Heavy-Chain Subclass of Bound Antiplatelet IgG in Autoimmune Thrombocytopenic Purpura

By K. Hymes, P. H. Schur, and S. Karpatkin

The gamma heavy-chain subclass of bound antiplatelet antibody was examined in six patients with autoimmune thrombocytopenic purpura (ATP) by a solid-phase radioimmunoassay. Monospecific antisera for γG1, γG2, γG3, and γG4 subclasses were employed in a "sandwich" technique, utilizing the binding of 125I-staphylococcal protein A. We have previously reported that serum antiplatelet antibody was restricted to the γG3 subclass in ATP. In contrast, all 4 IgG subclasses were found bound to platelets of ATP patients in the same distribution as that present in normal serum. It is suggested that the differences noted between serum antiplatelet IgG and platelet-bound IgG may represent different mechanisms of platelet injury.

It is now generally accepted that idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder. Platelets are destroyed by phagocytosis in the reticuloendothelial system (particularly spleen) following opsonization by IgG antibody, or possibly, attachment of immune complexes to their Fc receptor. Support for an IgG antibody being responsible for platelet damage is derived from several lines of evidence. The disease can be passively transferred across the placenta, and early in vivo studies on the passive transfer of this disease into volunteer recipients, by infusion of plasma from patients, revealed that the injurious factor was in the 7S IgG fraction of plasma and could be adsorbed to platelets. Other studies on the circulating antiplatelet antibody revealed that it could be neutralized with rabbit anti-human IgG antibody but not with anti-IgM, anti-IgA, or anti-IgD. More recent studies on the detection of bound anti-platelet antibody by a complement–red blood cell lysis inhibition technique, as well as a Fab-anti-Fab technique, a radioactive 125I-Coombs technique, and a solid-phase radioimmunoassay employing protein A, all reveal that IgG is present on the platelet surface of patients with ATP.

It is possible that there are at least two or more varieties of antiplatelet antibody in this disorder: one with high affinity, which binds to platelets, leads to thrombocytopenia, and correlates with disease activity; another with low affinity, which is found in the serum, binds poorly to platelets, and is present in patients with normal platelet counts and qualitative platelet functional defects. We have previously reported on the heavy-chain subclass of IgG in ATP serum and found it to be restricted to the γG3 subclass, employing monospecific antisera for the neutralization of γG1, γG2, γG3, and γG4. With the advent of quantitative techniques for the measurement of bound antiplatelet antibody, it became possible to examine the IgG subclasses of bound antiplatelet IgG.

This article reports our observations on the IgG subclasses of bound antiplatelet IgG in patients with severe autoimmune thrombocytopenic purpura. In contrast to that found in the sera of patients with ATP, bound antiplatelet antibody was found to have the same IgG subclass distribution as that found in normal serum.

MATERIALS AND METHODS

Six patients were studied with classical autoimmune thrombocytopenic purpura, who were known to have high levels of bound antiplatelet IgG. There was no history of neoplasm, sepsis, underlying disease, or drug ingestion. Bone marrow aspirates revealed increased megakaryocytes. ANA preparations for SLE were negative.

Preparation of Platelets

Blood was collected into EDTA Vacutainer test tubes (Becton-Dickinson Co., Rutherford, N.J.) and centrifuged at 150 g for 20 min at 4°C. The platelet-rich plasma (PRP) was removed and sedimented at 2000 g for 15 min. The platelet pellet was suspended in 5 ml of 1% ammonium oxalate (to remove red blood cells) and maintained at room temperature for 5 min. The platelets were then centrifuged and resuspended in 5 ml of a human Ringer–2 mM EDTA, pH 7.1, wash solution. This procedure was repeated thrice, prior to suspension in 1 ml of the same solution containing 10 mM benzamidine. A manual platelet count was obtained with phase optics.

Preparation of Soluble IgG-Platelet Extract

The platelet suspension was disrupted by four cycles of freeze-thawing in dry-ice acetone and 37°C water bath, and then sonicated in a Sonifer cell disruptor (Heat Systems–Ultrasonics, Plainview, N.Y.) at maximal intensity for 15 sec. The disrupted platelet...
BOUND ANTIPLATELET IgG SUBCLASSES IN ATP

Suspension was then centrifuged at 20,000 g for 20 min at 4°C, and the supernatant was employed for IgG detection or stored at −20°C. This supernatant fraction was shown to contain 7S IgG as determined by sucrose gradient analysis with known standards. Similar results were obtained following centrifugation at 100,000 g for 1 hr.

**Solid-Phase Radioimmunoassay**

This was performed, as described previously, employing rabbit anti-human IgG “sandwiched” to 125I-staphylococcal protein A. Briefly, the supernatant containing 7S IgG is applied in serial dilution to the wells of a plastic microtiter plate capable of adsorbing protein. Commercial rabbit anti-human IgG (γ heavy-chain specific) was obtained from Miles Research Products, Elkhart, Ind., and applied at a titer of 1:80. Monospecific anti-IgG subclass antisera were prepared as described below and applied at a titer of 1:4 for γG1, 1:8 for γG2, 1:20 for γG3, and 1:4 for γG4. Excess antibody was removed by washing with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline, 0.01 M, pH 7.4. 125I-staphylococcal protein A was then applied, 10,000 cpm/well, and incubated for 2 hr at room temperature. The wells were again washed, separated from their plastic plate, and assayed for radioactivity. Controls were run in the absence of platelet extract IgG and had “background” counts of 200–250 cpm for all subclass antisera. Similar counts were obtained when IgM or albumin was employed instead of IgG, as well as when rabbit anti-human IgM antibody was employed. Serial dilutions were assayed in duplicate.

A standard curve of highly purified IgG (Miles Research Products, Elkhart, Ind.) was run simultaneously with each group of platelet extracts. A standard curve of γG subclass IgG was constructed for each antisera from a commercially obtained Cohn fraction (Pentex, Elkhart, Ind.) that contained 70% γG1, 18% γG2, 9% γG3, and 3% γG4. Platelet IgG was expressed as nanograms per 10^6 platelets.

The mean platelet IgG obtained from 10 pooled controls (4–6 subjects per group) gave a value of 14.2 ± 3.7 (SD) ng/10^6 platelets. Five thrombocytopenic patients of nonautoimmune origin gave a value of 9.7 ± 3 ng/10^6 platelets. Percent subclass IgG of total IgG is given in parentheses.

**Iodination of Staphylococcal Protein A**

Staphylococcal protein A was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Na[125]I (17 Ci/mg) was obtained from New England Nuclear, Boston, Mass. Protein A was iodinated by the chloramine-T method, as described previously.

**Monospecific Antisera for IgG Subclasses**

Specific antisera directed against human heavy-chain subclasses were developed in monkeys and rabbits. The anti-sera were absorbed as necessary to make them specific with myeloma proteins coupled to Sepharose, to bromoacetyl cellulose, or “tree” myeloma proteins. Specificity was determined by immunoelectrophoresis, double diffusion in agar, and radial immunodiffusion. Antisera were then precipitated with 50% saturated ammonium sulfate and dissolved in their original volume of phosphate-buffered saline (PBS). These fractions were tested for their ability to specifically agglutinate cells coated with myeloma proteins of the appropriate subclass. The antisera were equally potent in their ability to agglutinate specifically sensitized red blood cells, but the anti-γG3 was somewhat more potent when employed in radial immunodiffusion.

**RESULTS**

The data in Table 1 indicate the total IgG/10^6 platelets as determined with rabbit anti-human γ heavy-chain antibody, and the subclass IgG/10^6 platelets as determined with monospecific antisera. The percent distribution of the subclasses are the same as that found in normal sera.

**DISCUSSION**

The binding of specific IgG heavy-chain subclasses to red blood cells are of relevance with regard to cell destruction. γG1 and γG3 fix complement and bind to Fc receptors of monocytes, whereas γG2 and γG4 are considerably less effective. Therefore, red blood cells coated with γG1 or γG3 are more likely to be destroyed by the reticuloendothelial system than are cells coated with γG2 or γG4. It was therefore of interest to find γG3 antiplatelet antibody in the sera of patients with ATP. This was in contrast to our studies.

<table>
<thead>
<tr>
<th>Patient (Age/Sex)</th>
<th>Total IgG</th>
<th>γG1</th>
<th>γG2</th>
<th>γG3</th>
<th>γG4</th>
<th>Subclass IgG</th>
</tr>
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<tbody>
<tr>
<td>65 F</td>
<td>1,124</td>
<td>1,000</td>
<td>70</td>
<td>50</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(89)</td>
<td>(6)</td>
<td>(4)</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>70 F</td>
<td>958</td>
<td>630</td>
<td>120</td>
<td>125</td>
<td>90</td>
<td>960</td>
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<tr>
<td></td>
<td></td>
<td>(86)</td>
<td>(12)</td>
<td>(13)</td>
<td>(9)</td>
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<tr>
<td>35 F</td>
<td>6,500</td>
<td>4,350</td>
<td>1,300</td>
<td>700</td>
<td>130</td>
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<tr>
<td></td>
<td></td>
<td>(67)</td>
<td>(20)</td>
<td>(11)</td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>25 M</td>
<td>750</td>
<td>440</td>
<td>160</td>
<td>90</td>
<td>30</td>
<td>720</td>
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<tr>
<td></td>
<td></td>
<td>(56)</td>
<td>(18)</td>
<td>(13)</td>
<td>(4)</td>
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</tr>
<tr>
<td>40 F</td>
<td>1,372</td>
<td>710</td>
<td>420</td>
<td>160</td>
<td>70</td>
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<tr>
<td></td>
<td></td>
<td>(51)</td>
<td>(31)</td>
<td>(12)</td>
<td>(5)</td>
<td></td>
</tr>
<tr>
<td>21 M</td>
<td>800</td>
<td>470</td>
<td>300</td>
<td>10</td>
<td>0</td>
<td>760</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(58)</td>
<td>(37)</td>
<td>(2)</td>
<td>(0)</td>
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</tr>
<tr>
<td>Average percent ± SEM</td>
<td>65 ± 5</td>
<td>21 ± 4</td>
<td>9 ± 2</td>
<td>4 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent in normal serum</td>
<td>70 ± 2</td>
<td>18 ± 3</td>
<td>8 ± 6</td>
<td>3 ± 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The normal level of total IgG/platelet is 14.2 ± 3.7 (SD) ng/10^6 platelets. Percent subclass IgG of total IgG is given in parentheses.
on the serum of systemic lupus erythematosus (SLE) patients, performed at the same time as our studies on the sera of ATP patients (with the same monospecific antisera), which revealed that all 4 γG subclasses were involved.

Our present observations on bound antibody IgG subclasses are not necessarily in disagreement with our earlier report, which examined serum antiplatelet antibody. It is reasonable to hypothesize that circulating antibody with, a priori, “lower affinity” may be restricted to the γG3 subclass, and may conceivably affect platelet function, whereas “high affinity” antibody, which is bound to the platelet, may contain all of the γG subclasses and affect platelet clearance.

It is of interest to postulate why γG3 may be the apparent predominant antiplatelet antibody in the serum, in contrast to that noted with bound antiplatelet antibody where all IgG subclasses were found. γG3 is known to aggregate easily. The hinge region of the γG3 molecule has recently been shown to be rich in sulfhydryl groups, which perhaps lead to preferential aggregation of γG3, following mild reduction and oxidation. It has previously been demonstrated that aggregated IgG preferentially binds to the Fc receptor and causes serotonin release. It is therefore possible that the technique employed for the measurement of serum antiplatelet antibody, the PF-3 immunoinjury technique, could have been measuring aggregated γG3 (either platelet specific or nonspecific). This would require, however, that patients with ATP have more γG3 or a plasma-serum environment that enhances γG3 aggregation. In this regard, it is of interest that approximately 20% of patients with ATP have elevated IgG levels.

An alternative possibility is that most of the γG subclasses found on the platelet membrane reflect nonspecific binding of immunoglobulin G. This could occur by either of two mechanisms. Immune complexes could bind nonspecifically to the platelet Fc receptor. In this regard, circulating immune complexes have recently been reported in ATP. It is of interest that patients with SLE and known immune complexes have a platelet “injury factor” composed of all the IgG subclasses. A second mechanism could result from platelet membrane injury by specific antiplatelet antibody (?γG3), which could conceivably promote the accessibility of membrane Fc receptors.

The manner in which all the subclasses of IgG bind to platelets has not been established. The applied technology does not differentiate IgG binding by its Fc receptor (as would be the situation with immune complexes) from IgG binding by its Fab domain. Experiments designed to answer this question are currently in progress.

ACKNOWLEDGMENT

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