Platelet-Bound IgM in Autoimmune Thrombocytopenia

By Johannes D. Nel, Kenneth Stevens, Aafke Mouton, and François J. Pretorius

Elevated levels of platelet-bound IgG (PA-IgG) are a feature of autoimmune thrombocytopenia (ATP), but it is well documented that this does not occur in all cases. This has led us to investigate the role of platelet-bound IgM (PA-IgM) in these patients using a quantitative enzyme-linked immunosorbent assay (ELISA). Forty-five determinations of PA-IgM and PA-IgG were done on 24 patients with ATP. Elevated levels of PA-IgM were found in 93.3% of the determinations, while PA-IgG was elevated in only 71.7.

CONSIDERABLE DIFFICULTY has been experienced in developing reliable methods for the detection and quantitation of platelet antibodies in autoimmune thrombocytopenia (ATP). In autoimmune hemolytic anemia, the immunoglobulins coating the red cell are readily detected by the Coombs test, but this technique is unsuitable for platelets since normal platelets aggregate spontaneously. It is clear that the use of highly accurate but more complicated quantitative methods is justified in investigating ATP.

The presence on the platelet surface of Fc receptors makes it difficult to distinguish between immune complexes adhering to these receptors and true autoantibodies attached to antigens. In the indirect methods used for quantitation of antibodies, this problem is overcome by the use of paraformaldehyde-fixed platelets but this procedure cannot be used in the direct methods of quantitation.1

The injurious factor in ATP has been identified as a 7S IgG,2 which was originally shown to be IgG3, but recently all subclasses have been incriminated.4 The role of the immunoglobulin class IgM in ATP has been considered unimportant in the past.16 Recently, however, in 80 patients with ATP, 17 were described with elevated platelet-associated IgM (PA-IgM) coexisting with elevated platelet-associated IgG (PA-IgG). In another 4 patients, elevated PA-IgM alone was found.7 Using the same semiquantitative fluorescent-labeled antiglobulin technique, others have reported the coexistence of increased PA-IgG and PA-IgM in a single patient with ATP.8

Out study deals with PA-IgG and PA-IgM in ATP showing that increased levels of PA-IgM are at least as common a phenomenon as increased PA-IgG in patients with chronic ATP.

MATERIALS AND METHODS

Patients

Forty-five determinations of PA-IgG and PA-IgM were performed on 24 patients with autoimmune thrombocytopenia. All patients at some time during the course of the illness had thrombocytopenia, normal or increased megakaryocytes in the bone marrow, normal hemoglobin and white cells, negative LE cell preparations and no demonstrable antibodies against DNA. Twenty-five healthy volunteers, mostly laboratory staff, were used to establish the normal values. Normal controls were run with each patient determination. Fifteen patients with thrombocytopenia associated with conditions in which an immune basis for the thrombocytopenia was not proved were also included in the study. This group consists of patients with acute leukemia (2), myelofibrosis (2), lymphoma (3), Cushing's syndrome (1), hypersplenism (1), acute hepatitis (1), bone marrow hypoplasia (4), and familial thrombocytopenic thrombopathia (1).

Preparation of Platelets

Blood (30 ml) was collected into glass tubes using EDTA as anticoagulant. Platelets were separated by differential centrifugation (200 g for 10 min). The platelet-rich plasma was then centrifuged at 750 g for 30 min and the residual red cells in the platelet button were lysed with 3 ml cold ammonium oxalate (10 g/liter). The platelets were washed 4 times (750 g for 30 min) in 0.15 M phosphate-buffered saline (PBS) pH 6.5 to reduce platelet aggregation.9 After the last wash, the platelet button was resuspended in complement fixation test buffer (CFT) containing 5% rabbit serum (CFT tablets obtained from Oxoid). The platelets were counted by phase-contrast microscopy, ensuring that no platelet aggregates were present and that the white cell contamination was less than 0.1%. The platelet counts were adjusted to 10 x 10^9/liter. Tests were usually done on the day of receiving the specimens. In a few instances, the platelets were stored at 4°C, but all tests were done within 48 hr.

Measurement of Platelet-Associated Immunoglobulins

Principles of the Original Method for IgG and C3

A modification of the enzyme-linked immunosorbent assay method (ELISA) as previously described was used.16 This is an

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antiglobulin consumption technique where peroxidase-conjugated specific antisera are reacted with platelets. The unbound fraction of the antisera is adsorbed onto serum-coated polystyrene balls (6.5 mm in diameter) suitable for the purpose. The details and the preliminary tests to exclude nonspecific binding have been reported previously.\textsuperscript{9} The unbound fraction attached to the polystyrene ball is then quantitated using an enzyme substrate color reaction, the intensity of the color produced being inversely related to the amount of IgG or C3 that was present on the platelets originally. By using titration curves with known quantities of IgG and C3, the amount of protein bound to the platelets was accurately quantitated. With this method, normal values of \(1.3 \pm 15.5\) ng IgG/10\(^6\) platelets and 0.22-0.96 ng C3/10\(^6\) platelets were obtained and a 2-30-fold increase in PA-IgG in patients with ATP was obtained.\textsuperscript{9} Recently, Hedge et al., who developed a similar technique to determine the level of PA-IgG, confirmed these findings.\textsuperscript{11}

Development of the PA-IgM Determinations

**Conjugated Reagents**

Horseradish peroxidase conjugated anti-human IgG and IgM antisera (rabbit) were used, the specificity of which is guaranteed by the manufacturers (Dakopatts, Copenhagen, Denmark).

**The Procedure**

**Titration Curves**

Separate doubling dilutions for IgG and IgM using a standard human serum (Behringwerke) were set up in duplicate in 65 mm × 11 mm glass tubes in 0.1 ml volumes of CFT buffer containing 5% rabbit serum. The concentration ranged from 400 ng to 3 ng per tube for IgG and from 80 ng to 0.3 ng per tube for IgM. For each curve, blank tubes and 100% free antibody tubes were included.

**Test Platelets**

A quantity of 0.1 ml platelet suspensions (10 × 10\(^6\)/liter) were set up in triplicate for both the IgG and IgM determinations. Of optimally diluted enzyme-linked antibody in CFT buffer containing 5% rabbit serum 0.2 ml was added to all tubes except the blanks. The optimum dilution for anti-IgG was 1:1000. For anti-IgM, a 1:2000 dilution was used to determine the normal values and a 1:1200 dilution for all the tests done on the patients with ATP, nonimmune thrombocytopenic patients, and normal controls (see Fig. 1).

All tubes were incubated for 2 hr in a 37°C waterbath. An antigen-coated polystyrene ball was then added to each tube and incubated for a further 2 hr. All tubes containing the balls were then washed 4 times with 0.15 M PBS, pH 7.2, by filling the tubes and then sucking them dry with a Pasteur pipette attached to a suction pump. One millilitre of freshly prepared substrate (see below) was then added to each tube. After 20 min, the reaction was stopped by adding 0.25 ml 2M H\(_2\)SO\(_4\) to each tube in the same order as the substrate had been added. The color produced was read in a Beckman model 24 spectrophotometer at 550 nm.

**The Substrate Solution**

A 0.1 M phosphate substrate buffer, pH 5.0, was used: 40 mg Orthophenylenediamine (Sigma) was dissolved in 100 ml buffer and 0.15 ml 30% H\(_2\)O\(_2\) was then added.

**Calculations**

The amount of the antiserum bound in the standard dilutions and onto the platelets was determined from the formula:

\[
\text{Percent antibody} = \frac{\text{OD test} - \text{OD blank}}{\text{OD maximum} - \text{OD blank}} \times 100
\]

The duplicate values for each dilution were averaged and then plotted as concentration (log scale) against the percent antibody binding.

The quantity of bound PA-IgG and PA-IgM was read from the standard curve after the mean percent bound antibody for the three tubes had been calculated. The advantage of having three platelet tubes is that in the event of spontaneous platelet aggregation developing during the washing procedure (a known tendency of platelets), this would not only result in a false platelet count but the aggregates would also be unequally distributed between the three tubes. The results would then differ widely, and the specimen would be recognized as being unsuitable.

**RESULTS**

The results for the PA-IgM from the 25 normal controls gave a mean of 2 ng IgM/10\(^6\) platelets with a range (mean ± 2 SD) of 0.5-4 ng IgM/10\(^6\) platelets (antiserum dilution 1:2000). With the 1:1200 diluted antiserum, the normal controls were always below the 4 ng IgM/10\(^6\) platelet level.

Tables 1 and 2 show the results of 45 platelet counts, PA-IgG, and PA-IgM determinations on 24 patients. Forty-two determinations (93.3%) showed an elevated PA-IgM and 32 (71.7%) an elevated PA-IgG. In 29 of the determinations (64.4%), both PA-IgG and PA-IgM were elevated. In 13 of the determinations (11 patients), i.e., 28.9% of all determinations, only elevated PA-IgM was present, but 7 of these 11 patients had elevated PA-IgG and PA-IgM values at some other period in the course of their illness (Table 1). Whether this finding was present in a further 3 patients of this group could not be determined, since only a single set of investigations was available. The 11th patient had an elevated PA-IgM without an elevated PA-IgG on 2 occasions while in remission. On only 3 occasions could elevated PA-IgG values without elevated PA-IgM values be shown (6.7% of all determinations). Overall, 93.3% of PA-IgM values were elevated as opposed to only 71.7% of PA-IgG values. Of all PA-IgM determinations 82.2% were at least elevated as opposed to only 82.2% of all determinations. Figure 2 illustrates the results of PA-IgG and PA-IgM determinations on the 15 patients with thrombocytopenia not due to ATP. Patients who had previously had ATP and who were in remission with normal PA-IgG and PA-IgM levels were not included in the study.

**Specificity Check on the Anti-IgM Antiserum**

Because ATP is associated with raised PA-IgG, the possibility that the raised PA-IgM levels were due to anti-IgM cross-reacting in vitro with IgG had to be excluded. Pure freeze-dried IgG (obtained from Pell Freeze) was reconstituted and diluted to concentra-
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Fig. 1. The dose-response curves at different dilutions of the conjugated anti-IgM antibody. The dotted line intersects the different curves at the 4-ng level, which is the upper limit of normal for PA-IgM. (©) Antiserum diluted 1:2000: the curve has a steep slope between 0.5 and 4 ng IgM, making it unsuitable for use in patients with ATP but suitable for establishing normal values. (©) Antiserum diluted 1:1200: the optimal dilution for use in patients with ATP, the accurate range being 4–40 ng/10⁶ platelets. (©) Antiserum diluted 1:500. Only values higher than 40 ng IgM/10⁶ platelets can be read with accuracy, limiting its use to strongly sensitized platelets.

The dose-response curves at different dilutions of the conjugated anti-IgM antibody. The dotted line intersects the different curves at the 4-ng level, which is the upper limit of normal for PA-IgM. (©) Antiserum diluted 1:2000: the curve has a steep slope between 0.5 and 4 ng IgM, making it unsuitable for use in patients with ATP but suitable for establishing normal values. (©) Antiserum diluted 1:1200: the optimal dilution for use in patients with ATP, the accurate range being 4–40 ng/10⁶ platelets. (©) Antiserum diluted 1:500. Only values higher than 40 ng IgM/10⁶ platelets can be read with accuracy, limiting its use to strongly sensitized platelets.

tions 2–30 times greater than the normal values that would occur on 10⁶ platelets. Such levels are comparable to those found in patients with ATP. These concentrations were subsequently used in place of the platelets and tested under the same laboratory conditions. In all these experiments, the anti-IgM antiserum gave zero readings at all concentrations of IgG.

DISCUSSION

The development of sensitive assays for quantitating PA-IgG has shown that the level is increased in most patients with ATP, although normal values are encountered in some patients with otherwise typical ATP. Here we record elevated PA-IgG levels in 71.9% of determinations. This agrees with the 67% of elevated levels reported recently using a similar ELISA method. However, several investigators, reviewed by McMillan, have found elevated levels of PA-IgG in as many as 90% of patients. In determining PA-IgM in ATP, raised levels have been found less frequently, and in the study of von dem Borne et al. using a fluorescence technique for the detection of PA-IgM, 21 positive results in 80 patients with ATP were found, that is 26.2% of the determinations. The lower sensitivity of the technique might explain the low percentage of patients with detectable PA-IgM.

In our series, the 93.3% elevated PA-IgM levels suggest that PA-IgM determinations are as important as PA-IgG determinations in the diagnosis and surveillance of patients with ATP. Our finding of 28.9% of increased PA-IgM determinations in patients with normal PA-IgG may account for the "antiplatelet factor" suggested to explain the discrepancy between PA-IgG levels and platelet survival in some patients reported recently.

As can be seen from Table 1, the fluctuation of the raised PA-IgG and PA-IgM values follows no definite pattern. Patient Pe, who has been followed for 5 yr and who has never been in remission, shows, over a period of 22 wk, wide fluctuations in the various immunoglobulin classes in spite of the platelet count and therapy remaining constant during this period. There is an
subclasses of IgG are involved and not only IgG3 as due to different responses to the subclasses of IgG, a previously reported. In addition, some patients with giving rise to steric hinderance. seemingly random fluctuations are difficult to explain similar situation possibly being present in ATP, since it correlate with the amount of hemolysis. This may be previously. We have not been able to confirm this but might be an artefact due to the large IgM molecule at still other times both values are elevated. These elevated, at other times the PA-IgM is elevated, while has recently been shown that, as in AIHA, all the immunoglobulin classes, i.e., sometimes the PA-IgG is positivity in immune hemolytic anemia (AIHA) where the degree of inverse correlation between PA-IgG levels and An

Table 1. The Results of 30 Platelet Counts, PA-IgG, and PA-IgM Determinations Done on 9 Patients

<table>
<thead>
<tr>
<th>Time</th>
<th>Patient</th>
<th>Platelet Count 10^9/Liter</th>
<th>PA-IgG ng/10^6 Platelets</th>
<th>PA-IgM ng/10^6 Platelets</th>
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<td>3</td>
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<td>Pe</td>
<td>6</td>
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<td>6</td>
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<td>39</td>
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apparently unpredictable fluctuation of these two immunoglobulin classes, i.e., sometimes the PA-IgG is elevated, at other times the PA-IgM is elevated, while at still other times both values are elevated. These seemingly random fluctuations are difficult to explain but might be an artefact due to the large IgM molecule giving rise to steric hinderance. An inverse correlation between PA-IgG levels and platelet count in patients with ATP was found previously. We have not been able to confirm this finding and a comparison might be made with autoimmune hemolytic anemia (AIHA) where the degree of positivity of the Coombs reaction does not necessarily correlate with the amount of hemolysis. This may be due to different responses to the subclasses of IgG, a similar situation possibly being present in ATP, since it has recently been shown that, as in AIHA, all the subclasses of IgG are involved and not only IgG3, as previously reported. In addition, some patients with ATP show normal levels of PA-IgG. Controversy still exists concerning the role of complement activation on the platelet membrane in ATP. Increased levels of C3 were reported in some studies, while we and others were unable to confirm this. The suggestion of a subset of patients with ATP in which the levels of platelet-associated C3 is elevated in the absence of increased PA-IgG complicates the issue further. Finally, host factors such as the compensated thrombolytic state must also be considered. In view of the complex situation that exists with many variables, it is unlikely that the platelet count would show a good inverse correlation with the level of a single immunoglobulin class.

In AIHA, the coexistence of IgG with IgM autoantibodies is uncommon. The chemical nature of the red cell antigens determines to a large extent the type of immune response and the different chemical nature of the antigens involved in ATP might thus be the reason for the nonparallel phenomenon. At present, it is not clear if PA-IgG in ATP is directed against a platelet antigen or whether it forms part of an immune complex, the IgG attaching to the platelet via its Fc receptor. In spite of evidence being presented in favor of immune complexes, there are strong indications that the antibody binds directly to a platelet antigen. The question now arises whether the IgM is directed against platelet membrane antigens or not. The relative roles of IgM and IgG in the pathogenesis of the disorder must still be elucidated. In this regard, the greater agglutinating power of IgM as compared to IgG might be important in determining the fate of the platelets.

A further possibility that must be considered is whether the IgM antibodies are directed against the
PA-IgG, acting as a rheumatoid-factor-type antibody. Rheumatoid factor can attach to various sites on the IgG molecule, the Fc site being the most frequent. After IgG has bound to its antigen, certain antigenic sites are apparently exposed. Rheumatoid factor can then attach to these and this forms the basis of the Rose Waaler test for rheumatoid factor. Rheumatoid factors with an antidiotypic specificity against the variable parts of the light and heavy chains have been described in lymphoproliferative disorders where they may possibly play a modulating role. It is of interest that rheumatoid factor can be anticomplementary, and it has been shown to be the result of a critical balance between IgG and anti-IgG IgM.

In a recent comprehensive study where PA-IgG determinations were done in 298 patients with various disorders including patients with ATP, it was concluded that increased PA-IgG was not confined to ATP. The hypothesis that increased PA-IgG is pathognomonic of immunologically mediated thrombocytopenia ought to be questioned. The results of the PA-IgG and PA-IgM determinations in the patient group with thrombocytopenia not due to ATP shown in Fig. 2 supports this view not only for PA-IgG but also for PA-IgM.

In the past, stress has been laid on the presence of PA-IgG in ATP. With this article we emphasize the hitherto unrecognized frequent involvement of PA-IgM in the complicated situation that exists.

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