An infant with severe neonatal alloimmune thrombocytopenia is described in whom an antibody directed at a new platelet-specific alloantigen, Ca (HPA-6b), is implicated. The new alloantigen is of low frequency in the population and was localized to platelet glycoprotein (GP) IIa. Immunoprecipitation studies using murine monoclonal antibodies specific for the GP complex IIb-IIIa and GPIIia alone (AP2 and AP3) suggest that the location of the Ca epitope on GPIIia may be near the binding site for AP3. Neonatal alloimmune thrombocytopenia associated with Ca is likely to be as severe as that seen in cases due to incompatibilities for the HPA-1 (P1a) and HPA-4 (Pen) platelet alloantigen systems, because each is located on GPIIia, a densely represented molecule on the platelet surface.

ANTIBODIES DIRECTED at platelet-specific alloantigens were first recognized in the late 1950s and early 1960s in patients with posttransfusion purpura (PTP) and neonatal alloimmune thrombocytopenia (NAT),1-4 To date, serologic studies have shown only a limited number of platelet alloantigen systems, most in the context of PTP and NAT cases. DNA sequence polymorphisms and consequent amino acid substitutions have been clarified for four of the five platelet alloantigen systems described so far. The systems HPA-1 (P1a, Zwb),5 HPA-2 (Ko),6 HPA-3 (Bak, Lek),7 and HPA-4 (Pen, Yuk)8 are each associated with single nucleic acid polymorphisms that correlate completely with serologic typing results. The fifth system, HPA-5 (Br, Zav, He), remains to be elucidated at the gene and protein level.

HPA-1 is an alloantigen system localized to platelet glycoprotein (GP) IIa, and its high frequency allele, HPA-1a (P1a1, Zwb1), provides the immunologic stimulus for most cases of NAT and PTP. NAT is a clinical syndrome characterized by marked thrombocytopenia in the fetus and neonate developing before or shortly after birth. It occurs when fetal platelets are destroyed after sensitization by transplacentally acquired maternal antibody directed against a platelet alloantigen inherited from the father. Estimates of the incidence of NAT range from 1 in 600 live births9 to 1 in 5,000.10 A recent prospective study of 5,000 pregnancies found the incidence of alloimmunization to HPA-1a to be 1 in 1,000, and the incidence of neonatal thrombocytopenia due to anti–HPA-1a to be 1 in 5,000.11 In addition to HPA-1a, a number of other platelet-specific alloantigens have been linked to the development of NAT, including HPA-1b (P1a2, Zwb2), HPA-2b (Ko2), HPA-3a (Bak4), HPA-3b (Bak5), HPA-4a (Pen4, Yuk5), HPA-4b (Pen6, Yuk7), and HPA-5b (Br7).12-23

We report here a case of NAT due to maternal antibody specific for a platelet alloantigen not previously described.

CASE REPORT

Nonidentical twins were delivered vaginally at 38 weeks gestational age to a 22-year-old Caucasian female with a history of two previous therapeutic abortions. The pregnancy was complicated by mild preeclampsia in the third trimester. Twin 1 (female) weighed 3.32 kg and had Apgar scores of 7 and 8 at 1 and 5 minutes, respectively. Twin 2, the index case, weighed 2.78 kg and had Apgar scores of 8 and 9 at 1 and 5 minutes, respectively. A few hours after delivery, twin 2 was noted to have petechiae over the anterior chest wall and back. A blood count showed a hemoglobin of 208 g/L, a total white blood cell (WBC) count of 27.4 × 10^9/L, and a platelet count of 5.1 × 10^9/L. A repeat platelet count was 38 × 10^9/L. The infant was transferred to the Neonatal Intensive Care Unit at The Hospital for Sick Children, Toronto.

Admission physical examination was normal apart from scattered petechiae on the infant’s trunk and extremities. The admission blood count was as follows: hemoglobin, 197 g/L; total WBC count, 28.02 × 10^9/L; neutrophil count, 22.98 × 10^9/L; and platelet count, 49 × 10^9/L. By day 2, the platelet count had decreased to 8 × 10^9/L and the infant was transfused with 1 U of random donor platelets. Posttransfusion, the platelet count increased to 114 × 10^9/L (Fig 1). On day 4 of life, a second platelet transfusion from a random donor was given with a similar good response. Significant thrombocytopenia (platelet count, <100 × 10^9/L) persisted for 3 weeks.

The clinical course of twin 1 is of interest. This infant manifested severe, persistent neutropenia without thrombocytopenia (Fig 2). During the period of severe neutropenia, an Escherichia coli urinary tract infection was treated successfully with intravenous broad spectrum antibiotics. Serologic studies on this infant and her parents confirmed maternal sensitization to the neutrophil-specific antigen, NA1, with transplacental passage of maternal anti-NA1 antibodies into the fetus as the cause for this infant’s neutropenia.

The mother of the twins did not, by either history or laboratory testing, have evidence of either autoimmune thrombocytopenia or neutropenia. Four years after the delivery of the twins, she delivered a male infant at term. This infant was unaffected by either thrombocytopenia or neutropenia (blood count on day 2 of life: hemoglobin, 176 g/L; total WBC count, 30.9 × 10^9/L; neutrophil count, 18.23 × 10^9/L; and platelet count, 301 × 10^9/L). The father of these infants is of Filipino descent.

MATERIALS AND METHODS

Samples. EDTA anticoagulated blood was obtained from the index case (twin 2) and his parents as well as from paternal grand-
parents, a paternal uncle, and the index case’s two siblings (twin 1, a sister, and the subsequent son). Serum was obtained from the mother.

Platelet typing. Platelets were isolated from the family’s blood samples and platelet typing for HPA-1, HPA-3, and HPA-4 platelet alloantigen systems was performed using an antigen-capture enzyme-linked immunosorbent assay (ACE). Well-characterized alloantisera or eluates derived from plasma collected from patients with PTP were used in assays for HPA-1a, HPA-4a (plasma), HPA-1b, and HPA-3a (eluate) serologic platelet typing. HPA-3b phenotyping was performed using allele-specific oligonucleotide hybridization using a probe specific for the HPA-3b form of the GPIlb gene.

Characterization of maternal platelet- and neutrophil-reactive antibodies. Using a standard microlymphocytotoxicity assay, maternal serum was tested against a 30-cell panel and found to have antibodies with specificity for HLA-A2, HLA-A9, and HLA-B40. The serum was referred to the American Red Cross, St Paul, Minnesota Regional Blood Services for evaluation of the neutropenia in twin 1. Lymphocytotoxicity in maternal serum was removed after absorption by random platelets. A granulocyte-specific antibody with specificity for the NA1 antigen was detected in the absorbed serum and an incompatibility for the NA1 antigen was confirmed by parental and twin 1 granulocyte typing.

ACE analysis. Maternal serum was evaluated for the presence of antiplatelet antibodies using the standard ACE and modified ACE (MACE) assays described previously. In the ACE, detergent-lysed platelets were added to microtiter wells that had been coated with a platelet glycoprotein-specific monoclonal antibody (MoAb). The plates were washed and patient serum added. After further washing, bound human IgG was detected using biotinylated murine MoAb HB 43 specific for the Fc portion of human IgG. Avidin-biotin–conjugated alkaline phosphatase was added and, after washing, substrate (p-nitrophenol phosphate) was added and the reactions read in an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech, Chantilly, VA).

The murine MoAbs AP2, specific for the platelet GPIlb-IIa complex, and AP3, specific for platelet GPIIIa, were used for GP target immobilizations. Specific to the AP3 immobilizations, detergent-lysed platelets were incubated in 2.5 mmol/L EDTA-Tris saline buffer, pH 7.4, at 37°C for 30 minutes to dissociate the GPIlb-IIia complex. EDTA-treated lysates were then added to 100 μL of AP2-linked sepharose beads and incubated for 2 hours at 22°C to remove remaining complexes. The supernatant fraction was then immobilized in both AP2 and AP3 ACES to determine specificity of reactions.

Immunoprecipitation analysis. Washed human platelets derived from a Ca⁺ individual were surface labeled with biotin, lysed in 1% Triton X-100, and subjected to immunoprecipitation analysis using anti–HPA-1a, anti-Ca alloantisera, or normal human serum. Immunoprecipitated proteins were run on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel under reducing conditions. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes, and these were incubated with streptavidin-conjugated horseradish peroxidase.
RESULTS

Platelet phenotyping. Table 1 shows the results of serologic testing using the AP2 ACE and MACE on family members and normal controls. All members of the family (mother, father, twin 1, twin 2, and the subsequent sibling) were heterozygous for HPA-3a and -3b antigens, whereas the mother and the other children lacked HPA-3a. The father and the affected twin were heterozygous for HPA-3a and -3b antigens, whereas the mother and the other children lacked HPA-3a. All members of the family were positive for HPA-4a; the mother and father were typed with both anti-HPA-4a and anti-HPA-4b typing sera and found to be homozygous for HPA-4a. It was assumed that all of the children were then also homozygous for HPA-4a.

Localization of the Ca epitope. The mother's serum was tested against the platelets of family members and against normal control platelets shown in Table 1. The strongest reactions were noted in the AP2 ACE against the father's platelet lysate and against the twin's platelet lysate. No reactions were noted against autologous platelets or platelets from the unaffected children. Similarly, maternal serum failed to react with the four normal donors in the panel. Maternal serum was also nonreactive when tested against platelets from 200 normal donors (data not shown). In the MACE, using AP2 to immobilize GPIIb-IIIa complexes from sensitized platelets, weak reactivity was shown only against the father's platelets. The AP2 ACE studies indicated that the Ca epitope is on GPIIb-IIIa. Additional family members were typed using maternal serum; the paternal grandfather was also found to be positive. These data are summarized in Fig 3.

Serologic identity of the Ca and Tu alloantigens. Serum Tu and Va (kindly provided by Dr R. Kekomaki, Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and Sr* (kindly provided by Dr V. Kiefel, Institut fur Klinische Immunologie und Transfusions-Medizin am klinikum der Justus-Liebig-Universitat, Giessen, Germany) were previously shown to react with epitopes localized to GPIIIa from respective paternal platelets in these cases of NAT. These sera were tested against Ca paternal platelets. Whereas sera Va and Sr* were nonreactive (data not shown), Tu was positive against Ca platelets in the AP2 ACE, as shown in Fig 4. Similarly, platelets positive for the Tu antigen (kindly provided by Dr R. Kekomaki, December 1991) were tested with serum Ca and were positive. Parallel testing of Ca serum against Sr* platelets and sera Tu and Sr* against Ca platelets using the monoclonal antibody immobilization of platelet antigens (MAIPA) assay32 showed similar results (Dr Volker Kiefel, personal communications, December 1991). Serum Ca was negative against Sr* platelets, and serum Tu but not serum Sr* reacted with Ca platelets. Serum Mo, a recently described private platelet alloantigen localized to a GPIIIa (A. von dem Borne, personal communication, December 1991), was also tested against Ca platelets and found negative (Volker Kiefel, personal communication, December 1991).

To further localize the antigen, paternal Ca platelets were lysed and exposed to EDTA at 37°C. The paternal Ca platelet lysate was added to AP2-linked sepharose beads to remove remaining GPIIb-IIIa complexes. The supernatant containing dissociated GPIIIa and GPIIb was then used in an AP3 as well as the AP2 ACE. As shown in Fig 5, undissociated paternal Ca GPIIb-IIIa complex immobilized with AP3 (specific for GPIIla) was less positive with Ca serum compared with complex immobilized with AP2 (specific for the complex of GPIIb-IIIa). Similar weaker reactivity was also observed after Ca lysate was dissociated and immobilized with AP3. Reactivity of serum Ca was absent when dissociated Ca platelet lysate was immobilized with AP2 (data not shown). These results suggested that Ca is located on GPIIIa, and that the epitopes for AP3 and Ca overlap to some degree.

To confirm the localization of the Ca antigenic epitope, immunoprecipitation of biotin-labeled surface proteins was performed using Ca alloantisera and paternal platelets. A

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Table 1. Serologic Testing of Ca Family Members and Controls

<table>
<thead>
<tr>
<th>Platelet Donor</th>
<th>MO</th>
<th>Fa</th>
<th>Tw1</th>
<th>Tw2</th>
<th>Son 2</th>
<th>Plt 1</th>
<th>Plt 2</th>
<th>Plt 3</th>
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Antibody detection

| ACE§  | Neg | 1+ | NT | Neg | 2+ | Neg | Neg | NT | Neg |
| MAC§  | Neg | 1+ | NT | NT | NT | Neg | Neg | NT | NT |

Abbreviations: NT, not tested; Mo, mother's platelet lysate; Fa, father's platelet lysate; Tw1, platelet lysate from twin 1; Tw2, platelet lysate from twin 2; Son 2, platelet lysate from younger, unaffected sibling; Plt 1 through 4, platelet lysate of normal controls.

* HPA-1 phenotype: a/a, HPA-1a/1a; a/b, HPA-1a/1b; b/b, HPA-1b/1b.
† HPA-3 phenotype: a/a, HPA-3a/3a; a/b, HPA-3a/3b; b/b, HPA-3b/3b.
‡ HPA-4 phenotype: a/a, HPA-4a/4a; a/b, typed only with HPA-4a typing sera.
§ ACE reactions using Ca serum versus family and control platelet lysates. Reactions were graded 1+ to 4+ according to elevations of the OD over the cutoff. The cutoff was established as the mean OD +4 SD of three negative control sera. 1+, >4 SD above mean; 2+, >8 SD above mean; 3+, >12 SD above mean; 4+, >16 SD above mean.
| MACE reactions using Ca serum versus family and control platelet lysates. |
NEW PLATELET-SPECIFIC ALLOANTIBODY, ANTI-CA

Fig 3. Pedigree of the Ca family. †, Proband; ●, individuals heterozygous for Ca antigen; ◻, individuals lacking Ca antigen reactivity as assessed by serologic testing (see text).

A band was evident in the same location as the positive control (anti-HPA-1a) at about 100 Kd molecular weight, supporting the presence of Ca on GPIIa and not GPIIb (Fig 6).

DISCUSSION

Serum Ca reacts with a new platelet alloantigen that appears to be serologically identical to the Tu antigen described by Kekomaki et al. The serum in this case reacted with GPIIb-IIIa from paternal and the affected son’s platelets. The only other identified platelet antigen incompatibility in this family was for HPA-3a. However, Ca serum did not react with the platelets from any of 200 other normal donors having various platelet antigen phenotypes, including HPA-3a.

Immunoprecipitation analysis and serologic testing localized the Ca antigen to GPIIIa. AP3, an anti-GPIIIa MoAb, interfered with the binding of the Ca antibody, suggesting that Ca binds to GPIIIa at or near the same epitope as AP3. Further studies are in progress to identify the genetic basis for the Ca antigen, and its apparent proximity to the AP3-binding site may aid in these investigations.
Serologic testing of family members with Ca serum showed autosomal inheritance. Antigen Ca is distinct from HPA-1, HPA-3, and HPA-4 antigen systems. The antigen is present in the population at a low frequency, but because several Tu-positive donors have been identified outside of the original family (Dr Riitta Kekomaki, personal communication, December 1991), this antigen is not a “private” specificity isolated to one family. It can therefore be provisionally designated HPA-6b, assuming it represents the low-frequency allele of a new platelet alloantigen system, HPA-6. It appears to have been associated with severe NAT (neonatal platelet count <8 \times 10^9/L in the present case). This is due perhaps to its localization to GPlIIa, which places it in a category of high-density NAT-related antigens (eg, HPA-1a and HPA-4a) that are associated with severe fetal thrombocytopenia and in utero intracranial hemorrhages.

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Neonatal alloimmune thrombocytopenia due to a new platelet-specific alloantibody