Platelet-directed Antibody in the Serum of Patients with Primary Thrombotic Thrombocytopenic Purpura

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ABSTRACT

Utilizing two platelet antibody assay systems, flow cytometric analysis and a microcytotoxicity assay, it was possible to demonstrate for the first time platelet-directed antibody in the sera of three of six patients with primary thrombotic thrombocytopenic purpura. These results lend support for an immune-mediated pathologic process in some patients with this disorder.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare disorder characterized by thrombocytopenia, microangiopathic hemolytic anemia, renal disease, fever, and fluctuating neurologic signs. The clinical picture is thought to be the result of diffuse platelet and platelet/fibrin thrombus formation in the microvasculature. Although the initial event leading to platelet aggregation and thrombus formation in these patients is unsettled, findings supportive of either primary endothelial cell injury or primary platelet aggregation as pathogenic mechanisms have been presented. A platelet aggregating factor in the plasma of some patients was first shown by Lian and co-workers and later confirmed by Kelton et al. The latter group suggested that the interaction of the aggregating factor with platelets may be accentuated by the presence of large multimers of factor VIII von Willebrand factor present in the plasma of these patients. In other studies deficient endothelial cell prostacyclin production and/or stabilization have been documented. Several groups have reported complement-dependent cytotoxic anti-endothelial antibodies in the serum of TTP patients. Finally, Burns and Zucker-Franklin reported three patients with TTP whose plasma caused spontaneous aggregation of normal platelets and whose sera caused immune destruction of cultured human endothelial cells.

Although no intrinsic platelet abnormality has been demonstrated in these patients, several investigators have found increased platelet membrane associated immunoglobulin (PAIgG), the level of which has been shown to corre-
late with disease activity. The pathologic significance of the elevated PAlgG is uncertain but may represent platelet specific antibody, non-specific absorption of IgG by damaged platelets or absorbed immune complexes. It was postulated by us that if this finding represented a specific immune response, unbound platelet antibody should be detectable in sera of patients during the course of their illness. Using both immunofluorescence flow cytometric analysis and platelet microcytotoxicity cross-matching antibody assay systems, it has been possible to demonstrate platelet specific antibody in the sera of three of six patients. Our results suggest an additional mechanism to account for the development of thrombocytopenia in some patients with primary TTP.

Materials and Methods

Patient Population

Six patients were seen over a five year period with a clinical picture compatible with TTP. The study group included four females and two males ranging in age from 21 to 50. All six had thrombocytopenia, microangiopathic hemolytic anemia, and fever. Renal impairment was noted in four; five had documented neurologic deficits during the course of their illness. None had any known underlying immunologic disorders that have been associated with TTP. Plasma and serum samples were obtained by venipuncture or at the time of plasmapheresis, but in all cases prior to the initiation of therapy.

Flow Cytometric Antibody Analysis

An indirect immunofluorescence platelet antibody assay was performed as previously described using the Ortho Spectrum III flow cytometer. In brief, an 0.01 mL aliquot of normal platelets at a final concentration of 250 × 10⁶ per L is incubated with 0.1 mL of either autologous serum from the target platelet donor or patient serum or dilutions of each for one hour at room temperature. After centrifugation and extensive washing, the platelet pellet is incubated with 0.05 mL fluorescein-conjugated goat F(ab′)2 antihuman IgG antiserum† for 15 minutes at room temperature. After repeat centrifugation and washing, the platelet pellet is resuspended in phosphate buffered saline to a final volume of 2 mLs. The amount of fluorescein conjugate bound in each platelet suspension is then measured using flow cytometric analysis. For this assay, region A on the spectrum III is defined as consisting of all 255 fluorescence intensity channels. The intensity of fluorescence, expressed as the mean value of region A (mean channel A), is proportional to the amount of serum IgG bound to the platelet membrane. The mean channel A reading for the test serum + platelet mixture is then compared to that of the autologous serum + platelet mixture and a ratio of relative fluorescence determined. In a previous report, it has been shown by us that a ratio greater than 1.3 of test serum/autologous serum is compatible with the presence of platelet directed antibody in the serum.

Microcytotoxicity Crossmatch Antibody Assay

A second approach to determine whether or not TTP serum contained platelet specific antibody employed a microcytotoxicity assay described by Lizak and Grumet. In this system, washed control blood group type O platelets

* Ortho Diagnostic Systems, Inc., Westwood, MA.
† Tago, Inc., Burlingame, CA.
from a healthy donor are first fluorescein labeled using carboxy-fluorescein diacetate. In the presence of an extraneous source of complement and anti-kappa light chains, platelet specific antibody in the serum will cause platelet lysis and liberation of the fluorescein dye. The assay was carried out in standard tissue typing trays and manually read using fluorescent microscopy. Wells were graded from 0 (no lysis) to 4 (complete lysis). However, for purposes of reporting, only wells with greater than 75 percent cell lysis were considered positive for the presence of antibody.

Autologous and non-immune thrombocytopenic sera served as negative controls; sera from a patient with idiopathic thrombocytopenic purpura and a patient with an anti-PIAI antibody served as positive controls. For both the indirect flow cytometric and the microcytotoxicity platelet antibody assays, donor platelets were HLA typed using standard techniques. In addition, the serum from each TTP patient was screened for the presence of anti-HLA reactivity using panels of HLA-typed lymphocytes prior to the indirect flow cytometric and microcytotoxicity crossmatch platelet antibody assays. Serum from patient #1 had 29 percent reactivity, i.e., 12 of 40 cells in the panel were lysed. Alloantibody specificity in this case included anti-HLA A2, A24, and probably B17. Serum from patient #3 had five percent reactivity, i.e., two of 40 cells underwent lysis. This patient's serum had HLA A2 and B17 antibodies present in low titer. Target cells used in the direct platelet antibody and the crossmatch assay were selected from donor platelets lacking these HLA antigens. The sera from the other four patients were non-reactive against all lymphocyte panels.

**Flow Cytometric Platelet Antibody Assay**

The sera from two of the six patients were positive for the presence of platelet specific antibody in this assay system (table II). Both of the positive assays in this series were on female patients. In one (patient #1), the ratio of test/autologous control mean channel A green fluorescence values of unadsorbed and lymphocyte-adsorbed serum samples were determined. The unadsorbed serum at a 1:20 dilution gave a mean fluorescence ratio of 2.15. Repeat assay following sequential adsorption of this patient's serum with lymphocytes from three donors who had A2, A24, and B17 HLA antigens, respectively, was still positive at a ratio of 1.66 (figure 1). The adsorbed serum sample was, however, unreactive in standard cytotoxicity testing against A2, A24 and B17 reference cells confirming that these anti-HLA antibodies had been completely removed. The sec-

‡ CBR Laboratories, Inc., Boston, MA.
<table>
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<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Fever</th>
<th>Deficit</th>
<th>Hematocrit</th>
<th>RBC Fragments*</th>
<th>Creatinine</th>
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<th>Nadir Platelets (10^9/L)</th>
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*Scoring for red blood cell fragments per high powered field: 1+ = 2 to 5; 2+ = 6 to 10; 3+ = 11 to 20; 4+ = >20.

Anti-platelet drugs included aspirin and/or dipyridamole. The total number of plasma exchanges are given.

Microcytotoxicity Crossmatch Assay

Similar to the flow cytometric assay, two of the six sera (patients #1 and #5) were also positive for the presence of platelet antibody in this system (table II). In both of these patients the reactions were highly positive with essentially complete lysis of all fluorescein-activated platelets in the assay wells. As noted previously, one of these sera (patient #1) was also positive in the flow cytometric assay system. The serum of patient #5 was reactive with target platelets from four different donors tested including platelets which were HLA A1, A25, B8, Bw22 and A30, B35 typed. The absence of circulating HLA antibodies in her serum would suggest that the reactivity noted was directed at platelet specific antigens.

The possibility that the positive results noted in each of these assay systems could be due to non-specific binding of circulating immune complexes to target platelets was examined by measuring immune complexes using a Clq binding assay. Values ranged from 1.7 to 9.6 μg IgG equivalents per mL with a mean value of 4.2 for the TTP group. These values are well within the reference range.
PLATELET ANTIBODIES IN THROMBOTIC THROMBOCYTOPENIC PURPURA

FIGURE 1. A comparison of the green fluorescence histograms generated on assay mixtures of donor platelets incubated with autologous control serum (A) and lymphocyte-adsorbed serum from patient #1 with thrombotic thrombocytopenic purpura (B) is shown. Each serum was assayed at a 1:20 dilution in phosphate buffered saline. The mean channel A green fluorescence values for the control and test sera were 12.9 and 21.5, respectively, for a ratio of test/control of 1.66.

range for normal control sera in this system and would make it unlikely that positive platelet antibody assay results seen in this study were secondary to presence of immune complexes.

Discussion

Utilizing two indirect platelet antibody assay systems, platelet antibody could be detected in the sera of three of six patients with primary TTP. Although the basis for the platelet reactivity of the sera in this study is not completely defined, it is unlikely that the findings noted were due to the presence of allo-antibodies resulting from previous sensitization with blood products. The HLA allo-antibodies were present in the sera of only two of the six patients examined. In one (patient #1), the serum was positive in both platelet antibody assay systems when target platelets did not have these HLA antigenic determinants present. In addition, complete adsorption of these HLA antibodies with lymphocytes had only minimal effect on the serum reactivity in the flow cytometric assay. The other patient who had allo-antibodies (HLA A2 and B17) in her serum was negative in both platelet antibody assays when target platelets lacking the HLA B2 and B17 antigens were used. No HLA A or B allo-antibody was detectable in the sera of the other two patients who had a positive platelet antibody assay result.

The possibility that the positive platelet antibody assay results were due to the presence of immune complexes was also unlikely since elevated levels of immune complexes were not found in any patient serum. This finding is in agreement with most other studies that have examined primary TTP using a variety of immune complex assay systems. It is equally unlikely that the reactivity seen in our study was due to non-specific absorption of IgG to damaged platelet membranes since platelets from normal donors and not patient platelets were used in all studies. It is probable that our results are reflective of an immune response with the development of platelet antibodies as part of the disease process in some patients with primary TTP. Although conjectural, the in vivo damage to the patient's platelets from the primary disease process could conceivably result in platelet antibody production from the exposure of basic membrane components shared by all platelets.

The role of specific platelet membrane glycoprotein components as antigenic determinants in auto- and allo-immune thrombocytopenias has been demonstrated by Devine and Rosse. It has recently been shown that cultured endothelial cells synthesize a protein immunologically similar to platelet glycoprotein IIb/IIIa complex. Another intriguing possibility is based on the finding in some patients with primary TTP of anti-endothelial cell antibodies which had HLA DRw reactivity. Cross-reactivity of this antibody with shared platelet antigens could thus account for a pathologic lesion to the endothelial cells, the findings of elevated platelet-associated immunoglobulin as reported by...
others, and for the presence of platelet-reactive antibody in the serum seen in this study. Most investigators, however feel that the HLA DR group is not expressed on platelets. Whether the serum antiplatelet antibodies in this study are directed to platelet specific antigens or reflect an autoimmune specificity to HLA antigens not previously thought to be shared by platelets can not be conclusively settled from these studies. The assay results following lymphocyte adsorption of the serum of one of the positive patients are consistent with the premise that these antibodies are directed at platelet-specific antigens.

It is unlikely and is not proposed by the present authors that the finding of platelet-directed antibodies in this study is reflective of a causative process in this disorder. At this point, these results serve to add to the diversity of abnormalities documented by others in this disorder and lend support for an immune-mediated pathologic process in some patients with this syndrome.

References