Acquired IgG Antibody Occurring in a Thrombasthenic Patient: Its Effect on Human Platelet Function

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In subagglutinating amounts, an IgG antibody isolated from the plasma of a polytransfused thrombasthenic patient (L) inhibited ADP-, epinephrine-, collagen-, and thrombin-induced aggregation of normal human platelets. The inhibition of ADP-induced aggregation was strongly diminished following the prior incubation of the antibody with control human platelet stroma but not with the stroma prepared from the platelets of two different thrombasthenic patients. The IgG(L) did not affect the binding of \(^{14}C\)-ADP to control human platelet membranes and did not inhibit the ADP-induced shape change.

NURDEN AND CAEN\(^1\,2\) reported glycoprotein abnormalities in membrane fractions isolated from the platelets of thrombasthenic patients of both subgroups.\(^3\) The major abnormality appeared to be a strongly reduced staining capacity for both carbohydrate and protein of glycoprotein II following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of isolated membranes. An altered expression of glycoproteins on the surface of thrombasthenic platelets was further indicated by the \(^{125}I\)-labeling patterns obtained following lactoperoxidase-catalyzed iodination procedures,\(^4\) and a relationship between the abnormal surface structure of thrombasthenic platelets and their defective function was previously suggested.\(^2\,4\)

Recent studies using SDS-PAGE procedures\(^5\) and lectin-affinity chromatography\(^6\) indicated that the platelet membrane glycoprotein composition is more complex than previously considered. Phillips and Poh Agin described a strong reduction in both glycoproteins IIb and III in thrombasthenic platelets and provided preliminary evidence that these glycoproteins are structurally distinct from each other.\(^7\)
This paper describes how an IgG antibody developed in a thrombasthenic patient recognizes a specific membrane site that seems implicated in those aspects of platelet function defective in thrombasthenia.

MATERIALS AND METHODS

The case report of patient (L) with Glanzmann thrombasthenia and the occurrence of the antiplatelet antibody were previously described. Isolation of IgG(L). The antibody was an IgG and was precipitated from the serum (L) by the addition of 40% w/v ammonium sulfate. The pellet was washed and resuspended in saline, and then dialyzed overnight at 4°C against 0.005 M phosphate buffer pH 8.1. The IgG was eluted through a DEAE-cellulose column with the same buffer, and then concentrated and dialyzed against 0.15 M NaCl. The protein concentration was determined and adjusted to 4 mg/ml.

Platelet aggregation. Platelet-rich plasma (PRP) was obtained by centrifuging human venous blood collected in 3.8% w/v sodium citrate (9 vol blood for 1 vol sodium citrate) at 120 g for 10 min at 15°C. Platelet-poor plasma (PPP) was obtained by centrifuging the remaining blood at 27,500 g for 15 min at 4°C. Citrated control human PRP (0.2 ml) with a platelet count adjusted to 3 x 10^8 platelets/ml with PPP was incubated for 60 sec in the cuvette of a Born MK II miniaggregometer (produced by the Dept. of Pharmacology, Royal College of Surgeons, London) in the presence of different concentrations (200, 135, or 100 μg/ml) of IgG(L) in saline, added 1 min before 10 μl of the following inducers (final concentrations): 2.5 μM ADP (Sigma, St. Louis), 2.5 μM epinephrine (Sigma), 20 μg collagen (Stago, Asnières, France), 0.12 NIH units thrombin (Hoffman-La Roche, Basle), 1.6 mg bovine fibrinogen (Kabi, Stockholm), and 2.0 mg ristocetin (Lundbeck, Copenhagen). The agglutinating activity of bovine fibrinogen preparations has been shown to be due to the presence of factor VIIIvWF protein, which is the agglutinating agent and will be specified as such in this paper. The velocity of aggregation was evaluated as previously described. Preliminary experiments determined that normal PRP was agglutinated by concentrations of IgG(L) ≥300 μg/ml; therefore 200 μg/ml IgG(L) was chosen as the highest subagglutinating concentration to be added.

Consumption of the antibody by control or thrombasthenic platelet stroma. Blood was collected from control humans and two thrombasthenic patients (other than (L)) into a heparinized tube of platelet-rich plasma and the remaining blood collected in 3.8% w/v sodium citrate (9 vol blood for 1 vol sodium citrate) at 120 g for 10 min at 15°C. The blood was centrifuged at 120 g for 10 min. The platelets were sedimented from the PRP by centrifugation at 2000 g for 10 min at 15°C. Platelet pellets were suspended in 0.015 M Tris HCl buffer, pH 7.4, containing 0.135 M NaCl, 0.053 M KCl, and 1.5 mM EDTA. These pellets were washed twice in this buffer and finally resuspended in the same buffer without EDTA. The platelets were disrupted by freeze-thawing five times and the stroma washed twice in saline (27,500 g, 15 min, 15°C). The stroma were resuspended at a concentration of 1 mg protein/ml in saline. Samples (0.2 ml) of this membrane-enriched fraction (non-diluted or diluted in saline 1:2 or 1:5) or 0.2 ml of saline (as control) were incubated with 0.2 ml of the 4 mg/ml IgG(L) solution for 120 min at 37°C, following an overnight incubation at 4°C. After centrifugation at 27,500 g for 15 min at 15°C, samples (10 μl) of the supernatants were tested for their residual inhibitory effect on ADP-induced aggregation of control PRP.

ADP-induced platelet shape change. PRP was prepared as described above and EDTA was added to give a final concentration of 2 mM. The ADP-induced platelet shape change was measured as the change in scattered light intensity as detected using a Born-Michal MK III aggregometer. ADP (1 μM final concentration) was added to PRP (3 x 10^8 platelets/ml) to which had been added IgG(L) (100 and 200 μg/ml) 1 min prior to the addition of the aggregating agent. All studies were performed at 37°C. The optical density change corresponding to the shape change was measured 6, 8, and 10 sec after the addition of ADP.

Clot retraction. The ADP-induced retraction of the reptilase clot was evaluated following the incubation of clotted samples for 15, 30, and 45 min as described by Kubise et al. Control PRP (0.7 ml) (300,000 platelets/ml) and 0.1 ml of 0.005 M Tris-HCl buffer pH 7.4 containing 0.145 M NaCl were pipetted into a glass test tube (5 mm diameter). The test tube was incubated for 2 min at 37°C in the presence or absence of IgG(L) (final concentrations 45, 22, or 9 μg/ml) before the addition of 0.1 ml ADP (5 x 10^-4 M final concentration). After a further
incubation for 2 min, 0.1 ml reptilase (Stago) was added; following the clot formation, clot retraction was measured and expressed as the percentage of the initial length of the clot.

The thrombin-induced clot retraction was studied using suspensions of washed platelets.\textsuperscript{15} Human platelets were isolated from PRP and washed according to the method of Mustard et al.\textsuperscript{16} Aliquots (0.7 ml) of the washed platelet suspension were incubated in the presence or absence of IgG(L) (final concentrations 45, 22, or 9 µg/ml), 0.1 ml of 1\textsuperscript{°} human fibrinogen, and 0.1 ml of thrombin at 20 NIH units/ml. Mixing of the reagents was performed as quickly as possible by gentle shaking. Samples were incubated in a water bath at 37°C for 30 min and the volume of fluid separated from the clot was measured by means of a 1-ml syringe as evaluated by Niewiarowski.\textsuperscript{15}

Effect of IgG(L) on the binding of $^{14}C$-ADP to isolated platelet membranes. The binding of $^{14}C$-ADP to isolated platelet membranes was measured as previously described by Legrand and Caen.\textsuperscript{17} The platelet membranes were prepared by the glycerol lysis technique of Barber and Jamieson.\textsuperscript{18} The membrane fraction recovered at the upper interface of the 30\textsuperscript{o} sucrose solution was washed by centrifugation at 100,000 g for 1 hr through a 10\textsuperscript{o} sucrose solution. The membranes were then resuspended at a concentration of 0.2 mg protein/ml in a solution of 0.01 M Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.001 M EDTA and then incubated at 37°C for 10 min with IgG(L) (150, 300, 400, or 600 µg/ml). These concentrations are greater than those used for inhibition of aggregation, since under our experimental conditions these amounts did not agglutinate the membrane preparations. Then various concentrations of $^{14}C$-ADP (550 mCi/mmol; Radiochemical Center, Amersham, England) were added and incubations were continued for 30 min in a shaking water bath as described by Nachman and Ferris.\textsuperscript{19} The unbound $^{14}C$-ADP was separated from membrane-bound $^{14}C$-ADP by vacuum filtration on 0.8-µm Millipore filters (AAWP 02500). Radioactivity on the filters (membrane-bound $^{14}C$-ADP) was measured as previously described.\textsuperscript{17}

RESULTS

\textit{ADP-, collagen-, epinephrine-, and thrombin-induced aggregation with IgG(L).} IgG (L) added to control human PRP at different concentrations (200, 133, or 100 µg/ml) 60 sec before the addition of ADP (2.5 \(\mu\)M) collagen (20 µg), epinephrine (2.5 \(\mu\)M) or thrombin (0.12 U NIH) inhibited both the velocity (Table I) and the intensity of the observed aggregation. The degree of inhibition observed was related to the concentration of antibody used. When control IgG was added under the same conditions no inhibition occurred.

\textit{Bovine factor VIII\textsubscript{vWf}–induced agglutination and ristocetin-induced aggregation with IgG(L).} IgG(L) modified neither platelet agglutination as induced fol-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Inducer & \multicolumn{3}{|c|}{IgG (Final Concentration µg/ml)} & \hline
(Final Concentration) & Control & 200 & 133 & 200 \hline
ADP (2.5 \(\mu\)M) & 0 & 10 & 30 & 75 \hline
Collagen (20 µg) & 0 & 15 & 35 & 80 \hline
Epinephrine (2.5 \(\mu\)M) & 0 & 15 & NT & 60 \hline
Thrombin (0.12 U NIH) & 0 & 18 & 38 & 60 \hline
Bovine fibrinogen (containing factor VIII\textsubscript{vWf}) (1.6 mg) & 0 & 0 & 0 & 0 \hline
Ristocetin (2 mg) & 0 & 0 & 0 & 0 \hline
\hline
\end{tabular}
\caption{Inhibition (%) of Platelet Aggregation by Control IgG and IgG(L)}
\end{table}

Results are expressed as the percentage inhibition of the velocity of aggregation as observed in the absence of the IgG(L). NT, not tested.
Results expressed as the mean of the intensity of the optical density change (scattered light in arbitrary units) recorded 6, 8, and 10 sec after the addition of ADP.

Clot retraction (%) was measured 15, 30, and 45 min after clot formation.

Consumption of IgG(L) activity by control or thrombasthenic platelet stroma. Table 2 shows that incubation of IgG(L) with normal platelet stroma reduced its ability to inhibit aggregation caused by 1 μM ADP. Consumption of the antibody activity was directly related to the concentration of the control platelet stroma used. Following incubation with stroma prepared from the platelets of two thrombasthenic patients [different from patient (L)] much of the antibody activity was recovered in the supernatant.

Effect of IgG(L) on the ADP-induced shape change. The ADP (1 μM)-induced shape change of control PRP was not modified in the presence of two different concentrations of IgG(L) (Table 3). Addition of the IgG(L) itself did not induce a shape change.

Effect of IgG(L) on clot retraction. At a concentration of 45 μg/ml IgG(L) completely inhibited ADP-induced reptilase clot retraction. This retraction was strongly inhibited at a concentration of 22 μg/ml and was slightly inhibited at 9 μg/ml IgG(L) (Table 4). Similar results were obtained when clot retraction was studied using a suspension of washed platelets, human fibrinogen, and thrombin.

Effect of IgG(L) on the binding of 14C-ADP to isolated platelet membranes. Preincubation of the membranes with increasing concentrations of IgG(L) (up to 600 μg) did not modify the subsequent binding of 14C-ADP. The affinity constant as determined by varying the concentrations of 14C-ADP in the medium (from 0.4 to 8 μM) remained unchanged even when the membranes had been preincubated with the maximal dose of 600 μg IgG(L) (Fig. 1).

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**Table 2. Consumption (%) of IgG(L) by Control and Thrombasthenic Platelet Stroma**

<table>
<thead>
<tr>
<th>Platelet Stroma</th>
<th>Final Concentration (μg/ml)</th>
<th>Control Platelet Stroma (Mean of Three Experiments)</th>
<th>Th1 Platelet Stroma</th>
<th>Th2 Platelet Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>80</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>61</td>
<td>74</td>
<td>76</td>
</tr>
<tr>
<td>250</td>
<td>-</td>
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<td>74</td>
</tr>
<tr>
<td>500</td>
<td>-</td>
<td>20</td>
<td>75</td>
<td>76</td>
</tr>
</tbody>
</table>

Th1 and Th2: two thrombasthenic patients different from patient (L). Residual inhibitory effect of IgG(L) following incubation with saline or with different amounts of stroma isolated from normal or thrombasthenic platelets (see Materials and Methods). Results are expressed in terms of the percentage inhibition of the velocity of 1 μM ADP-induced aggregation by unadsorbed antibody.

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**Table 3. Effect of IgG(L) on ADP-induced (1 μM) Shape Change of Control PRP**

<table>
<thead>
<tr>
<th>μg/ml</th>
<th>IgG(L)</th>
<th>0</th>
<th>14</th>
<th>100</th>
<th>14</th>
<th>200</th>
<th>13</th>
</tr>
</thead>
</table>

Results expressed as the mean of the intensity of the optical density change (scattered light in arbitrary units) recorded 6, 8, and 10 sec after the addition of ADP.

**Table 4. Reptilase Clot Retraction (%) Induced by ADP in the Presence of Different Concentrations of IgG(L)**

<table>
<thead>
<tr>
<th>IgG(L) (μg/ml)</th>
<th>Time (min)</th>
</tr>
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<tbody>
<tr>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>0</td>
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</table>

Clot retraction (%) was measured 15, 30, and 45 min after clot formation.

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Following the addition of bovine fibrinogen (1.6 mg/ml) containing factor VIII_vWF nor platelet aggregation as induced by ristocetin (2 mg/ml) (Table 1).

Consumption of IgG(L) activity by control or thrombasthenic platelet stroma. Table 2 shows that incubation of IgG(L) with normal platelet stroma reduced its ability to inhibit aggregation caused by 1 μM ADP. Consumption of the antibody activity was directly related to the concentration of the control platelet stroma used. Following incubation with stroma prepared from the platelets of two thrombasthenic patients [different from patient (L)] much of the antibody activity was recovered in the supernatant.
AGGREGATION: ANTIBODY INHIBITION

DISCUSSION

Thrombasthenia is an inherited bleeding disorder characterized by a long bleeding time and a defective platelet function. It has been shown that thrombasthenic platelets fail to aggregate in the presence of ADP, collagen, thrombin, or epinephrine, but agglutinate in response to bovine factor VIII, and aggregate in the presence of ristocetin. More recently, in the presence of rabbit subendothelium an absence of platelet thrombus formation was reported despite the covering of the subendothelial surface by a monolayer of adhering platelets. IgG(L) is an antiplatelet antibody that developed in a polytransfused thrombasthenic patient. IgG(L) inhibited ADP-, collagen-, thrombin-, and epinephrine-induced aggregation of normal human platelets as studied in the aggregometer. It did not modify bovine factor VIII-induced agglutination or ristocetin-induced aggregation. Previous studies showed that this antibody did not inhibit platelet adhesion to subendothelium but did inhibit the normally observed thrombus formation, resulting in a platelet interaction with subendothelium similar to that observed with thrombasthenic platelets. In the presence of IgG(L) the ADP-induced retraction of the reptilase clot using normal PRP and the thrombin-induced clot retraction using washed platelets and fibrinogen were both inhibited. The antibody therefore induced thrombasthenialike functional defects in normal human platelets.

Prior incubation with control human platelet stroma strongly reduced the inhibitory effect of the antibody, while incubation with the stroma prepared from the platelets of two thrombasthenic patients did not affect its activity. These results are in agreement with the finding of Degos et al. that the antigenic determinant recognized by the antibody (L) is absent or strongly modified in thrombasthenic platelets. The platelets of patient (L) have a typical throm-
basthenialike surface defect involving membrane glycoproteins. SDS-PAGE analysis of the immunoprecipitate obtained following the addition of rabbit anti-human γ-globulin to an incubation of IgG(L) with Nonidet P-40 solubilized 125I-labeled normal human platelets suggested that IgG(L) recognized a 125I-labeled (and therefore surface oriented) membrane constituent of apparent mol wt 120,000 daltons. It therefore seems that this component, which migrates with the glycoprotein II family on SDS-PAGE (Degos Li unpublished observations), plays a central role in the mechanism of human platelet aggregation as induced by physiologic aggregation-inducing agents.

The exact mechanism by which the platelet site recognized by IgG(L) is implicated in platelet aggregation remains unknown. One possibility was that the antibody (L) was directed against membrane receptors involved in the induction of the aggregation. However, in the presence of IgG(L) the binding of 14C-ADP to control platelet membranes was normal. Thrombasthenic platelets also bind 14C-ADP within the normal kinetic parameters and with a normal affinity. These results suggest that the component reacting with the antibody does not play a role in the initial binding of ADP to platelets. It is also known that ADP is able to induce the shape change in thrombasthenic platelets. The antibody (L) did not inhibit the ADP-induced shape change of control human platelets, indicating that the platelets were able to respond to the stimulus following its interaction with the receptor and that the aggregation defect in thrombasthenia appears to be at a stage subsequent to the initial interaction with the physiologic stimulus.

Macfarlane and Mills, using highly purified ATP, showed that this compound completely inhibited ADP-induced aggregation but did not inhibit epinephrine- and thrombin-induced primary aggregation of human platelets, which, according to these authors, therefore occurs by a mechanism independent of ADP. Using serial dilutions of the antibody (L) or of the aggregation inducers, we found a similar inhibition for ADP-, epinephrine-, and thrombin-induced aggregation. We therefore conclude that the antibody acts at a late stage in the mechanism of aggregation common for each of these aggregation inducers.

The recently reported finding of two glycoprotein deficiencies in thrombasthenic platelet membranes raises the question of the antigenic relationship between these two glycoproteins. It is interesting that IgG(L) inhibited clot retraction as well as aggregation. This may well imply that the membrane component recognized by IgG(L) played a central role in both of these physiologic processes. The specificity of the action of the antibody was shown by its lack of inhibition of platelet agglutination by bovine factor VIII, aggregation by ristocetin, or adhesion to subendothelium. Further studies on the nature of the membrane antigenic determinant involved in the interaction of IgG(L) are in progress.

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