Platelet Cold Agglutinins

By Stanley P. Watkins, Jr., and N. Raphael Shulman

A "cold" platelet agglutinin is described which is present in plasma and serum, is greater than 100,000 in molecular weight, appears to be a protein of the immunoglobulin type, is adsorbed by platelets in the absence of bivalent cations, and reacts only at temperatures below 34°C. It agglutinates homologous and autologous platelets but appears to have no effect on in vivo platelet function. It is seen in patients with systemic disease and can cause spuriously low platelet counts.

The purpose of this report is to describe a phenomenon of platelet agglutination that was responsible for spuriously low platelet counts in five patients. The agglutination was caused by an abnormal protein that attached to platelets at temperatures below 34°C and had the physical and immunologic characteristics of a gamma globulin. This protein had no demonstrable effect on the function of platelets in vivo.

The clumping agent was discovered because platelet counts obtained by an automatic electronic particle counter on five patients were not only inexplicably low in view of the patients' lack of symptoms of thrombocytopenia, but also varied from day to day beyond physiologic possibilities. Counts performed microscopically on the same patients were normal if blood was diluted directly from a fingerstick or if anticoagulated blood was not allowed to cool below 34°C. Since the platelets in microscopic counts done on blood that had cooled to room temperature were clumped, an attempt was made to identify the agglutinin.

Materials and Methods

Anticoagulants were used as follows: sodium heparin 50 U./ml. whole blood; tri-sodium citrate one part 1.4 M (40 per cent) to 100 parts whole blood; disodium ethylenediamine tetraacetate (EDTA) one part 0.27 M (10 per cent) to 15 parts whole blood; sodium oxalate one part 0.075 M (1 per cent) to nine parts whole blood. All blood was drawn in plastic syringes.

Platelet agglutination was evaluated by observing the number of platelet clumps in a standard blood cell counting chamber under phase microscopy. The whole blood or mixtures of platelets and protein fractions were diluted 1 to 100 in 1 per cent ammonium oxalate and mixed in a rotary agitator for five minutes prior to observation in the counting chamber. The number of free and clumped platelets could be estimated easily because the clumps usually consisted of two to five platelets and often more than 10.

Platelet-rich plasma was obtained by centrifuging anticoagulated whole blood for four minutes at 1000 × g. Platelet buttons for concentrated platelet suspensions were made by centrifuging platelet-rich plasma for 10 minutes at 2000 × g. Resuspensions to the desired concentration were made in ABO-type specific plasma.

Serum was obtained by allowing blood to clot in glass for 24–36 hours at 37°C. Serum was fractionated on G-200 Sephadex columns by standard technique using 0.15 M NaCl containing 0.004 M EDTA as eluting fluid. The three peaks of material were concentrated.
### Table 1.—Clinical and Laboratory Summary of Patients Evaluated

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex and Age</th>
<th>Diagnosis</th>
<th>Range of Platelet Counts at 22–22°C</th>
<th>Actual * Platelet Count</th>
<th>Total Serum Protein Gm./100 ml.</th>
<th>Alba Glob.</th>
<th>a1</th>
<th>a2</th>
<th>β</th>
<th>γ</th>
<th>Cryoprotein</th>
<th>Agglutinin Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M 39</td>
<td>Metastatic malignant melanoma</td>
<td>67–92,000</td>
<td>298,000</td>
<td>5.7</td>
<td>3.3</td>
<td>0.2</td>
<td>0.5</td>
<td>0.7</td>
<td>1.0</td>
<td>0</td>
<td>1:2</td>
</tr>
<tr>
<td>2</td>
<td>M 26</td>
<td>Felty's syndrome</td>
<td>52–112,000</td>
<td>284,000</td>
<td>7.2</td>
<td>2.7</td>
<td>0.5</td>
<td>0.7</td>
<td>0.6</td>
<td>2.7</td>
<td>4+</td>
<td>1:8</td>
</tr>
<tr>
<td>3</td>
<td>F 91</td>
<td>Metastatic squamous cell carcinoma</td>
<td>24–62,000</td>
<td>200,000</td>
<td>6.0</td>
<td>3.2</td>
<td>0.4</td>
<td>0.7</td>
<td>0.7</td>
<td>1.0</td>
<td>0</td>
<td>1:2</td>
</tr>
<tr>
<td>4</td>
<td>F 60</td>
<td>Felty's syndrome</td>
<td>12–36,000</td>
<td>195,000</td>
<td>9.2</td>
<td>2.18</td>
<td>0.2</td>
<td>0.44</td>
<td>0.58</td>
<td>5.8</td>
<td>2+</td>
<td>1:32</td>
</tr>
<tr>
<td>5</td>
<td>M 60</td>
<td>Recovery phase of serum hepatitis</td>
<td>12–100,000</td>
<td>100,000</td>
<td>7.5</td>
<td>2.9</td>
<td>0.4</td>
<td>0.5</td>
<td>0.7</td>
<td>3.0</td>
<td>2+</td>
<td>1:8</td>
</tr>
</tbody>
</table>

* * Performed on blood processed at 37°C as described in Materials and Methods.
to one half the original volume and reeleduted by the same procedure for further purification prior to use in the agglutination mixtures. The final concentration of protein was adjusted to 3 mg./ml. in the > 300,000 mol. wt. peak; 25 mg./ml. in the < 300,000 to > 100,000 mol. wt. peak; and 60 mg./ml. in the < 100,000 mol. wt. peak. In mixtures of platelets and purified proteins the final platelet concentration was 10^5/mm.3 and protein concentrations half those listed in the previous sentence.

Protein in the > 100,000 < 300,000 mol. wt. peak was precipitated by mixing with an equal volume of saturated ammonium sulfate. The precipitate was separated by centrifugation and dissolved in water. Both the supernatant and precipitate solution were dialyzed against 1000 volumes of 0.15 M NaCl and concentrated by ultrafiltration to the original volume of serum from which the protein had been obtained.

Adsorption studies were performed by adding 0.1 ml. of platelets of various concentrations suspended in EDTA plasma to 0.3 ml. of plasma containing the agglutinin. After incubation for one hour at room temperature the samples were centrifuged 10 minutes at 400 × g., and the agglutinin in the supernatant plasma was titered. In titering, 0.3 ml. of the supernatant plasma was mixed with 0.05 ml. of platelet-rich plasma containing 3.0 × 10^5 platelets/mm.3, and agglutination was evaluated as described above. An agglutinin that titered 1/32 as defined in Results was decreased to approximately 1/8 when adsorbed with platelets at 10^5/mm.3 final concentration and completely neutralized by adsorption with 2 × 10^6 platelets/mm.3.

Rabbit anti-human gamma globulin was used to determine the specificity of the agglutinin after it was eluted from platelets. The agglutinin in 0.6 ml. of serum was adsorbed with 3 × 10^9 platelets for 30 minutes at room temperature. Control human serum was treated in the same manner as agglutinating serum. Platelets used for adsorption were removed by centrifuging at 2000 × g. for 10 minutes and were suspended in 0.3 ml. of 0.15 M NaCl. The suspensions were heated at 50°C for 15 minutes, then centrifuged for 15 minutes at 2000 × g. (keeping the temperature at 50°C). One aliquot of each supernatant fluid was mixed with one-third volume of rabbit anti-human gamma globulin serum and another aliquot with one-third volume of normal rabbit serum. After the four mixtures were incubated 30 minutes at 34°C and 30 minutes at 5°C, they were centrifuged at 25,000 × g. for 10 minutes. This removed visible precipitate in the mixtures with antiglobulin serum. The supernatant fluids were then incubated with one-third volume of normal barium sulfate adsorbed human serum to neutralize excess rabbit anti-human gamma globulin. Precipitates that formed at this stage were removed by centrifuging at 25,000 × g. for 10 minutes. These preparations were then tested for platelet agglutinins as described above.

Cryoprotein was separated from the serum by allowing blood to clot at 37°C and then incubating the serum for 48–72 hours at 4°C. Precipitate that formed in the cold was separated by centrifugation, resuspended in a volume of normal serum equal to the volume of serum from which the precipitate was obtained, and tested for platelet agglutinins.

The presence of fibrinogen degradation products was evaluated by using the immunologic techniques of Ferreira and Murat.

RESULTS

The patients with inexplicably low and variable platelet counts whom we studied, are listed in Table 1. Two patients had metastatic carcinoma but no detectable serum protein abnormalities, and three patients had diseases associated with high immunoglobulin levels and cryoproteins.

Platelet clumps that developed in the blood of these patients are shown in Fig. 1. Agglutination was discernible within several minutes at room temperature in the patients’ whole blood or in mixtures of the patients’ serum or serum fractions with suspensions of normal platelets. The number of agglutinated platelets increased progressively for more than one hour (Table 2). Maximum agglutination was noted in samples kept below 31°C, but no agglutination occurred above 34°C. After agglutination occurred at temperatures below 34°C,
Fig. 1.—Platelet Agglutination. Platelet clumps in cell counting chamber under phase microscopy at room temperature. Whole blood at 1/100 dilution in 1 per cent ammonium oxalate from patient 4, Table 1.

Table 2.—Rate of Platelet Agglutination at Room Temperature (22°C)

<table>
<thead>
<tr>
<th>Incubation Time at Room Temperature (22°C)</th>
<th>Minutes</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>50</th>
<th>80</th>
<th>360</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of agglutinated platelets (× 10^3/mm.³)</td>
<td></td>
<td>0</td>
<td>17</td>
<td>120</td>
<td>156</td>
<td>174</td>
<td>198</td>
</tr>
</tbody>
</table>

Whole blood from patient 4, Table 1, was allowed to stand at room temperature for the indicated times. Agglutinated platelets were calculated by subtracting the number of non-agglutinated platelets from the total present before incubation. An aliquot of the same blood kept at 37°C showed no evidence of clumping over this time interval.

It could not be reversed by warming the agglutinates to 37°C. In order to obtain accurate platelet counts on the patients in Table 1 it was necessary to keep their blood samples above 34°C by warming the syringes, sample bottles and diluting fluid used in cell counts. After blood was diluted in counting pipettes, the remainder of the counting procedure could be done at room temperature without agglutination occurring.

The types and amounts of anticoagulants listed in Materials and Methods had no effect on the rate or degree of agglutination. Siliconized or plastic surfaces had no effect on agglutination compared to glass. All subsequent agglutination experiments described were performed in glass test tubes with EDTA as an anticoagulant.

Sera from four of the five patients and from normal controls were fractionated by Sephadex filtration as described in Materials and Methods. The aggluti-
nating protein was found in the >100,000--<300,000 mol. wt. peak in patients 1, 3 and 4, and in the > 300,000 mol. wt. peak in patient 5. As with whole serum, agglutination of platelets by the serum fractions occurred only at temperatures below 34°C. Fractions prepared identically from normal serum did not agglutinate platelets.

The agglutinating protein could be adsorbed out by platelets and was precipitated by 50 per cent ammonium sulfate (see Materials and Methods). The agglutinin found in patient 4 could be analyzed best with specific antisera because of its high titer. This agglutinin was eluted from platelets and mixed with rabbit antihuman 7S gammaglobulin as described under Materials and Methods. After the precipitate resulting from this mixture was removed and excess antiglobulin neutralized (see Materials and Methods), there was no platelet agglutinating activity remaining.

Three of the five patients were noted to have a cryoprotein in their sera. There was no detectable decrease in the platelet agglutinin titer when cryoprotein was separated from the patients' sera as described in Materials and Methods. Moreover, the cryoprecipitate, redissolved in normal serum, did not agglutinate platelets.

None of the sera or fractionated material that agglutinated platelets contained detectable fibrinogen degradation products, when tested as described in Materials and Methods.

DISCUSSION

A number of substances of low molecular weight are known to agglutinate platelets in vitro, namely thrombin,4-6 adenosine diphosphate,7,8 triethyl tin,9 adenosine triphosphate, norepinephrine, 5-hydroxytryptamine and epinephrine hydrochloride.6,10-12 The agglutinin we are describing is a high-molecular-weight substance (> 100,000 mol. wt.) as determined by G-200 Sephadex filtration. The agglutinin is not dependent on the presence of calcium or magnesium ions for activity, and the type of anticoagulant used has no effect on agglutination. Thus, it differs from the thrombocyte agglutinating factor described by Brinkhous et al.13 and from ADP.8 In 1967, Yamazaki et al.,14 described a platelet-clumping substance present in heparinized rabbit plasma after intravenous injection of agar, bacterial endotoxin or adrenalin. The same authors15 noted that a similar platelet-clumping substance appeared in acidified (pH 5) plasma obtained from normal rabbits. The substance described by Yamazaki et al. was not present in plasma to which citrate, oxalate or EDTA had been added, and was also absent in serum. Kowalaski,16 in 1967, noted that low concentrations of fibrinogen degradation products caused platelets to agglutinate. Fibrinogen degradation products could not be detected in the sera we studied, using a highly sensitive flocculation test.3 The cold agglutinin described herein is present in serum providing blood is kept at 37°C during clotting, does not require acid pH for activity, and reacts in the absence of divalent cations, is adsorbed on platelets, has physicochemical properties consistent with proteins of the immunoglobulin type, and in one instance was proved to be a 7S gammaglobulin by immunologic techniques.

The cold agglutinin appears to have no effect on platelets in vivo, probably
because it does not attach at temperatures above 34°C. Platelet cold agglutinins have been described in thrombocytopenic states, but most platelet antibodies react best at 37°C. Extensive surgical procedures were performed on four of the five patients studied and none had abnormal bleeding, or abnormal bleeding times (Ivy technique). All five patients had systemic disease.* The agglutinin appears to be a nonspecific manifestation of disease similar to the occurrence of cryoprecipitate which was present in three of the five patients.

The agglutinin is of clinical importance in that it may be responsible for spuriously low and widely fluctuating platelet counts, especially if counts are performed by an electronic particle counter. If one suspects that a low or fluctuating platelet count is erroneous, visual rather than electronic techniques should be employed, and the determination made on blood obtained from a fingerstick or on venous blood kept at 37°C.

REFERENCES


*Two additional patients have been observed: one with liposarcoma and one with a metastatic islet cell tumor of the pancreas.
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