Antibodies to Platelets in Patients With Anti-Phospholipid Antibodies

By Henk Jan Out, Philip G. de Groot, Marja van Vliet, Gijsbert C. de Gast, H. Karel Nieuwenhuis, and Ronald H.W.M. Derksen

Binding of anti-phospholipid antibodies to circulating platelets and its consequences on platelet activation and aggregation was investigated in 11 patients with anti-phospholipid antibodies. Seven patients had mild thrombocytopenia. Nine healthy donors served as controls. Binding to platelets was investigated by performing enzyme-linked immunosorbent assays (ELISAs) with phospholipids as antigen on platelet eluates. Platelet activation was measured by flow cytometry using monoclonal antibodies to an activation-specific lysosomal membrane protein. Findings in ELISA were compared with results of a conventional immunofluorescence method to detect platelet autoantibodies. In seven patients antibodies to negatively charged phospholipids were present in platelet eluates. In all thrombocytopenic patients and controls the platelets were not activated and aggregation was not impaired. There was a positive concordance of 50% between the results of immunofluorescence and ELISA. No apparent relation was found between the results of ELISA or immunofluorescence and platelet counts. It is concluded that anti-phospholipid antibodies can bind to circulating platelets. This binding is not associated with measurable aggregation abnormalities nor with platelet activation characterized by exposure of lysosomal membrane proteins. More studies are necessary to determine the exact role of anti-phospholipid antibodies in the pathogenesis of thrombocytopenia and thrombosis.

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Patients

Eleven patients with anti-phospholipid antibodies agreed to enter this study after informed consent. Patient characteristics are given in Table 1. There were nine women and two men (median age 32 years, range 26 to 64 years). Seven patients had systemic lupus erythematosus (defined as having at least four criteria according to the American Rheumatism Association), three had lupus-like disease (less than four criteria), and one patient had a primary antiphospholipid syndrome, characterized by a history of thrombosis. Seven patients had mild thrombocytopenia (between $92 \times 10^9/L$ and $144 \times 10^9/L$). The platelet counts of the other four patients ranged from $204 \times 10^9/L$ to $272 \times 10^9/L$. Patient 10 did not have the lupus anticoagulant and patient 5 had no anticardiolipin antibodies. Patients 5, 7, and 10 were treated with prednisone (daily dose 10, 5, and 20 mg, respectively). Only patient 5 used immunosuppressive drugs (azathioprine, 50 mg/d). Patients 3, 4, 7, 8, and 9 had a history of thrombosis and received oral anticogulant treatment.

Nine healthy donors served as controls in the anti-phospholipid antibody ELISAs on platelet eluates and the immunofluorescence tests.

Samples

One hundred milliliters of whole blood was obtained by venipuncture. Fifty-five milliliters were collected into plastic tubes containing anticoagulant. Platelet counts of the other four patients were $109/L$.

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Platelet-rich plasma was prepared by centrifugation at 2,000g for 15 minutes. All tests were performed within 12 hours after blood collection.

Platelet Counts

Platelets were counted in a hemocytometer according to Turk using phase-contrast microscopy after addition of a solution of lidocain in dimethyl sulfoxide (Plaxan, Ets, Fiers, Belgium) to induce erythrosis.

Lupus Anticoagulant

Samples were considered lupus anticoagulant-positive when at least two of the three following criteria were fulfilled:
1. A prolonged partial thromboplastin time using a 1:1 mixture of patient and pooled normal plasma with thromboplastin derived from human brain;
2. A progressive increase in the partial thromboplastin time of test plasma upon dilution of thromboplastin; and
3. An increased kaolin clotting time-index, calculated from the kaolin clotting time of control and test plasma, mixed at different ratios.

Platelet Eluates

Eluates were prepared from platelets of the patient and the normal control with a modification of the elution method. Washed packed platelets were mixed with one part of phosphate-buffered saline-bovine serum albumin (PBS-BSA) 0.2% and two parts of ether, by vigorous shaking for 2 minutes. The mixture was incubated for 30 minutes at 37°C in a waterbath, and the shaking was often repeated. Subsequently, the mixture was centrifuged for 10 minutes at 2,800g. After centrifugation, three layers were present, consisting of ether, stroma, and the eluate.

Anti-Phospholipid Antibody ELISA

Sera and platelet eluates were assayed by ELISA for the presence of antibodies against the negatively charged phospholipids cardiolipin (CL), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and the zwitterionic phosphatidyethanolamine (PE), phosphatidylethanolamine (PE), and sphingomyelin (SM; all from Sigma, St Louis, MO). Twenty-five microliters of phospholipid solution (for CL, 48 μg/mL in ethanol 70%; for other phospholipids, 50 μg/mL in ethanol 70%) was added to 96-well polystyrene assay plates and coated on the surface by evaporation under nitrogen. The uncoated areas were blocked by addition of 150 μL PBS containing 10% fetal calf serum (FCS) for 2 hours at room temperature, washed three times with PBS, and then 50 μL of 1:100 dilutions (in PBS/10% FCS) of test serum or 50 μL of undiluted platelet eluate was added in duplicate. After incubation for 1 hour at room temperature the plates were washed three times with PBS. Alkaline phosphatase-conjugated antibodies (goat antihuman IgG or IgM alkaline phosphatase; Tago, Burlingame, CA) were diluted in PBS/10% FCS (1:2,000 for IgG; 1:1,500 for IgM) and 50 μL was added to each well. After incubation for 3 hours at room temperature the plates were washed again with PBS. Then 100 μL substrate (0.6 mg p-nitrophenyl phosphate/mL diethanolamine buffer pH 9.8; Sigma) was added. The color reaction was stopped by addition of 50 μL 2.4 mol/L NaOH after 15 minutes for the sera and after 75 minutes for the eluates. Color development was read at 405 nm in a Titertek multiscan photometer (Flow Laboratories, Helsinki, Finland).

Results were obtained by subtracting the optical density (OD) values of the normal controls from OD values of the patients. Cut-off levels (low positive, medium positive, high positive) were established by comparison with internationally validated standards for the cardiolipin ELISA. Accordingly, low positive findings were defined as differences in OD between patients and controls of at least 0.1, medium positive results were obtained when the difference exceeded 0.3, and high positive findings were defined as a difference in ODs of more than 0.7.

Aggregation Studies

Platelet aggregation studies were performed on platelet-rich plasma at 37°C after stimulation with 0.625, 1.25, 2.5, and 5.0 μmol/L of ADP (DADE Diagnostics, Aquada, Puerto Rico); 0.25, 0.5, 1, 2, and 4 μg/mL of equine collagen (Hormon Chemie, Munich, Germany); 0.38, 0.765, and 1.53 mmol/L of arachidonic acid (Bio/Data, Hatboro, PA) and different concentrations (range 0.1 to 0.4 mg/mL) of ristocetin (H. Lundbeck & Co, Copenhagen, Denmark; all final concentrations). These concentrations included threshold concentrations for secondary aggregation and the release reaction. The aggregation studies were performed in platelet-rich plasma (0.5 mL, stir speed: 900 rpm). Aggregation was measured by two techniques; firstly, by measuring the change in light transmission in an aggregometer (PAP-4; Bio/Data), designated as optical aggregation, and secondly, by measuring the disappearance of single platelets according to a modification of the method described by Frojmovic et al., designated as single platelet disappearance. For the last mentioned procedure, 100 μL samples were collected from the aggregating suspension after 15 seconds of ADP stimulation and after 120 seconds of collagen and ristocetin stimulation. These samples were immediately mixed with 9 volumes of 0.5% glutaraldehyde in PBS (15 mmol/L sodium phosphates buffered saline-BSA) 0.2% and two parts of ether, by vigorous shaking for 2 minutes. The mixture was
Flow Cytometry

In the seven thrombocytopenic patients and normal controls, flow cytometric analysis was performed to detect activated platelets in vivo with a murine monoclonal antibody, designated RUU-SP 2.28, that binds to a 53-Kd lysosomal membrane protein expressed on the surface of human blood platelets after activation.\textsuperscript{1,2} Freshly paraformaldehyde-fixed unstimulated platelets were washed twice with PBS/5 mmol/L EDTA and diluted to a concentration of 3 x 10^7/mL. Twenty microliters of platelet suspension was incubated with 20 μL of monoclonal antibody (MoAb) (5 μg/mL) for 30 minutes at room temperature. Isotype-matched MoAbs were used as an aspecific control. After washing, platelets were stained with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Central Laboratory of Blood Transfusion, Amsterdam, the Netherlands) for 30 minutes at room temperature. Subsequently, the platelets were washed twice with PBS/0.2% BSA, EDTA, and resuspended in 2 mL PBS for analyses.

From each sample, 5,000 platelets were analyzed in a FACSCAN flowcytometer (Becton Dickinson, Mountain View, CA) at a wavelength of 488 nm (200 mW laser power). Fluorescence and scatter signals were calibrated daily using 2-μm Calibrite beads (Becton Dickinson). Light scatter and fluorescence data were obtained at logarithmic settings and analyzed using the FACSCAN software. Platelets were distinguished from other cells on the basis of their forward and side scatter. Gating was set such that 90% of the particles in a sample were accepted for fluorescence analysis.

IF Tests

Antibodies to platelets were measured by IF on paraformaldehyde-fixed platelets in suspension, essentially as described by Von dem Borne et al.\textsuperscript{14} In short, indirect IF was performed as follows. Normal donors platelets were washed three times in a solution of Na2EDTA in PBS, pH 7.4 (EDTA-PBS), containing 0.1% BSA, fixed with 1% paraformaldehyde in PBS for 5 minutes at room temperature, washed again twice, and resuspended in EDTA-PBS-0.1% BSA to a final platelet concentration of 3 x 10^7/mL. Platelet suspension (0.1 mL) and 0.1 mL of patient serum or platelet eluate were mixed and incubated for 30 minutes at 20°C. The platelets were then washed three times and 0.1 mL FITC-labeled antiglobulin reagent (Central Laboratory of Blood Transfusion), appropriately diluted in PBS-0.2% BSA, was added. After another incubation for 30 minutes at room temperature, the platelets were washed twice, resuspended in a mixture of glycerol-PBS (3:1, vol/vol), and examined under the fluorescence microscope. Results were graded as negative (−), weakly positive (+), medium positive (+++), and highly positive (+++), based on fluorescence intensity and number of fluorescent cells.

Direct IF was performed on the patient’s own platelets. Platelets were prepared in the same way as those of the donors and incubated directly with the diluted FITC-labeled antiglobulin (anti-IgG and anti-IgM; Central Laboratory of Blood Transfusion, and Dakopatts, Glostrup, Denmark).

The following experiments were performed: (1) direct IF on platelets from the patient, (2) indirect IF of eluates from normal donor platelets, and (3) indirect IF with patient serum on platelets from normal donors. The presence of autoantibodies to platelets was established when all three tests were positive. When (1) and/or (3) were positive and (2) was negative, positive results were considered to be caused by immune complexes on the platelet membrane, not associated with the presence of autoantibodies to platelets.\textsuperscript{15}

Statistics

The Student’s t-test was used where appropriate.

RESULTS

Anti-Phospholipid Antibody ELISAs

The sera and platelet eluates of the patients were screened with seven phospholipids for the presence of anti-phospholipid antibodies by ELISA (Tables 2 and 3).

Sera. One patient (patient 5) with the lupus anticoagulant did not have antibodies to phospholipids as measured by ELISA, whereas the other six thrombocytopenic patients and four patients with normal platelet counts had antibodies primarily to the anionic phospholipids (PS, CL, PA, PI). Three patients also had antibodies to PE (patients 3, 10, and 11) and one to SM (patient 11) in the IgG-ELISA, and two patients had antibodies to PC (patients 2 and 9) and three to SM (patients 2, 3, and 9) in the IgM-ELISA.

Positive titers of anti-phospholipid antibodies to zwitterionic phospholipids were all weakly positive and distinctly lower than the values found for anionic phospholipids.

Eluates. Of the seven thrombocytopenic patients the IgG-ELISA was positive for PS in two, for CL in three, for PA in one, and for PI in three patients. The IgM-ELISA was positive in only one thrombocytopenic patient for PI. Of four patients with normal platelet counts, the IgG-ELISA was positive for PS in two, for PA in one, and for PI in two patients. Each non-thrombocytopenic patient was positive in the IgM-ELISA for PS, CL, PA, and PI.

Thus, in the eluates of seven patients anti-phospholipid antibodies were found directed against the negatively charged phospholipids PS, CL, PA, and PI and not to the zwitterionic PC, PE, and SM. Antibodies to PI were most pronounced. In all patients with positive anti-phospholipid
Table 3. Results of IgM Anti-Phospholipid Antibody-ELISAs on Sera and Platelet Eluates as Compared With IF

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<th>Anionic Phospholipids</th>
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<td>Thrombocytopenic Patients</td>
<td>++</td>
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<td>Non-Thrombocytopenic Patients</td>
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For the ELISAs: = OD,,,,,, - OD,,,,, > 0.1 and < 0.3; ++ = OD,,,,,, - OD,,,,, > 0.3 and < 0.7; +++ = OD,,,,,, - OD,,,,, > 0.7.

Platelet Activation

Flow cytometric analysis using MoAbs against the lysosomal activation-dependent membrane protein demonstrated 4.9% (SD 1.7%) activated platelets in the thrombocytopenic patients versus 4.3% (SD 1.2%) in controls. A typical fluorescence histogram is shown in Fig 1.

Platelet Function

ADP 5 μmol/L, collagen 2 μg/mL, and ristocetin 0.4 mg/mL induced irreversible aggregation tracings in all patients and controls, with a maximal change in light transmission ranging from 50% to 100%.

Mean concentrations (SD) of ADP yielding 50% of platelet aggregation as measured with the single platelet disappearances were 0.87 μmol/L (SD 0.65) for the patients and 0.86 μmol/L (SD 0.49) for the controls. For collagen, these values were 0.56 μg/mL (SD 0.87) and 0.52 μg/mL (SD 0.42). Mean ristocetin concentrations (SD) yielding 50% of platelet aggregation in patients and controls were 0.33 mg/mL (SD 0.02) and 0.28 mg/mL (SD 0.10), respectively. All differences between patients and controls were not significant (Student's t-test).

Correlation IF and ELISA

IgG. Autoantibodies to platelets as established by IF were found in two of seven thrombocytopenic (29%) and two of four patients with normal platelet counts (50%). Of these, three patients (75%) demonstrated anti-phospholipid antibodies in the eluates. Conversely, of six patients with positive anti-phospholipid antibody titers of the IgG class, three were also positive in IF tests (Table 2).

IgM. Positive IF tests for IgM were found in four of seven thrombocytopenic patients (57%) and in none of the patients with normal platelet counts. Of these, one had anti-phospholipid antibodies in its eluate (25%). Of two patients with positive anti-phospholipid antibodies ELISAs in the eluates, one was positive in IF tests (Table 3).

When both IgG and IgM were taken together, six of seven thrombocytopenic patients (86%) and two of four patients with normal platelet counts (50%) had autoantibodies to platelets by IF. Of these, four had anti-phospholipid antibodies of identical isotype in their eluates (50%). Four of seven patients with anti-phospholipid antibodies in platelet eluates (57%) had positive IF results.

DISCUSSION

This study demonstrates the existence of binding of anti-phospholipid antibodies to circulating platelets in seven of eleven patients (64%), which is in accordance with recent studies of Hasselaar et al., who demonstrated cross-reactions of antibodies isolated by cardiolipin-vesicles to gel-filtered platelets. Aggregation was not impaired and the platelets were not activated, as assessed by flow cytometric analysis using MoAbs directed against an activation-dependent lysosomal membrane protein.

Full externalization of negatively charged phospholipids has been demonstrated to take place after activation with...
strong agonists (collagen and thrombin). In our experiments, it is unlikely that this has happened. However, platelet activation without lysosomal membrane protein exposure yielding maximal fibrinogen receptor-site expression and maximal alpha- and dense granule release may also be possible after weak activation. Therefore, a partial externalization of negatively charged phospholipids without lysosomal release as the explanation for the binding we found cannot be excluded. Also, binding may be directed at the small amounts of PI and PS normally present in nonactivated platelets.

Cross-reactivity of anti-phospholipid antibodies to negatively charged phospholipids was more pronounced in the sera than in the eluates. Remarkably, anti-PI antibodies were found in all positive eluates, suggesting that PI is the primary target. However, titers of anti-phospholipid antibodies in the platelet eluates were low and the predominance of anti-PI antibodies may reflect different sensitivities of the anti-phospholipid ELISAs.

Three patients with anti-phospholipid antibodies in their sera did not have these antibodies in their platelet eluates. In two of these patients (patients 1 and 8) this finding might be explained by the rather low titters in the sera. Differences in affinity to platelets between patients because of an intrinsic heterogeneity of anti-phospholipid antibodies may also account for these discrepancies.

Binding of anti-phospholipid antibodies to platelets does not necessarily result in thrombocytopenia. Probably, the balance between platelet clearance and subsequent production will determine the platelet counts in patients with anti-phospholipid antibodies on their platelets. Whether enhanced clearance of these platelets will take place cannot be answered, because we felt it was not ethically justified to perform platelet survival tests requiring radioactively labeled platelets or bone marrow studies in patients with only mild thrombocytopenias. However, three patients (patients 2, 3, and 7) had bone marrow studies performed on them in the past and marrow aspirates did not show hypoplastic bone marrow, suggesting that peripheral platelet destruction is the cause for the low platelet counts in these patients.

The overall positive concordance of IF and ELISA was 50%, suggesting that both tests may in part detect the same antibodies. Differences in sensitivity between both assays may be responsible for discordant results. Moreover, most patients suffered from systemic lupus erythematosus, a disease with a marked prevalence of different autoantibodies. Autoantibodies other than anti-phospholipid antibodies may therefore be responsible for positive IF results and the thrombocytopenia in these patients.

In conclusion, binding of anti-phospholipid antibodies to circulating platelets is proven by this study. This binding does not induce platelet activation associated with lysosomal granule release. Also, such activation is not mandatory for binding. No abnormalities in aggregation were seen. However, more studies are needed to ensure the nature of the primary epitope on platelets and to determine the role of anti-phospholipid antibodies in thrombocytopenia or thrombosis.

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