Effects of the Lupus Anticoagulant in Patients with Systemic Lupus Erythematosus on Endothelial Cell Prostacyclin Release and Procoagulant Activity


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A disturbance in endothelial cell (EC) function may be pathogenetic in the thrombotic tendency of patients with the lupus anticoagulant (LA). The ability of serum from normal subjects and patients with systemic lupus erythematosus (SLE), with and without the LA, to modulate the release of prostacyclin (PGI₂) and the expression of procoagulant activity by cultured human EC was investigated. Only the 10% and 20% serum concentrations from patients with SLE-LA produced a significantly greater inhibition of 6-keto-prostaglandin F₁₆ (6-keto-PGF₁₆) release (the stable metabolite of PGI₂) than control serum. However, when patients with SLE-LA having Raynaud's phenomenon were excluded from this group, there was then no significant difference between the effect of the patient and control serum. Serum from patients with SLE ± LA caused a significant increase in EC procoagulant activity compared to healthy controls. The two-stage partial thromboplastin time expressed in seconds decreased from 66 (normal) to 34 (SLE - LA) and 31 (SLE + LA), but there was no significant difference between the patients with and without the LA. The significantly increased EC procoagulant activity induced by serum from patients with SLE ± LA may account for the observed increased incidence of thrombotic events in patients with SLE. Our data suggest that factors other than decreased prostacyclin release are responsible for the altered hemostasis observed in patients with SLE + LA. J Invest Dermatol 90:744–748, 1988

The lupus anticoagulant (LA) is an acquired autoantibody (usually IgG or IgM) that acts as an inhibitor of the prothrombinase complex, thus prolonging the in vitro one- and two-stage assays that measure coagulation via the extrinsic and intrinsic pathways [1]. Paradoxically the presence of the LA is not associated with a hemorrhagic diathesis but appears to produce a hypercoagulable effect.

The prevalence of one or more thrombotic events has been reported to be as high as 50% in patients with SLE + LA and 10% in patients with SLE - LA, although the criteria for the thromboembolic phenomena were poorly defined [2,3]. The cause of the thrombotic tendency is not known, but because histopathology of affected vessels does not show an intense perivascular inflammatory cell infiltrate or a vasculitis, it is possible that disturbed endothelial cell thromboreisistance may be responsible.

Studies have shown that antiphospholipid antibodies, like the lupus anticoagulant, bind to phospholipids other than cardiolipin and perhaps they should now be termed anti-phospholipid antibodies [4,5]. Recently, Gharavi et al [6] examined the binding of antibodies present in patients with SLE and the LA and found that binding to phosphatidylserine (PS) and phosphatidylinositol (PI) was nearly equal to cardiolipin. Interestingly, there was no binding to phosphatidylcholine (PC). Arachidonic acid (AA), required for the synthesis of prostacyclin (PGI₂), is liberated by hydrolytic cleavage from its esterified form in the 2-acyl position of cell membrane lipids by the phospholipases A₂ and C [7,8]. It has been reported that plasma from patients with SLE inhibits endothelial cell PGI₂ release and this might contribute to the thrombogenic tendency. Although PC is thought to be the major source of AA within endothelial cells it is possible that antiphospholipid antibodies like LA, which are associated with increased thrombotic events, can interfere with the liberation of AA and therefore inhibit the synthesis of PGI₂. Unfortunately, earlier reports showing in vitro inhibition of endothelial cell PGI₂ production by plasma from patients with SLE utilized non-human endothelial cells, rat aortic rings, and bovine aortic endothelial cells [9–12]. Moreover, the LA may also interfere with the normal hemostatic balance associated with the endothelial cell membrane by increasing the expression of procoagulant activity and further promote a thrombotic event.

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Abbreviations:
AA: arachidonic acid
BSS: balanced salt solution
EC: endothelial cell
6-keto-PGF₁₆: 6-keto-prostaglandin F₁₆
LA: lupus anticoagulant
M199: medium 199
PC: phosphatidylcholine
PGF₂: prostaglandin E₂
PGF₂₆: prostaglandin F₂₆
PGI₂: prostacyclin
PI: phosphatidylinositol
PS: phosphatidylserine
SLE: systemic lupus erythematosus
TXB₂: thromboxane B₂

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We considered it important, therefore, to establish whether the presence of the LA could modulate 1) the release of PGL₁ by cultured human endothelial cells; and 2) the procoagulant activity of these treated cells.

MATERIALS AND METHODS

Subjects Twenty-two patients, attending the Departments of Dermatology and Rheumatology at The Middlesex Hospital, each of whom satisfied the American Rheumatism Association revised criteria for the classification of SLE [13] and 9 age- and sex-matched healthy normal controls were selected. All subjects studied were white. The patients with SLE who were all receiving the same type of treatment were subdivided into those with and without the lupus anticoagulant. This was defined by either a prolonged prothrombin time or a prolonged activated partial thromboplastin time that did not correct with 50% control plasma, and a prolonged dilute Russell viper venom time which did correct with washed platelets [14-16]. Serum was then obtained from 9 patients with SLE + LA (9 women, mean age 36, age range 26 to 46), 13 patients with SLE - LA (12 women, 1 man; mean age 36, age range 21 to 46) and 9 normal healthy volunteers (7 women, 2 men; mean age 38, age range 18 to 54). Of the 9 patients with SLE + LA, 4 had had spontaneous abortions, 5 had suffered from venous thromboses, renal involvement, and thrombocytopenia (<100,000 x 10⁹/L), and one experienced Raynaud’s phenomenon. Four patients in the SLE - LA group suffered from Raynaud’s phenomenon. To provide a larger control group for some experiments, serum was collected from 10 patients with systemic sclerosis (9 women and 1 man aged 21 to 56 years with a mean age of 46 years), 8 patients with primary Raynaud’s phenomenon (8 women aged 18 to 49 with a mean age of 39 years) and 7 patients with sero-positive rheumatoid arthritis (6 women and 1 man aged 37 to 63 with a mean age of 49). Anti-cardiolipin antibodies were detected using the ELISA method described by Gharavi et al [6]. Cardiolipin, alkaline phosphatase conjugated goat anti-human IgG, IgM, and IgA, alkaline phosphatase substrate and bovine serum albumin were purchased from Sigma Ltd., UK. Adult bovine serum was purchased from Imperial Laboratories, UK and microporous plates from Flow Laboratories Ltd., UK. Anti-cardiolipin antibodies were only detected in patients in the SLE + LA group; 4 had IgM, 3 had IgG, and 2 patients had both IgM and IgG.

Endothelial Cell Cultures Human umbilical vein endothelial cells were grown in culture using the method described by Jaffe [17]. Briefly, umbilical veins from individual cords were cannulated, washed with balanced salt solution (BSS, pH 7.4), and filled with 0.1% collagenase (Clistroidium Histolyticum, Boehringer Mannheim GmbH, West Germany). After a 12-min incubation at 37°C, detached cells were obtained by flushing with Medium 199 (M199) and were centrifuged at 250 x g for 5 min. The cell pellet was resuspended in M199 containing 100 units/ml penicillin, 50 µg/ml streptomycin, amphotericin B, glutamine (Gibco), and 20% pooled human serum and were grown in 24-well plastic cluster plates (Sterilin Ltd., UK) in humidified 5% CO₂/95% air at 37°C. In all experiments, the cells used were primary cultures at confluence and they were identified as endothelial cells by their “cobblestone” appearance and by positive uniform staining for factor VIII antigen.

Incubations of Cultured Endothelial Cells with Serum Confluent primary cultures were washed once with M199 and then were incubated with either serum free media or media containing 1, 5, 10, or 20% serum from each patient for 24 h in humidified 5% CO₂/95% air at 37°C. At the end of each incubation, the cells were washed with M199 and following stimulation with the calcium ionophore A23187 10 µM (Sigma Ltd., UK) for 2 h, the supernatants were collected and kept frozen at -20°C for the radioimmunoassay of 6-keto-PGF₁α. The endothelial cells were trypanized (0.25% trypsin), centrifuged at 500 x g for 1 min, resuspended in 200 µl of BSS, and after addition of 10 µl of methylene blue, the cells were counted in an improved Neubauer Chamber (Weber, England).

Radioimmunoassay of 6-Keto-PGF₁α The antibody to 6-keto-PGF₁α (donated by Dr. M. Greaves, Department of Haematology, Royal Hallamshire Hospital, Sheffield, UK) was incubated with the samples overnight at 4°C. Cross-reactivities of the antiserum with other major eicosanoids (PGF₂α, PGF₃α, TXB₂) were less than 1%. The tracer was 6-keto-[³H]PGF₁α (Amersham International PLC, Amersham, UK) and the standard was 6-keto-PGF₁α (Wellcome Laboratories, Beckenham, UK). Unbound tracer was separated from bound by the addition of dextran/charcoal. The assay was linear over the concentration range 0.1 to 2.5 ng/ml and the limit of sensitivity was 39 pg/ml. Concentrations of 6-keto-PGF₁α in the supernatant were expressed as ng/10⁶ cells.

Assay of Procoagulant Activity Human umbilical vein endothelial cells were grown to confluency as described above in 35-mm diameter plastic Petri dishes (Sterilin Ltd., UK). The cells were washed twice with M199 and were overlaid with either 1,500 µl M199, or 1,300 µl M199 and 300 µl of patient or control serum and then incubated for 6 h in 5% CO₂/95% air at 37°C. At the end of the incubation, the cells were washed twice with M199, overlaid with 500 µl fresh M199 and scraped off with a rubber policeman. The cells were kept at -20°C and at time of use were freeze-thawed 3 times to ensure complete disruption of the cell membrane. The procoagulant activity of the endothelial cell suspensions was then determined using a one-stage clotting assay; 100 µl of pooled normal human plasma was pipetted into glass tubes and allowed to equilibrate in a water bath for 30 sec at 37°C; 100 µl of the cell lysate was added and thoroughly mixed and synchronously with adding 100 µl of 0.025 M CaCl₂, a stopwatch was started. The tubes were tilted through 90° every 2 sec and the time taken for a clot to form was measured. All estimations were duplicated and the means calculated.

In selected experiments, the procoagulant activity was also determined using a two-stage clotting assay. This involved adding and mixing 100 µl of each of the cell lysate and pooled human plasma, which were then incubated in a waterbath for 9 min 30 sec at 37°C; 100 µl of the platelet substitute (Diagnostic Reagents Ltd., Oxford, UK) was added and after a further 30 sec, 100 µl of 0.025 M CaCl₂, at which time the stopwatch was started and the tubes were tilted through 90° every 2 sec until a visible clot formed. Again all estimations were performed in duplicate and the means calculated.

To confirm that the endothelial cell procoagulant activity was activating both the intrinsic and extrinsic coagulation pathways, cultured endothelial cells were incubated with normal human serum and assays were performed in which the normal human plasma was replaced with plasma deficient in specific factors. Factor VIII- and IX-deficient plasmas were obtained from patients genetically deficient in these factors; factor X-deficient plasma was from Diagnostic Reagents Ltd., Oxford and artificially depleted factor VII-deficient plasma was prepared using the method described by Giddings [18]. All these factor deficient plasmas had specific activities of less than 0.01 IU/ml.

In order to standardize the thromboplastin assays in the laboratory, pooled normal human plasma produced a prothrombin time of 15 ± 0.75 seconds and a kaolin partial thromboplastin time of 40 ± 2.5 seconds.

Statistical Analysis Differences in 6-keto-PGF₁α production following addition of serum were analyzed by comparing the effect of one serum concentration from patients with SLE ± LA against the same concentration from normal individuals using the Mann Whitney test. In view of a previous study showing that serum from patients with Raynaud’s phenomenon inhibits the release of PGL₁ from cultured human endothelial cells [19], statistical analysis was performed inclusive and exclusive of patients with SLE and Raynaud’s phenomenon. Differences in procoagulant activity between the groups were evaluated by the Mann-Whitney Test.
RESULTS

6-keto-PGF<sub>1α</sub> Production There was a concentration-dependent inhibition of 6-keto-PGF<sub>1α</sub> release with all the serum tested, but only the 10 and 20% serum from patients with SLE - LA produced a significantly greater inhibition than control serum (p < 0.05 and p < 0.02, respectively) (Fig 1). This statistical difference was no longer evident when 4 patients in the SLE - LA group with Raynaud’s phenomenon were excluded from the analysis. There was a trend for serum from patients with SLE + LA to cause a greater inhibition of PGF<sub>2α</sub> release than normal controls, but this did not reach statistical significance (p = 0.15) and this result was not affected by exclusion of one patient with Raynaud’s phenomenon in the SLE + LA group. There was no significant difference between the patients having SLE with or without the lupus anticoagulant and similarly there was no significant difference between the combined effects of the SLE serum [(SLE + LA) + (SLE - LA)] and control serum. The percentage inhibition of 6-keto-PGF<sub>1α</sub> release by the different sera as compared with serum-free medium after a 24-h incubation is shown in Figure 2.

Endothelial Cell Procoagulant Activity The mean procoagu-
ulant activity of the endothelial cell lysate following incubation with medium 199 alone was 70 and 110 sec, using the one- and two-stage clotting assays, respectively. All the serum, whether from controls or patients, significantly increased the endothelial cell procoagulant activity compared with M199 alone, as judged by a shortening of the clotting times (p < 0.001). Using the one-stage clotting assay, only the serum from patients with systemic sclerosis produced a significant change from control serum, with prolongation of the clotting times (p < 0.05). Serum from patients with SLE ± LA caused a significant shortening of the two-stage clotting assay (p < 0.001) (Table I). Comparison of the results of serum from patients with SLE who did or did not have the LA, there was a significant difference using the one-stage assay (p < 0.001) but not with the two-stage assay. None of the patients with SLE - LA studied had Raynaud’s phenomenon and exclusion of the one patient in the SLE + LA group did not alter the statistical analysis.

To ensure that the assays were indeed measuring the activities of the intrinsic and extrinsic coagulation pathways selected experiments were performed using factor-deficient plasma and lysate of endothelial cells incubated with control serum (Table II). Only the factor X-deficient plasma prolonged the one-stage clotting assay, whereas plasma deficient in either factor VIII, IX, or X prolonged the two-stage clotting assay.

DISCUSSION

There have been many reports documenting the paucity or absence of inflammation at sites of thrombosis in patients with the lupus anticoagulant. These have included cases with main blood vessel occlusions [20], discrete cutaneous lesions [21], and widespread cutaneous necrosis [22]. Proposed explanations for the venous and arterial thrombotic tendency have focused on dysfunction of the

Table I. Effects of Serum from Different Patient Groups on Expression of Endothelial Cell Procoagulant Activity

<table>
<thead>
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<th>One-Stage Coagulation Assay</th>
<th>Two-Stage Coagulation Assay</th>
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<tr>
<td>Medium 199 alone</td>
<td>70 ± 6 (n = 5)</td>
<td>110 ± 10 (n = 5)</td>
</tr>
<tr>
<td>Normal subjects</td>
<td>37 ± 2 (n = 11)</td>
<td>66 ± 3 (n = 10)</td>
</tr>
<tr>
<td>Systemic lupus erythematosus - LA</td>
<td>39 ± 1 (n = 9)</td>
<td>34 ± 1* (n = 9)</td>
</tr>
<tr>
<td>Systemic lupus erythematosus + LA</td>
<td>31 ± 1 (n = 9)</td>
<td>31 ± 2* (n = 9)</td>
</tr>
<tr>
<td>Primary Raynaud’s Phenomenon</td>
<td>41 ± 2 (n = 8)</td>
<td>64 ± 6 (n = 6)</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>49 ± 4* (n = 10)</td>
<td>63 ± 3 (n = 10)</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>41 ± 2 (n = 7)</td>
<td>78 ± 6 (n = 5)</td>
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Results are expressed in seconds as means ± SEM of duplicate experiments.
Compared with normal controls.
* p < 0.001.
† p < 0.05.
Table II. Effects of Factor-Deficient Plasma on the Measurement of Procoagulant Activity of Endothelial Cells Incubated with Normal Control Serum

<table>
<thead>
<tr>
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<th>One-Stage Clotting Assay (seconds)</th>
<th>Two-Stage Clotting Assay (seconds)</th>
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<tbody>
<tr>
<td>Factor VII-deficient</td>
<td>36 ± 4</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>Factor VIII-deficient</td>
<td>35 ± 3</td>
<td>&gt;120</td>
</tr>
<tr>
<td>Factor IX-deficient</td>
<td>36 ± 4</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Factor X-deficient</td>
<td>&gt;300</td>
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endothelium and these include inhibition of prostacyclin release from blood vessels [9–12], increased von Willebrand factor activity and increased tissue plasminogen activator release [23], inhibition of protein C activation in vivo [24], interference with antithrombin III activity [25], and inhibition of prekallikrein activation [26].

Although samples of plasma were used in the coagulation tests for detection of the lupus anticoagulant, patients’ sera were used in all the in vitro endothelial cell studies. The rationale for selecting serum was that citrated plasma in the presence of calcium in the tissue culture medium clots and it was believed to be unwise to use heparin as an anticoagulant since it acts as a mitogen for endothelial cells.

The experiments showed that there was a significant difference in the inhibition of PGI2 release between the serum from normal volunteers and patients with SLE without the LA, but there was no difference with the serum from patients with SLE having the LA. When patients in the SLE − LA group having Raynaud’s phenomenon were excluded from the analysis there was no longer a significant difference. The rationale for excluding the patients with Raynaud’s phenomenon was that serum from patients with primary Raynaud’s phenomenon and patients with Raynaud’s phenomenon in association with systemic sclerosis inhibits the release of PGI2 from cultured human endothelial cells [19] and it was thought possible that serum from patients with Raynaud’s phenomenon and SLE might induce a similar effect. Although only four such patients were included, it appears that such an inhibition does occur and a larger study with a greater number of patients is required to confirm this.

The demonstration that serum from patients with SLE + LA does not inhibit PGI2 release contrasts with the results of earlier studies and this may be explained by differences in methodology. First, Carreras et al [9] measured PGI2 formation by a bioassay and this cannot take into account the extent of hydrolysis of PGI2 to its inactive metabolite 6-keto-PGF1α. Second, Carreras et al [9] and Carreras and Vernylen [11] used rings of rat aorta as a source of endothelial cells, which, unlike human endothelial cells, spontaneously release PGI2. In addition Carreras et al [10] and Elias and Eldor [12] incubated the patients’ plasma with the bovine endothelial cells and then measured the production of 6-keto-PGF1α in the plasma supernatant. This induces many variables into the radioimmunoassay measurement, which is evidenced by their high normal plasma levels of 6-keto-PGF1α (100 to 381 pg/ml) [27].

The question whether serum from patients with SLE + LA can modulate endothelial cell procoagulant activity also was examined. Under normal circumstances, endothelial cells present a thromboresistant luminal surface and do not express procoagulant activity because any thromboplastin (apoprotein III) which is present exists in a protected or cryptic form or near the cell surface [28]. However, when endothelial cells are stimulated either physically or with agents such as thrombin [29], endotoxin [30], or interleukin-1 [31] procoagulant activity is increased. This process requires de novo protein and RNA synthesis because it may be inhibited by cycloheximide and actinomycin D.

Activation of the extrinsic pathway by the phospholipid-apoprotein III complex (tissue factor) is achieved either directly by activation of factor X or indirectly by the activation product of factor VII, factor VIIa [32]. This explains why in the present study factor VII-deficient plasma did not alter the one-stage clotting assay, whereas factor X-deficient plasma did. Osterud and Rappaport [33] showed that tissue factor could also enhance thrombin via the intrinsic pathway by catalyzing factor IX activation. In the present study, factor VIII- and factor IX-deficient plasma prolonged the two-stage clotting assay, but the requirement for factor VIII appeared to be smaller because factor VIII-deficient plasma did not prolong the clotting time as much as factor IX-deficient plasma. Clearly, therefore, there are complex interactions between the two pathways on release of procoagulant activity that eventually result in the activation of factor X. This, then, reacts with factor V and calcium to produce the prothrombin activator.

Serum from patients with systemic sclerosis significantly decreased endothelial cell procoagulant activity, whereas the effect of serum from patients with rheumatoid arthritis was not significantly different from control serum. These findings may in part explain the infrequency of thrombotic events in these patients. Indeed, despite the numerous histologic studies of vascular disease in patients with systemic sclerosis [34–36], there have only been a few case reports documenting arterial thrombosis [37,38].

Tannenbaum et al [39] showed that serum from patients with SLE increased endothelial cell production of tissue factor compared with healthy controls. However the serum used had previously been heat-inactivated at 56°C for 60 min and this may have caused formation of aggregated IgG. These authors showed separately that increasing concentrations of aggregated IgG stimulated tissue factor formation, and because patients with active lupus tend to have higher concentrations of serum immunoglobulins, it is possible that Tannenbaum et al [39] may just have been measuring the effects of the increased amount of aggregated IgG present. Furthermore, the patients in that study were not subdivided into those with and without the LA.

In this study, there was a nonsignificant increase in procoagulant activity following incubation with serum from patients with SLE + LA using the one-stage coagulation assay, whereas using the two-stage coagulation assay both groups of SLE patients significantly increased the procoagulant activity. The reason for the difference between the assays may have been due to the longer incubation in the two-stage assay allowing greater activation of the factors. Alternatively, the incubation with the patients’ sera may have induced the production of an enzyme capable of cleaving Hageman factor (factor XII) and this would only be demonstrated by analysis of the intrinsic coagulation pathway [40].

The cause of the increased procoagulant activity remains to be elucidated, but perhaps it may be related to the binding of IgG to endothelial cells causing some perturbation of the endothelial cell membrane [41,42]. The disparity between the increased procoagulant activity and the normal release of PGI2 may be related to the binding properties of the respective antibodies. The preferential binding of anti-cardiolipin antibodies to phosphatidylerine and phosphatidylinositol but not to phosphatidylcholine, from which arachidonic acid is principally liberated, may allow normal release of PGI2. Conversely, other antibodies including the F(ab′)2 fragments of SLE-IgG described by Cines et al [41] may cause greater perturbations of the endothelial cell membrane and in some way stimulate the synthesis of procoagulant activity. Clearly, further work is required to elucidate the biochemical consequences of the binding of anti-cardiolipin and anti-phospholipid antibodies to cell membranes. Until then, it can be concluded that patients with SLE do have a thrombotic tendency that may be caused by increased procoagulant activity. However, the lack of specificity of this finding in patients with SLE + LA suggests that additional factors may be superimposed to produce the pathologic thromboses observed in those patients.

REFERENCES

20. Asherson RA, Mackworth-Young CG, Harris EN, Gharavi AE, Hughes GRV: Multiple venous and arterial thromboses associated with the lupus anticoagulant and antibodies to cardiolipin in the absence of SLE. Rheumatol Int 5:91 – 93, 1985
36. Rodnan GP, Myerowitz RL, Justh GO: Morphologic changes in the digital arteries of patients with progressive systemic sclerosis (scleroderma) and Raynaud phenomenon. Medicine 59:393 – 408, 1980