Clin. exp. Immunol. (1988) **73**, 456–460

Anticardiolipin antibodies in systemic sclerosis: immunological and clinical associations

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(Accepted for publication 4 May 1988)

SUMMARY

Anticardiolipin antibodies of IgG/IgM class were detected in seven of 28 patients with systemic sclerosis including five of 16 patients severely affected by extensive visceral disease. This severely affected sub-group also showed significant elevations of plasma levels of von Willebrand factor antigen in 10 cases and serum C_{1q} binding activity in seven cases respectively. This triple association raises the possibility that multiple immunological mechanisms are involved in the pathogenesis of systemic sclerosis and its vascular lesions.

Keywords anticardiolipin antibodies immue complexes von Willebrand factor antigen β-thromboglobulin systemic sclerosis

INTRODUCTION

The occurrence in systemic sclerosis of Raynaud's phenomenon, abnormalities of nailbed capillaries (Maricq, LeRoy & D'Angelo, 1980), telangiectasia in areas of involved skin and a distinctive arterial lesion (Norton & Nardo, 1970) all suggest that vascular injury may be important in the pathogenesis of the disorder. The recent finding that elevated plasma levels of von Willebrand factor antigen correlated with extensive visceral involvement by the disease (Greaves *et al.*, 1988) provides support for this view. Platelet abnormalities have also been described (Kallenberg *et al.*, 1982) and the disorder has, like the related connective tissue disease of systemic lupus erythematosus, a high incidence of both circulating immune complexes (Pisko *et al.*, 1979; Van der Meulen *et al.*, 1979; Hughes *et al.*, 1983) and autoantibodies (Bernstein, Steigerwald & Tan, 1982; Catoggio *et al.*, 1983). In systemic lupus erythematosus, the presence of anticardiolipin antibodies identifies a subgroup of patients who are predisposed to vascular occlusion (Harris *et al.*, 1983) because of the possible effects of these antibodies on vascular endothelium and/or platelets. The present study reports the detection of these autoantibodies in a subpopulation of patients with systemic sclerosis and relates them to plasma levels of von Willebrand factor antigen, β-thromboglobulin, circulating C_{1q} binding immune complexes and the clinical extent and severity of the disease.

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MATERIALS AND METHODS

Patients

Twenty-eight patients with systemic sclerosis were investigated (24 women, 4 men; mean age 52·8 ± 14·7 years). Raynaud's phenomenon and acrosclerosis were constant features in all patients who were investigated by a standard protocol which enabled points to be awarded for cutaneous and visceral involvement and so produce a disease score for each patient (Greaves *et al.*, 1988). On this basis the patients were subdivided into categories of 'severe disease' (disease score six or more–13 women, 3 men; mean age 52·6 ± 12·7 years) and 'mild disease' (disease score five or less–11 women, 1 man; mean age 48·3 ± 14·4 years), this latter group containing patients with the CREST syndrome and anticentromere antibody. The duration of disease, as determined from the onset of Raynaud's phenomenon, in 'severely' and 'mildly' affected subgroups was 14·2 ± 9·1 years and 15·8 ± 6 years respectively.

Detection of Anticardiolipin antibodies by ELISA

Thirty microlitres of cardiolipin in ethanol (50 μg of cardiolipin/ml) was added to each well of a rigid 96-well microtitre plate. The cardiolipin was evaporated overnight at 4°C and the plate was washed three times with phosphate-buffered saline (PBS). Seventy-five μl/well of 10% adult bovine serum (ABS) was added to each well and incubated at room temperature for 1 h. The ABS was discarded and the plate washed once with PBS. Fifty microlitres of either a standard or test plasma diluted in 10% ABS were added to each well and incubated at room temperature for 3 h. The wells were washed three times with PBS after which 50 μl of a 1/1000 dilution of alkaline phosphatase

Table 1. Anti-cardiolipin antibodies C_{1q}-binding and von Willebrand factor antigen in patients with systemic sclerosis (SS)

	Patient Groups			
	Total SS ($n=28$)	Mild SS ($n=12$)	Severe SS ($n=16$)	P
Anticardiolipin antibodies (mean ± s.d.)				
IgG (Ref. range 0–5·2 u)	3·55 ± 2·97	2·70 ± 1·75	4·21 ± 3·58	NS
IgM (Ref. range 0–4·4 u)	2·10 ± 2·04	1·85 ± 1·49	2·31 ± 2·42	NS
C_{1q}-binding (mean ± s.d.) (Ref. range 0·8–13·8%)	12·27 ± 14·18	6·25 ± 2·73	17·44 ± 17·84	<0·05
von Willebrand factor antigen (mean ± s.d.) (Ref. range 0·5–1·5 u/ml)	1·43 ± 0·60	1·03 ± 0·37	1·72 ± 0·56	<0·001

NS not significant.

conjugated goat antiserum was added to each well and the plates left for 1·5 h. The plates were washed three times in PBS and 50 μl of well mixed freshly prepared p-nitrophenyl phosphate solution (1 μg/ml in diethanolamine buffer pH 9·8) was added to each well. After incubating for 30 min ± 15 min in the dark at 37°C in a 'wet box' the absorbence was measured at 405 nm using a Titertek Multiscan. Results were expressed in anticardiolipin units calculated against IgG and IgM anticardiolipin standards kindly donated by Dr. N. Harris, the Rayne Institute, London, to give reference ranges of 0–5·2 u and 0–4·4 u for IgG and IgM antibodies, respectively, based on determinations (mean ± 2 s.d.) in 40 normal laboratory staff and blood donors. The specificity for cardiolipin was confirmed by the absence of any consistent reactivity of the assay with sera containing either high titre rheumatoid factors or myeloma para proteins.

C_{1q} binding assay

This was based on the method of Zubler & Lambert (1976), using C_{1q} prepared from fresh normal human serum and iodinated by lactoperoxidase. The method was modified by the use of 4% PEG to precipitate macromolecular-bound C_{1q}, used heat aggregated (63°C for 30 minutes) Cohn fraction II (Blood Products Laboratories, Elstree, Herts., UK) to construct standard curves (0·1 mg/ml to 3·0 mg/ml) and gave a reference range of 0·8–13·8%.

Von Willebrand factor antigen

This was determined by an enzyme-linked immunosorbent assay which was standardized by both MRC and internal standards to give a reference range of 0·5–1·5 units/ml (Short *et al.*, 1982).

β-thromboglobulin

This was detected in platelet-poor plasma by a radioimmunoassay (Amersham International plc, Amersham, UK) based on the method of Ludlam & Cash (1976) to give a reference range of 10–60 ng/ml.

Platelet factor 4

This was measured in the same platelet-poor plasma sample used to assay β-thromboglobulin by a radioimmunoassay (Abbott Labs, North Chicago, Illinois, USA) based on the method of Handin, MacDonough & Lesch (1978) to give a reference range of 0–11 ng/ml.

Table 2. Titres of anti-cardiolipin antibodies (s.d. above mean value of reference range) in relation to the severity of disease in 28 patients with systemic sclerosis (SS)

Anti-cardiolipin antibodies (IgG/IgM)	Patient groups		
	Total SS ($n=28$)	Severe SS ($n=16$)	Mild SS ($n=12$)
>2 s.d.	7	5	2
>3 s.d.	4	3	1
>5 s.d.	3	3	0

Statistical Analysis

Comparison of anticardiolipin antibody titres, C_{1q} binding activity and levels of von Willebrand factor antigen, β-thromboglobulin: platelet factor 4 ratios in the 'severe' and 'mild' patient sub-groups was made by Student's *t*-test.

RESULTS

Anticardiolipin antibodies

The titres of anticardiolipin antibodies in the total group of patients with systemic sclerosis and in the mildly and severely affected subgroups are shown in Table 1. While the mean value in all the patient categories fell within the limits (mean ± 2 s.d.) of the normal reference range, seven patients had elevated levels of IgG and/or IgM anticardiolipin antibodies. These elevations were greater than 2 s.d. above the mean of the reference range in three cases, greater than 3 s.d. in one case and greater than 5 s.d. in a further three cases (Table 2). Five of these seven cases were in severely affected patients, including all three cases with the highest titre of anticardiolipin antibody. By contrast, only two of the mildly affected subgroup had elevated levels of anticardiolipin antibody (Table 2).

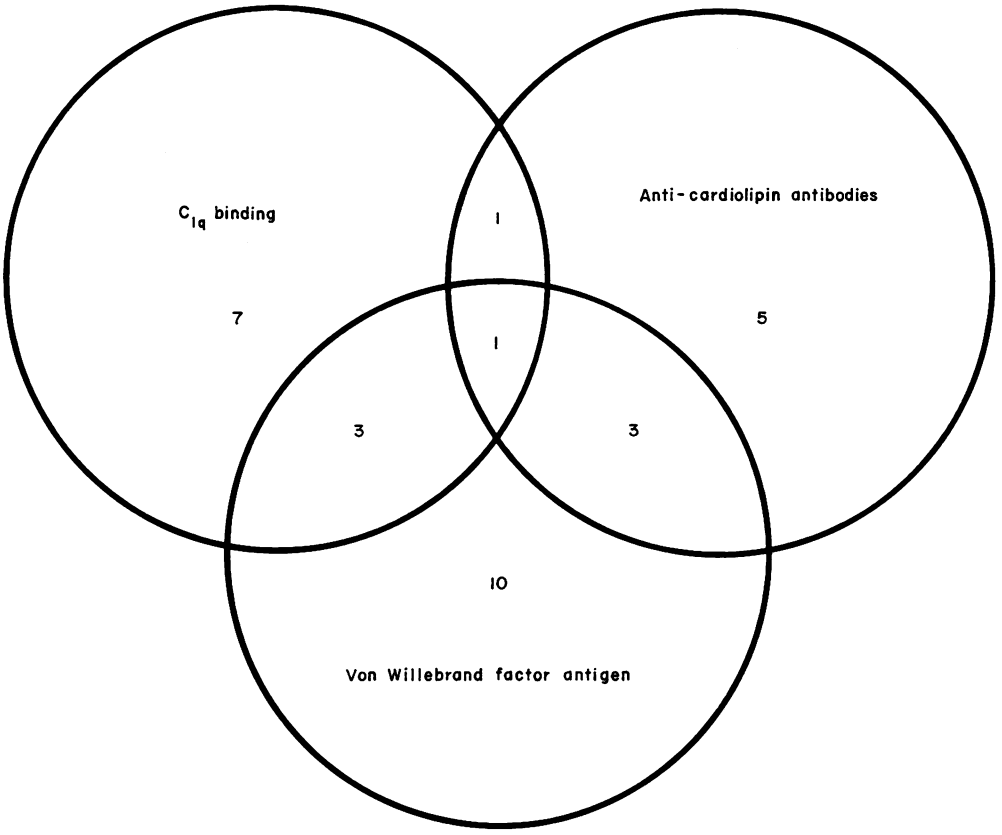

Fig. 1. The association between raised levels of von Willebrand factor antigen, C_{1q} binding and anti-cardiolipin antibodies in 16 patients with severe systemic sclerosis.

C_{1q} binding activity

The results for C_{1q} binding activity in the total group of patients with systemic sclerosis and in the mildly and severely affected subgroups are all summarized in Table 1. Increased C_{1q} binding activity was found in seven patients with systemic sclerosis, the increases being confined to patients severely affected by the disease who had mean levels of $17{\cdot}44 \pm 17{\cdot}84\%$ in contrast to the $6{\cdot}25 \pm 2{\cdot}73\%$ of the mildly affected subgroup ($P < 0{\cdot}05$).

Von Willebrand factor antigen

Elevated plasma levels of von Willebrand factor antigen showed a similar relation to extensive visceral disease, 10 of the 16 patients in the severely affected subgroup having elevated levels (mean $1{\cdot}72 \pm 0{\cdot}56$), in contrast to only two of the 12 mildly affected patients (mean $1{\cdot}03 \pm 0{\cdot}37$; $P < 0{\cdot}001$).

β-thromboglobulin and platelet factor 4

Plasma levels of β-thromboglobulin and platelet factor 4 did not differ in the two sub-groups of patients with systemic sclerosis. The mean ratio of β-thromboglobulin: platelet factor 4 was $6{\cdot}1 \pm 2{\cdot}9$ in the severely affected subgroup and $5{\cdot}4 \pm 2{\cdot}7$ in the mildly affected patients. Plasma levels of β-thromboglobulin were found to be elevated in nine patients overall but seven of these patients had parallel increases in plasma levels of platelet factor 4, suggesting spurious *in vitro* platelet activation occurring as a result of difficulties with venesection. However, two cases showed persistent elevation of β-thromboglobulin levels in the absence of any concomitant rise of platelet factor 4, both patients having extensive visceral disease and elevated plasma levels of von Willebrand factor antigen. One of these patients had the highest titre of anticardiolipin antibody in the study, albeit associated with a residual serum creatinine of 140 μmol/l from a previous episode of scleroderma renal crisis while the other one had a persistently elevated serum C_{1q}-binding activity of 40–60% and normal renal function.

Correlation between anticardiolipin antibodies, C_{1q} binding activity, von Willebrand factor antigen and clinical features

The correlation between elevated levels of von Willebrand factor antigen, C_{1q} binding activity and anticardiolipin antibodies in the 16 patients with extensive visceral disease is summarized in Fig. 1. In this severely affected subgroup, four of the five patients with anticardiolipin antibodies also had elevated levels of von Willebrand factor antigen, including the three patients with the highest titre of anticardiolipin antibodies, two of whom had received treatment for scleroderma renal crisis. Overall, elevated C_{1q} binding activity and/or anticardiolipin antibodies were present in seven of the 10 patients with elevated levels of von Willebrand factor antigen. However, in view of the small numbers, the trends revealed by these figures did not reach statistical significance.

DISCUSSION

The validation of the assay used to detect anticardiolipin antibodies in this study has reliably established that raised levels of these antibodies are present in 20 to 25% of patients with systemic sclerosis. In particular, the possibility that false

positive results have been produced by the hyperglobulinaemia (Lenzi, Rand & Spiera, 1986) that can be a feature of systemic sclerosis, has been reasonably excluded by the methodology used in the assay. The study is the most comprehensive to date, enlarging on preliminary reports of occasional and generally low titre anticardiolipin antibodies in patients with systemic sclerosis (McHugh *et al.*, 1987; Baguley *et al.*, 1987). The association between anticardiolipin antibodies, especially in the higher titres that this investigation has revealed, elevated plasma levels of von Willebrand factor antigen and extensive visceral disease is a particularly important aspect of the study as it suggests that anticardiolipin antibodies may be involved in the pathogenesis of some aspects of systemic sclerosis. Although none of the patients had a history of major thrombosis, the possibility that anticardiolipin antibodies may be capable of causing vascular damage by other means than the overt thrombotic tendency associated with their presence in systemic lupus erythematosus, has to be considered. While experiments suggesting that the anticardiolipin antibodies found in patients with systemic lupus erythematosus may inhibit prostacyclin production by vascular endothelium (Carreras & Vermylen, 1982; Schorer & Watson, 1987) have not been uniformly reproducible (Rustin *et al.*, 1987; Petraiuolo, Bovill & Hoak, 1987) there is evidence that these autoantibodies can react with phospholipid antigens in platelets (Cortelazzio *et al.*, 1987; Khamashta *et al.*, 1987). Such a reaction could result in the release of platelet activation products such as β-thromboglobulin, transforming growth factor β and serotonin which can either have an inhibitory effect on vascular endothelium (Heimark, Twardzik & Schwartz, 1986) and/or a stimulatory effect on fibroblasts with the resulting development of both vascular lesions and perivascular collagen deposition. Although earlier reports of elevated β-thromboglobulin levels in patients with systemic sclerosis (Kallenberg *et al.*, 1982) were not controlled by simultaneous measurement of platelet factor 4 levels to exclude false positive values resulting from difficulty with venesection, it is relevant to note that two patients in this study have shown some evidence of *in vivo* platelet activation. While one of these patients had the highest titre of anticardiolipin antibodies in the study, associated with slight renal impairment from a previous episode of scleroderma renal crisis, the other had high levels of circulating C_{1q} binding complexes. As immune complexes may also cause platelet activation through their ability to react with platelet Fc receptors (Penttinen, 1977), it is particularly interesting to note that both these patients had not only grossly elevated levels of von Willebrand antigen but also severe and extensive visceral disease.

This investigation has also re-emphasised that systemic sclerosis is a disease characterized by circulating immune complexes and that these complexes, as in previous reports (Pisko *et al.*, 1979; Hughes *et al.*, 1983), are associated with extensive visceral involvement. It is, however, the association between such circulating complexes, anticardiolipin antibodies and elevated plasma levels of von Willebrand factor antigen in severely affected patients that is perhaps the most important finding of this study. Such a triple association, revealed by what have been broadly based investigations, not only strengthens the case for an immune pathogenesis, but also suggests that multiple pathogenetic processes may be involved in the disorder. Support for such a concept is provided by the related connective tissue disease of systemic lupus erythematosus, which may show association or 'overlap' with systemic sclerosis (Tuffanelli & Winkelmann, 1962), in which differing disease manifestations are produced by either autoantibodies (with resulting haemolysis, thrombocytopenia or leucopenia) or immune complexes (with resulting vasculitis and nephritis) or by a thrombotic tendency that is associated with generally very much higher titres of anticardiolipin antibodies. In systemic sclerosis the pathogenesis of the vascular lesion that seems central to the progression of the disease may similarly have a multifactorial basis. Although earlier reports of albumin-associated serum factors which are cytotoxic for vascular endothelium (Kahaleh & LeRoy, 1983) have either not been substantiated or found to be due to oxidative artefacts (Blake *et al.*, 1985), sera from up to 20% of patients have been shown to be capable of causing an antibody-dependent cellular cytotoxicity of vascular endothelium (Penning *et al.*, 1984). The responsible serum factor was associated with IgG containing fractions and may well be an autoantibody to vascular endothelium. In addition, platelet activation, even if occurring only intermittently, may well be a central effector mechanism. Such platelet activation with release of β-thromboglobulin, transforming growth factor β and serotonin could result not only from endothelial damage with exposure of collagen in vessel walls but also from the circulating immune complexes and anticardiolipin antibodies revealed by this investigation. This multifactorial view of the immunopathogenesis of systemic sclerosis not only provides a stimulus for the further investigation of platelet, plasma and serum interactions with vascular endothelium but also carries a therapeutic implication for the combined use of antiplatelet and immunosuppressive therapy.

ACKNOWLEDGMENTS

This work was supported by grants from the Wellcome Trust, the Trent Regional Health Authority and the Trustees of the United Leeds Hospitals.

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