Platelet-Associated Complement C3 in Immune Thrombocytopenic Purpura

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Platelet-associated C3 (PA-C3) was measured with a quantitative immunofluorescence assay. With this assay, PA-C3 levels were determined for 78 normal volunteers, 30 patients with immune thrombocytopenic purpura (ITP), and 20 patients with nonimmune thrombocytopenias. Platelet-associated IgG (PA-IgG) levels were also measured with our standard quantitative immunofluorescence assay. All patients with nonimmune thrombocytopenias and ITP in remission had normal PA-C3 levels. Twenty-four patients with active ITP were classified into 3 groups: 9 (38%) with increased PA-IgG and normal PA-C3 levels, 10 (42%) with elevated PA-C3 and PA-IgG levels, and 5 (20%) with increased PA-C3 values only. A direct correlation was found between PA-C3 and PA-IgG levels. PA-IgG levels were higher in the group of patients with elevated PA-C3 levels than in those with normal values. Platelet survival studies showed reduced survival times of 1.5–2.5 days for the 5 patients with elevated PA-C3 levels only. Elevated PA-C3 levels returned to normal in 7 ITP patients whose platelet counts increased in response to corticosteroid therapy or to splenectomy. Therefore, PA-C3 and PA-IgG assays can be used to identify patients with ITP, to follow their response to therapy, and to classify them into immunologic subgroups similar to red cell classification by Coombs' testing in immune hemolytic anemia.

THE IMMUNOLOGIC BASIS of idiopathic thrombocytopenic purpura (ITP) has been long established. An autoantibody of the immunoglobulin-G class is responsible for the immune platelet destruction in most cases of ITP. Increased levels of platelet-associated immunoglobulin-G (PA-IgG) have been recently demonstrated in the majority of patients with ITP, and an inverse relationship exists between the PA-IgG level and the platelet count in these patients. In some patients, the IgG autoantibody apparently activates complement on the platelet surface leading to elevated levels of both platelet-associated IgG and C3. However, 10%–30% of patients with ITP show no increase in PA-IgG levels. This observation may be partially explained by the recent finding of patients with ITP who have elevated platelet levels of only C3 or IgM.

In the present study, platelet-associated C3 (PA-C3) was measured by a quantitative immunofluorescence assay similar to that reported previously by our group for the determination of PA-IgG. This report describes the method of the PA-C3 assay, analyzes variables, and applies the assay to a study of normal controls and patients with immune and nonimmune thrombocytopenias. Our findings allow a classification of ITP into 3 separate groups based on levels of PA-IgG and PA-C3. Moreover, we confirm the previous finding of a subgroup of ITP patients with elevated levels of PA-C3 only and now demonstrate reduced platelet survival times in these patients.

MATERIALS AND METHODS

Patients

The following groups of subjects were studied. (1) Thirty adult patients with immune thrombocytopenic purpura (ITP) who represented consecutive cases between March 1980 and July 1981 seen by the Memorial Hospital Hematology Service or referred only for PA-C3 and PA-IgG testing. The group included 16 females and 14 males ranging in age from 16 to 88 yr. The diagnosis of ITP was made according to established criteria: thrombocytopenia (platelets <110,000/cu mm), a normal bone marrow with adequate or increased megakaryocytes, absence of splenomegaly, normal coagulation studies, and no other obvious cause for the thrombocytopenia. Six patients had acute ITP, 18 chronic ITP (i.e., present >3 mo), and 6 chronic ITP in remission (i.e., platelets >150,000/cu mm while either receiving therapy or not for previously diagnosed ITP). Among the 24 patients with active ITP at the time of platelet testing, 15 were receiving no therapy, 6 had taken prednisone for less than 1 wk, and 3 had received prednisone for more than 1 wk. Two patients in remission were taking low-dose, alternate-day prednisone and one was receiving daily azathioprine. The platelet counts in the patients with acute ITP ranged from 4000 to 50,000/cu mm and for the chronic ITP patients with active disease ranged from 25,000 to 108,000/cu mm. Four patients with acute and 11 with chronic ITP showed a response to therapy while being followed by us.

(2) Twenty patients aged 22–82 yr with nonimmune thrombocytopenia (platelets 18,000–120,000/cu mm) due to bone marrow infiltration by malignant disorders (10), to bone marrow hypofunction from chemotherapy (8), or to congestive splenomegaly due to cirrhosis (2).

(3) Seventy-eight normal controls consisting of adult volunteers aged 16–86 yr with platelets >150,000/ cu mm.

The study was approved by the institutional Human Experimentation Committee, and informed consent was obtained from all volunteers and patients according to the principles of the Declaration of Helsinki before drawing blood samples or performing platelet survival measurements.

Preparation of Platelets

Blood was anticoagulated with 1 part acid citrate dextrose solution (ACD, U.S.P. Formula “A”) to 6 parts whole blood. Platelet-
rich plasma (PRP) was separated by centrifugation at 180 g for 8 min. All experiments were performed at 21°C. Platelets were pelleted by centrifugation at 1800 g for 10 min and were washed twice with phosphate-buffered saline (PBS) (pH 7.2) containing 15% ACD (PBS-ACD, pH 5.2). Contaminating erythrocytes and leukocytes were separated by two centrifugations at 300 g for 3 min. Finally, the platelets were resuspended in PBS and allowed to stand for 30–45 min before the platelet assays were performed. There was no clumping of platelets with this procedure. Red cell contamination was always kept at <0.5% and leukocyte contamination at <0.1% due to their effect on total fluorescence of the specimen at higher levels.

Determination of Platelet-Associated C3 (PA-C3)

Fluorescein isothiocyanate (FITC) conjugated anti-human C3 goat serum (protein content of 8.9–11.6 mg/ml, antibody content of 0.63–0.80 mg/ml, and fluorescein/protein concentration ratio 3.6:5.6) was obtained from Meloy Laboratories (Springfield, Va.). The antibody formed a precipitin arc on immunoelectrophoresis with C3 and C3b (kindly provided by Drs. Carl H. Hammer and Melvin Berger, National Institute of Health, Bethesda, Md., and prepared according to previously described methods20); and it also reacted with C3d (personal communication, Meloy Laboratories). The antiserum was aliquoted and stored at −70°C until used. Frozen antiserum gave results comparable with fresh antiserum stored at 4°C for 2 wk, whereas antiserum stored at 4°C for longer than 2 wk showed progressive loss of fluorescence compared with frozen material.

Platelets suspended in PBS were adjusted to a concentration of 1.0 ± 0.05 × 10^10/cu mm for the PA-C3 assay. To 0.5 ml of this platelet suspension were added 10 μl of the antiserum, which gave a final fluorescent antibody concentration of 77–98 nM. After thorough mixing, the incubation was continued for 18 min. The platelets were washed twice with PBS-ACD, transferred to new test tubes after the first washing step (to eliminate contamination by the FITC-antibody adhering to the test tube walls), and finally were resuspended in 3 ml of PBS. Platelet counts were again performed at this stage and always ranged between 70% and 85% of the initial counts. A few minutes before measurement of the platelet fluorescence derived from bound anti-C3 antibody, 4 mg of sodium dodecyl sulfate (SDS) were added to each sample to obtain a transparent solution. This concentration of SDS had no quenching effect on the fluorescence.19 Fluorescence was determined in a fluorospectrophotometer (MPF-44A, Perkin-Elmer Corp., Norwalk, Conn.) with excitation wavelength 493 nm and emission measured at a wavelength of 516 nm. The “apparent” quantity of platelet-bound anti-C3 antibody was calculated from the known FITC/protein ratio of the antibody and the fluorescence of a standard amount of antibody measured with each set of assays. The “true” value of bound antibody was then determined from the above by subtracting nonspecifically trapped and nonspecifically bound antibody, which were quantified as described previously.15,24 The former was 1.1 × 10^4 ml/10^10 platelets and the latter amounted to 0.05 fg/platelet. The quantity of “true” bound antibody was then divided by the number of platelets and expressed in femtograms (10^-15g) per platelet (and termed the “platelet-associated C3 level” in this report). All assays were performed in duplicate.

Determination of Antibody/Antigen Binding Ratios

Fluorescein isothiocyanate-conjugated C3b (FITC-C3b) was kindly provided by Dr. Gordon D. Ross (University of North Carolina School of Medicine, Chapel Hill, N.C.). Conjugation with FITC was performed at pH 9.5 for 16 hr at 4°C.21 FITC-conjugated C3b was gel filtered through Sephadex G25 (Pharmacia Fine Chemicals, Upsala, Sweden) and purified by Pevikon block electro-phoresis. Purity was evaluated from densitometer tracings of SDS-polyacrylamide gels stained with Coomassie blue. The C3b constituted >98% of the total protein (50 μg) applied to the gels. Analysis of FITC-C3b by SDS gels and by ultracentrifugation revealed no aggregates. The molar ratio of FITC-C3b was determined by measuring the optical density of the fluorophor at 495 nm (ε19 = 2.44 × 10^4) and the amount of protein by the method of Lowry et al.23 The FITC-C3b molar ratio was in the range of 1.66–2.17. FITC-C3b was stored in small aliquots at −70°C until used.

Available platelet binding sites for C3b (PA-C3b) were determined as is to be reported in detail elsewhere. Briefly, platelet suspensions prepared as described above at a concentration of 1.25 ± 0.05 × 10^10 cells/cu mm were incubated at 21°C for 18 min with FITC-C3b at a concentration ranging from 15 to 78 nM. After 2 washings and resuspension in PBS, the FITC-C3b-labeled platelets were incubated for 18 min with rhodamine-B isothiocyanate (RITC) conjugated anti-human C3 goat serum (protein content of 17.8 mg/ml, antibody content of 1.44 mg/ml, RITC/protein absorbance ratio 0.38, Atlantic Antibodies, Westbrook, Me.). This antibody had the same precipitin characteristics on immunoelectrophoresis as the FITC-conjugated anti-C3 antibody (from Meloy Laboratories). The sample was then processed as described above with the FITC-conjugated antibody for the PA-C3 assay. The excitation wavelength for RITC was 556 nm and emission was measured at 580 nm.

Direct and Indirect Platelet-Associated IgG (PA-IgG) Levels

PA-IgG assays were performed with the quantitative immunofluorescence techniques we previously described.15,24 The direct PA-IgG assay measures the amount of IgG on the platelet surface, while the indirect assay quantifies the amount on the platelet surface after incubation with test serum (i.e., determines the amount of serum platelet antibody).

51Cr-Sodium-Chromate-Labeled Platelet Survival Measurements

Isologous platelet survival studies were performed as originally described by Morrison and Baldini25 with modifications as we reported previously.24 The normal range determined in a concurrent control group was 7.2–9.8 days for platelet survival times.24

Statistical Analysis

The Student’s t test, linear regression analysis by the method of least squares, and correlation coefficients were performed according to standard methods.26

RESULTS

Experimental Variables in the PA-C3 Assay

Washing steps. Platelets were washed 0–4 times with PBS-ACD, incubated with the FITC-conjugated anti-C3 antibody, and processed as described above. A reduction in the amount of bound antibody was seen with platelets washed up to 2 times, after which a plateau was achieved. In other experiments, platelets that had been washed twice were incubated with the FITC-conjugated antibody and then washed 0–4 times before measurement of fluorescence. Again, the amount of bound antibody decreased progressively.
until the second washing step, after which it was constant. These studies showed that loosely bound and trapped C3 or FITC-conjugated antibody were removed by two washing steps, which were used routinely for the assay. These results are consistent with those found previously for the PA-IgG assay.\(^5\)

**Time of incubation.** The amount of FITC-conjugated antibody that interacted with platelet-associated C3 was measured as a function of the incubation time. A period of steep rise in the binding of antibody was seen during the first 6 min of incubation and this was followed by a slower, steady rate of binding with the slowest incline after 15–18 min. Based on the results of these studies, an 18-min incubation time was routinely used (for reasons as indicated with the PA-IgG assay).\(^\)\(^5\)

**Variation of antibody/antigen binding ratios.** To determine the optimal antibody/antigen ratio, both were labeled with a different fluorophor. The antigen was FITC-conjugated C3b and the antibody was RITC-conjugated anti-human C3 antibody. Both fluorescent markers have emission spectra that can be measured independently, and no significant energy transfer occurs between the two fluorophors.\(^5\)\(^\)\(^5\) The antibody/antigen ratio was evaluated as a function of the amount of antibody in the reaction mixture and as a function of the amount of antigen present on the platelet surface. First, platelets were incubated with FITC-conjugated C3b at a concentration of 15 nM, and the amount bound to platelets was determined with the RITC-conjugated antibody in a series of concentrations ranging from 0 to 500 nM. Under these conditions of antibody excess, the antibody/antigen ratio increased progressively in a linear fashion to an antibody level of 250 nM, after which it began to plateau. The antibody/antigen ratio was 1.0 at a free antibody concentration of 115 nM. Therefore, for the PA-C3 assay, the antibody concentration was maintained in the range of 77–98 nM, ensuring that the antibody/antigen ratio did not exceed 1.0. In the second set of experiments, platelets were incubated with FITC-C3b in concentrations ranging from 15 to 78 nM, and platelet-bound antigen was determined with RITC-conjugated antibody at a fixed concentration of 97 nM. When the antigen was present in excess, the antibody/antigen ratio could be depicted as a hyperbolic curve with a peak of the curve of 1.0 at a FITC-C3b concentration of 0.74 fg/plt.

**Platelet-Associated C3 (PA-C3) Levels for Normal Subjects and Patients With Nonimmune Thrombocytopenia**

PA-C3 measurements in 78 normal volunteers ranged from 0.10 to 0.33 fg/plt with a mean of 0.21 fg/plt (Fig. 1). The normal range for PA-C3 levels was 0.21 ± 0.12 fg/plt (mean ± 2 SD). Twenty patients with nonimmune thrombocytopenia due to bone marrow failure or congestive splenomegaly had PA-C3 levels within the normal range (Fig. 1). None of the normal subjects or patients with nonimmune thrombocytopenia had an elevated direct or indirect PA-IgG level.

**PA-C3 and PA-IgG Levels in Patients With Immune Thrombocytopenic Purpura (ITP)**

The PA-C3 levels determined in 30 patients with ITP are shown in Fig. 1. The levels ranged between 0.14 and 1.00 fg/plt. Among the 24 ITP patients with active disease, 15 had elevated PA-C3 levels. There was no significant difference in PA-C3 levels between the patients with acute ITP and with chronic ITP. Classification of ITP patients based on PA-C3 and direct PA-IgG levels is shown in Table 1. The 24

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<tr>
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*All patients in remission.*
patients with active disease could be grouped into 3 categories: those with increased PA-IgG levels and normal PA-C3 levels (n = 9, 38%), those with both elevated PA-IgG and PA-C3 levels (n = 10, 42%), and patients with elevated PA-C3 values only (n = 5, 20%). All six patients with chronic ITP in remission had normal levels of PA-C3 and PA-IgG. A correlation (r = 0.59, p < 0.05) was observed between the levels of PA-IgG and PA-C3. In addition, the mean PA-IgG level for ITP patients with both increased PA-IgG and PA-C3 levels was significantly greater than for patients with elevated PA-IgG and normal PA-C3 levels (mean ± SEM, 2.96 ± 0.46 versus 1.88 ± 0.32 fg/plt, respectively; p < 0.05).

Sequential PA-C3 levels were performed for 7 patients while they were treated with corticosteroids or splenectomy (Fig. 2). In two patients with only increased PA-C3 levels, the PA-C3 values returned to normal when their platelets rose from 12,000 to 72,000/cu mm and from 60,000 to 165,000/cu mm, respectively. (The other 3 patients with only elevated PA-C3 levels have not been treated, since their platelets have ranged between 60,000 and 80,000/cu mm. For 5 patients with increased PA-C3 and PA-IgG levels, their platelets increased above 115,000/cu mm with prednisone therapy (3 patients) or after splenectomy (2 patients). In all 5, the PA-C3 levels decreased to normal concomitant with the increased platelet counts. However, their elevated PA-IgG levels returned to normal only 1–4 mo later.

**Platelet Survival Studies in ITP Patients With Only Elevated PA-C3 Levels**

$^{51}$Cr-labeled platelet survival studies were performed in the 5 patients with ITP who showed increased PA-C3 (0.34–1.00 fg/plt) but normal PA-IgG levels. The platelet survival times in these 5 patients were reduced to 1.5–2.5 days. All 5 patients also had normal indirect PA-IgG levels, indicating that alloimmunization was not the cause of the reduced survival times. Platelet survival studies were performed in only 5 other patients with active ITP. Due to the limited number of these studies, no correlations could be made between platelet survival times and the levels of PA-C3 or PA-IgG.

**DISCUSSION**

Our quantitative immunofluorescence assay proved to be a rapid and reliable method for measuring PA-C3, as it was previously reported to be for the determination of PA-IgG levels. With the PA-C3 assay, the antibody/antigen binding ratio varied with the concentration of both antibody and antigen. Therefore, for the same reasons as with the PA-IgG technique, in the PA-C3 assay, a fixed antibody concentration was used that maintained the antibody/antigen binding ratio at nearly 1.0. We recognize that the method of our assay only determines an antibody/antigen ratio for C3b as the antigen. However, a similar binding ratio was presumed for the antibody and other complement components (C3d, etc.) on the platelet surface, since complement components are not known to form aggregates on cell surfaces. We chose not to calculate the values of PA-C3 from a normal standard curve since, as with the PA-IgG assay, it may be nonrepresentative for ITP patients. Rather, determination of PA-C3 levels was based on daily standards of the fluorescein-labeled antibody of a known fluorescein/protein concentration. Finally, leukocyte contamination was reduced to an extremely low level to prevent falsely elevated PA-C3 levels due to antibody attachment to C3 on leukocytes.

In this study we could classify patients with ITP into 3 groups: those with only elevated PA-IgG levels (38%), those with increased levels of both PA-IgG and PA-C3 (42%), and those with only elevated PA-C3 values (20%). This classification is similar to that of immune hemolytic anemia based on Coombs' testing, which includes 25% of cases with only a positive anti-C3 Coombs' test. Three prior studies of ITP patients found no case with an elevated PA-C3 level. However, the qualitative immunofluorescence assays used in two of these studies were probably too insensitive to detect the levels of PA-C3 in ITP. These assays showed negative staining for PA-IgG on normal platelets, which by our quantitative technique
was less than 1.35 fg/plt. Therefore, it is not unexpected that these assays would be negative for the PA-C3 levels we found in ITP that were 1.0 fg/plt or less. In contrast, two studies found increased PA-C3 levels for 11/16 and 12/21 patients with ITP. However, only the latter study found any patients with ITP (3/21) with only an increased PA-C3 level.

C3 fixation to platelets may possibly be produced by an IgG complement-fixing antibody, another class of antibody, or an immune complex. Cases of ITP with elevated levels of only PA-C3 could be due to a potent complement-fixing IgG antibody present on the platelet surface in amounts measured within the normal range, to a low-affinity complement-fixing IgM antibody or immune complex, or to an IgM complement-fixing antibody, as suggested by cases of ITP with elevated platelet-bound IgM. ITP due to elevated PA-C3 probably represents the 10%-30% of ITP cases reported with normal PA-IgG levels (20% in our study). Our platelet survival studies suggest that increased C3 alone on platelets in ITP may play a primary pathogenetic role in this disorder, similar to C3 coating alone on erythrocytes in immune hemolytic anemia.

We found a moderate correlation (r = 0.59) between the levels of IgG and C3 bound to platelets in ITP and a significantly higher mean level of PA-IgG for ITP patients with an elevated PA-C3 than for those with normal PA-C3 levels, confirming similar previous observations. These findings are expected if the antiplatelet IgG antibody fixes complement, since C1 fixation to a cell membrane requires juxtaposition of two IgG antibody molecules, and this condition is more easily met when large amounts of IgG are on the platelet surface.

ITP platelets that are coated with higher levels of IgG antibody show shorter survival times and greater hepatic destruction. However, one of our patients with a markedly elevated PA-IgG level (3.45 fg/plt) and an increased PA-C3 responded promptly to splenectomy (Fig. 2), suggesting that hepatic destruction of platelets is not prominent in all cases of ITP with high levels of PA-IgG. Presently, it is not known if increased platelet-bound C3 magnifies the rate and degree of platelet destruction for a given amount of bound IgG as it does with erythrocytes in immune hemolytic anemia. The present study included too few survival measurements to address this point. Also, the principal site of platelet destruction in cases of ITP with only elevated PA-C3 levels remains to be determined.

Our patients who responded to therapy showed a return to normal of PA-C3 levels long before PA-IgG levels became normal. The only previous study to sequentially measure PA-C3 levels found a simultaneous reduction in both PA-IgG and PA-C3 levels. These different observations remain unexplained and require further investigation.

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