Effect of Heterologous Antibody on Human Platelets

By Robert W. Colman and Alan D. Schreiber

The effect of heterologous anti-human platelet antibody on human platelet function was examined in the presence and absence of whole plasma as an in vitro model for antibody-induced immune damage to cells. Heterologous IgG anti-human platelet antibody mediated platelet aggregation and released serotonin from both platelets in plasma and from platelets isolated by gel filtration and increased the availability of platelet acid phosphatase in a dose-response fashion. Antiplatelet antibody failed to release β-glucuronidase (lysosomal enzyme marker) or cause lactic dehydrogenase loss (cytolysis). The effect of the antiplatelet antibody on platelets proceeded in the absence of complement. The active molecule in the anti-platelet antiserum was isolated in the IgG fraction and all three indicators of platelet injury were mediated by purified monomeric IgG. Thrombin was not required for the antibody-mediated effects, as three thrombin inhibitors failed to block the reaction. EDTA was an effective inhibitor, suggesting a cation requirement; however, as little as 38 μM calcium was sufficient for effective platelet aggregation and release. The inability of acetylsalicylic acid to inhibit the effect of the antiplatelet antibody suggests that heterologous antibody (IgG) induced platelet alteration proceeds by a different mechanism than that mediated by ADP and epinephrine and does not involve endogenous platelet prostaglandin synthesis.

Immunoglobulin interaction with platelets may result in thrombocytopenia as in drug reactions and autoimmune disease. Additionally, antigen–antibody complexes may trigger platelet aggregation and thrombosis as in renal allotransplant rejection. Immunologic damage to platelets may also augment the inflammatory reaction through chemotactic inducing factor(s), vascular permeability enhancing substances or proteolytic enzymes.

Studies examining antibody-induced platelet alterations primarily involve rabbit platelets. Several differences, however, have been observed between the rabbit platelet and the human platelet. Studies involving human platelets have been primarily concerned with the effect of aggregated IgG or IgG formed soluble immune complexes.

In this study we investigate the quantitative effect of heterologous anti-human platelet antisera and highly purified IgG antiplatelet antibody on platelets in plasma and on platelets isolated by gel filtration. The effects on platelet aggregation, the release reaction, lysosomal stability, and cytolysis are assessed and compared with the effects of the platelet-aggregating agents ADP, epinephrine, collagen, and thrombin.
MATERIALS AND METHODS

ADP and epinephrine bitartrate (Sigma Chemical Co., St. Louis, Mo.), soluble skin collagen (Worthington Biochemicals, Freehold, N.J.), monospecific goat antisera to rabbit IgG and rabbit IgM (Cappel Laboratories, Downingtown, Pa.), monospecific antisera to human IgG (Behring Diagnostics, Inc., Woodbury, N.Y.), tosyl-L-arginine methyl ester (Cyclo Chemical Corp., Los Angeles, Calif.), acetylsalicylic acid (Fisher Scientific Company, King of Prussia, Pa.), hirudin (Sigma), heparin (Upjohn, Kalamazoo, Mich.), Na$_{125}$I (New England Nuclear, Cambridge, Mass.) were obtained as indicated. Protein was quantitated by Folin analysis$^{13}$ and alkaline disc gel electrophoresis$^{14}$ performed as indicated.

Platelet Preparation

Platelet-rich plasma (PRP). Human blood from normal volunteers was collected into anticoagulant solution (one part 3.8$,\text{,}$ sodium citrate to nine parts whole blood), centrifuged at 23°C for 10 min at 170 g and the supernatant PRP removed by aspiration and used for the studies. Whole blood and plasma were exposed only to plastic surfaces. The platelet count of PRP varied from 200,000 to 350,000/μl during these studies. The white blood cell count was 3 and the red blood cell count was 2/100,000 platelets, as determined by light microscopy.

Platelet-poor plasma (PPP). Blood was collected as for PRP, centrifuged at 2500 g for 10 min, and the supernatant PPP harvested. The platelet count was less than 20,000 platelets/μl.

Gel-filtered platelets. Platelets were filtered through Sepharose 2B using a modification of the method of Tangen et al.$^{15}$ PRP (3.5 ml) was applied to the top of a 2.5 x 9.0-cm plastic column previously equilibrated with a modified Tyrode’s buffer, pH 7.4, containing no added calcium$^*$ and containing 0.3$,\text{,}$ crystalline human serum albumin (Pentex, Miles Laboratories, Kankakee, Ill.), dextrose 0.1$,\text{,}$ and Mg$^{2+}$ (2 mM). Fractions containing platelets appeared at the void volume of the column and were pooled so as to contain 150,000-250,000 platelets/μl. There were no detectable leukocytes and only 1 erythrocyte/100,000 platelets in the pool. Platelet separation from plasma proteins was confirmed by several observations. The absorbance at 280 μm of the platelet suspension after centrifugation at 2500 g for 15 min was less than 0.02 after subtraction of the solution blank and neither fibrinogen (MW 330,000) as measured by staphylococcal clumping$^{16}$ nor low density lipoprotein as measured by immunoassay$^{17}$ was detected in the supernatant following removal of platelets by sedimentation. In addition, gel filtration effectively separated the effluent containing the platelets from the first component of complement (C1) (MW 800,000) as assessed by effective molecule titration$^{18}$ and IgG (MW 160,000) as measured by radial immunodiffusion.

Prior to each experiment, 3 mg/ml human 95$,\text{,}$ clottable fibrinogen (Kabi, Stockholm, Sweden) were added to the gel-filtered platelet suspension.

Platelet Function and Components

Platelet aggregation was carried out as previously described$^{20}$ in the Chronolog aggregometer. The transmittance of PRP was arbitrarily set at 0 and that of PPP at 100. The per cent aggregation is defined as the transmittance after aggregation is complete. In the aggregometer platelet agglutination by antibody is not distinguishable from aggregation. When measured in the aggregometer, platelet clumping is referred to as aggregation. Antibody-induced platelet clumping on microtiter plates (see below) is referred to as agglutination. These are operational terms and do not imply mechanism.

β-glucuronidase was assayed according to the method of Fishman et al.$^{21}$ using phenolphthalein glucuronide as a substrate.

Acid phosphatase was assayed by the method of Brandenberger and Hanson$^{22}$ using o-carboxyphenyl-phosphate as a substrate. Measurements were made only on platelets in plasma.

Lactic dehydrogenase (LDH) determination was made as previously described.$^{23}$

*The concentration of calcium$^{19}$ present in the buffer derived from trace amounts in the reagents was 38 μM. In contrast, Tyrode’s buffer, as usually constituted, contains 2 mM (2000 μM) calcium.
HETEROLOGOUS ANTIBODY

$^{14}$C-serotonin release from platelets was assessed by a modification of the method of Jerushalmy and Zucker.\textsuperscript{24} Platelet-rich plasma was incubated with 8 $\mu$Ci of $^{14}$C-serotonin for 30 min at 37° C at which time 80\%-90\% of the radioactivity was bound to the platelets. The per cent released into the supernatant following platelet sedimentation was equal to:

$$\frac{\text{cpm in supernatant after incubation} - \text{cpm in supernatant prior to incubation}}{\text{total cpm in PRP} - \text{cpm in supernatant prior to incubation}} \times 100$$

**Preparation of Rabbit Anti-Human Platelet Antiserum**

Human blood (60 ml) was collected into 2 ml of 5\% ethylenedinitrilotetraacetate (EDTA) in plastic tubes (Falcon Plastics, Oxnard, Calif.). Platelet-rich plasma was prepared by sedimentation at 200 g at 23° C for 6 min. The washing solution (platelet buffer) was comprised of 65 mg KH$_2$PO$_4$, 550 mg of NaH$_2$PO$_4$·H$_2$O, 600 mg NaCl, 2000 mg sucrose, and 2500 mg gelatin adjusted to pH 6.5 in 1 liter of H$_2$O. After three washes, the sedimented platelets were resuspended to 2 ml in platelet buffer, mixed with an equal volume of complete Freund’s adjuvant and injected into the hind footpads of New Zealand white rabbits. The animals were boosted with incomplete Freund’s adjuvant containing an equal volume of platelets, as above, 2 wk after immunization and phlebotomized 2 wk subsequently. The blood was allowed to clot (25° C) for 1 hr and the rabbit antiplatelet antiserum harvested after centrifugation at 2500 g for 20 min and incubated for 30 min at 58° C. Although fresh rabbit antiplatelet antiserum contained approximately 500,000 hemolytically effective C1 molecules/ml, heating the antiserum at 58° C for 30 min destroyed 100\% of the hemolytically effective rabbit C1.

**Measurement of Antibody-induced Platelet Changes**

Two-tenths milliliter of an appropriate dilution of heat-inactivated serum and 0.3 ml PRP or gel-filtered platelets were mixed, and the reaction was begun by stirring in a Chronolog aggregometer at 37° C. Aggregation was measured as indicated below during a 10-min period. The tube was then transferred to an aluminum block maintained at 37° C by circulating water and stirred at the same speed as the aggregometer, 1200 rpm, for an additional 35 min. Following this incubation, the platelets were centrifuged at 2000 g for 4 min, and aliquots of the supernatant were withdrawn for measurements of $^{14}$C-serotonin, $\beta$-glucuronidase, and lactic dehydrogenase. The activity of the enzyme released into the plasma supernatant was corrected by subtracting the concentration of enzyme present in PPP. No correction was needed for gel-filtered platelets. Acid phosphatase was not released into plasma and no increase in the supernatant was found when compared to PPP. Acid phosphatase was measured only in experiments with PRP and the contribution of plasma acid phosphatase was accounted for by subtracting the amount in PPP. Since acid phosphatase activity was associated entirely with the platelets and not released into the supernatant, detection of its activity is referred to as acid phosphatase availability rather than release. The $^{14}$C-serotonin release was calculated as described above.

In all experiments comparing the effect of ADP and epinephrine with that of antiplatelet antibody, the modified Tyrode’s buffer containing a low concentration of calcium (38 $\mu$M) was employed.

Total enzyme activity for all three enzymes was measured after exposure to 2\% Triton X-100. This concentration of nonionic detergent did not inhibit the enzymatic activity. The concentration of each enzyme using this agent was as high or higher than that obtained by sonication, homogenization, or multiple freeze-thaw cycles.

**Platelet Agglutination**

Antiplatelet antibody-agglutinating activity was determined using a microtiter platelet agglutination technique. Platelets were obtained from whole human blood in the manner indicated above in the preparation of antiplatelet antiserum. The platelets were resuspended to a concentration of 800,000/µl and 25 µl added to serial dilutions of rabbit antiplatelet antiserum or normal rabbit serum diluted in modified Tyrode’s buffer with or without 0.01 M EDTA in a total volume of 75 µl. The plates (Cook Engineering Co., Alexandria, Va.) were then mixed and incubated at 37° C for 30 min and then at room temperature; agglutination was determined at 30 60 min and scored.
from 1+ to 4+. The titer was scored as the reciprocal of the dilution that produced 1+ agglutination. The agglutination titer for the rabbit antiplatelet antiserum used in these studies corresponded to a relative antibody concentration of 16 in the platelet function studies (Figs. 1-3). The presence of 0.01 M EDTA did not alter the agglutination titer. Rabbit serum resulted in 0 to trace agglutination when undiluted.

**Complement Components**

Rabbit Cl was quantitated by an effective molecule titration utilizing guinea pig and human components. Several dilutions of rabbit antiplatelet antiserum were incubated at 30°C for 10 min with sheep red blood cells sensitized with rabbit hemolytic antibody and the fourth component of human complement (EAC4). The hemolytic reaction sequence was brought to completion with addition of partially purified guinea pig C2 for 12 min at 30°C and fresh guinea pig serum diluted 1:15 with veronal-buffered saline containing 0.04 M EDTA for 1 hr at 37°C. The number of hemolytically effective Cl molecules in the rabbit antiserum was then determined.

**Isolation of Rabbit IgG Antiplatelet Antibody**

Sixty milliliters of rabbit antiplatelet antiserum was dialyzed for 6 hr with 0.0035 M phosphate buffer, pH 7.8, sedimented at 1000 g, and the supernatant harvested and applied to a 5 x 100-cm column of quanternary aminomethyl (QAE) Sephadex equilibrated with the same buffer. The effluent obtained by washing with 600 ml of equilibrating buffer contained IgG but no detectable IgM by Ouchterlony analysis. The effluent was concentrated to the starting volume, dialyzed for 6 hr with 0.0035 M phosphate buffer pH 6.0, and applied to a sulphopropyl (SP) Sephadex (5 x 50-cm) column equilibrated with the same buffer. The column was washed with 600 ml of equilibrating buffer and eluted with a linear salt gradient of 1500 ml of equilibrating buffer and 1500 ml of 0.0035 M phosphate buffer pH 6.0 containing 0.3 M potassium chloride. The column was run at 50 ml/hr and 10-ml fractions were collected. Rabbit IgG eluted as a single protein peak at an ionic strength equivalent to that of 0.02 M NaCl.

**Radioiodinated IgG**

Radioiodination of highly purified IgG fractions was accomplished by the method of McConahey et al. followed by Sephadex G-50 chromatography. Equal volumes of radiolabeled IgG (7.5 µg) were incubated with 1 x 10^9 washed human platelets, erythrocytes, or granulocytes at 37°C for 30 min. Granulocytes were isolated by Ficoll-Hypaque density gradient centrifugation of whole human blood followed by sedimentation with 3% dextran and removal of contaminating erythrocytes with 0.2% NaCl. Contamination with mononuclear cells was <5%. The platelets, erythrocytes, or granulocytes that had been incubated with IgG were washed twice with modified Tyrode's buffer containing 0.01 M EDTA, and the amount of radiolabel adsorbed was determined with a gamma scintillation counter (Nuclear Chicago, Chicago, Ill.) The number of IgG molecules per cell was calculated from the protein content of the IgG fraction and the per cent protein adsorbed per cell.

**Statistics**

The results are expressed as mean ± SEM. Comparisons were evaluated using the Students’ t test for unpaired samples.

**RESULTS**

**Effect of Platelet-aggregating Agents on Platelets in Plasma and Gel-filtered Platelets**

Aggregation of and ^14C-serotonin release from gel-filtered platelets was similar to that of platelets in plasma (Table I) with the aggregating agents ADP and epinephrine (p > 0.7). The presence of a low calcium concentration (38 µM) in the modified Tyrode’s buffer solution, rather than the 2 mM calcium present in the usual Tyrode’s buffer, facilitated aggregation and release. Aggregation of
Table 1. Effect of ADP, Epinephrine, and Collagen on the Aggregation and $^{14}$C-Serotonin Release of Platelets in Plasma and Gel-filtered Platelets

<table>
<thead>
<tr>
<th>Agent</th>
<th>Platelet-rich Plasma</th>
<th>Gel-filtered Platelet-rich Plasma (no added Ca$^{2+}$)</th>
<th>Platelet-rich Plasma</th>
<th>Gel-filtered Platelet-rich Plasma (no added Ca$^{2+}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (8–25 μM)</td>
<td>71.6 ± 2.0</td>
<td>74.0 ± 2.0</td>
<td>55.1 ± 3.2</td>
<td>54.5 ± 4.2</td>
</tr>
<tr>
<td>Epinephrine (10–50 μM)</td>
<td>73.8 ± 3.7</td>
<td>71.0 ± 2.0</td>
<td>70.8 ± 8.0</td>
<td>66.1 ± 3.4</td>
</tr>
<tr>
<td>Collagen (22 μg/ml)</td>
<td>59.1 ± 5.0</td>
<td>72.6 ± 2.3</td>
<td>55.8 ± 10.8</td>
<td>56.5 ± 6.3</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SEM percent aggregation and $^{14}$C-serotonin release for each agent. The range of concentration of each agent for all of the separate donors is given. Each mean represents experiments from at least nine separate normal donors. For each experiment a single concentration of agent was used. This concentration gave a maximum percent aggregation in platelet-rich plasma and was then employed in evaluating the response in the gel-filtered platelets of that donor.

gel-filtered platelets with collagen was significantly increased compared to collagen aggregation of platelets in PRP ($p < 0.001$), but no difference in $^{14}$C-serotonin release was observed. To test if gel filtration had altered the stability of lysosomal granules, the release of $\beta$-glucuronidase, a lysosomal acid hydrolase, was studied with the aggregating agents ADP and epinephrine (Table 2). Both agents released only minimal amounts of $\beta$-glucuronidase from platelets. No significant differences were noted between gel-filtered platelets and platelets in their own plasma.

Effect of Rabbit Anti-Human Platelet Antiserum on Platelets in Plasma and Gel-filtered Platelets

Platelet aggregation. Rabbit anti-human platelet antiserum induced aggregation of human platelets in a dose-related fashion (Fig. 1). At a relative antibody concentration of 16, platelet aggregation of approximately 55% was seen. A fourfold increase in antibody concentration did not further significantly increase the degree of aggregation in PRP. The amplitude of the aggregation in PRP was increased when compared to that with normal rabbit serum ($p < 0.001$); in addition, aggregation with normal serum averaged 2%, and was usually under 5%. A similar pattern was observed with gel filtered platelets when they were incubated with rabbit anti-human platelet antiserum. With increasing

Table 2. Effect of ADP, Epinephrine, and Antiplatelet Antibody on $\beta$-Glucuronidase and LDH Release

<table>
<thead>
<tr>
<th>Agent</th>
<th>$\beta$-Glucuronidase Release (%)</th>
<th>LDH Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>4.0 ± 1.4</td>
<td>8.4 ± 2.8</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>16.1 ± 10.6</td>
<td>8.3 ± 1.6</td>
</tr>
<tr>
<td>Antiplatelet antibody</td>
<td>3.8 ± 2.0</td>
<td>5.0 ± 2.6</td>
</tr>
<tr>
<td>8*</td>
<td>3.8 ± 2.0</td>
<td>5.0 ± 2.6</td>
</tr>
<tr>
<td>16</td>
<td>2.5 ± 2.1</td>
<td>7.2 ± 1.7</td>
</tr>
<tr>
<td>32</td>
<td>7.5 ± 3.5</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>4.7 ± 2.4</td>
<td>4.0 ± 4.0</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SEM percentage $\beta$-glucuronidase or LDH release of the total platelet $\beta$-glucuronidase or LDH as determined by Triton lysis. All experiments are in platelet-rich plasma. For ADP and epinephrine the number of donors and concentrations used are the same as in Table 1. For antiplatelet antibody the means represent five experiments.

*Relative concentration of antiplatelet antibody.
concentrations of antiserum, platelet aggregation progressively increased. Similar results were obtained with a second rabbit anti-human platelet antiserum. Platelet aggregation was maximal at 5 min at each antibody concentration examined.

$^{14}$C-Serotonin release. The release of $^{14}$C-serotonin from human platelets incubated with rabbit antiplatelet antiserum also progressively increased in relation to the logarithm of the antiserum concentration (Fig. 2). At a relative antibody concentration of 16 or greater, release was markedly increased when compared to that of normal rabbit serum alone whether PRP or gel-filtered
platelets were used. The effect of the antiplatelet antiserum on gel-filtered platelets was similar to that on platelets in plasma. Similar results were also obtained with a second antiserum prepared in a different rabbit. Unlike platelet aggregation, $^{14}$C-serotonin release progressively increased with time reaching a maximum between 25 and 45 min at the higher antibody concentrations tested. This slow loss of platelet serotonin contrasted with the rapid loss of serotonin with ADP and epinephrine and may reflect either a difference in the mechanism of release or an inhibitory effect of the antibody on the platelet re-uptake of released serotonin.

**β-Glucuronidase and LDH release.** Incubation of human platelets in plasma with ADP, epinephrine, or rabbit antiplatelet antiserum resulted in release of only small amounts of β-glucuronidase (Table 2). No consistent dose response was observed, and no significant differences were noted between the antiserum and normal rabbit serum. Stirring with modified Tyrode’s buffer for up to 45 min at 37°C released ≤ 2% β-glucuronidase.

With ADP, epinephrine, and antiplatelet antibody LDH loss was less than 10%, (Table 2). No differences were noted between gel-filtered platelets and PRP. Stirring with modified Tyrode’s buffer for 45 min released ≤ 6% LDH.

**Acid phosphatase availability.** In the absence of antiplatelet antiserum less than 2% of the normal acid phosphatase was detected in PRP. After exposure to ADP and epinephrine, virtually all acid phosphatase activity was associated with the platelets and 19%–25% became available. Incubation of platelets in plasma with antiplatelet antiserum resulted in 65%–75% acid phosphatase availability (Fig. 3). Normal rabbit serum failed to increase the availability of acid phosphatase.
Table 3. Effect of Thrombin Inhibitors on Antibody-induced Platelet Aggregation and Release

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Concentration</th>
<th>Aggregation (%)</th>
<th>14C-Serotonin Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control† (modified Tyrode's buffer)</td>
<td>—</td>
<td>58</td>
<td>67</td>
</tr>
<tr>
<td>Tosyl-L-arginine methyl ester</td>
<td>5 mM</td>
<td>54</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>55</td>
<td>73</td>
</tr>
<tr>
<td>Hirudin</td>
<td>1.6 NIH U/ml</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td>Heparin</td>
<td>2.0 U/ml</td>
<td>67</td>
<td>81</td>
</tr>
</tbody>
</table>

*Mean of at least two determinations with each inhibitor.
†The relative antiplatelet antibody concentration was 16. Normal rabbit serum gave 10% aggregation and 13% 14C-serotonin release.

Effect of Inhibitors on Antibody-mediated Platelet Aggregation and 14C-Serotonin Release

In these experiments a relative antiplatelet antiserum concentration of 16 was employed, since this concentration of antiserum was just sufficient for a maximal response (Figs. 1 and 2) and therefore inhibition could be readily detected. The requirement for thrombin in the antibody-mediated platelet alterations was studied by examining the effect of three thrombin inhibitors on the interaction. Two-tenths milliliter of diluted antiserum was mixed with 0.3 ml PRP and incubated with 10 μl of modified Tyrode's buffer or selected inhibitory compounds dissolved in modified Tyrode's buffer for 5 min at 37°C in the aggregometer cuvette. The reaction was then started by stirring the solution at 1200 rpm and the per cent aggregation and 14C-serotonin release determined. The antiserum incubated with the modified Tyrode's buffer control produced 58% aggregation and released 67% of the serotonin from the platelets (Table 3). None of the three thrombin inhibitors, tosyl-L-arginine methyl ester, hirudin or heparin, interfered with the effect of the rabbit antiplatelet antiserum on platelets. These inhibitors were present in concentrations shown in separate experiments to inhibit the effect of thrombin (1 NIH U/ml) on fibrinogen in plasma.

Acetylsalicylic acid (ASA) is known to inhibit the second phase of aggregation and the release reaction initiated by ADP and epinephrine. The effect of various concentrations of ASA was studied on the antibody-induced aggrega-

Table 4. Effect of Acetylsalicylic Acid on Antibody-induced Platelet Aggregation and Release

<table>
<thead>
<tr>
<th>Acetylsalicylic Acid* (mM)</th>
<th>Aggregation (%)</th>
<th>14C-Serotonin Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiplatelet Antibody†</td>
<td>Epinephrine (50 μM)</td>
</tr>
<tr>
<td>0</td>
<td>52</td>
<td>80</td>
</tr>
<tr>
<td>0.05</td>
<td>46</td>
<td>15</td>
</tr>
<tr>
<td>0.20</td>
<td>42</td>
<td>17</td>
</tr>
<tr>
<td>1.0</td>
<td>46</td>
<td>16</td>
</tr>
<tr>
<td>4.0</td>
<td>47</td>
<td>22</td>
</tr>
</tbody>
</table>

*Mean of two experiments.
†The relative antiplatelet antibody concentration was 16. Normal rabbit serum gave 8% aggregation and 2% 14C-serotonin release.
Table 5. Effect of EDTA on Antibody-induced Platelet Aggregation and Release

<table>
<thead>
<tr>
<th>EDTA (mM)</th>
<th>Aggregation (%)</th>
<th>Epinephrine (50 μM)</th>
<th>14C-Serotonin Release (%)</th>
<th>Epinephrine (50 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiplatelet Antibody</td>
<td></td>
<td>Antiplatelet Antibody</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>44</td>
<td>74</td>
<td>69</td>
<td>56</td>
</tr>
<tr>
<td>0.5</td>
<td>42</td>
<td>63</td>
<td>53</td>
<td>11</td>
</tr>
<tr>
<td>2.0</td>
<td>26</td>
<td>22</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>10.0</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.0</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean of two experiments
†The relative antiplatelet antibody concentration was 16. Normal rabbit serum gave 10% aggregation and 2% 14C-serotonin release.

EDTA has been shown to prevent completely platelet aggregation and 14C-serotonin release by ADP and epinephrine. A dose-dependent inhibition by EDTA of both platelet aggregation and 14C-serotonin release was evident when platelets were exposed to epinephrine. A similar dose-related inhibition was found for aggregation and 14C-serotonin release by antiplatelet antibody (Table 5). At concentrations of ≥ 10 mM EDTA, complete inhibition of platelet aggregation and 14C-serotonin release were observed for the antibody-mediated reactions.

Isolation of Rabbit IgG From Anti-Human Platelet Antisera

IgG isolated from rabbit antiplatelet antisera by sequential QAE-Sephadex and SP-Sephadex chromatography, as described in Materials and Methods, contained the major portion of activity capable of aggregating platelets and releasing 14C-serotonin from both plasma platelets and gel-filtered platelets and exposing platelet acid phosphatase of plasma platelets. Rabbit IgG eluted on Sephadex G-200 at the same volume as aldolase (MW 160,000) (Fig. 4). Similarly, the activities resulting in platelet aggregation, platelet 14C-serotonin release, and platelet acid phosphatase availability eluted tube for tube coincident with monomeric IgG. This highly purified IgG antiplatelet antibody (900 μg/ml) revealed a single band corresponding to IgG on alkaline disc gel electrophoretic analysis. Similar concentrations of a control preparation of monomeric human IgG isolated from serum in an identical manner to the rabbit IgG antiplatelet antibody, but not containing antiplatelet antibody activity, had no effect on either gel filtered or plasma platelets.

The highly purified IgG antiplatelet antibody was radioiodinated with 125I and used to sensitize equal numbers of human platelets, erythrocytes or granulocytes. While only 30 IgG molecules were bound per erythrocyte, 3500 IgG molecules were bound per platelet. The granulocyte enriched fraction adsorbed 250 IgG molecules per cell. Thus, the IgG antibody had primarily platelet specificity. Controls with radiolabeled, highly purified IgG not possessing anti-
Fig. 4. Sephadex G-200 gel filtration of rabbit antiplatelet antibody obtained by QAE and SP Sephadex chromatography. Fractionation was performed by upward flow at 20 ml/hr and 8-ml fractions were collected. The column was equilibrated with 0.0035 M phosphate buffer, pH 7.8, containing 0.12 M KCl. The capacity of each fraction to aggregate platelets (x—x), release platelet serotonin (a—a), and increase platelet acid phosphatase availability (c—c) is shown. IgG (○—○) was assessed by Ouchterlony analysis.

platelet antibody activity gave < 10^−5, the adsorption seen with the IgG anti- platelet antibody. The capacity of the granulocyte fraction to adsorb antibody may reflect common antigens on the platelet and granulocyte surface, or may be due to the few contaminating lymphocytes and platelets in the granulocyte-enriched fraction.

DISCUSSION

As an in vitro model for antibody induced cellular immune damage, we have examined the effect of heterologous IgG antiplatelet antibody on human platelets in the presence of plasma as well as by using purified components. Human platelets were effectively isolated from plasma proteins by Sepharose 2B gel filtration and interacted at 37°C with rabbit antiplatelet antisera or the IgG fraction isolated from the antiplatelet antisera.

The functional integrity of these gel filtered platelets was similar to that of platelets in the presence of plasma (PRP). Gel filtration in modified Tyrode's buffer produced platelets which aggregated and released serotonin upon the addition of ADP and epinephrine to a similar extent as platelets in platelet-rich plasma (Table 1). Small amounts of calcium may be required for the reactivity of gel-filtered platelets, since 38 μM Ca^{2+} were present in the modified Tyrode's solution used in these experiments.

The process of gel filtration did not appear to render the platelet more susceptible to alteration as incubation of gel-filtered and plasma platelets with ADP and epinephrine did not cause significant β-glucuronidase release (Table 2). Since β-glucuronidase is a lysosomal marker, this indicates no appreciable lysosome labilization. ADP and epinephrine also failed to release LDH from both plasma platelets and gel-filtered platelets (Table 2), indicating no detectable platelet cytolysis.

Heterologous anti-human platelet antisera were able to cause platelet aggregation (Fig. 1), serotonin release (Fig. 2), and increased availability of platelet
acid phosphatase (Fig. 3). In all three determinations quantitative aspects were similar with relative antibody concentrations of 16 able to cause significant amounts of aggregation and serotonin release. Acid phosphatase is a membrane enzyme similar to platelet factor 3 in its biologic behavior. Similarly, anti-platelet antibody has been shown to increase the availability of platelet factor 3.\(^{33}\)

Heterologous anti-human platelet antiserum has been previously shown to produce visible platelet aggregation and total nucleotide release.\(^{34}\) However, in the latter study, dose dependent quantitative data were not reported. Anti-platelet antibody does not cause lysosomal enzyme release or platelet cytolysis (Table 2). Thus, heterologous antiplatelet antibody seems to produce release from only the dense granules (serotonin), similar to the effect seen with ADP, epinephrine and collagen.

The active principle in the antiplatelet antiserum fractionated on Sephadex G-200 coincident with monomeric IgG (Fig. 4), indicating that aggregated IgG or IgG-containing immune complexes\(^{10,12}\) were not responsible for the effect. The effect of the antiplatelet antibody on platelets was complement independent, since the reaction was supported in a system involving gel-filtered platelets and purified IgG antibody or heat-inactivated antiplatelet antiserum having no detectable Cl by effective molecule titration. These experimental conditions precluded activation of both the classic and the properdin complement pathways.

Evidence that heterologous antibody (IgG) induced human platelet alteration may proceed by a different pathway than that mediated by ADP and epinephrine is indicated by the observation that acetylsalicylic acid does not inhibit the antibody-mediated effects at concentrations that completely inhibit epinephrine-induced serotonin release (Table 4). The failure of acetylsalicylic acid, a known inhibitor of prostaglandin synthesis,\(^{36}\) even at high concentrations to inhibit the antibody-induced second wave of aggregation and \(^{14}\)C-serotonin release indicates that endogenous prostaglandins, endoperoxide intermediates, or thromboxanes\(^{35}\) are not involved. This observation indicates that the antibody-induced platelet alteration proceeds by a pathway different from that mediated by ADP, epinephrine or collagen, which involves prostaglandin synthesis.\(^{37}\)

The antibody effect is reminiscent of that of thrombin since aspirin does not inhibit thrombin-induced aggregation and release (except at very low concentrations of thrombin). However, unlike thrombin, antibody fails to cause significant release of β-glucuronidase. Moreover, antibody-induced aggregation does not require thrombin participation because thrombin inhibitors do not impair the reaction (Table 3).

The fact that as little as 2 mM EDTA partially inhibited the effect of both epinephrine and antiplatelet antibody (Table 5) suggests a cation requirement for both pathways. The failure of 10 mM EDTA to inhibit platelet agglutination, as determined by a technique used to assess agglutination of erythrocytes,\(^{38}\) suggests that the platelet alterations observed are not due to the agglutinating effect of the antibody.

Most of the studies involving the immunologic effects on human platelets
have involved the effect of either preformed IgG containing immune complexes or aggregated IgG.\textsuperscript{10,12} Exposure to these reactants results in release of nucleotides and platelet aggregation without loss of cytoplasmic or lysosomal enzymes.\textsuperscript{10,34} These results are similar to the effect that we observe with IgG antiplatelet antibody (Fig. 4). Monomeric IgG not possessing antiplatelet antibody activity has no effect. Whether or not platelet immune injury mediated by preformed antigen–antibody complexes proceeds by a mechanism similar to that mediated by anti-platelet antibody remains to be determined. Similarly, if human antiplatelet antibody affects human platelets in a manner similar to heterologous IgG antibody is unknown.

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\section*{REFERENCES}

38. Schreiber AD, Frank MM: The role of antibody and complement in the immune clearance and destruction of erythrocytes. II. J Clin Invest 51:589, 1972
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