Single Point Mutation in Human Glycoprotein IIIa Is Associated With a New Platelet-Specific Alloantigen (Mo) Involved in Neonatal Alloimmune Thrombocytopenia


Here we describe a new platelet-specific alloantigen that was identified in a case of neonatal alloimmune thrombocytopenia. This antigen has provisionally been called "Mo." By studying the Mo family, it was shown to be inherited in an autosomal dominant manner. Immunoprecipitation and Western blot analysis showed that the antigen resides on platelet glycoprotein IIIa (GP IIIa). Genomic analysis, performed by applying polymerase chain reaction and sequencing, showed a C→G substitution of base pair 1267 of the coding region of the DNA for GP IIIa, resulting in a substitution of Proline⁴⁰⁷ by Alanine⁴⁰⁷. That this substitution is associated with the antigen could be demonstrated by restriction fragment length polymorphism analysis of cDNA, prepared from platelet RNA, and of genomic DNA. It was confirmed by dot-blot hybridization with allele-specific oligonucleotides. All family members, also those being Mo antigen-positive, were healthy. None of them appeared to suffer from increased tendency of bleeding or thrombosis. Thus, the Mo mutation does not lead to significant platelet dysfunction in vivo with heterozygous carriers. One of 450 random healthy blood donors who were tested was positive for the Mo antigen. Typing was performed by the classical serologic methods as well as by DNA analysis.

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MATERIALS AND METHODS

Case report. The family in which this case of NAIT occurred will be called Mo. After an uneventful second pregnancy, a boy was born to a healthy mother. The Apgar scores were 9 and 10 after 1 and 5 minutes, respectively. Petechiae developed 3 hours postpartum; otherwise the boy was healthy. There were no signs of infection and on physical examination there was no hepatomegaly or splenomegaly. The petechiae worsened and the boy was admitted to a neonatal intensive care unit with extensive hematomas. Four hours after delivery he vomited fresh and old blood. There also was melena with fresh blood admixed. Laboratory investigation at that time showed a hemoglobin (Hb) count of 13.1 mmol/L, hematocrit (Ht) 0.61, leukocyte count 36.6 × 10⁹/L (differential 86% neutrophils, from which 26% were stabs, 5% lymphocytes, 8% monocytes and 1% eosinophils). Activated partial thromboplastin time (APTT) was 34.2 seconds, PTT 12.6 seconds, fibrinogen 2.1 g/L, and platelets 3 × 10⁹/L. Because of the leukocytosis with 26% stabs in combination with thrombocytopenia, he was suspected of having a septicaemia and treated with antibiotics. However, during the next 2 days the platelet count did not increase. Blood cultures remained negative. Gastrintestinal tract bleedings were most pronounced during the first 2 days, for which recurrent washings with cold saline were performed. Under suspicion of a neonatal alloimmune thrombocytopenia, he was given a transfusion of maternal platelets, from which the maternal plasma had been removed and substituted by normal donor plasma. After transfusion, the platelet count was 56 × 10⁹/L. When the platelet count decreased to 15 × 10⁹/L the following day, he was treated with high-dose intravenous Ig (IVIg) (0.4 g/kg) for 3 days. The platelet count stabilized and started to increase slowly to 46 × 10⁹ in the following weeks. There were no signs of intracranial bleedings and an echography of the brain showed no abnormalities. The first child of these parents was born healthy and had not shown signs of an increased bleeding tendency. At the first control in the outpatient clinic, the platelet count was 311.

Blood. Blood samples from both parents and the child and later also from different family members were analyzed. The platelets of the parents were typed for platelet alloantigens to show potential incompatibilities. The following antisera were used: anti–HPA-1a (IgG-anti-Zw⁺/P⁺), anti–HPA-1b (IgG-anti-Zw⁻/P⁻), serum 1072 containing IgM alloantibodies against the HPA-2b antigens, and serum 9725 containing IgM alloantibodies against the HPA-2a antigen, anti–HPA-3a (IgG-anti-Bak⁺), anti–HPA-3b (IgG-anti-Bak⁻), and anti–Sr (IgG-anti-Sr⁺). The latter two sera were gifts from Prof Dr Mueller-Eckhardt (Giessen, Germany). As a negative control serum, the serum of a nontransfused male with blood-group AB was
The arrow indicates the child with NA1T caused by anti-lgG antibodies (Jackson), diluted in incubation buffer, was placed in the 4°C (wt/vol) for 30 minutes at 4°C. To remove unsolubilized material, the lysate was centrifuged at 13,000g for 30 minutes at 4°C. Thirty-

Moa.

NP-40 [wt/vol], 0.05% Tween-20 [vol/vol], and 0.2% BSA [wt/vol]).

pL of ice-cold incubation buffer (Tris buffer supplemented with 1% NP-40 [wt/vol], supplemented with 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.02 mg/mL trypsin inhibitor, and 5 mmol/L N-ethylmaleimide (NEM) for 30 minutes at 4°C. The lysate was centrifuged for 30 minutes at 13,000g at 4°C. Fifty microliters thereof was placed in triplicate in the wells of a microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) precoated with MoAb. The platelets were subsequently washed three times in PBS/BSA and solubilized in 100 μL of immunoprecipitation buffer (IPB) containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40 [wt/vol], and 5 mmol/L N-ethylmaleimide (NEM) for 30 minutes at 4°C. The lysate was centrifuged for 30 minutes at 13,000g at 4°C. Fifty microliters of a 10% suspension of polyclonal goat-anti-mouse IgG (CLB, GM 17D) or MoAb mouse-anti-human IgG (CLB MH16-1) covalently linked to Sepharose-4B beads (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were added to the lysate and incubated for 3 hours at 4°C. The beads were washed three times with IPB at 4°C. The pellet was resuspended in 50 μL of sodium dodecyl sulfate (SDS) sample mix and heated at 95°C for 5 minutes. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gels. The gels were dried and autoradiographed with the help of an intensifier screen.

Western blotting. Platelets for immunoblotting analysis were washed and solubilized in a buffer composed of 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L NEM, 1 mmol/L PMSF, and trypsin inhibitor (0.02 mg/mL). The lysates were centrifuged at 13,000g for 30 minutes. The platelet proteins were separated on 7.5% SDS-PAGE under nonreducing conditions (SDS) sample mix and heated at 95°C for 5 minutes. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gels. The gels were dried and autoradiographed with the help of an intensifier screen.

Western blotting. Platelets for immunoblotting analysis were washed and solubilized in a buffer composed of 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L NEM, 1 mmol/L PMSF, and trypsin inhibitor (0.02 mg/mL). The lysates were centrifuged at 13,000g for 30 minutes. The platelet proteins were separated on 7.5% SDS-PAGE under nonreducing conditions and subsequently electrophoretically transferred to nitrocellulose according to Towbin (1979). The blot was then preincubated for 1 hour at room temperature in PBS with 3% fetal calf serum (FCS) and cut into strips. The strips were incubated with the different sera (1:20) diluted in 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% Tween-20 (pH 8.0) [TBST]), or the MoAbs (1:500 diluted also in TBST), and were washed three times with TBST. Second incubations were with alkaline phosphatase-conjugated goat-anti-human IgG or alkaline phosphatase-conjugated goat-anti-mouse IgG (1:7,500; Promega, Madison, WI). The nitrocellulose strips were washed three times again with TBST. The alkaline phosphatase reaction was visualized by using the Proto Blot immunoscreening system (Promega) with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates.

Table 1. Platelet Antigens, Blood Groups of the Parents

<table>
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<th>Family Member</th>
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used. The maternal serum was tested with paternal platelets (cross-match) and panel platelets (blood-group A, HPA-1a, b; HPA-2a, b; HPA-3a, b; HPA-4a, b; HPA-5a, b) and with platelets from the father's relatives. Blood from family members was drawn by veno puncture after informed consent was obtained.

Monoclonal antibodies (MoAbs). The following MoAbs were used: MB45, CD42b against GP Ibα; CLB-thromb/1 (C17), CD61 against GP IIIa; CLB-thromb/4 (10G11), CD49b against VLA chain; Y2/5I, CD61 against GP IIIa; MB9, CD41 against GP IIb; SW16, an MoAb against platelet GP V. All MoAbs except SW16 have been described elsewhere.

Platelets and the indirect platelet immunofluorescence test (PIFT). Determination of the phenotype on platelets was performed by PIFT on chloroquine-treated platelets as described before, except for HPA-5a and b, which were tested for in the MAIPA (see below).

MoAb immobilization of platelet antigen assay (MAIPA). The MAIPA was performed according to Kiefel et al. with minor modifications. In a typical experiment, 5 × 10⁶ freshly isolated packed platelets were resuspended in 50 μL of an appropriate dilution of MoAbs together with 40 μL of human serum and incubated for 30 minutes at 37°C. The cells were then washed three times in phosphate-buffered saline/bovine serum albumin (PBS/BSA). After pelleting, the platelets were solubilized in 50 μL ice-cold Tris buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) supplemented with 1% NP-40 [wt/vol] for 30 minutes at 4°C. To remove unsolubilized material, the lysate was centrifuged at 13,000g for 30 minutes at 4°C. Thirty-five microliters of supernatant was collected and transferred into 50 μL of ice-cold incubation buffer (Tris buffer supplemented with 1% NP-40 [wt/vol], 0.05% Tween-20 [vol/vol], and 0.2% BSA [wt/vol]). Fifty microliters thereof was placed in triplicate in the wells of a microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) precoated with goat-anti-mouse IgG (7.5 g/mL; Jackson ImmunoResearch, West Grove, PA) in 0.1 mol/L NaHCO₃/Na₂CO₃, pH 9.6, overnight at 4°C. The plate was incubated overnight at 4°C. After five washings, 50 μL alkaline phosphatase-conjugated goat-anti-human IgG or IgM antibodies (Jackson), diluted in incubation buffer, was placed in the wells and incubated for 2 hours at 4°C, followed by another five washings. Finally, the bound conjugate was demonstrated by adding Sigma-104 phosphatase substrate (Sigma Chemicals, St Louis, MO) dissolved in diethanolamine buffer, pH 10. The extinction was measured in a Titertek reader (ITK Diagnostics BV, Uithoorn, The Netherlands) at 405 nm.

Immunoprecipitation. Platelets, 5 × 10⁶, radiolabeled with 125I and iodogen as catalyst, were incubated with 50 μL of human serum or 50 μL of MoAb. The platelets were subsequently washed three times in PBS/BSA and solubilized in 100 μL of immunoprecipitation buffer (IPB) containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40 [wt/vol], supplemented with 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.02 mg/mL trypsin inhibitor, and 5 mmol/L N-ethylmaleimide (NEM) for 30 minutes at 4°C. The lysate was centrifuged for 30 minutes at 13,000g at 4°C. Fifty microliters of a 10% suspension of polyclonal goat-anti-mouse IgG (CLB, GM 17D) or MoAb mouse-anti-human IgG (CLB MH16-1) covalently linked to Sepharose-4B beads (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were added to the lysate and incubated for 3 hours at 4°C. The beads were washed three times with IPB at 4°C. The pellet was resuspended in 50 μL of sodium dodecyl sulfate (SDS) sample mix and heated at 95°C for 5 minutes. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gels. The gels were dried and autoradiographed with the help of an intensifier screen.
Isolation of genomic DNA and platelet RNA. Genomic DNA was isolated from mononuclear leukocytes using the methods as previously described. Platelet RNA was prepared according to the procedure of Chomczynski and Sacchi, as modified by Lyman et al.

Amplification of genomic DNA and platelet mRNA. Platelet RNA of two members of the Mo family (the father of the child with NAI T and the father’s brother) both serologically typed Mo+ and a non-related donor (Mo−) were isolated. Oligonucleotides for the polymerase chain reaction (PCR) were the following: primer 1, TAGAATTCAACATTAGGTGTTCATCCCTG (nucleotides 1172 to 1193 extended with a HindIII site at its 5’ end); primer 2, ATATTGCTATATCCCACACTCAAGG (nucleotides 1409 to 1427 extended with an EcoRI site at its 5’ end), and primer 1^t used in combination with 2 to amplify genomic DNA, beginning at map position 21365 of the sequence as published by Zimrin et al and is complementary to noncoding DNA. TAGAATTCTTTCTTTTCATCCAGGTGAC (extended with HindIII site at its 5’ end). PCR was performed with the Perkin-Elmer Cetus Thermal Cycler (Norwalk, CT) with 0.7 DNA and 20 pmol of each of the PCR primers using 2 U Taq-DNA polymerase (Promega) in a buffer recommended by the manufacturer, in a total volume of 50 μL. Thirty-three cycles were performed as follows: 1-minute denaturation at 95°C, 2-minute primer annealing at 62°C, and a 2½-minute extension at 72°C. Amplified fragments were purified by agarose electrophoresis, cleaved with the HindIII and EcoRI restriction endonucleases and were inserted into puc 18 with T4 DNA ligase. The insert-containing puc-18 preparations were used to transform DH5α Escherichia coli competent cells in the presence of ampicillin and X-gal. Nucleotide sequences were determined by the dideoxy-chain-termination method using Sequenase according to the manufacturers’ recommendations (US Biochemical Corp, Cleveland, OH). Bsp-1286 restriction digests were performed under conditions recommended by the manufacturer (Biolabs, New England, Beverly, MA). Furthermore, we performed, with overlapping sets of primer DNA, analysis of the entire GP IIIa molecule; from all regions, at least six nucleotide sequences were determined.

Allele-specific oligonucleotide dot-blot hybridization to amplified DNA. Allele-specific oligonucleotides in this study had nucleotide sequences 5’ GGCTGTCCCCAGG 3’ (probe A), 5’ GGCTGTGCCCAGG 3’ (probe B), as discussed later. Oligonucleotides were radiolabeled using [γ-32P]adenosine triphosphate (ATP) and T4 polynucleotide kinase (Boehringer, Mannheim, Germany). After PCR, 10-μL portions of the reaction mixtures were diluted with 100 μL of water and boiled for 3 minutes, whereafter 110 μL, 20 times
PLATELET-SPECIFIC ALLOANTIGEN Mo⁺

PCR strategy:

\[ \text{cDNA} \]

\[
\begin{array}{c}
\text{GP IIIa} \\
5' \quad 1172-1193 \\
3' \quad 1409-1427
\end{array}
\]

\[ 270 \text{ bp} \]

\[ 100 \text{ bp} \]

\[ 170 \text{ bp} \]

\[ \text{Bsp 1286} \]

\[ \text{M} \]

\[ \text{1} \quad \text{Mo}^- \]

\[ \text{2} \quad \text{Mo}^+ \]

\[ \text{3} \quad \text{Mo}^+ \]

Fig 3. (A) Diagrammatic representation of the GP IIIa mRNA molecule, which contains the Mo⁺ polymorphism. The locations of the two oligonucleotide primers used for the PCR are shown. (B) Amplified 270-bp GP IIIa fragments were digested with the restriction enzyme Bsp 1286 and subsequently analyzed by electrophoresis through a 1.5% Agarose gel. The gel contains the digested samples of an Mo⁺ individual (lane 1) and two Mo⁺ individuals (lanes 2 and 3).

Concentrated, SSC (1× SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0) were added to the denatured DNA samples. For each sample, two 100-μL portions were spotted onto zeta-probe using a BioRad (Richmond, CA) dot-blot apparatus. The filters were prehybridized in 5× concentrated Denhardt’s, 5× concentrated SSC, 5 mmol/L EDTA, 10 mmol/L Na₂HPO₄, pH 7.0, at 37°C for 1 hour and then were hybridized to either probe A or probe B by adding 100 ng probe to the prehybridization mix at 37°C. The filters were then washed five times with 3× concentrated SSC for 15 minutes each time at 1°C higher than 37°C. Autoradiography was performed for at least 6 hours at −70°C using Kodak XAR film (Eastman Kodak, Rochester, NY) with an intensifying screen.

RESULTS

Serologic investigations. The phenotyping of the parents' platelets showed no incompatibilities for HPA-1, -2, -3, or
-5 alloantigens. The results are shown in Table 1. The direct test on platelets of the mother was weakly positive using an anti-Ig or an anti-IgM conjugate but negative with an anti-IgG conjugate. Cross-matching with maternal serum and paternal platelets gave strongly positive results, also after chloroquine treatment (which dissociates HLA antigens of the surface of the platelets). These antibodies were of the IgG class. The Mo serum did not react with any of the typed panel platelets, suggesting the presence of alloantibodies against a private or a low-frequency antigen.

To investigate the occurrence of the Mo antigen in the family, platelets from the available family members were tested in the PRT.

**Family study.** The Mo family tree is depicted in Fig 1. As shown in this figure, all 9 members of the first generation, 14 members of the second generation, and 5 members of the third generation, to which the propositus belongs, could be studied. All members of this large family were healthy and none had a history of a bleeding tendency or a tendency for thrombosis and/or embolism. There was no previous case of NAIT in the family. Routine blood counts were normal. From the segregation pattern in the family, it can be concluded that the Mo antigen is inherited as an autosomal dominant allele (Fig 1). Because all members of the family were HPA-1 (a+, b−), an association with the HPA-1 antigens cannot be excluded or proven. There is no apparent coinheritance with any of the other HPA antigens tested and there is no relation with the Sr private antigen.12 The results of the MAIPA localize the Mo antigen on GP IIb/IIIa, because the maternal serum gave strongly positive reactions in combination with C17 (MoAb against GP IIb/IIIa) when tested with paternal platelets, whereas with the other MoAbs (MB45, 10G11, MB9, SW16) the results were negative. Typing results in the Mo family in the MAIPA were in concordance with those obtained in the PFTT.

**Immunohematological investigations.** To further localize the Mo antigen we performed immunoprecipitation and Western blotting. The Mo antibodies precipitated predominantly GP IIIa (Fig 2). Although GP IIb could be precipitated from the lysate by specific antibodies, few GP IIb were coprecipitated by the Mo antibodies. Dissociative conditions during the precipitation together with a poorer iodine labeling of GP IIb probably contributed to this. However, the immunoblotting experiments demonstrate more unequivocally that an epitope on GP IIIa is recognized by the Mo antibodies (Fig 2).
Genetic analysis of the Mo antigen. We analyzed the cDNA coding for GP IIIa of two Mo⁺ donors and one unrelated Mo⁻ donor. After PCR (270 bp) with primers 1 and 2, subcloning and sequence analysis, a single base change C → G at base 1267 was observed. This C → G mutation changes a CCC codon for proline into a GCC that codes for alanine at amino acid 407 of the mature GP IIIa DNA. No other base-pair changes in the entire GP IIIa cDNA were noticed.

RFLP analysis. The C → G substitution created a recognition site for the restriction enzyme Bsp 1286, which cleaves at 5'-GTGCC-3' but not at 5'-GCCGC-3' sequences. Digestion of the PCR fragments (primers 1 and 2) with Bsp 1286 yielded the fragments of expected size (Fig 3).

For family analysis on genomic DNA, we used primers 1', 2, which yielded a PCR fragment of 228 bp. From the mononuclear leukocytes of seven family members (four Mo⁺+ and three Mo⁻), genomic DNA was isolated, amplified, and restriction fragment length polymorphism (RFLP) analysis was performed with Bsp 1286. The results after digestion were in agreement with those of the RFLP analysis performed on cDNA (Fig 4).

Allele-specific oligonucleotide (ASO) analysis. ASO typing using dot-blot hybridization analysis was also performed, using oligonucleotide probes specific for the Mo⁺ and Mo⁺⁺ sequence to probe the PCR products of cDNA and genomic DNA, from six Mo⁺⁺ and two Mo⁺ family donors (Fig 5). These data were fully concordant with the RFLP analysis results.

Until now, we have tested 450 random blood donors for expression of the Mo antigen. Initially, typing was performed in the PIFT and MAIPA techniques. One of them appeared to be positive for the Mo antigen; this was confirmed by DNA analysis. This implies that Mo⁺ is a low-frequency antigen.

DISCUSSION

Alloimmunization of a mother by fetal platelet-specific alloantigens may provoke a fetal and/or neonatal alloimmune thrombocytopenia. Most commonly, alloantibodies against HPA-1a are involved. Recently, alloantibodies against a “private” platelet alloantigen “Sr⁺” were described by Kroll et al.12 Here we report a new “private” platelet alloantigen involved in NAIT, different from Sr⁺, called Mo⁺.

We identified the platelet glycoprotein on which this Mo⁺ antigen resides to be GP IIIa by means of MAIPA, immunoprecipitation, and immunoblotting. Introduction of molecular biologic techniques has given insight in the genetic basis of platelet alloantigen systems. So far, four systems have been shown to be associated with single base-pair substitutions in the coding DNA for their respective glycoproteins. Because the possibility existed that more than one amino acid substitution may be responsible in the formation of an alloantigen, we sequenced the entire coding region of GP IIIa. As the only difference with the published sequence and with that of a control donor, we found a C → G substitution at base pair 1267 of the mature DNA giving rise to a Proline⁶⁰⁷/Alanine⁶⁰⁷ polymorphism. This could be demonstrated by direct sequencing of the GP IIIa cDNA. The presence of the
mutation could also be shown by RFLP analysis of the by PCR-obtained fragments derived either from cDNA or genomic DNA. Because there is only one amino acid substitution in the entire GP IIla, we believe that this is the cause of the antigenicity of the Mo antigen. However, the antigenic epitope does not need to be identical.

The alloantigen "Mo" has a restricted population expression; i.e., only one unrelated donor was found to be positive. This was shown by classical serologic methods as well as by DNA analysis.

Because none of the family members positive for the Mo antigen had signs of an altered hemostasis, it seems likely that heterozygosity does not affect the physiologic function of GP IIb/IIIa. However, it is notable that of the nine platelet alloantigens described thus far, five, including the Mo antigen, reside on GP IIb/IIIa. It seems that in vivo mutagenesis does not per se lead to clinically noticeable platelet dysfunction. However, more detailed analysis may show more subtle dysfunctions. The genomic basis of the antigen polymorphisms makes such studies feasible.

In conclusion, we describe a new platelet alloantigen that was identified in a case of NAIT on membrane glycoprotein IIIa.

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Single point mutation in human glycoprotein IIla is associated with a new platelet-specific alloantigen (Mo) involved in neonatal alloimmune thrombocytopenia

RW Kuijpers, S Simsek, NM Faber, R Goldschmeding, RK van Wermerkerken and AE von dem Borne