Platelet-bound Complement (C3) in Immune Thrombocytopenia

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The fixation of complement to the circulating platelet in immune thrombocytopenia was detected by measurement of one of the complement components, C3, on the surface of platelets from patients with idiopathic thrombocytopenic purpura (ITP) and systemic lupus erythematosus (SLE) using the anti-C3 consumption assay. The surface IgG was determined simultaneously using the previously described anti-IgG consumption assay. Washed platelets from normal controls had 3.5 fg (10³ g) of C3, or about 11,000 molecules per platelet, an amount comparable to the IgG (4.1 fg, or 15,000 molecules, per platelet). For most patients with ITP both C3 and IgG were increased on the platelet surface, although for 5 of 16 patients only IgG was increased. Two patients with SLE and thrombocytopenia had an increase in both C3 and IgG. Six patients with SLE who were not thrombocytopenic had normal amounts of membrane-bound C3 and IgG. In 5 patients, 3 with ITP and 2 with collagen vascular disease, both surface immunoproteins decreased with successful treatment of the thrombocytopenia.

The thrombocytopenia in “idiopathic” thrombocytopenic purpura (ITP) is usually due to immune destruction of the platelets. Infusions of plasma from such patients can cause thrombocytopenia in normal recipients.¹ The plasma factors responsible for the destruction of the platelets appear to be antibodies in that they are found among the 7S immunoglobulins of the plasma and are absorbed to normal platelets,²³ and the amount of surface immunoglobulin (IgG) present on the platelets of patients with ITP is increased and is inversely related to the platelet count.⁴ Thus, IgG antibody appears to play an important role in the pathogenesis of this disorder.

A similar role for IgG antibody has been postulated in autoimmune hemolytic anemia of the warm antibody type. In some circumstances, IgG antibody seen in this syndrome is able to fix complement. When this occurs, the rate of hemolysis may be greater for a given amount of bound IgG.⁵ Analysis for the presence or absence of complement on the red cell surface may yield valuable information concerning the antigens and antibodies involved.

Complement binding by antibodies in ITP has been difficult to assess. Although complement fixation by certain alloantibodies (anti-Pl₄ and anti-HLA antibodies)⁶ and drug-related antibodies⁷ has been demonstrated, complement fixation has not been demonstrated with the serum of patients with ITP in in vitro assays.² In none of these instances has there been direct demonstration...
of the fixation of complement components to the platelet surface by antigen antibody reactions, either in vivo or in vitro.

During the activation of complement by immune reactions at the membrane surface of the target cell, the third component of complement (C3) is fixed to the membrane and, in the case of the red cell, it is easily detectable. In order to investigate the role of complement binding in vivo in the immune destruction of platelets in patients with ITP and thrombocytopenia due to systemic lupus erythematosus (SLE), we have devised a method for the detection and quantitation of C3 on the platelet surface using a modification of the quantitative antoglobulin consumption test, which we had previously used to quantitate surface-bound IgG on platelets. We have found that C3 is present on the platelets of many patients with ITP and the amount present is proportional to the amount of bound IgG. In no instance was C3 fixed when IgG was not fixed. The amount of both IgG and C3 correlated inversely with the platelet count, both initially and during the induction of remission. These studies indicate that the antibody in ITP fixes complement in vivo.

**MATERIALS AND METHODS**

**Patient Material**

Platelets from patients in the following categories were studied: (1) Normal controls consisted of adult volunteers with platelet counts greater than 150 x 10⁹/liter who were not taking medicines and who had no clinical illnesses. (2) Thrombocytopenic controls consisted of 10 adult patients with platelet counts less than 100 x 10⁹/liter. Two of these patients had lymphoma, 2 had metastatic solid tumors, 2 had myeloproliferative diseases, 1 had aplastic anemia, 2 had acute myelogenous leukemia without antecedent platelet transfusion, and 1 had alcohol-induced acute thrombocytopenia. (3) Each of the 16 patients with ITP by definition had a platelet count less than 100 x 10⁹/liter, a bone marrow with an increased or normal number of megakaryocytes, the absence of a palpable spleen, and no explainable cause for the thrombocytopenia (e.g., drugs, disseminated intravascular coagulation syndrome, sepsis, or collagen vascular disease): 12 of these patients were studied prior to any specific therapy; 4 were studied after prednisone had been initiated for 2-3 days. (4) Eight patients with SLE were studied. All of these patients had a clinical syndrome suggestive of the diagnosis as well as at least one supporting serologic test. Two of the patients had severe thrombocytopenia with platelet counts less than 20,000; 6 patients had platelet counts greater than 100,000. (5) Six patients with a variety of clinical problems related to abnormalities of the platelet and with platelet counts of less than 100 x 10⁹/liter were studied. Their clinical data are presented in Table 1.

**Buffers and Complement Reagents**

Veronal-buffered saline (VBS) and buffered 0.015 M ethylenediaminetetraacetic acid, disodium salt (EDTA-VSB) were prepared as outlined.

**Table 1. Platelet-bound Immunoproteins in Various Platelet Disorders**

<table>
<thead>
<tr>
<th>Illness</th>
<th>Platelet Count (x 10⁹/liter)</th>
<th>IgG (fg/platelet)</th>
<th>C3 (fg/platelet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug reaction*</td>
<td>3</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Heparin-induced TP</td>
<td>50</td>
<td>6.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Quinine-induced TP</td>
<td>4</td>
<td>45</td>
<td>14</td>
</tr>
<tr>
<td>von Willebrand disease</td>
<td>160</td>
<td>3.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Glanzman thrombasthenia</td>
<td>150</td>
<td>7.8</td>
<td>0.8</td>
</tr>
<tr>
<td>PNH†</td>
<td>110</td>
<td>4.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Mucosal and dermal exfoliation while taking allopurinol and quinidine.
†Paroxysmal nocturnal hemoglobinuria.
Platelets

Platelet-rich plasma was harvested after differential centrifugation from fresh blood that had been drawn by sterile venipuncture into a 10\(^{9}\) EDTA solution. A platelet button was obtained by high-speed centrifugation, and these platelets were washed and resuspended three times in EDTA-VBS using a total volume of six times the original platelet-rich plasma volume. The final platelet dilutions were in VBS only. Platelet counts were done by phase microscopy using siliconized chambers with a reproducible accuracy of 10\(^{9}\). White cell contamination varied with the in vivo platelet count, ranging from less than 0.1\(^{\%}\), for counts greater than 40 \(\times 10^{9}\) liter to 3\(^{\%}\), for counts less than 5 \(\times 10^{9}\) liter.

Assay for Membrane-bound C3

Membrane-bound C3 was measured by a modification\(^9\) of the anti-C3 consumption test of Borsos and Leonard.\(^10\) In this test, a concentration of anti-C3 was determined that was capable of lysing cells coated with human C3 in the presence of excess guinea pig complement. When this amount of anti-C3 was preincubated with platelets with C3 bound to the membrane or with fluid-phase C3, the amount of lysis was reduced in inverse proportion to the amount of C3 capable of absorbing the anti-C3 from solution. From knowledge of the inhibition of lysis by known amounts of fluid-phase C3, the amount of C3 present on the platelets could be calculated.

Purified C3 was obtained from human serum by the method of Nilsson and Müller-Eberhard\(^11\) as modified by Logue et al.\(^9\) Antibody to human C3 was obtained by immunizing rabbits, was rendered specific as described by Logue,\(^9\) and was absorbed with aggregated bis-diazotized aggregated IgG and sheep red cells with anti-C3. This solution did not lyse a population of IgG-coated sheep cells in the presence of guinea pig complement. A dilution that would lyse about 70\(^{\%}\) of the C3-coated sheep cells was used. Sheep cells coated with human C3 (EC3) were prepared by incubating washed sheep cells in normal human serum containing heterophile antibody for 20 min at 30\(^\circ\)C, washing three times with VBS, incubating for 20 min with 0.02 M 2-mercaptoethanol, washing three times with VBS, and incubating with trypsin for 30 min at 37\(^\circ\)C. The cells were washed in Isogever and suspended at a concentration of 4.4 \(\times 10^{9}\) cells/ml. The EC3 reagent cells did not lyse in the presence of the anti-IgG antiserum (as prepared for use in the membrane-bound IgG assay described below), and excess guinea pig complement.

The assay required three 1 hr incubation steps in a test tube at 37\(^\circ\)C with the successive addition of these components: (1) 0.1 ml each of anti-C3 and either fluid-phase C3 or washed platelets, both at a known concentration; (2) 0.1 ml of the EC3 suspension; and (3) 0.2 ml of guinea pig serum, a source of complement at a 1/20 dilution. The reaction was stopped with the addition of 5 ml Isogever to each tube, the cells were removed by centrifugation, and the amount of free hemoglobin in the supernatant fluid was measured by the optical density (OD) at 412 nm.

The inhibition of lysis (I) was calculated as:

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I = 1 - \frac{OD - CB}{OD_m - CB}
\]

where CB, the cell blank, is the optical density of the supernatant fluid in the tube with diluent replacing the anti-C3, and OD\(_m\) is the optical density of the supernatant fluid in the tube containing no added C3. Two curves were drawn, one relating increasing lysis inhibition with an increasing concentration of fluid-phase C3 and the other with an increasing concentration of washed platelets. Thus the amount of C3/platelet in femtograms (10\(^{-15}\) g) could be calculated by numerical or graphic methods.

Assay for Membrane-bound IgG

The surface-bound IgG was demonstrated simultaneously on the same sample of blood used for the above platelet C3 determinations, as described by Dixon et al.\(^5\)

RESULTS

Platelet-associated IgG and C3 decreased rapidly after one washing in a volume of EDTA-VBS buffer equivalent to the initial plasma volume but re-
mained constant after washing with 3–6 such volumes. In 21 normal volunteers, the platelet-bound IgG was $4.1 \pm 2.9 \text{ fg/platelet}$ (mean ± 1 SD) and for C3, $3.5 \pm 3.3 \text{ fg/platelet}$ (Fig. 1). The platelet-bound protein was defined as increased if it was greater than 2 SD above the mean established for the normal volunteers.

Of the 16 patients with ITP, 14 had an elevation of IgG, and 9 of these had an elevated C3 (Fig. 1). For these patients, a proportional relationship was found between the platelet surface IgG and C3 (Fig. 2). No patient with ITP had an elevated platelet-bound C3 without an increase in the surface-bound IgG.

Among the 6 nonthrombocytopenic SLE patients, 2 had a mild elevation of
the measured proteins, 1 with IgG alone and the other with C3 alone. Of these 6 patients, 4 had been taking prednisone for some other manifestation of their illness. Both of the thrombocytopenic SLE patients had an elevation of both IgG and C3.

For patients with a variety of other platelet disorders, IgG and C3 were measured as reported in Table 1. Both were elevated in a patient with quinine-induced thrombocytopenia. Both were also elevated in a patient who experienced thrombocytopenia, severe exfoliative dermatitis, and mucositis. A drug reaction was likely as this patient had been started on allopurinol and quinidine only 2 wk earlier, and the skin disease and thrombocytopenia cleared with the addition of prednisone and withdrawal of both drugs. The patient was not rechallenged with either agent.

During treatment, the surface-bound C3 fell as the platelet count rose. The clinical course of a patient with ITP in whom splenectomy was not possible because of obesity is shown in Figure 3. The platelet-bound C3 and IgG fell slightly with therapy with the addition of cyclophosphamide to the prednisone, but only with vincristine did these immunoproteins become normal, as did the platelet count.

The clinical response of 4 other patients in whom the platelet count improved is shown in Figure 4: 2 had ITP, and 1 had SLE. The fourth had rheumatoid arthritis; she was given gold injections and developed bleeding and thrombocytopenia, which reversed when she was treated with prednisone and British anti-Lewisite. Eight months later, while on no specific therapy, she again developed thrombocytopenia, this time responding to prednisone alone. Of these 4 patients, a beneficial response occurred in 2 with the addition of vincristine to a therapeutic program that had previously failed; for 2 of these patients, two therapeutic maneuvers were used simultaneously.

**DISCUSSION**

These studies present for the first time direct measurements of the presence of one of the complement components, C3, on platelets, particularly in ITP. We
have measured small amounts of C3 on normal platelets, a mean of 3.5 fg, or 11,000 molecules, per platelet. The amount of C3 on normal platelets is comparable to the amount of IgG found on such platelets (4.1 fg, or 15,000 molecules, per platelet). Whether or not these small amounts of IgG and C3 have any significance in the destruction of the normal platelet is not known.

As shown previously and confirmed in the present series, nearly all patients with ITP have measurably increased amounts of IgG on the platelet membrane surface.4 In the present study, abnormally large amounts of platelet-bound C3 were detected in some patients (9 of 16) but not in others (5 of 16). In the former group, the amount of IgG bound was generally greater than in the latter group, and the amount of C3 fixed was roughly proportional to the amount of IgG bound. The fixation of complement when large amounts of IgG are bound might be expected since the conditions necessary for the fixation of the first component of complement (C1), i.e., the juxtaposition of two antibody molecules at the surface,12 are more easily met than when the amount of IgG on the surface is small.

In 3 of 5 patients with ITP on whose platelets C3 was not detected, the amount of IgG bound was relatively small; this could account for our inability to detect C3. However, in two instances the amount of IgG present on the platelets was as great as that on the platelets of the patient on whose platelets C3 was detected. Therefore, some explanation other than low IgG concentration must be posited for the lack of C3 binding in these patients. The antibodies may be of an IgG subclass that does not fix complement (IgG4 or IgG2). Karpatkin et al. have stated that the antibodies in ITP are nearly always IgG3,13 the subgroup most efficient in complement fixation. In these patients, the antigens with which these antibodies interacted may be placed on the membrane such that two antibody molecules cannot achieve sufficiently close juxtaposition for fixation of C1. This mechanism has been postulated to explain the inability of antibodies of the Rh system to fix complement, since these antigens

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*It is possible but unlikely that the inhibition of anti-IgG and anti-C3 noted in these studies was due to material released from the platelet other than their respective antigens, IgG and C3. Studies using other antibodies have not demonstrated any nonspecific inhibition of the test system by normal or ITP platelets.
are few in number and widely spaced. To date, the antigens with which the antibodies in ITP interact are not identified and their relative density on the platelet surface has not been determined. At the present time we have no explanation for the lack of C3 fixation by these antibodies.

In autoimmune hemolytic anemia the fixation of IgG alone to the red cell surface is apparently sufficient to bring about the destruction of the red cell, but the rate of destruction of the red cell is thought to be greater when C3 is fixed along with IgG than when the same amount of IgG alone is fixed. In experimental animal studies in vivo, the clearance of red cells sensitized with a given amount of antibody is greater when complement is fixed than when it is not. From the present studies it is clear that IgG alone is able to bring about the destruction of platelets at an increased rate; it is not possible to determine whether the fixation of C3 effects more destruction in the presence of an equal amount of fixed IgG.

In SLE, both immune hemolytic anemia and thrombocytopenia occur frequently. When the antibody is directed against the red cell, complement is always affixed to the red cell surface. Therefore, the fixation of complement to platelets by antibodies in this disorder was anticipated. In our series, patients with SLE and thrombocytopenia had both IgG and C3 on the surface, whereas those who were not thrombocytopenic had normal amounts of IgG and C3. Those patients who responded to therapy had a reduction in both.

A certain proportion of patients with autoimmune hemolytic anemia have C3 but not IgG bound to the red cell surface as detected by the direct antiglobulin test. We did not find any instances in the present series in which C3 was fixed to the platelet without the fixation of abnormally large amounts of IgG. This finding indicates that in immune thrombocytopenia such antibodies are probably uncommon, if they exist.

Complement fixation in vitro has been demonstrated in certain platelet-antibody interactions (drugs, alloimmune antibodies, SLE), but, in general, tests have failed to demonstrate complement fixation in vitro with the series from patients with ITP. This observation has led to the conclusion that these antibodies are not capable of fixing complement, a conclusion at variance with the present data, which suggest fixation of complement in vivo. This discordance may occur because the complement fixation test is relatively insensitive in the detection of the fixation of small amounts of complement. Furthermore, complement fixation to circulating platelets in vivo occurs over a period of time with the accumulation of inactive C3 on the membrane, whereas in the in vitro test only relatively short periods of time are allowed for fixation of complement. An analogous difficulty in detecting fixation of complement in vitro is encountered in autoimmune hemolytic anemia when the antibody is IgG and present in relatively low concentrations.

The importance of complement fixation to the platelets in the pathogenesis of ITP is at this time uncertain. As pointed out above, it may alter the survival of the platelet. Furthermore, in certain animals, fixation of complement in vitro has been shown to alter the function of the platelet. The fixation in vivo may be responsible for the subtle functional changes observed in ITP. Further work is necessary in order to elucidate the diagnostic and physiologic significance of complement fixation in ITP.
REFERENCES

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TW Hauch and WF Rosse