Plasma From Systemic Lupus Erythematosus Patients With Antiphospholipid Antibodies Promotes Platelet Aggregation

Studies in a Perfusion System


The possible platelet-aggregating effect of plasma from systemic lupus erythematosus (SLE) patients (n=19) was investigated under flow conditions. Aliquots of the SLE plasmas with (n=10) or without (n=9) anticardiolipin antibodies (ACAs) were added to anticoagulated blood (1:20, vol/vol). Plasma from normal donors was used as a control. Blood was incubated for 15 minutes at 37°C and then perfused through annular chambers containing denuded arterial segments. Perfusions were performed for 10 minutes at a shear rate of 800 sec⁻¹. The interaction of platelets with vessel subendothelium (SE) was morphometrically evaluated in thin sections. In control experiments, the percentage of the SE covered with platelets was 23.6±4.3% (mean±SD). Large aggregates (more than 5 μm in height) covering 11.8±5.7% of the exposed SE were noted. The deposition of platelets was statistically increased (38.5±7.6%, p<0.01 versus control) in the presence of SLE plasmas with demonstrated antiphospholipid antibodies (APAs). The formation of large aggregates was also augmented (30.3±5.9%, p<0.01 versus control). A similar response was obtained after addition of affinity-purified immunoglobulin G and immunoglobulin M fractions from two patients with ACAs. SLE plasmas with no detectable APAs did not influence the morphometric parameters studied. Results of the present study indicate that the presence of APAs in SLE plasma promotes platelet aggregation under flow conditions. These observations may help to explain the pathophysiology of the thrombotic events occurring in patients with APAs. (Arteriosclerosis and Thrombosis 1992;12:196-200)

Patients with systemic lupus erythematosus (SLE) are known to suffer from arterial or venous thrombotic complications. The prolonged activated partial thromboplastin time found in plasmas from some of these patients has given rise to the term “lupus anticoagulant” (LA). The presence of anticardiolipin antibodies (ACAs) in plasmas from patients with SLE or other diseases has been associated with a higher risk of thrombosis. At present, it is well established that ACAs, like the LA, may bind negatively charged phospholipids other than cardiolipin. Thus, both groups of antibodies have generally been grouped under the term antiphospholipid antibodies (APAs). The clinical associations of APAs have raised the question of whether these APAs play a pathophysiological role in the development of thrombosis.

Low platelet counts have been reported in SLE patients. Although this has been ascribed to an immunologic problem, there is a consistent relation between moderate reductions of platelet count and development of thrombotic events in patients with LA. Despite indirect evidence that platelets are implicated in the thrombotic complications occurring in SLE patients, the role of platelets in the pathophysiology of these events in APA-positive patients has yet to be established.

The present study was undertaken in an attempt to elucidate whether the addition of plasma derived from patients with SLE to normal anticoagulated blood might have a proaggregating effect on platelets. The role of the presence of APAs in SLE plasma was also investigated. Experiments were performed
in a perfusion system with flowing blood, and the interaction of platelets with the subendothelium was morphometrically evaluated.

**Methods**

**General**

Human blood anticoagulated with citrate-phosphate-dextrose (final concentration of citrate in blood, 19 mM) was obtained from normal volunteers by venipuncture. Platelet count and hematocrit were always in the normal range, that is, from 2 to $3.5 \times 10^5$ platelets/μl and from 42% to 48%, respectively.

**Patients**

The study, approved by the Human Experimental Committee of the Hospital Clinic, was carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained from all the participants. Nineteen SLE patients were included in this study. All patients with APAs included in this study ($n=10$) had a history of thrombotic events, assessed clinically or confirmed by venogram (see Table 1 for details). The remaining nine were SLE patients with no detectable APAs and without a history of thrombosis.

None of the patients had either thrombocytopenia or positive Coombs’ test at the time the study was performed. None of them had taken aspirin (or other drugs that might affect platelet function) in the previous 2 weeks.

**Detection of Lupus Anticoagulant by Coagulation Assays**

Prothrombin time, activated partial thromboplastin time, diluted Russell's viper venom time, and tissue thromboplastin inhibition test were assessed. The positive value for each test was defined as mean plus three standard deviations above the mean normal value of a control group consisting of 100 healthy blood donors. To rule out any defect in a coagulation factor, each assay was also performed with a mixture of patient and control plasma (1:1, vol/vol). LA was defined as the absence of normalization of the coagulation time by the control plasma in each assay. All the tests were performed for each patient.

**Detection of Anticardiolipin Antibodies by Enzyme-Linked Immunosorbent Assay**

ACAs were determined according to a method previously described with minor modifications. In brief, flat-bottomed wells of microtiter plates (Nunc, Roskilde, Denmark) coated with cardiolipin (Sigma Chemical Co., St. Louis, Mo.) were exposed to dilutions of the patients’ sera and then incubated for 1 hour at room temperature and then for a further 16 hours at 4°C. Wells were washed with phosphate-buffered saline (PBS) and incubated for 1 hour at 37°C with a 1:10,000 (vol/vol) dilution of the first antibody (goat anti-human immunoglobulin G [IgG] or IgM; Tago Inc., Burlingame, Calif.). After washing with PBS, wells were incubated for 1 hour at 25°C with a 1:1,000 (vol/vol) dilution of alkaline phosphatase–conjugated secondary antibody (rabbit anti-goat IgG; Sigma). Plates were then washed with diethanolamine buffer (DEA), pH 9.8, followed by exposure in the dark at room temperature to a 1 mg/ml solution of p-nitrophenyl phosphate freshly prepared in DEA. After 1 hour, the reaction was stopped by addition of 3 M NaOH. The optical absorbance was read at 405 nm. Results were expressed as a binding index with respect to optical absorbance values of normal pooled serum. Values were considered positive when the logarithms of the binding indexes were greater than the 98th percentile of the cumulative distribution of the control group. A semiquantitative value according to a progressive scale was given for antibody titers (see Table 1).

**Affinity Purification of Anticardiolipin Antibodies**

Serum from each of two different patients with high levels of ACAs was obtained in sufficient quantity to guarantee purification of immunoglobulins (patients 2...
Patients were evident in six patients (1, 2, 3, 4, 5, and 7). Affinity purification of the ACAs of these patients was achieved after absorption of patients' sera on cardiolipin liposomes, as previously described.13

Perfusion Studies

One-ml aliquots of the SLE plasmas were added to 19 ml of anticoagulated blood obtained from normal donors with matched blood group. Pooled plasma from normal isogroup donors was used as a control. In both cases, blood was incubated for 15 minutes at 37°C before perfusion.

Perfusions were carried out at 37°C in perfusion chambers, as described by Baumgartner and Muggli.14 Enzymatically denuded rabbit aorta segments15 were mounted on the plastic rods of the perfusion chambers and exposed to recirculated blood. Flow was obtained by pumping the blood through a hemodialysis blood pump (Renal Systems, Minneapolis, Minn.) at the appropriate flow rates to produce a wall shear rate of 800 sec⁻¹. After perfusion for 10 minutes, the segments were rinsed with PBS, fixed in glutaraldehyde/formaldehyde solution (2%:3%, vol/vol), and embedded in JB-4 compound (Poly-science, Warrington, Pa.) as previously described.16 Three-micron sections were obtained from plastic blocks, stained with toluidine blue, and used for morphometric evaluations.

Morphometric Evaluation

Platelets interacting with subendothelium were evaluated according to the method described by Baumgartner and Muggli.14 Studies were designed so that the person performing the morphometric studies was not aware of the experimental design. A specially developed computer program was used.16 Platelets or groups of platelets were classified as follows: contact (C), platelets that were attached but not spread on the subendothelium; adhesion (A), platelets that were spread on the subendothelium or that formed layers of less than 5 μm in height; and thrombi (T), platelet aggregates of 5 μm or more in height. All of these basic parameters are expressed as a percentage of the total length of the vessel screened. The total covered surface (CS) was obtained by adding the previous basic parameters (C+A+T).

Statistical Analysis

Results of the present study are expressed as mean±SD. The coefficient of variation of the morphometric parameters in perfusion studies calculated in our laboratory is 22%. Wilcoxon's test for paired data was used for statistical comparisons. A probability level of 0.05 was considered statistically significant.

Results

Patients

Table 1 describes the APA pattern of the SLE patients who were the subjects of our study. LAs were evident in six patients (1, 2, 3, 4, 5, and 7).

Ten of the patients showed ACAs. IgM-subclass ACAs were found to be positive in patients 1, 3, 4, 6, 7, 8, 9, and 10. IgG-subclass ACAs were found to be positive in patients 2 and 5. Patient 3 was found to be positive for both IgG and IgM ACAs.

Platelet Interactions With Subendothelium

Effects of plasma. As shown in Figure 1, the total surface of the vessel covered by platelets (%CS) in control experiments was 23.6±4.3%. Under our experimental conditions, aggregates of more than 5 μm (%T) were found covering 11.8±5.7% of the exposed subendothelium. Because the surface of the vessel covered by contact platelets in our experiments was always less than 1%, the percentage of the vessel covered by adherent platelets (%A) can be easily inferred from the bar diagram.

The addition of 1:20 (vol/vol) of plasma from SLE patients with no detectable APAs had no apparent effect on the parameters that quantify platelet-subendothelium interactions. The total CS and the presence of aggregates remained essentially at the levels observed in control experiments (Figure 2a). However, the addition of plasma from SLE patients with demonstrated APAs resulted in a clear change of the pattern of platelet interaction (Figure 2b). The total CS rose to 38.5±7.6% (p<0.01 versus control), and there was a marked increase in the presence of platelet aggregates (30.3±5.9%, p<0.01 versus controls; see Figure 1).

Effects of affinity-purified antibodies. The addition to citrated blood of increasing concentrations (9, 12, 15, and 18 μg/ml) of purified immunoglobulin fractions from patients 2 and 7 (IgM and IgG, respectively) resulted in a progressive increase in platelet-subendothelium interactions. A statistically significant increase of the same magnitude as that observed with plasma (p<0.01) in the total CS and in the presence

![Figure 1. Bar diagram represents morphometric values obtained in perfusion experiments with anticoagulated blood. A statistically significant increase (p<0.01) both in total platelet deposition (covered surface) and in the formation of large aggregates (thrombi) was observed in the group of perfusions that contained a 1:20 (vol/vol) proportion of plasma from patients with systemic lupus erythematosus (SLE) with positive anticardiolipin antibodies (APA).](image-url)
of platelet aggregates was observed after addition of 12 μg/ml of the IgM fraction from patient 2. A slightly higher concentration (15 μg/ml) of the IgG fraction from patient 7 was required to achieve a similar level of statistical significance (p<0.01).

Discussion

Results of this study present ex vivo evidence that plasma from SLE patients with ACAs promotes platelet aggregation on denuded arterial surfaces.

Although there is indirect evidence that platelets have a role in the thrombotic events that occur in SLE patients, this thrombotic tendency has not been reproduced experimentally. In fact, LA still remains a laboratory finding primarily characterized by a prolongation of coagulation tests.

Several investigators have suggested that LA may interfere with coagulation tests by interacting with different phospholipid components involved in the blood clotting process. However, the mechanism involved in the pathophysiology of the thrombotic events occurring in some patients remains unknown.

Prevention of the vascular production of prostaglandin I2 has been proposed as a mechanism through which APAs may promote the development of arterial thrombosis in some patients. Under experimental conditions, the enzymatically digested vascular surface exposed to the perfusates is unable to produce prostaglandin I2. Thus, it seems reasonable to relate the proaggregating effect observed for plasmas containing APAs to their action on blood components and very likely on platelets.

The mechanism through which ACA-positive plasmas might promote platelet aggregate formation is not clear. Recent data suggest a frequent occurrence of cross-reactivity between antibodies to anionic phospholipids and platelets. It is possible that the activation of platelets by components of the subendothelial structures results in the exposure of negatively charged phospholipids on the platelet surface, allowing the binding of ACAs. It has also been suggested that ACA-positive sera might increase thromboxane formation in platelets. It is known that treatment with aspirin—a drug that effectively blocks thromboxane A2 production in platelets—is effective in the prevention of repeated abortion, a clinical condition in which the presence of APAs has been found to be consistently increased.

All the patients with ACAs who were involved in our study had a previous history of thrombosis. A highly sensitive enzyme-linked immunosorbent assay technique was used for the detection of ACAs. The fact that plasmas with no detectable ACAs did not modify the interaction of platelets with subendothelium gives indirect evidence of the heterogeneity of the mechanisms involved in the pathophysiology of the thrombotic events that develop in SLE patients. The results of our experiments support the hypothesis that ACA-positive plasmas promote platelet aggregation. However, due to the presence of anticoagulant (citrate) in the perfusates we cannot rule out the possibility that ACA-negative SLE plasma may promote blood clotting through interactions with coagulation factors.

Results obtained with purified immunoglobulins from two patients indicate that this fraction could be responsible for the proaggregating response we observed in our experimental system. However, a word of caution is required in this respect. A recent study by McNeil et al. suggests that APAs require the presence of a cofactor (β2-glycoprotein I) to bind to phospholipids. According to this study, APAs might interfere with the above-mentioned cofactor, predisposing a prothrombotic diathesis. It is very likely that the immunoglobulin fraction we have obtained retains some β2-glycoprotein I activity, as this cofactor is not completely removed during the purification process we used. Studies are already in progress using more complex purification methods in patients with ACAs with or without a history of thrombosis. Results of these studies should allow us to confirm the relative importance of APAs in the changes in platelet reactivity that we observed.

In summary, our results indicate that the presence of APAs in SLE plasma promotes platelet aggregation under flow conditions. These findings may help us to understand the pathophysiology of the thrombotic events that occur in patients with APAs. Prospective studies are required to confirm whether patients with APAs are at higher risk of arterial thrombosis. If this were the case, some benefit could be derived from treatment with antiplatelet drugs.
References


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G Escolar, J Font, J C Reverter, A López-Soto, M Garrido, R Cervera, M Ingelmo, R Castillo and A Ordinas