Effect of Anti-P1\textsuperscript{A1} Antibody on Human Platelets. I. The Role of Complement

By Douglas B. Cines and Alan D. Schreiber

We studied the interaction of complement with human platelets. Complement was activated by IgG anti-P1\textsuperscript{A1} antibody obtained from 3 patients with the post-transfusion purpura syndrome. We used a heparin-plasma buffer system that permits complement activation and also preserves platelet function. With this system complement activation was efficient, and platelet immune alteration was extensive. Anti-P1\textsuperscript{A1} antibody was effective only in the presence of complement, in which case both platelet lysis and serotonin release (release reaction) in the absence of lysis were observed. Platelet lysis, as assessed by \(^{51}\text{Cr}\) loss, required 10-fold more antibody than was necessary to induce platelet aggregation and release of \(^{14}\text{C}\text{-serotonin.}\ This platelet release reaction required an intact classic complement sequence through C6. The extent of platelet serotonin release paralleled the depletion of C1 and C4 from platelet-rich plasma. Concentrations of antibody insufficient to induce platelet aggregation and serotonin release could still activate C1 and deposit increased C3 on the platelet surface. These studies demonstrated that complement activation by anti-P1\textsuperscript{A1} antibody can alter human platelets in a nonlytic system. Several phases of complement-mediated human platelet alteration are possible, depending on the concentration of anti-P1\textsuperscript{A1} antibody.

Platelet immune alteration occurs in several human disorders and may be mediated by either antiplatelet antibodies\textsuperscript{1-4} or IgG containing immune complexes.\textsuperscript{5,7} The complement system may be involved as well. Such immune alterations commonly result in thrombocytopenia caused by phagocytosis of IgG-coated platelets.\textsuperscript{6,9} However, qualitative platelet abnormalities may also result.\textsuperscript{10-12} Complement activation by immunologic stimuli has been shown to cause lysis of human platelets.\textsuperscript{13} However, in the absence of lysis, a role for platelet alteration mediated by the classic complement pathway has not been established in man.\textsuperscript{14,15}

We studied the interaction between human platelets and complement under conditions in which complement is activated by a potent isoantibody (anti-P1\textsuperscript{A1} antibody) obtained from patients with the post-transfusion purpura syndrome.\textsuperscript{16,17} We studied the nature of the platelet–complement interaction and the complement component requirement for the release reaction. We observed that anti-P1\textsuperscript{A1} antibodies can mediate several phases of complement interaction with human platelets.

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Supported by American Heart Association Grant 75-711, and by Grants HL-18827 and CA-15236 and by Special Fellowship AI-05477 (Dr. Cines). Dr. Schreiber is a Leukemia Scholar of the Leukemia Society of America.


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Blood, Vol. 53, No. 4 (April), 1979 567
MATERIALS AND METHODS

Fresh frozen guinea pig serum (Rockland, Gilbertsville, Pa.), sodium heparin (Riker Laboratories, Northridge, Calif.), ADP and epinephrine bitartrate (Sigma Chemicals, St. Louis, Mo.), soluble skin collagen (Worthington Biochemical, Freehold, N.J.), crystalline human serum albumin (Pentex, Miles Laboratories, Kanakakee, Ill.), fibrinogen (Kabi, Stockholm, Sweden), 51Cr-sodium dichromate, 14C-serotonin, and Na211 (New England Nuclear, Cambridge, Mass.) were obtained as indicated. Isotonic veronal-buffered saline (pH 7.4) containing 0.1% gelatin and 0.00015-M CaCl2 (VBS), guinea pig C1, and guinea pig C2 were prepared as previously described. Highly purified C5 was the generous gift of Dr. Ulf Nilsson (School of Dental Medicine, University of Pennsylvania). The human complement components C3 and C6 through C9 were obtained from Cordis Laboratories, Miami, Fla. Complement intermediates were prepared as previously described. Individual components were assayed by effective molecule titration.

Platelet-Rich Plasma (PRP)

Human blood from normal volunteers was collected into either citrate or heparin. The citrate buffer contained 1 part 3.2%-3.8% sodium citrate to 9 parts whole blood; the heparin buffer contained 1.3-2.5 units of heparin per milliliter of whole blood. Anticoagulated whole blood was centrifuged at 23°C, and the supernatant PRP was removed by aspiration.

Platelet-Poor Plasma (PPP)

Blood was collected as for PRP and centrifuged at 2500 g for 10 min (23°C), and the supernatant PPP was harvested as previously described.

Gel-Filtered Platelets

Platelets were filtered through Sepharose 2B using a modified Tyrode's buffer (pH 7.4) containing no added calcium. Fractions containing platelets appeared at the void volume of the column and were pooled so as to contain 200,000-250,000 platelets/μl. In experiments where ADP was used as a control, human 95% clottable fibrinogen (3 mg/ml) was added to the gel-filtered platelet suspension. Gel-filtered platelets were diluted with either modified Tyrode's buffer or heparinized PPP (1.5 units heparin/ml).

Platelet Aggregation and 14C-Serotonin Release

Platelet aggregation and platelet 14C-serotonin release were carried out as previously described.

Platelet 51Cr Loss

The loss of 51Cr from human platelets was assessed by a modification of the method of Aster and Enright. PRP was obtained from blood anticoagulated with 0.3 ml of 5% EDTA per 10 ml of whole blood. The PRP from 10 ml of whole blood was incubated with 100 μCi of 51Cr-sodium dichromate for 30 min at room temperature and washed five times with modified Tyrode's buffer containing 0.01-M EDTA. The platelets were suspended to 300,000/μl with PPP containing heparin (1.3-2.5 units/ml) and 5-mM magnesium. The percentage of 51Cr released into the supernatant following platelet sedimentation at 12,000 g for 4 min was then determined. All radioactivity was measured in a gamma counter (Nuclear Chicago, Des Plaines, III.).

In selected experiments PRP was incubated with 14C-serotonin and/or 51Cr as described. The radiolabeled PRP was gel-filtered to remove the 51Cr and 14C that were not platelet-associated. These radiolabeled gel-filtered platelets were then diluted to their final concentration with either buffer or heparinized PPP (1.5 units heparin/ml).

Interaction of Platelets With Anti-P1 Antibody and Complement

The antiplatelet antibodies employed in these studies were obtained from 3 patients with the post-transfusion purpura syndrome. The identity of the antibody in each patient plasma was shown to be directed at the P1 antigen by Dr. Scott Murphy of Jefferson Medical College or by Dr. Richard Aster of the Milwaukee Blood Center. In experiments that assessed the requirement for complement or individual complement components, we used partially purified anti-P1 antibody. Partially purified
anti-P1\textsuperscript{44} antibodies were isolated by QAE Sephadex and Sephadex G-200\textsuperscript{24} or by affinity chromatography using anti-IgG-coupled Sepharose\textsuperscript{23}.

In order to assess the interaction of human platelets with anti-P1\textsuperscript{44} antibody and human complement, it was necessary to establish an anticoagulant buffer system that did not contain chelating agents and that permitted both effective complement activation and the functional study of human platelets. To this end we employed a buffer system that contained low (but anticoagulant) concentrations of heparin. We compared such concentrations of heparin with anticoagulant concentrations of citrate (0.32\% with 2-mM Mg\textsuperscript{2+}) in regard to inhibition of complement activity in whole plasma. Using a hemolytic antibody titration that employs sheep erythrocytes coated with limited amounts of rabbit antibody and excess guinea pig complement,\textsuperscript{14} we observed that as little as 0.12\% citrate inhibited lysis of the antibody-coated erythrocytes threefold more effectively than did heparin at 1 U/ml. Using a Cl effective molecule titration, heparin (1 U/ml) was observed to depress Cl activity only in the presence of limiting amounts of Cl. This inhibition was readily overcome by the addition of small amounts of Cl (200 U/ml). Since the concentration of Cl in plasma\textsuperscript{26} is approximately 100,000 U/ml, very little inhibition of Cl by heparin was observed. However, citrate (0.12\%) was observed to be a potent inhibitor of Cl, producing 90\% inhibition in a Cl effective molecule titration even in the presence of added Cl. Therefore, in our studies with platelets, antibody, and complement, a low concentration of heparin (usually 1.6 U/ml) was employed in plasma to permit more effective complement activation. This concentration of heparin also showed little inhibition of Cl activity in whole plasma.

Heparin-anticoagulated PRP (0.3 ml) radiolabeled with either \textsuperscript{14}C-serotonin or \textsuperscript{51}Cr or both were incubated with 0.2 ml of anti-P1\textsuperscript{44} antibody dilution (adjusted to pH 7.6) for 45 min at 37\°C in a Chronolog aggregometer and stirred with a magnetic stirrer at 1200 rpm. When platelets were gel-filtered prior to interaction with the antibody source, the gel-filtered platelets (150 \mu l) were incubated at 37\°C with 0.2 ml of anti-P1\textsuperscript{44} antibody in the presence of 150 \mu l of heparin-autologous plasma for 45 min at 37\°C. These sensitized platelets were then examined either for release of \textsuperscript{14}C-serotonin or \textsuperscript{51}Cr as described or for the presence of C3 (vide infra). With the use of a shaking 37\°C water bath, antibody-mediated serotonin release did not require stirring but it was augmented by stirring, whereas ADP- and epinephrine-mediated serotonin release required stirring. Following incubation at 37\°C, all reactions were terminated by the addition of 50 \mu l of 5\% EDTA to prevent serotonin release during centrifugation, and the radioactivity released was determined. In each experiment, controls were performed with ADP or epinephrine, and in selected experiments controls were performed with collagen.

\textsuperscript{14}C-Serotonin Release by Complement-Deficient Plasmas

The requirement for specific complement components in the antibody-mediated release reaction was studied using plasma obtained from a patient with hereditary angioedema (Cl inhibitor deficiency) with < 1\% normal plasma C4\textsuperscript{27} and in selected experiments with plasmas obtained from patients with congenital deficiencies of C3, C5, C6, and C7. Plasma deficient in C3 was the generous gift of Drs. Steven Osofsky (Duke University Medical Center) and Barry H. Thompson (Kessler Air Force Base, Mississippi); plasma deficient in C5 was the gift of Dr. Stephen Rosenfield (University of Rochester School of Medicine and Dentistry, Rochester, N.Y.); four plasmas deficient in C7 were the gifts of Drs. Henry Gewurz (Rush-Presbyterian Medical Center, Chicago, Ill.) and Terrence Lee (North Carolina Memorial Hospital, Chapel Hill, N.C.). Plasma deficient in C6 and the platelet-rich plasma of 3 patients deficient in C7 were kindly provided by Dr. Lee. Plasma from an additional patient with congenital C6 deficiency was a gift from Dr. John Leddy (University of Rochester School of Medicine and Dentistry, Rochester, N.Y.). Deficient plasmas were collected in either citrate or EDTA and dialyzed against the modified Tyrode's buffer at 4\°C for 24 hr before use.\textsuperscript{14}Cr- or \textsuperscript{14}C-serotonin-labeled gel-filtered platelets obtained in whom plasma was observed, however, citrate (0.12\%) was observed to be a potent inhibitor of Cl, producing 90\% inhibition in a Cl effective molecule titration even in the presence of added Cl. Therefore, in our studies with platelets, antibody, and complement, a low concentration of heparin (usually 1.6 U/ml) was employed in plasma to permit more effective complement activation. This concentration of heparin also showed little inhibition of Cl activity in whole plasma.

Quantitation of IgG and C3 per Platelet

An assay has been developed in order to readily measure the amount of IgG antibody and C3 on the platelet surface.\textsuperscript{28} This assay is a modification of the IgG (gamma) and C3 (nongamma) Coombs test
commonly employed to detect IgG or C3 on the erythrocyte surface. Here, the antiglobulin reagent (anti-IgG or anti-C3) is radiolabeled with $^{125}$I, adsorbed twice with normal erythrocytes, and incubated with platelets.

In the indirect IgG Coombs test, 10$^5$ washed platelets in EDTA (obtained from erythrocyte ABO type 0 donors) were incubated with the antibody source for 45 min at 37°C, washed three times, and resuspended to 1 ml. The antibody-coated platelets were then incubated with $^{125}$I anti-IgG. To allow for complement fixation the indirect C3 Coombs test was performed using heparinized PRP (approximately 1.5 U/ml heparin and 10$^5$ platelets). The platelets were incubated with limiting concentrations of antibody (45 min at 37°C), and the reaction was terminated with 50 μl of 5% EDTA. These platelets were then washed and incubated with $^{125}$I anti-C3. In both indirect tests, following incubation with the $^{125}$I antiglobulin reagent, the platelets were washed four times with Tyrode’s EDTA buffer and resuspended, and the amount of platelet-associated radioactivity was determined in a gamma scintillation counter.

Although the results could be expressed as platelet-associated radioactivity, we employed a standard curve that uses erythrocytes coated with a known number of IgG antibody or C3 molecules to estimate the number of IgG or C3 sites per platelet.28 The standard curve relating erythrocyte-associated radioactivity to the amount of IgG or C3 per erythrocyte had a slope approximating 1.0. By comparing the adsorption of $^{125}$I (anti-IgG or anti-C3) radioactivity onto platelets with that adsorbed onto erythrocytes coated with a known amount of IgG or C3, the amount of platelet-associated IgG or C3 was quantitatively determined. This platelet IgG or C3 is designated as antiglobulin (anti-IgG or anti-C3) combining sites, rather than as molecules of IgG or C3. One anti-globulin combining site per platelet adsorbs an amount of radioactive anti-IgG or anti-C3 equivalent to that adsorbed by one molecule of IgG anti-erythrocyte antibody or C3 per erythrocyte. Using this assay, 2.4 ± 2.5 X 10$^3$ anti-IgG combining sites per platelet are present on normal platelets,28 a portion of which represents nonspecific interaction of radiolabeled protein with the platelet surface. Low concentrations of anti-P1$^A$ antibody that do not increase the measured platelet-associated IgG above the normal range are capable of causing platelet serotonin release (see Results). Therefore, values of platelet-associated IgG < 10 X 10$^3$ anti-IgG combining sites per platelet represent a calculation based on serial twofold dilutions, assuming a continued linear relationship between antibody added and IgG deposited per platelet.

RESULTS

Effect of Antibody Concentration

Anti-P1$^A$ antibody caused the release of $^{14}$C-serotonin and the loss of $^{51}$Cr from human platelets in a dose-dependent manner (Fig. 1). Only 1.5 X 10$^3$ anti-IgG combining sites per platelet were required for $^{14}$C-serotonin release. These quantitative data were similar for all three anti-P1$^A$ antibodies. This low amount of IgG per platelet was sufficient to activate the classic complement pathway (Fig. 2) and significantly increase the level of platelet-associated C3 (Table 1). However, approximately 1.2 X 10$^4$ anti-IgG combining sites per platelet were required to
elicit significant $^{51}$Cr loss (Fig. 1). Maximal serotonin release was obtained with 3–6 $\times$ $10^5$ anti-IgG combining sites per platelet, and this occurred in the absence of $^{51}$Cr loss. Under these experimental conditions, 30% $^{51}$Cr loss was achieved with the highest antibody concentration.

The time course of antibody-induced serotonin release is shown in Fig. 3. At high antibody concentrations ($1.2 \times 10^6$ anti-IgG combining sites per platelet), serotonin release was rapid and was associated with $^{51}$Cr loss. $^{51}$Cr loss was associated with diminution in the number of intact platelets without detectable platelet aggregation. At concentrations of 1.5 or 3.0 $\times$ $10^5$ anti-IgG combining sites per platelet, at least 20 min at 37°C were required before the formation of visible platelet aggregates and the release of serotonin.

**Complement Requirement for Platelet Lysis**

Complement was required for antibody-mediated platelet $^{51}$Cr loss. When washed $^{51}$Cr-labeled platelets were exposed to partially purified anti-P1Al antibody in the absence of a complement source, i.e., in the absence of plasma or in the presence of heat-inactivated plasma, platelet $^{51}$Cr loss was reduced to less than 5%. The addition of whole plasma as a complement source restored the capacity of the anti-P1Al antibody to cause $^{51}$Cr loss from the radiolabeled platelets.

**Complement Requirement for Platelet Serotonin Release**

Complement activation occurred during 14C-serotonin release caused by nonlytic concentrations of antibody (Fig. 2). Anti-P1Al antibody was added to platelets in the presence of a plasma at 37°C for 45 min. The platelets were sedimented, and the supernatant plasma was harvested and assessed for depletion of the first (C1) and fourth (C4) complement components. As shown, consumption of C1 occurred concurrently with the release of platelet serotonin. In a similar fashion the depletion

| Table 1. Increase in Platelet-Associated C3 Caused by Anti-P1Al Antibody |
|---------------------------------|-----------------|
| Platelets + normal plasma ($N = 9$) | $8,780 \pm 1640^*$ |
| Platelets + anti-P1Al plasma ($N = 2$) | A 13,600 |
|                                | B 49,000 |

*Results expressed as mean $\pm$ 2 SD of the mean. In the absence of plasma, platelets carried $6550 \pm 1250$ C3 sites. Anti-P1Al antibody was employed at a concentration calculated to cause, $4.8 \times 10^5$ (A) and $9.6 \times 10^5$ (B) anti-IgG combining sites per platelet. The mean of two experiments is shown. Almost identical quantitative data were obtained with all three anti-P1Al antibodies.
of C4 paralleled the release of serotonin (not shown). Significant C1 consumption was also observed at concentrations of antibody insufficient to mediate the release reaction; platelet C3 deposition occurred at these low antibody concentrations (Table 1). In the absence of a complement source, partially purified anti-P1\(^{A1}\) antibody did not mediate platelet serotonin release.

In order to further assess the role of the classic complement pathway in the release reaction occurring at nonlytic antibody concentrations, we used the plasma of a patient with congenital C1 inhibitor deficiency who had less than 1% hemolytically active plasma C4 but normal plasma levels of C1 and the terminal components C3-9 characteristic of such hereditary angioedema patients. When partially purified anti-P1\(^{A1}\) antibody isolated from all three anti-P1\(^{A1}\) patient plasmas was incubated with gel-filtered platelets and C4-deficient plasma, no \(^{14}\)C-serotonin release was observed at antibody concentrations ranging from \(3.0 \times 10^3\) to \(24 \times 10^3\) anti-IgG combining sites per platelet (Table 2). Thus an intact classic complement pathway was necessary for antibody-mediated serotonin release at the antibody concentrations employed. However, gel-filtered platelets released

### Table 2. Role of Complement in the Platelet Release Reaction Mediated by Anti-P1\(^{A1}\) Antibody

<table>
<thead>
<tr>
<th>Complement Source (Plasma)</th>
<th>24</th>
<th>12</th>
<th>6</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-inactivated (N = 3)</td>
<td>64%†</td>
<td>8.2%</td>
<td>5.7%</td>
<td>--</td>
</tr>
<tr>
<td>C4D (N = 2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3D (N = 2)</td>
<td>--</td>
<td>--</td>
<td>7.8</td>
<td>&lt;1</td>
</tr>
<tr>
<td>C5D (N = 3)</td>
<td>8.8</td>
<td>6</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>C5D + C5 (N = 2)</td>
<td>--</td>
<td>--</td>
<td>50%</td>
<td>--</td>
</tr>
<tr>
<td>C5D (N = 2)</td>
<td>--</td>
<td>--</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>C7D PPP (N = 4)</td>
<td>53</td>
<td>31</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>C7D PRP (N = 3)</td>
<td>44</td>
<td>28</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

*Radiolabeled gel-filtered platelets (0.150 ml) were incubated with partially purified anti-P1\(^{A1}\) antibody (0.2 ml) in the presence of each complement source (0.150 ml) at 37°C for 45 min. Percentage \(^{14}\)C-serotonin release was determined as described in the text.

†All results are expressed as mean percentage of \(^{14}\)C-serotonin released as compared with a concurrent normal plasma control. Under the experimental conditions, \(^{14}\)C-serotonin release for 19 experiments with normal plasma as the complement source was 80%, 61%, 53%, and 24% at the four different antibody concentrations shown.

‡D = deficient plasma; N = number of experiments.


\[14\text{C}-\text{serotonin}\] when normal plasma was substituted for C4-deficient plasma as the complement source. When heat-inactivated plasma was employed as the complement source, serotonin release was observed only at the highest antibody concentration (Table 2).

Additional experiments were performed with a single anti-\(\text{PlA}^1\) antibody to determine which complement components were necessary to effect the antibody- and complement-mediated release reaction (Table 2). Only small amounts of \(14\text{C}-\text{serotonin}\) were released by antibody from normal gel-filtered platelets resuspended in plasmas selectively deficient in C3 and C5. The addition of purified C5 (33 \(\mu\)g/ml) restored 50% of the activity to the C5-deficient plasma. No release occurred in plasmas from 2 patients selectively deficient in C6. In experiments using C7-deficient heparinized PRP (3 patients), the anti-\(\text{PlA}^1\) antibody released significant (but less than maximal) amounts of \(14\text{C}-\text{serotonin}\). Similarly, significant \(14\text{C}-\text{serotonin}\) release was observed using normal washed platelets resuspended in four separate C7-deficient plasmas. Significant \(^{51}\text{Cr}\) release did not occur in any experiments using plasmas deficient in C3, C5, C6, or C7.

**Role of Anticoagulant in Complement Activation**

As noted above, complement activation by antibody-coated erythrocytes was more efficient in a heparin-plasma buffer than in a citrate-plasma buffer. Similar results were observed with antibody-coated platelets. We studied the consumption of C1 during antibody-mediated platelet damage (Table 3) in the heparin- and citrate-plasma buffer systems. At all five antibody concentrations studied, C1 consumption was more effective in the heparin-plasma buffer system. Since most previous studies of antibody–platelet interaction have been performed in a citrate-plasma buffer, we also compared the efficiency of antibody-mediated serotonin release in our heparin-plasma buffer with that in citrated plasma (Fig. 4). Similar results were obtained with citrate concentrations of 0.32% and 0.42%. An approximately 10-fold higher concentration of IgG per platelet was necessary to cause \(14\text{C}-\text{serotonin}\) release in the presence of citrate. In each buffer system \(14\text{C}-\text{serotonin}\) release in the absence of antibody and with normal allogeneic plasma alone was less than 7%.

We also explored whether or not the concentration of heparin employed in our studies increases the sensitivity of platelets to aggregate and to release serotonin on exposure to nonimmunologic stimuli. We compared the smallest (threshold) concentrations of ADP, epinephrine, and collagen required to elicit platelet aggregation and \(14\text{C}-\text{serotonin}\) release from normal PRP in heparin and in citrate. There was no increase in platelet sensitivity in the presence of heparin.

### Table 3. Percentage C1 Consumption in Heparin (1.6 U/ml) or Citrate (0.32 %) Buffer

<table>
<thead>
<tr>
<th>IgG Sites (\times 10^{-3})/Platelet</th>
<th>C1 Consumption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrate</td>
</tr>
<tr>
<td>24*</td>
<td>44</td>
</tr>
<tr>
<td>12</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>1.5</td>
<td>4</td>
</tr>
</tbody>
</table>

*The mean of at least two experiments with each antibody concentration is shown.*
threshold concentrations necessary for aggregation by ADP or collagen were similar in citrate buffer and heparin buffer. Collagen-induced release of serotonin occurred at threshold doses of aggregation in both citrate and heparin. In some donors, more ADP or epinephrine was required for serotonin release in heparin than in citrate; with some platelet donors, release of \(^{14}\text{C}\)-serotonin in heparin did not occur with ADP.

**DISCUSSION**

We assessed the interaction of complement with human platelets by using anti-Pi\(^{\text{A1}}\) antibodies obtained from 3 patients with the post-transfusion purpura syndrome. Using this naturally occurring isoantibody, we defined three phases of complement–platelet interaction: platelet lysis, platelet serotonin release, and complement activation in the absence of a release reaction.

In order to preserve platelet function and to permit complement activation, we used a low-dose heparin-plasma buffer system. The interaction of heparin with platelets is complex,

but there has been little study of the effect of low concentrations of heparin as a single anticoagulant in vitro. In agreement with others,\(^{30}\) we found no enhancement of platelet aggregation or of the release reaction by nonimmunologic stimuli in the presence of heparin. This may reflect the inhibitory effect of physiologic concentrations of Ca\(^{++}\) on the platelet release reaction induced by ADP.\(^{31}\) Previous studies, including that by Deykin and Hellerstein, have suggested that antibody-mediated serotonin release is facilitated in heparinized plasma.\(^{46}\) Our data suggest that more efficient classic complement pathway activation in heparin may explain these results (Table 3 and Fig. 4). Although heparin, at the concentrations employed, appears inhibitory toward early complement component activation,\(^{32-34}\) this effect of heparin is overcome by the presence of the excess Cl available in normal plasma. Anticoagulant doses of citrate, which are commonly employed for platelet studies, were less optimal for complement activation (Table 3) and were less effective in supporting the complement-dependent release reaction (Fig. 4).

The effect of the complement–platelet interaction was dependent on the amount of anti-Pi\(^{\text{A1}}\) antibody employed. At high concentrations of antibody, complement activation caused platelet lysis (Fig. 1). With moderate concentrations of antibody, complement caused release of platelet \(^{14}\text{C}\)-serotonin in the absence of lysis. Low
concentrations of the anti-P1\textsuperscript{A1} antibodies activated the classic complement pathway and increased the amount of C3 on the platelet surface in the absence of observable in vitro platelet alteration. All these effects of anti-P1\textsuperscript{A1} antibody were mediated through classic complement pathway activation. Neither platelet lysis nor platelet aggregation and \textsuperscript{14}C-serotonin release in the absence of lysis was produced by purified antibodies under these experimental conditions in the absence of a complement source or in the presence of C4-deficient plasma. Heat-inactivated plasma, which contains the heat-stable component C4, did permit serotonin release at the highest antibody concentration. This might reflect, in part, interaction of plasma C4 with C1 or other components reported to be present on the platelet membrane.\textsuperscript{35,39}

Previous studies with human platelets have suggested that human isoantibodies or autoantibodies can cause complement-independent platelet aggregation or nucleotide release.\textsuperscript{36-38} Heterologous antibodies have also been observed to cause a complement-independent release reaction involving either human\textsuperscript{24} or rabbit\textsuperscript{40} platelets. Several plasma factors may be involved in the deposition of complement components on human platelets.\textsuperscript{39,41} It appears that some of these components remain on the normal washed platelet surface.\textsuperscript{35,39} It is therefore difficult to be certain of an entirely complement-independent release reaction.\textsuperscript{35,43} Our results indicate a requirement for an exogenous source of C4, C3, C5, and C6 to mediate the release reaction caused by some anti-P1\textsuperscript{A1} antibodies. This suggests that some platelet-associated complement components are either insufficient in amount or function to be effective under the experimental conditions.

Previous investigators have observed antibody-mediated human platelet aggregation\textsuperscript{4} and \textsuperscript{14}C-serotonin release\textsuperscript{37} in the presence of plasma. The absolute requirement for exogenous complement, including the individual components involved, was not explored. A requirement for C3 has been noted previously for the release by antibody of histamine from rabbit platelets\textsuperscript{40} and in one previous study of \textsuperscript{14}C-serotonin from human platelets.\textsuperscript{42} However, in the latter study platelet lysis could not be excluded, and the experiments were not performed under conditions that optimally preserved platelet function. The use of gel filtration and heparin provide a more efficient system to assess platelet functional alteration by complement. This is also the case with complement activation initiated by platelet autoantibodies (unpublished observation).

Activation of the alternative complement pathway by zymosan requires complement\textsuperscript{44,45} and specifically appears to require C7, perhaps as the C567 complex, for significant platelet alteration to occur. This effect requires a zymosan particle, fibrinogen, and γ-globulin, whereas fluid-phase activation of the complement sequence has not been reported to cause significant damage to human platelets.\textsuperscript{44} Our studies with a single antibody have shown significant release in C7-deficient plasma and from C7-deficient PRP. Because of the presence of trace amounts of C7 (1%) in the antibody preparation, we can only conclude that there is a limiting role for C7 in this reaction.

In this study we used a monospecific antibody that did not cause platelet release in the absence of complement. Thus we were able to explore quantitatively the effect of complement on the nature of the subsequent platelet alteration. Several phases of complement interaction with human platelets were possible. High concentrations of antibody caused platelet lysis. Moderate concentrations of
antibody caused the release of \textsuperscript{14}C-serotonin from platelet granules in the absence of lysis. Still lower concentrations of antibody activated the classic complement pathway and bound measurable C3 to the platelet surface. This degree of C3 deposition was unassociated with detectable in vitro platelet alteration, but it may induce a subtle platelet functional alteration or permit platelet interaction with C3-receptor-bearing cells.

ACKNOWLEDGMENT

The authors thank Dr. Sanford Shattil of the University of Pennsylvania and Dr. Richard Aster of the Milwaukee Blood Center for their gift of anti-P1\textsuperscript{14} plasma and Dr. Scott Murphy of Jefferson Medical College and Dr. Richard Aster for determining the P1\textsuperscript{14} antibody activity in the antisera. We also thank Dr. Chester Zmijewski of the Tissue Typing Laboratory, Department of Pathology, University of Pennsylvania, for examining the sera for the presence of anti-HLA antibodies, and we thank Ms. Patricia McDermott for her encouragement and advice.

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