Neonatal Alloimmune Thrombocytopenia: Detection and Characterization of the Responsible Antibodies by the Platelet Immunofluorescence Test

By Albert E. G. Kr. von dem Borne, Eleonore F. van Leeuwen, L. Elly von Riesz, Chris J. van Boxtel, and C. Paul Engelfriet

Platelet immunofluorescence, together with other serologic tests on platelets, lymphocytes, and granulocytes, was used to investigate the sera of 38 mothers with newborns who suffered from thrombocytopenia. In sera of 33 mothers, platelet-specific IgG alloantibodies were demonstrable. Three sera also contained HLA antibodies, of which two were only detectable in the lymphocyte cytotoxicity test. Two other sera contained granulocyte-specific alloantibodies. In sera of 2 mothers, antibodies were found that reacted with all cell types in all tests. However, after further analysis, it became clear that platelet-specific alloantibodies were probably also present in these 2 sera. In 29 cases, the specificity of the platelet alloantibodies was anti-Zw* = PI*. One serum contained antibodies directed against a new antigen, Bk*. This new antigen was defined after the investigation of the family and a small-scale population study. Two other sera had platelet antibodies with still undefined specificities. In all positive sera, IgG platelet alloantibodies were detected, and sometimes IgM antibodies were also present. The IgG antibodies were mostly of the IgG1 subclass, but sometimes IgG3 and/or IgG4 was also found. In a few sera, only IgG3 antibodies were detected. In our series, we found no increased frequency of blood group ABO compatibility between mother and child, although it has been described by others and is well known to occur in rhesus alloimmunization. Of all the tests used, the platelet immunofluorescence test, especially the test on paraformaldehyde-fixed platelets in suspension, gave the best results in the detection of platelet antibodies in neonatal alloimmune thrombocytopenia.

Neonatal alloimmune thrombocytopenia (NAITP) is caused by immunization of the mother against antigens present on the platelets of her child. The antibodies belong to the IgG class, are most directed against platelet-specific antigens, and usually show anti-Zw* = PI* specificity. Occasionally, the antibodies seem to have anti-HLA specificity.

For the serologic detection of IgG HLA antibodies, sensitive methods are available, such as the lymphocyte cytotoxicity test and the immunofluorescence test on lymphocytes. However, the detection of platelet-specific antibodies is often difficult. Such antibodies do not cause platelet agglutination and seldom bind complement. The only methods currently available for the detection of this kind of antibody are the antiglobulin consumption test and the blocking test with platelet-specific complement-fixing antiserum. The former technique is insensitive. Limitations of the latter technique are that platelet-specific complement-fixing antisera are scarce and that antibodies with a specificity other than that of these antisera cannot be recognized.

Recently, Soulier et al. developed a radioactive indirect antiglobulin test and showed that this test could be applied for the detection of platelet-specific IgG antibodies in sera from mothers with a newborn with NAITP. For the same purpose, platelet immunofluorescence microphotometry and recently immunofluorescence on paraformaldehyde-fixed platelets in suspension were introduced into our laboratory.

In this report the results obtained in cases of NAITP with both fluorescence techniques are described and compared with those obtained with other serologic methods.

MATERIALS AND METHODS

Patients

In typical cases of NAITP, the mother is healthy and does not suffer from thrombocytopenia. Pregnancy and labor are uncomplicated. The newborn is apparently normal, but develops thrombocytopenic purpura shortly after delivery. The disease is transient, and no other perinatal pathology is present.

Only cases that fulfilled these criteria are included in this report. A history of neonatal purpura in older siblings was taken as strong further evidence in favor of the diagnosis. Altogether 38 cases were evaluated. In 6, there was a history of one or more affected siblings.

Sera

In all 38 cases, the mother's serum, obtained within 2 wk after delivery, was investigated. Cord serum from the newborn was seldom available.

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Cells

The serum of the mother was tested with lymphocytes, granulocytes, and platelets from the father and from random blood group 0 donors. When ABO incompatibility existed between the serum of the mother and the cells of the father, the serum was absorbed with normal blood group A and/or B red cells, or with AB substance, before use. Cells from the baby were not tested routinely, because a sufficient quantity of fresh blood was hardly ever available. This was not a major limitation of our study, because in The Netherlands, paternity is seldom questionable (Nijenhuis, personal communication). The specificity of platelet antibodies was determined with platelets obtained from donors typed for the Zw and Ko antigens. The specificity of HLA antibodies was tested with a panel of 35-39 HLA-A, B, and C typed lymphocytes.

Lymphocytes and platelets were isolated from heparinized blood, granulocytes, and platelets from EDTA blood.

Reagents

For the immunofluorescence test (IFT) on platelets, we used polyspecific sheep anti-human Ig labeled with fluorescein isothiocyanate (FITC) (code number SH 17-01-F08), monospecific rabbit anti-human IgG1 (KH 161-51-A1), anti-IgG2 (KH 162-05-A3), anti-IgG3 (KH 163-45-A1), and anti-IgG4 (KH 164-46-A1), in conjunction with horse anti-rabbit Ig labeled with FITC (PK 17-2-F4), all products from our own laboratory. The preparation of the IgG subclass-specific antisera has been described elsewhere. For the IFT on lymphocytes, we used monospecific rabbit anti-human IgG labeled with FITC (KH 16-103-F4), and for the IFT on granulocytes, F(ab)2 fragments of polyspecific sheep anti-human anti-immunoglobulin labeled with FITC (S26-H17-1-F3), also a product of our laboratory. The optimal dilution of the antoglobulin reagents was determined by chessboard titration with various dilutions in saline of antibody-containing sera and normal AB sera. The optimal dilution was the dilution that gave maximal fluorescence with the antibody-containing sera, but no fluorescence with the normal AB sera.

For the antoglobulin consumption test, we used a specially selected polyclonal rabbit anti-human IgG serum, produced in our laboratory. This serum had a titer of 1:512 in the antiglobulin test with IgG anti-D-sensitized rhesus D-positive (OR,R) red cells in a 5% suspension in saline. The IgG anti-D serum used was obtained from a rhesus-negative volunteer, who had been immunized with rhesus D-positive (OR,R) red cells.

For the complement-fixation test, sheep red cells, rabbit anti-sheep hemolysins, and guinea-pig complement from the Netherlands National Institute of Health were used in dilutions and proportions prescribed by the manufacturer.

Platelet Agglutination Test (PAT)\textsuperscript{17,18}

Platelets, obtained from EDTA blood by differential centrifugation, were washed three times with EDTA-PBS (9 mM Na\textsubscript{2}HPO\textsubscript{4}, 26.4 mM Na\textsubscript{2}HPO\textsubscript{4}, 140 mM NaCl, pH 6.8-7.0) and resuspended in this medium to a concentration of 5 x 10\textsuperscript{5} /liter. EDTA was added to the serum under investigation (EDTA serum: 9 volumes of serum and 1 volume of 55 mM Na\textsubscript{2}EDTA in PBS). One drop of platelet suspension and two drops of serum were mixed in the wells of a Kline plate (Cotig, Paris), covered with a plastic cover, and rotated on a Kline shaker at 80 rpm for 30 min at 4°C. The reaction was read microscopically and graded from 0 to 4+.

Platelet Antiglobulin Consumption Test (PAGCT)\textsuperscript{19}

A pellet of 1.5 x 10\textsuperscript{5} platelets from EDTA blood was washed once with EDTA-PBS and resuspended in 0.2 ml of EDTA serum. The mixture was incubated for 1 hr at 37°C. The platelets were spun down, washed 6 times with EDTA-PBS and resuspended in 0.1 ml of rabbit anti-human IgG. After incubation for 6 min at 20°C and centrifugation, the supernatant was titrated (twofold dilutions) against a 5% (v/v) suspension of IgG anti-D-sensitized rhesus D-positive (OR,R) red cells in saline. A reproducible fall in titer of at least two dilution steps was considered as positive. Normal EDTA serum from blood group AB donors served as a control.

Platelet Complement Fixation Test (PCFT)\textsuperscript{20}

Sera were heat-inactivated and mixed with one part veronal buffer containing Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (3.1 mM diethyl barbituric acid, 1.8 mM sodium diethyl barbiturate, 0.5 mM MgCl\textsubscript{2}, 0.15 mM CaCl\textsubscript{2}, 147 mM NaCl, pH 7.4). Platelets from heparinized blood were washed three times in saline (150 mM NaCl) and suspended in this solution to a concentration of 2 x 10\textsuperscript{5} /liter. A quantity of 0.09 ml of twofold dilutions of guinea-pig serum in veronal buffer, 0.03 ml serum, and 0.03 ml platelet suspension, were mixed and incubated for 1 hr at 37°C. To this incubation mixture, 0.03 ml of 3% (v/v) suspension of sensitized sheep red cells in veronal buffer was added. After another incubation for 30 min at 37°C, the mixture was centrifuged and the percentage of hemolysis measured photometrically.

Platelet Immunofluorescence Microphotometry (PIFMP)\textsuperscript{12,13}

Platelets from EDTA blood, washed in EDTA-PBS, were suspended in PBS to a concentration of 3 x 10\textsuperscript{5} /liter. One drop of platelet suspension was put on a slide, dried with a fan at 20°C and fixed for 10 min with acetone. The platelets were incubated with three drops of serum for 30 min at 37°C, in a wet plexiglass chamber. The slides were washed three times in PBS for 5 min at 20°C. Three drops of optimally diluted (see under Reagents) FITC-labeled antoglobulin were applied, followed by another incubation for 30 min at 20°C, and washing. The preparations were mounted in glycerol-PBS (three parts of glycerol and one part of PBS) and covered with a coverslip. Fluorescence per platelet was measured with a Leitz Orthoplan microscope, with a Xenon lamp and a Leitz phasemeter attachment. Fluorescence of 20 platelets was measured and the mean calculated. Normal AB serum was used as a control. Fluorescence values of more than twice the coefficient of variation above the mean value of normal serum were considered as positive.

Platelet Suspension Immunofluorescence Test (PSIFT)\textsuperscript{14}

Platelets prepared from 10 ml of EDTA blood, washed in EDTA-PBS, were fixed in 3 ml of 1% (w/v) paraformaldehyde for 2 min at 20°C. After fixation, the platelets were washed again twice and resuspended in EDTA-PBS, containing 0.2% (w/v) bovine serum albumin (BSA), to a concentration of 4 x 10\textsuperscript{8} /liter. Two drops of serum (0.1 ml) and two drops of platelet suspension were mixed and incubated for 30 min at 20°C or 37°C. The platelets were again washed three times with EDTA-PBS-BSA and incubated with two drops of optimally diluted FITC-labeled antoglobulin for 30 min at 20°C. After two more washings with EDTA-PBS-BSA, the platelets were resuspended in one drop of glycerol-PBS, mounted on a slide, covered, and examined under the fluorescence microscope. Normal AB serum served as a control. Fluorescence was graded from 0 to 4+. The test was considered as positive when at least a 1+ reaction
was obtained. With AB serum, a negative reaction was always found.

**Lymphocyte Cytotoxicity Test (LCT)**

Lymphocytes were prepared from heparinized blood by carbonyl-iron treatment, dextran sedimentation of the red cells, and Ficoll-Isopaque gradient centrifugation, as previously described. The test was performed with the NIH standard method.

**Lymphocyte Immunofluorescence Test (LIFT)**

Lymphocytes, prepared as described above, were washed twice in PBS and resuspended to a concentration of 10⁷/liter. Two drops of serum and two drops of lymphocyte suspension were mixed and incubated for 30 min at 37°C. After again washing three times in PBS, the lymphocytes were incubated for 30 min at 4°C with optimally diluted FITC-labeled antiglobulin. For the dilution, PBS containing 0.1 M Na2Na2 (PBS-NaNa2) was used. After two washings with PBS-NaNa2 at 4°C, four drops of paraformaldehyde 1% (w/v) in PBS were added, followed by another incubation for 5 min at 20°C. After two more washings, the lymphocytes were resuspended in one drop of glycerol-PBS, mounted on a slide, covered, and examined under the fluorescence microscope. Normal AB serum served as a control. A test was considered to be positive when more than 30% of the lymphocytes showed at least a 1+ reaction. In the normal AB serum control, there was always a weak fluorescence of less than 30% of the cells, probably due to monocytes and B lymphocytes present in the lymphocyte suspensions.

**Granulocyte Immunofluorescence Test (GIFT)**

Granulocytes were prepared from EDTA blood by dextran sedimentation of the red cells, Ficoll-Isopaque gradient centrifugation of the leukocyte-rich supernatant, and lysis of the red cells in the granulocyte-rich pellet with NH₄Cl solution. The granulocytes were washed twice in PBS, fixed for 5 min at 20°C with 1% (w/v) paraformaldehyde in PBS, and washed once in PBS-BSA (0.2%, w/v, BSA in PBS). The granulocytes were resuspended in PBS-BSA to a concentration of 10⁶/liter. Two drops of serum and two drops of cell suspension were mixed and incubated for 30 min at 37°C. After three washings with PBS-BSA, the granulocytes were incubated with two drops of optimally diluted FITC-labeled antiglobulin reagent and incubated for 30 min at 20°C. After they had been washed again twice, the granulocytes were suspended in one drop of glycerol-PBS, mounted on a slide, covered, and examined under the microscope. Normal AB serum was again the negative control, which gave no or only very weak fluorescence. The fluorescence was evaluated as outlined above.

**Platelet Typing**

Platelet antigens were typed in the PAT with agglutinating anti-Zw, anti-Ko, and anti-Ko sera and in the PSIFT with nonagglutinating anti-Zw+ sera.

**RESULTS**

**Serologic Findings**

In the first 11 cases of neonatal alloimmune thrombocytopenia (NAITP), the serum from the mother was tested with platelets, lymphocytes, and granulocytes from the father and from random donors, in all the techniques described above (Table 1). In two sera, no antibodies were detected in any of these techniques. With seven sera, a positive immunofluorescence test (IFT) was obtained only with platelets. This indicated that platelet-specific antibodies were present. One serum also contained lymphocytotoxic antibodies. With two sera, positive reactions were found not only with platelets, but also with lymphocytes and granulocytes.

From this first series of investigations, it became clear that for the diagnosis of alloimmunization against platelet-specific or HLA-A, B, and C antigens, the immunofluorescence tests, together with the lymphocytotoxicity test (LCT), were superior to the other techniques, which failed to detect the antibodies in most sera (Table 1). The first 11 sera were tested in 2 platelet IFTs, immunofluorescence microphotometry, and suspension immunofluorescence. Both tests were positive with 8, and negative with 2 sera. With one serum, the microphotometric method gave a negative result, whereas the suspension method was positive. So the latter test appeared to be more reliable than the former. Therefore, we restricted our further studies to the suspension IFT on platelets, the IFTs on lymphocytes and granulocytes, the LCT, and the platelet agglutination test (PAT). The PAT was included, because we had found in earlier studies that this test, although insensitive for the detection of IgG-platelet antibodies, was more sensitive for IgM-platelet antibodies than the platelet IFT.

The results with all sera studied, those of the first series included are summarized in Table 2. To exclude autoantibodies, the serum of the mother was also tested with her own cells, with negative results in all. In the sera of three mothers, no antibodies of any kind were found. In one of the three cases, cord blood serum of the baby was examined as well, with a negative

<table>
<thead>
<tr>
<th>Number of Cases</th>
<th>PAT</th>
<th>PAGCT</th>
<th>PCFT</th>
<th>PIFMP</th>
<th>PSIFT</th>
<th>LAT</th>
<th>LCT</th>
<th>IFT</th>
<th>GIFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No antibodies</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Platelet-specific</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>Polyreactive</td>
</tr>
</tbody>
</table>

*Results are given as the number of sera showing a positive reaction.

Abbreviations are: PAT, platelet agglutination test; PAGCT, platelet antiglobulin consumption test; PCFT, platelet complement fixation test; PIFMP, platelet immunofluorescence microphotometry; PSIFT, platelet suspension immunofluorescence test; LAT, leukoagglutination test; LCT, lymphocyte cytotoxic test; IFT, lymphocyte immunofluorescence test; GIFT, granulocyte immunofluorescence test.
result. In the other two, the platelets of the mother, the father, and the child were typed for the antigens of the Zw and Ko system. In only one, was there Zw<sup>a</sup> incompatibility between the mother, the child, and the father, the mother being homozygous Zw<sup>b</sup>.

In 32 sera, platelet-specific alloantibodies were detected, with a titer ranging from 1/2 to 1/128. In 3 of these 32 sera, alloantibodies reacting with lymphocytes (two in the LCT, one in the LIFT) were also found and in two of them alloantibodies reacting with granulocytes. The granulocyte-specific antibodies were not only demonstrable in the IFT, but also in the leukocyte agglutination test. The leukocyte antibodies in these 3 sera did not interfere in the platelet IFT. Thus, the specificity of the platelet antibodies could be established without preceding absorption of the sera with leukocytes.

With the sera of three mothers, positive reactions were obtained with all three cell types. Thus, the nature of the antibodies here was not immediately clear. In two cases, the mother’s serum was absorbed with the husband’s leukocytes. However, these antibodies were so strong that, even after multiple absorptions, they still reacted with all the three cell types of the husband. Acid eluates of the leukocytes, used for the absorption, also contained polyreactive antibodies. The two sera contained “polyspecific” antibodies as shown in the LCT test with the lymphocytes of our HLA-typing panel. These sera were tested in the LCT and the immunofluorescence test with lymphocytes and platelets from random donors. One serum reacted with the lymphocytes and the platelets from 20 of 25 donors, only with the platelets from one and not with the lymphocytes and the platelets from 4. The other serum reacted with the lymphocytes and the platelets from 10 donors and only with the platelets from 2. This finding indicated that platelet-specific antibodies were also present in these sera. Unfortunately, further studies were not possible, because no more serum was available.

The serum of the third mother contained specific anti-HLA-B7 and B17 (see later). When tested with lymphocytes, granulocytes, and platelets of HLA-B7 and B17 negative donors, only a positive reaction with platelets was found. Thus, it is likely that also this serum contained platelet-specific antibodies. The specificity of these antibodies is still under investigation.

### Specificity of the Platelet-Reactive Alloantibodies

Sera that contained platelet-specific alloantibodies were tested in the PSIFT with a panel of platelets from donors typed for the antigens of the Zw and Ko system. When specific HLA antibodies were present, platelets from HLA-compatible donors were used. The results are shown in Table 3.

In 29 cases, the antibodies were directed against the Zw<sup>a</sup> = Pt<sup>44</sup> antigen. In 28, the platelets of the father were typed and found to be Zw<sup>a</sup>(+) and Zw<sup>b</sup>(-). In 25, the platelets of the mother were also typed and found to be Zw<sup>a</sup>(-), Zw<sup>b</sup>(+). i.e., positive for the allelic antigen Zw<sup>b</sup> = Pt<sup>45,46</sup>. In one other case, the antibodies were too weak to establish specificity. However, it is likely that here too the antibodies were anti-Zw<sup>a</sup>, because the father was Zw<sup>a</sup>(+), Zw<sup>b</sup>(-), the mother Zw<sup>a</sup>(-), Zw<sup>b</sup>(+), and the child Zw<sup>a</sup>(+), Zw<sup>b</sup>(+). Moreover, all three were Ko(b+), Ko(a-).

In one case, the platelet antibodies were directed against a new platelet antigen, which we called Bak'. This new antigen was characterized (by using the serum of the mother in the PSIFT) as the result of the investigation of the family (P.B.) and by determining the frequency of the new antigen in the Netherlands population. The results are shown in Fig. 1 and Table 4.

### Table 2. Reaction Pattern Obtained With the Sera of All 38 Mothers of Newborns With Thrombocytopenia

<table>
<thead>
<tr>
<th>Number of Cases</th>
<th>PAT</th>
<th>PSIFT</th>
<th>LCT</th>
<th>LIFT</th>
<th>GIFT</th>
<th>Specificity Indicated by the Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No antibodies</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>Platelet-specific</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Polyreactive</td>
</tr>
</tbody>
</table>

Abbreviations are: PAT, platelet agglutination test; PSIFT, platelet suspension immunofluorescence test; LCT, lymphocyte cytotoxic test; LIFT, lymphocyte immunofluorescence test; GIFT, granulocyte immunofluorescence test.

In 29 cases, the antibodies were directed against the Zw<sup>a</sup> = Pt<sup>44</sup> antigen. In 28, the platelets of the father were typed and found to be Zw<sup>a</sup>(+) and Zw<sup>b</sup>(-). In 25, the platelets of the mother were also typed and found to be Zw<sup>a</sup>(-), Zw<sup>b</sup>(+). i.e., positive for the allelic antigen Zw<sup>b</sup> = Pt<sup>45,46</sup>. In one other case, the antibodies were too weak to establish specificity. However, it is likely that here too the antibodies were anti-Zw<sup>a</sup>, because the father was Zw<sup>a</sup>(+), Zw<sup>b</sup>(-), the mother Zw<sup>a</sup>(-), Zw<sup>b</sup>(+), and the child Zw<sup>a</sup>(+), Zw<sup>b</sup>(+). Moreover, all three were Ko(b+), Ko(a-).

In one case, the platelet antibodies were directed against a new platelet antigen, which we called Bak'. This new antigen was characterized (by using the serum of the mother in the PSIFT) as the result of the investigation of the family (P.B.) and by determining the frequency of the new antigen in the Netherlands population. The results are shown in Fig. 1 and Table 4.

### Table 3. Specificity of the Platelet-Reactive Antibodies in 35 Cases of Neonatal Thrombocytopenia

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Zw&lt;sup&gt;a&lt;/sup&gt; (anti-Pt&lt;sup&gt;44&lt;/sup&gt;)</td>
<td>28</td>
</tr>
<tr>
<td>Anti-Zw&lt;sup&gt;b&lt;/sup&gt; + anti-HLA-B8</td>
<td>1</td>
</tr>
<tr>
<td>Anti-Bak' + anti-HLA-A3</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>Unknown + anti-HLA-B7 + B17</td>
<td>1</td>
</tr>
<tr>
<td>Indeterminable</td>
<td>1</td>
</tr>
<tr>
<td>Polyreactive</td>
<td>2</td>
</tr>
</tbody>
</table>


distribution of the new antigen, Bak\(^*\), in this family and the Zw and Ko phenotypes of the various members. Lod-score analysis of the P.B. family, and of other families investigated for different reasons, showed that no linkage existed between the gene coding for Bak\(^*\) and the Zw and Ko genes (unpublished results). By typing more than a hundred normal donors, the phenotype frequency of the new antigen was determined. As shown in Table 4, this differed considerably from the phenotype frequency of the Zw and Ko antigens, established by Weerdt et al.\(^{17,18}\) More detailed information about this new antigen will be published elsewhere.

In two further cases, platelet-specific alloantibodies with other, not yet defined, new specificities were found. By typing the platelets for Zw and Ko antigens and for the new Bak\(^*\) antigen, incompatibility for these antigens was excluded. Further genetic studies have still to be done.

Sera that contained lymphocytotoxic alloantibodies were tested in the LCT with the lymphocytes of our HLA-typing panel. The results are shown in Table 3. As mentioned, two sera reacted positively with the majority of the cell samples, which indicated "polyspecificity." Crossabsorption studies were not done. Because no reaction occurred with the mother's own lymphocytes, the presence of autolymphocytotoxins was excluded. One anti-Zw\(^*\) serum and the anti-Bak\(^*\) serum contained specific HLA antibodies, anti-HLA-B8 and anti-HLA-A3, respectively, that were only demonstrable in the LCT and not in the IFT. One serum with platelet-specific antibodies of yet unknown specificity also contained anti-HLA-B7 and B17. In case of the anti-Bak\(^*\) serum and the serum with antibodies of unknown specificity, we performed cross-absorption studies to prove that we were dealing with separate antibodies. Leukocytes from the father removed the HLA antibodies, but not the platelet-specific antigens.

### Table 4. Platelet-Specific Antigens: Frequency in the Netherlands Population

<table>
<thead>
<tr>
<th>System</th>
<th>Antigen</th>
<th>Phenotype Frequency (%)</th>
<th>Gene Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zw</td>
<td>Zw(^*)</td>
<td>97.6</td>
<td>0.855</td>
</tr>
<tr>
<td>Zw</td>
<td>Zw(^*)</td>
<td>26.8</td>
<td>0.155</td>
</tr>
<tr>
<td>Ko</td>
<td>Ko(^*)</td>
<td>14.3</td>
<td>0.0743</td>
</tr>
<tr>
<td>Ko</td>
<td>Ko(^*)</td>
<td>99.4</td>
<td>0.9225</td>
</tr>
<tr>
<td>Bak</td>
<td>Bak(^*)</td>
<td>90.8</td>
<td>0.696</td>
</tr>
</tbody>
</table>

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Fig. 1. Results of platelet typing in the P.B. family. Positivity or negativity for Bak\(^*\) is shown, as well as the Zw and Ko phenotype of the various family members.
specific antibodies, while platelets from the father removed both antibody types.

The specificity of the granulocyte-specific alloantibodies, found in two sera, is still under study.

**Class and Subclass Identification of the Alloantibodies**

The immunoglobulin class of the platelet antibodies was determined with monospecific FITC-labeled antimmunoglobulin reagents. All but one of the cases with antibodies were tested (Table 5). All sera contained IgG antibodies, three sera also contained weak IgM antibodies. IgA antibodies were not found. One serum was too weak to be analyzed. The IgM-antibody-containing sera, two with anti-Zw	extsuperscript{*} antibodies and one with anti-Bak	extsuperscript{*}, were repeatedly negative in the PAT. Thus, a greater sensitivity of the PAT for detecting IgM antibodies was not found with these sera.

Of the platelet-specific alloantibody-containing sera, 26 were tested with monospecific IgG subclass antisera (Table 6). IgG1 antibodies were found in most sera, sometimes in combination with IgG3 and/or IgG4, never with IgG2. In IgG3 antibodies alone were encountered sometimes, but never IgG2 and IgG4 alone. Of the 26 sera, 7 were too weak to be analyzed in this way.

**Platelet Alloimmunization and Blood Group System**

In 31 cases of NAITP, in which the mother's serum contained platelet alloantibodies, the ABO blood groups of the child, the mother, and the father were determined. From this study, it appeared that ABO compatibility was not found more often than expected. The results of this statistical study are depicted in Table 7. Also, when only primiparae were tested, the frequency of ABO compatibility did not differ from normal.

**DISCUSSION**

In the study reported here, platelet-reactive alloantibodies were detected in the sera of 35 of 38 mothers whose infants had neonatal thrombocytopenia. Van der Weerdt, who used platelet agglutination, anti-globulin-consumption, and complement-fixation techniques, found platelet antibodies in 3 out of 10 cases, and Shulman et al., who used agglutination, complement fixation, and blocking techniques, in 48 of 112 cases. So, in comparison with other published series, we obtained positive results far more often. This was clearly due to the use of the immunofluorescence test. Platelet agglutination was always negative, and anti-globulin consumption and complement fixation were positive with only few sera. Moreover, immunofluorescence was always positive when one of the other tests for platelet antibodies was positive.

In one case of NAITP, the antibodies were only detected in the platelet suspension IFT and not in the microphotometric IFT. A possible explanation for this finding is that, in the latter method, acetone fixation was used, which may have resulted in the destruction of the antigen in question, Bak	extsuperscript{*}.

The negative results with 3 of the 38 sera may be due to insufficient sensitivity of the IFT, but of course, it is also possible that other causes than alloimmunization were responsible for the thrombocytopenia.

Recently, Soulier et al. described their results with a sensitive indirect radioactive antiglobulin test on platelets in four cases of NAITP. They, too, could not detect antibodies in all sera; one of the four sera was negative. Although this method was not routinely applied in our laboratory, we found with sera containing anti-Zw	extsuperscript{*} or anti-HLA-A2 antibodies that its sensitivity was the same as that of our suspension immunofluorescence test. Thus, it is not likely that a higher percentage of positive results would have been found in NAITP had the radioactive method been used.

In the majority of our patients (27 of 38), only platelet-specific alloantibodies were detected in the serum. In some (6 of 38), alloantibodies with different cell specificities were also found. The sera of three patients also contained specific HLA antibodies. In two of these three, the HLA antibodies were only detected in the lymphocyte cytotoxic test and not in the immunofluorescence tests. The sensitivity for HLA antibodies of the IFT on lymphocytes, granulocytes, and platelets is usually the same as that of the LCT. However, HLA-typing sera may sometimes

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Number</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
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<tbody>
<tr>
<td>Anti-Zw	extsuperscript{*}</td>
<td>28</td>
<td>28</td>
<td>2*</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Bak	extsuperscript{*}</td>
<td>1</td>
<td>1</td>
<td>1*</td>
<td>0</td>
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<tr>
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<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polyreactive</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Weak in all three.
show so-called CYPFLUN reactions, i.e., positive cytotoxicity and negative fluorescence. This was also found with the above two sera. This phenomenon, not yet explained, might be due to the presence of low-affinity HLA antibodies, which are washed away by the many washings necessary for the IFT.

The serum of one patient contained lymphocyte-specific alloantibodies of yet uncertain nature, only demonstrable in the lymphocyte IFT. Two sera also contained granulocyte-specific alloantibodies, detectable in the leukoagglutination test and the granulocyte IFT. The newborns in question did not suffer from neonatal granulocytopenia, perhaps because the titer of the antibodies was too low ($\frac{1}{2}$-$\frac{1}{4}$). However, this finding indicates that a combined alloimmune thrombocytopenia and granulocytopenia can be expected to occur sometimes in the newborn.

In 2 of our 3 cases of NAITP, the mother’s serum contained polyreactive alloantibodies, probably polyclonal antibodies. However, by testing these sera with platelets, lymphocytes, and granulocytes from many random donors, we obtained evidence that they also contained platelet-specific allo-antibodies. Thus, in none of our cases could we attribute NAITP solely to anti-HLA immunization.

We confirmed that the platelet-specific antibodies in NAITP are nearly always anti-Zw. Anti-Zw was described, for the first time by Van Loghem et al. and later independently by Shulman et al. under the name anti-P1, in patients with posttransfusion purpura. So, the importance of the antibodies was too low ($\frac{1}{2}$-$\frac{1}{4}$). However, this finding indicates that a combined alloimmune thrombocytopenia and granulocytopenia can be expected to occur sometimes in the newborn.

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As was to be expected, IgG antibodies were present in all sera. In three sera, weak IgM antibodies were also found. IgA antibodies were not encountered. When the IgG antibodies were analyzed for the subclasses, IgG1 was detected most often, sometimes together with IgG3 and/or IgG4. In a few sera, IgG3 alone was found. Therefore, the immunochemical composition of platelet alloantibodies in NAITP does not seem to differ from that of red cell alloantibodies in hemolytic disease of the newborn.

Recently, Gratwohl and Shulman published results, indicating that, as in rhesus alloimmunization, ABO compatibility between the mother and the child facilitates platelet alloimmunization of the mother. However, our results on ABO typing in NAITP do not support this theory. The frequency of ABO compatibility in our series was not different from that encountered in normal pregnancies, even when only first pregnancies were analyzed.

In conclusion, immunofluorescence on platelets, especially the suspension IFT, was found to be a suitable test for the detection of platelet antibodies in neonatal thrombocytopenia. Moreover, immunochromatographic characterization and the determination of the specificity of the antibodies was also possible. The reproducibility of the test is high. It has the advantage of simplicity and is applicable on a routine scale. This opens the possibility of a more prompt diagnosis of platelet alloimmunization as well as for the selection of suitable donors for platelettransfusion therapy.

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