serum, (●——●) IgM positive serum.

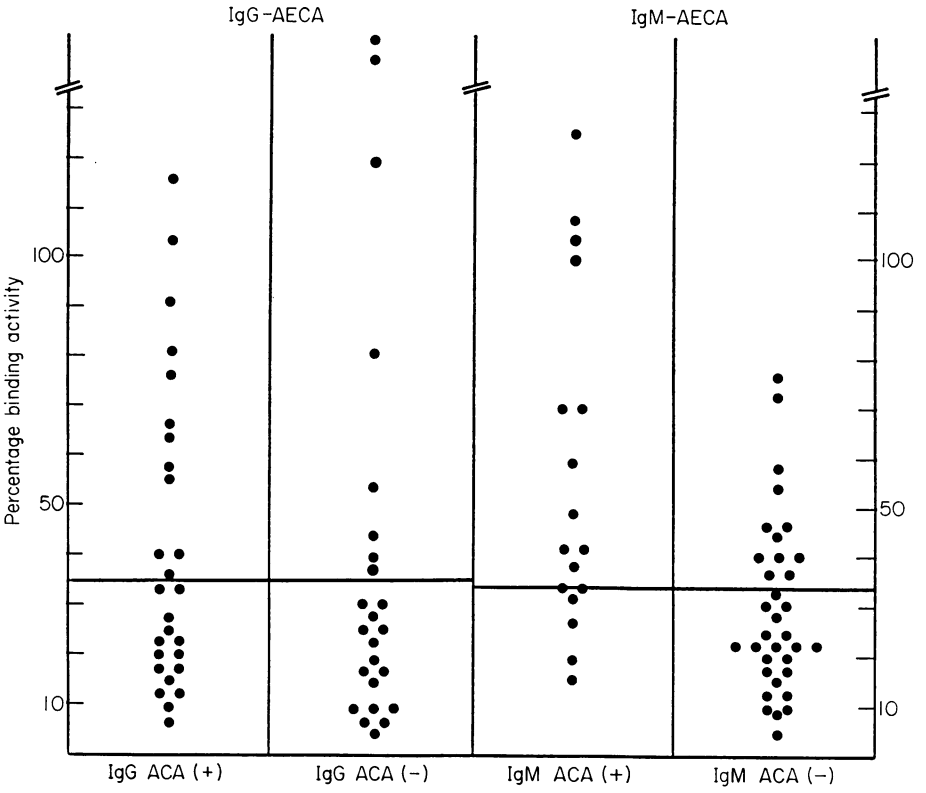

Fig. 2. Prevalence of IgG or IgM anti-endothelial cell antibodies (AECA) in ACA positive or negative SLE sera. The horizontal line represents the mean values + 3 s.d. of percentage binding activity to endothelial cells of 16 normal sera (see Materials & Methods).

Table 1. Percentage inhibition of anti-cardiolipin (ACA), anti-phosphatidyl serine (APSA) and anti-endothelial cell (AECA) activities by cardiolipin liposomes in 18 SLE sera. Inhibition of less than 10% was taken as not significant

Serum No.	Patient	ACA		APSA		AECA	
		IgG	IgM	IgG	IgM	IgG	IgM
1	BR	94·0	–	92·6	–	< 10·0	–
2	CA	73·0	74·0	78·0	49·0	50·1	42·3
3	BL	89·0	93·0	93·0	86·0	–	18·5
4	BT	–	97·0	–	51·0	–	49·9
5	RO	93·0	98·0	95·0	93·0	–	13·2
6	GA	95·0	–	ND	ND	10·1	–
7	BO	96·3	–	ND	ND	< 10·0	< 10·0
8	BE	81·9	92·5	ND	ND	–	< 10·0
9	LO	89·3	97·2	ND	ND	< 10·0	–
10	SC	–	74·4	ND	ND	–	< 10·0
11	BO	–	74·6	ND	ND	–	< 10·0
12	PR	93·5	76·1	ND	ND	< 10·0	–
13	FA	81·4	77·0	ND	ND	–	< 10·0
14	GE	90·0	85·6	ND	ND	< 10·0	–
15	AL	87·9	–	ND	ND	< 10·0	< 10·0
16	SP	82·8	95·2	ND	ND	49·3	–
17	MA	74·5	76·0	ND	ND	< 10·0	–
18	MU	78·5	82·7	ND	ND	19·0	–

– indicates sera which displayed negative binding to cardiolipin, phosphatidylserine and endothelial cells for the corresponding isotypes. ND not done.

Table 2. Studies with affinity purified anti-cardiolipin antibodies. The endothelial cell binding of the affinity purified anti-cardiolipin antibodies, Cohn fraction II (Cohn F.II) and normal human IgM were expressed as ng bound of ^{125}I-labelled affinity purified goat anti-human IgG or IgM

	SERUM		AFFINITY PURIFIED	
	ACA	AECA	IgG-AECA	IgM-AECA
MA	+	+	8·78	2·76
SP	+	+	11·31	7·86
MU	+	+	18·92	7·61
CA	+	+	5·05	ND
KE	+	−	6·37	0·81
RE	+	−	4·65	0·73
DU	+	−	0·75	3·21
COHN F.II			3·94	—
NORMAL IgM			—	3·12

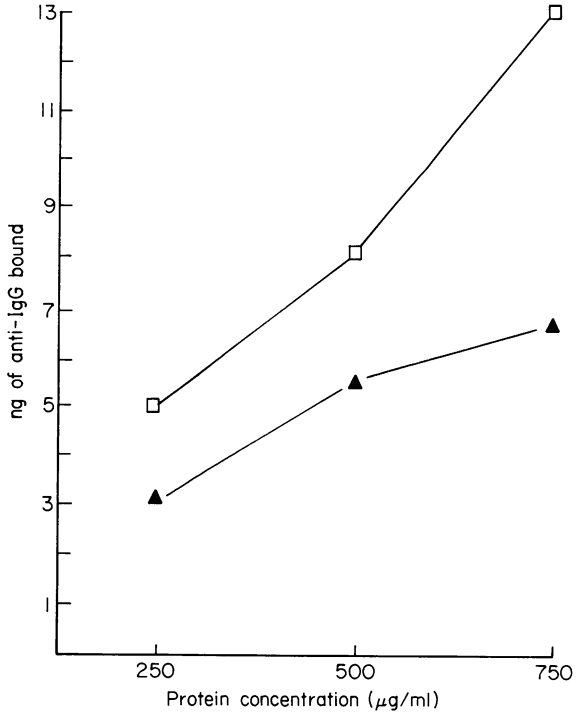

Fig. 3. Endothelial cell binding activity of CA affinity purified anti-cardiolipin preparation at different antibody concentrations. The binding activity is expressed as ng of anti-IgG bound. The control is represented by Cohn fraction II at comparable concentrations. □——□ CA affinity purified ▲—▲ Cohn fraction II.

Studies with affinity purified anti-cardiolipin antibodies

Finally, affinity purified preparations eluted from cardiolipin liposomes were tested for their ability to bind to unfixed human endothelial cells. For this purpose we selected four SLE sera positive for ACA and AECA, three of which displayed clear inhibition of the endothelial cell binding after absorption with cardiolipin liposomes, and three additional sera highly positive for ACA but negative for AECA. As shown in Table 2, endothelial cell binding activity was eluted for the four ACA and AECA positive sera. By contrast, the affinity purified fractions obtained from the other three sera did not display endothelial cell binding activity with the exception of the KE serum.

The affinity purified anti-cardiolipin fractions displayed an increase in endothelial cell binding by increasing their antibody concentrations in comparison to the control immunoglobulin preparations (Fig. 3). Furthermore, to rule out the possibility that binding occurs via Fc receptors, endothelial cells were incubated with purified rabbit IgG in order to block the Fc receptors. The cells were pre-incubated with three different concentrations of rabbit IgG (50, 500 and 5,000 μg/ml) for one hour at 37°C, washed three times with HBSS and then tested with two different affinity purified anti-cardiolipin preparations. This treatment did not affect the binding of the affinity purified fractions (affinity purified fraction alone: 4·90 and 7·00 ng of anti-IgG bound respectively; after cell treatment with 5,000 μg/ml of rabbit IgG: 5·00 and 6·70 ng bound respectively).

DISCUSSION

The Carreras' hypothesis suggests that anti-phospholipid antibodies (LLAC) can affect endothelial cell functions by reacting with antigenic determinants present on their surface (Carreras *et al.*, 1981; Carreras & Vermylen, 1982). In order to investigate the possible cross-reactivity between anti-phospholipid antibodies and endothelial surfaces, we studied 51 SLE sera for anti-endothelial cell antibodies and for anti-phospholipid antibodies identified by the cardiolipin radioimmunoassay. Using a cell surface radioimmunoassay we confirmed the presence of anti-endothelial cell antibodies in SLE patients, finding a prevalence of 39·2% for IgG and of 45·1% for IgM AECA.

Our findings are quite comparable with the data of Shingu & Hurd (1981) who found 50% of positivities for IgG-AECA in SLE sera using indirect immunofluorescence on human cultured endothelial cells. More recently Hashemi, Douglas-Smith & Izaguirre (1987) using a cellular ELISA assay, demonstrated that the amount of IgG binding to EC in SLE and normal sera overlapped considerably although the mean values were higher in SLE. On the other hand the same authors found a statistically significant increase in IgM endothelial cell binding activity from SLE sera. In addition Cines *et al.* (1984), studying sera from active SLE patients, showed a significantly increased IgG-binding activity in 24 out of 27 sera tested.

Little is known in the literature about the association between AECA and clinical or laboratory parameters in SLE patients. In this regard we were unable to correlate the presence of AECA with the clinical and laboratory parameters investigated, including the signs associated with the presence of anti-phospholipid antibodies (Harris, Gharavi & Hughes, 1985). Our data contrast with the findings by Le Roux *et al.* (1986) who found an association between IgG-AECA, detected by cell

radioimmunoassay, and LLAC in a smaller series of SLE patients. This discrepancy could be due to different selection criteria, because in the Le Roux's series the prevalence of LLAC was unusually higher (40%) than in previous reports (6–15%) (Harris, Gharavi & Hughes, 1985; Shapiro & Thiagarajan, 1982; Derksen & Kater, 1985; Petri *et al.*, 1987).

Again no correlation was found between positivities for anti-cardiolipin and for anti-endothelial cell assay, in agreement with very recent findings by McCarty & Kazava (1988). These data seem to suggest that the production of ACA and AECA may be sustained by different pathogenetic mechanisms. However, to investigate if anti-phospholipid antibodies can participate in the whole anti-endothelial cell binding activity, absorption experiments with cardiolipin liposomes were performed in 18 sera positive both for ACA and AECA. We found that cardiolipin liposome suspensions, able to inhibit to a great extent the reactivity against negatively charged phospholipids (cardiolipin and phosphatidylserine), gave partial inhibition of anti-endothelial cell antibodies in seven samples.

These results suggest that, at least in some SLE sera, only a part of the whole anti-endothelial cell activity is due to antibodies which recognize phospholipid structures on the endothelial cell membranes. On the other hand we showed that affinity purified anti-cardiolipin preparations from SLE sera bind endothelial living cells and that the binding does not occur via the Fc receptors.

It is useful to speculate how some antibody preparations eluted from cardiolipin liposomes react with endothelial cells, although no correlation was found between AECA and anti-cardiolipin levels in SLE sera and AECA was not inhibited by cardiolipin liposomes in most sera examined.

The lack of correlation between anti-phospholipid and anti-endothelial cell antibodies, as well as the incomplete inhibition of anti-endothelial activity by cardiolipin absorption are not surprising since AECA are probably a heterogeneous antibody population directed against different antigenic determinants (Cines et al., 1984; Morales & Stastny, 1977; Simionescu & Simionescu, 1986) which include membrane phospholipid structures recognized by the cross-reacting antibodies. In addition, antibodies eluted from cardiolipin liposomes are polyclonal and probably contain sub-populations that bind phospholipids with different affinities, some of which are present on endothelial cell surfaces. It is possible that polyclonal antibodies eluted from cardiolipin liposomes have higher concentrations of antibody sub-populations binding endothelial cell surfaces than is normally present in patient's serum. This may explain why an AECA negative serum can give affinity purified anti-cardiolipin antibodies displaying anti-endothelial cell binding. On the other hand the polyclonal composition of the affinity purified preparations can also account for the fact that only certain sub-populations react with endothelial cells. This could be the reason why the increase in antibody concentration is not associated with a marked increase in endothelial binding.

Finally, the presence of varying concentrations of sub-populations of anti-cardiolipin antibodies able to bind endothelial cell surfaces may explain the contradictory results in the literature with respect to the effects of anti-cardiolipin positive sera on endothelial cell functions (Izaguirre *et al.*, 1987; Hasselaar *et al.*, 1987; Schorer, Wickham & Watson, 1988). Anyway the finding that anti-phospholipid antibodies can react with surface structures on intact human endothelial cells might represent the basis to explain one of the pathogenetic mechanisms by which these antibodies affect the coagulation process *in vivo* according to the Carreras' hypothesis. This does not exclude the possibility that other pathogenetic mechanisms can be mediated by the anti-phospholipid antibodies, as suggested by their reactivity with negatively charged phospholipids exposed on the surface of activated platelets (Khamashta et al., 1988).

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