Max\textsuperscript{a}, A New Low-Frequency Platelet-Specific Antigen Localized on Glycoprotein IIB, Is Associated With Neonatal Alloimmune Thrombocytopenia

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We have identified a new platelet-specific alloantigen, Max\textsuperscript{a}, responsible for a typical case of neonatal alloimmune thrombocytopenic purpura. The maternal serum reacted strongly with paternal platelets in the platelet immunofluorescence test, whereas platelet alloantigen typing showed that no known human platelet antigen (HPA)-system was involved. In the monoclonal antibody (MoAb)-specific immobilization of platelet antigens (MAIPA) assay, the new antigen was located on the platelet membrane glycoprotein (GP) IIB-IIla complex, but immunoprecipitation and immunoblot experiments to further localize the antigen failed. However, in the MAIPA assay, the binding of the anti-Max\textsuperscript{a} antibodies from the maternal serum was blocked by two anti-GPIIb MoAbs. Thus, the antigen appeared to be located on GPIIb. Analysis of the family led to the identification of six additional Max\textsuperscript{a} individuals. Three of these six individuals and the father were tested in the platelet aggregation test and were found to be normal. In the analysis of normal donors, three of 500 were typed positive for the new platelet-specific antigen, indicating a phenotype frequency of 0.6% in the normal population. Platelet RNA was isolated from the newborn's Max\textsuperscript{a} father and from a healthy donor phenotyped as Max\textsuperscript{-}, reverse-transcribed, and the entire GPIIb coding region was amplified by polymerase chain reaction. Subsequent nucleotide sequence analysis showed a single G \rightarrow A substitution at position 2,603, predicting a valine \rightarrow methionine amino acid substitution at position 837 of the mature glycoprotein. This mutation abolished a BatfI restriction site at the cDNA level and a BstNl restriction site at the genomic DNA level, respectively. The genetic association between the new antigen and this point mutation was confirmed by allele-specific restriction analysis on cDNA and on genomic DNA, as well as by allele-specific primer amplification on genomic DNA. The new mutation is 19 bp upstream of the mutation underlyng the HPA-3 system. Therefore, we also evaluated the association between Max and the HPA-3 polymorphism. So far, all Max\textsuperscript{+} individuals were also found to be HPA-3b, whereas 50 HPA-3a individuals were all Max\textsuperscript{-}. This may indicate that Max\textsuperscript{a} is a variant of the HPA-3b allele.

The platelet membrane GPIIb-IIla complex, also known as \alpha IIb\beta3, belongs to the integrin family of heterodimeric adhesion receptors involved in cell-cell and cell-matrix interactions.\textsuperscript{1} The GPIIb-IIla complex binds ligands containing the Arg-Gly-Asp (RGD) sequence such as fibrinogen, von Willebrand factor, fibronectin, and vitronectin. It plays a prominent role in primary hemostasis.\textsuperscript{2}

The GPIIb-IIla complex carries the majority of the human platelet alloantigen systems and most of these systems are situated on GPIIb-IIla human platelet antigen-1 (HPA-1), -4, -6, -7, -8 and the private antigen Gro\textsuperscript{a}. Only the HPA-3 system (Bak) is located on GPIIb.\textsuperscript{3,4} The private platelet antigen Va\textsuperscript{a} was also shown to reside on the GPIIb-IIla complex,\textsuperscript{10} but has not yet been linked to a specific subunit. To date, only two other platelet antigen systems are known to be carried by other platelet membrane glycoproteins: GPIb\alpha and GP Ib\alpha bear the HPA-2 and HPA-5 system, respectively.\textsuperscript{5}

The molecular basis of all human platelet polymorphisms found so far is a single base-pair mutation leading to a single amino acid substitution in the polypeptide chain of the relevant glycoprotein.\textsuperscript{5,7,11-14}

These alloantigenic epitopes are responsible for the immune responses underlying three well-characterized clinical syndromes: neonatal alloimmune thrombocytopenic purpura (NATP) posttransfusional purpura (PTP), and refractoriness to platelet transfusions. In the first two bleeding disorders, the increased platelet destruction is caused by the development of antibodies against platelet-specific antigens in sensitized subjects. In the case of platelet transfusion refractoriness, HLA antibodies are usually involved, but sometimes platelet-specific alloantibodies can also be implicated.\textsuperscript{15}

In the white population, neonatal alloimmune thrombocytopenia is usually caused by alloantibodies directed against the HPA-1a antigen.\textsuperscript{16,17} Less frequently the immune response is induced by a sensitization against the HPA-5b antigen,\textsuperscript{18} whereas only rarely it is caused by other platelet-specific alloantigens.\textsuperscript{5,8,19-22}

In this report, we describe a new low-frequency platelet alloantigen located on GPIIb and responsible for a case of NATP. The genetic characterization showed that a single base-pair difference at GP IIb cDNA position 2,603 was associated with the new platelet antigen, named Max\textsuperscript{a}. This base change leads to a valine \rightarrow methionine polymorphism at amino acid position 837 of the mature heavy chain GP IIb, near the polymorphism underlying the HPA-3 system.\textsuperscript{23} The new alloantigen was found in three of 500 white donors, indicating a phenotype frequency of 0.6% and a calculated genotype frequency of 0.03. Thus, it might be a relevant
alloantigen in other cases of NATP, PTP, and refractoriness to platelet transfusion.

CASE REPORT

After an uneventful first pregnancy, a 32-year-old white woman gave birth to a healthy girl who was admitted to a neonatal intensive care unit after a few hours because of petechiae and extensive hematomas. On physical examination, no hepatomegaly, splenomegaly, signs of infections, or neurologic damage was found. There was neither hematuria nor melena. Laboratory tests showed a Hb level of 10.6 mmol/L, Ht 48%, a total white blood cell (WBC) count of 18.8 x 10⁹/L, with a normal differential and a platelet count of 6 x 10⁹/L. Coagulation parameters were within normal range. An echo-encephalography showed a 2.5-cm diameter right parietal hematoma.

She was given a transfusion of random donor platelets and intravenous IgG (0.4 g/kg for 3 days), which resulted in a good platelet increment (platelet count 147 x 10⁹/L). Shortly after transfusion, she developed generalized convulsions that disappeared with anticonvulsant therapy. The following day two additional platelet transfusions from random donors were given, both with a good response. After 20 days, she was discharged in good clinical condition. At that time, platelet count was 140 x 10⁹/L, whereas the diameter of parietal hematoma had decreased to 0.6 cm. Neurologic signs did not reoccur when anticonvulsant therapy was discontinued. After discharge, platelet count remained normal.

MATERIALS AND METHODS

Blood Samples

Blood samples from the parents of the child, all available family members, and from healthy normal donors were collected by venipuncture and anticoagulated in EDTA. No material from the child was available.

For further studies, informed consent was obtained. The Blood-bank of Utrecht (Utrecht, The Netherlands) supplied blood from 500 Dutch random donors.

Platelet Serology and Characterization of Maternal Antibodies

Platelet phenotyping was performed in the platelet immunofluorescence test (PIFT) on chloroquine-untreated and -treated platelets, and in the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay as previously described. Serum from a nontransfused male with blood group AB was used as negative control.

The maternal serum was tested in the PIFT and in the MAIPA assay with paternal platelets and with a panel of typed blood group 0 donor platelets positive or negative for the following antigens: HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -7a, -7b, Gro⁺⁺, and Gro⁺⁻.

In competitive binding studies, 5 x 10⁹ platelets were preincubated for 15 minutes with 50 μL of MoAb (1:100) and for an additional 30 minutes with the maternal serum (1:15). After two wash-steps with phosphate-buffered saline (PBS)/10 mmol/L EDTA/0.2% w/vol bovine serum albumin (BSA), the platelets were incubated for 30 minutes with fluorescein-isothiocyanate (FITC)-labeled sheep polycolonal antihuman-Ig (SH 17F, CLB, Amsterdam, The Netherlands). Two additional wash-steps were performed before fluorescence intensity was measured by FACScan.

Monoclonal Antibodies and Human Antiseras

The following monoclonal antibodies (MoAbs) were used: MB45 against GPIb; CLB-thromb/1 (C17) against GPIb-IIIa; CLB-thromb/4 (10G11) against GPIb-IIIa; 11A (from Dr Y. Bai, Beijing, China); PL249-A19 (from Dr C. Kaplan, Paris, France), and MB9 against GPIb; Y2/51 (from Dr. J. Cordell, Oxford, UK), VIPL 2 (from Dr. W. Knapp, Vienna, Austria), and BL-E2 (from Dr H. Fiebig, Leipzig, Germany) against GPIIa; W6/32 (from Dr S. Meuer, Heidelberg, Germany) against HLA-class I.

The following human antisera were used: anti-HPA-1a (IgG anti-Zw/P(A)); anti-HPA-1b (IgG anti-Zw/P(B)); anti-HPA-2a (IgM anti-Ko); anti-HPA-2b (IgM anti-Ko); anti-HPA-3a (IgG anti-Bak); anti-HPA-3b (IgG anti-Bak); anti-HPA-4a (IgG anti-Yuk'); anti-HPA-5a (IgG anti-Br'); anti-HPA-5b (IgG anti-Br'); anti-HPA-6b (IgG anti-Tu'); anti-HPA-7b (IgG anti-Mo'); anti-Va⁺ (IgG anti-Va⁺); anti-HPA-8b (IgG anti-Sf) and anti-Gro⁺ (IgG anti-Gro⁺).

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The anti-HPA-3b, anti-HPA-5a, and anti-HPA-8b sera were gifts from Professor Mueller-Eckhardt, Giessen, Germany. The anti-HPA-4a serum was provided by Dr Furuhata, Matsumata, Japan. Anti-HPA-6b and anti-Va⁺ sera were gifts from Dr Kekuniuki, Turku, Finland.

For immunochemical experiments, the antibodies in the mother’s serum were purified by absorption and elution from reactive platelets. Briefly, antigen positive donor platelets in a suspension of 1 x 10⁹ cells/mL were incubated with an equal volume of the maternal serum for 30 minutes at 37°C. The platelets were pelleted and afterwards mixed with one volume of PBS/BSA 0.2% and two volumes of ether by vigorous shaking. After incubation of the platelet suspension at 37°C for 30 minutes and centrifugation at 2,800g for 10 minutes, the buffer layer was collected.

Immunoochemical Investigations

Immunoprecipitation and Western blotting experiments were performed as previously described. Platelet Function Studies

Platelet aggregation was studied in four Max⁺⁺ subjects of the newborn’s family and in two healthy Max⁺⁺ donors. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation of blood collected in 3.8% (wt/vol) sodium citrate (blood/anticoagulant = 9/1) at 200g for 15 minutes and at 3,000g for 20 minutes, respectively. The platelet count in the PRP was adjusted to 300 x 10⁹/L with PPP. Platelet aggregation was studied by the densitometric method of Born with a Platelet Aggregation Profiler (model PAP-4, BioData Corporation Hatboro, PA) at 37°C under continuous stirring. The following agonists were used: adenosine diphosphate (ADP; Boeringher Mannheim, Germany) 1.5 mg/mL. Anti-HPA-7b (IgG anti-Mo'); anti-HPA-8b (IgG anti-Sf) and anti-Gro⁺ (IgG anti-Gro⁺).

To evaluate the functional effect of anti-Max⁺⁺ antibodies on Max⁺⁺ platelets, one volume of the PRP from Max⁺⁺ individuals, containing 600 x 10⁹ platelets/mL, was incubated with an equal volume of plasma from a healthy Max⁺⁺ donor or with anti-Max⁺⁺ plasma for 30 minutes at 37°C. After stimulation with ADP 1 μmol/L and collagen 2 μg/mL, platelet aggregation was recorded, as described above.

Isolation of Platelet RNA and cDNA Amplification

Platelet RNA, prepared from fresh platelets according to the procedure of Chomzynsky and Sacchi as modified by Lyman et al, was isolated from the Max⁺⁺ father and from an unrelated healthy Max⁺⁺ donor. The cDNA was synthesized with 200 U of Superscript II (GIBCO BRL, Grand Island, NY) in a total volume of 20 μL containing 7.5 μL of RNA template, 30 optical density (OD) units of pol(N)6 (Pharmacia, Piscataway, NJ), 0.5 mmol/L of deoxyribonucleotide triphosphate (dNTP) mix (deoxadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate).
deoxynucleosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate) (Promega), 10 mmol/L DTT, 30 U of RNAsin (Promega, Madison, WI), and the 5x reaction buffer according to the instructions of the manufacturers. After incubation for 1 hour at 37°C and 10 minutes at 95°C, 50 μL of water was added to the cDNA mixture.

Seven oligonucleotide primer sets were used in the polymerase chain reaction (PCR) for amplifying the entire coding region of GPIIb from nucleotide location 8 to 3243 (according to Poncz et al24). The PCR was performed on 1 μg of cDNA template in a total volume of 50 μL. The reaction mixture contained 100 pmol of each primer, 0.2 mmol/L of each dNTP, 1.0 mmol/L MgCl₂, and 2 U of Taq DNA polymerase (Promega) in the appropriate buffer.

Thirty-five cycles of amplification were performed in a Thermal cycler (Perkin Elmer Cetus, model 480, Norwalk, CT) with denaturation at 95°C for 1 minute, annealing at 50°C for 1.5 minutes and extension at 72°C for 2.5 minutes. An aliquot of each PCR sample was then separated by electrophoresis on a 2% agarose gel containing ethidium bromide to verify the presence of products of the expected size.

Amplified products were directly subcloned into the vector pGEM-T (Promega), without any previous purification, and then transformed into JM109 high efficiency competent cells (Promega). Recombinant colonies were selected by blue-white screening on indicator plates. Nucleotide sequence analysis was determined by the dideoxy chain termination method with the TaqTrack Sequencing System according to the manufacturer's recommendations (Promega) and the appropriate primers. From all seven fragments, at least eight subclones were sequenced.

Isolation and Amplification of Genomic DNA

Genomic DNA from the father, all available family members, three unrelated donors phenotyped Max⁺, and 50 Max⁻ donors was isolated from peripheral blood leukocytes by the method described by Ciulla et al28. A 448-bp region of the GPIIb gene, containing the Max polymorphic nucleotide, was amplified with primer Bak1 (GPIIb bases 13838-13857 numbering according to Heidenreich et al29) and primer Bak2 (GPIIb bases 14199-14183). The PCR was performed with 1 μg of template, 20 pmol of each primer, 0.2 mmol/L of each dNTP, 1.5 mmol/L MgCl₂, and 2 U of Taq DNA polymerase in the appropriate buffer. Thirty-five cycles of 1 minute at 95°C, 1 minute at 62°C, and 2.5 minutes at 72°C were performed. Again, 5 μL of PCR-amplified products were analyzed on 2% agarose gel.

Allele-Specific Restriction Enzyme Analysis (ASRA)

ASRA on cDNA. Primers 6A (nucleotide 2380-2398 of GPIIb cDNA) and 6B (nucleotide 2894-2876) were used on cDNA to obtain PCR products containing the Max polymorphism. These 530-bp products were then tested for the presence of specific BstYI sites following the recommended conditions of Boehringer Mannheim. Digested PCR products were further analyzed on 2% agarose gels.

ASRA on genomic DNA. The BstNI (Biolabs, Beverly, MA) digestion was performed with the 448-bp fragment encompassing the Max polymorphic site, obtained with the PCR described above. The reaction was carried out under conditions recommended by the manufacturers and the obtained DNA fragments were analyzed on 10% acrylamide gels. Analysis of the HPA-3 polymorphism was performed as described earlier30 with a Fok I ASRA.

Allele-Specific Primer Amplification (ASPA)

Antisense oligonucleotide specific primers selected for the ASPA, also called PCR sequence-specific primers (PCR-SSP), were Max-G and Max-A corresponding to position 13,958 to 13,940 of the GPIIb genomic DNA coding for Max⁺ and Max⁻, respectively. Primer Bak1, positioned in the intron upstream of exon 26, carrying the Max polymorphism, was used as universal sense primer. A primer set amplifying a 371-bp fragment of the 4th exon of p22 phox gene, encoding the α-chain of cytochrome b₅₅₃, served as a control (primers 2221 and 222230). PCR was performed with 1 μg of genomic DNA in a total volume of 50 μL containing 20 pmol of each primer, 2 U of Taq DNA polymerase in the appropriate buffer, 1.0 mmol/L MgCl₂ and 0.2 mmol/L of each dNTP. After 5 minutes of DNA denaturation at 95°C, 34 cycles of 1 minute at 95°C, 1 minute at 62°C, and 1.5 minutes at 72°C were performed.

RESULTS

Serologic Investigations

The maternal serum reacted strongly with paternal platelets in the PIFT and when HLA-class I antigens were removed from the platelet surface by chloroquine treatment. Maternal serum did not react with a panel of typed donor platelets and with her own platelets. The antibodies present in the maternal serum were of the IgG class.

Platelet phenotyping of the parents did not show incompatibilities for the known platelet-specific alloantigens HPA-1 to HPA-7, for the antigen Sr⁺ (HPA-8b) or the private alloantigens Va⁺ and Gro⁺ (Table 1).

In the MAIPA assay, the maternal serum gave a positive reaction only when used in combination with MoAbs directed against GPIIb and GPIIIa (Fig 1), but not with MoAbs against other GPs (Ia, Ila, Ib), strongly suggesting that the new antigen, Max⁺, is located on the GPIIb-IIIa complex. Furthermore, of the MoAbs directed against the components of this complex (11A, PL249-A19, MB9 against GPIIb and Y2/51, VIPL2, BL-E2 against GPIIIa), MoAb MB9 against GPIIb was unable to induce a positive reaction. It suggested that the Max⁺ epitope is located near the epitope recognized by this MoAb, and thus on GPIIb. This was confirmed by immunofluorescence inhibition studies. MoAb MB9 only partially inhibited the binding of anti-Max⁺ antibodies in this assay. However, MoAb 11A, also directed against GPIIb, induced complete inhibition of binding. MoAbs 11A did not, or only marginally, inhibited in the MAIPA assay.

A total of 500 unrelated donors were tested by PIFT and MAIPA for the expression of the Max⁺ antigen and three were found to be positive. These three donors were all phenotyped as HPA-3a/3b.

Family Studies

Family investigations were performed by PIFT, MAIPA assay, and by ASRA with BstNI at genomic DNA level (see below). All family members were healthy, none had a history of bleeding and/or thrombosis, and all routine blood counts were within the normal range. From the inheritance pattern of the Max⁺ antigen in the newborn’s paternal family, depicted in Fig 2, it is evident that the new antigen segregates as an autosomal codominant character. As determined by ASRA (see below), all Max⁺ subjects were heterozygous for the new antigen.
Table 1. Platelet Phenotyping of the Parents

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Platelet phenotyping was performed by PIFT and by MAIPA assay.
Abbreviation: NT, not tested.

Platelet Function Studies

Because the Max antigen is located on the GPIIb-IIIa complex, which plays a primary role in platelet function, we evaluated the platelet aggregation in Max+ family members of the first and second generation (the father, grandparents, and both uncles of the newborn). Platelet aggregation in response to ADP and collagen and ristocetin-induced agglutination were normal in all Max+ subjects, as compared with platelet aggregation of two Max- normal donors (data not shown).

When platelet function was evaluated in presence of anti-Max antibodies, a weak inhibition of platelet aggregation (<20%) was found in all Max+ individuals but also in Max- individuals. Thus, this inhibition was probably due to an aspecific effect (data not shown).

Immunohistochemical Investigations

To further localize the Max antigen, immunoprecipitation studies with 125I-labeled platelets and Western blotting experiments were performed. Although GPIIb could be precipitated from the lysate of Max+ platelets by specific MoAb or anti-HPA-3a serum, no platelet membrane glycoprotein was detected on precipitation with either the maternal serum or anti-Max antibodies purified by absorption and elution. Anti-Max antibodies also failed to react with GPIIb in the immunoblot (data not shown).

Fig 1. Reactivity of the maternal serum containing anti-Max antibodies tested in the MAIPA assay with Max+ paternal platelets, measured as increase of optical density. MoAbs directed against the major platelet membrane GPs, as well as different MoAbs against the GPIIb-IIIa complex or one of its components, were used.

Fig 2. Pedigree of the Max family: The Max genotyping is depicted. Half-filled black symbols: Max+ individuals; open symbols: Max- subjects. The arrow indicates the child with NATP caused by anti-Max antibodies.

Genetic Characterization of the Max Antigen

Because the binding of anti-Max antibodies was inhibited by two anti-GPIIb MoAbs, we assumed that the epitope was localized on GPIIb. Therefore, the entire coding region of GPIIb cDNA was amplified in seven overlapping fragments. When analyzed on 2% agarose gels, the PCR products from the father showed the same mobility as products obtained from normal donors. After subcloning, nucleotide sequence analysis showed a nucleotide difference at base number 2,603 (numbered according to Poncz et al.27 Fig 3). The G at position 2,603 was found in all eight clones obtained from the control donor and in five of nine clones obtained from the father. In the other four clones from the father, the normal G was changed into an A. This nucleotide difference predicts a valine → methionine substitution at amino acid position 837 of the mature GPIIb. Sequence analysis of the remaining regions did not show additional nucleotide differences.

ASRA

Studies on cDNA. The G → A substitution at position 2603 of the cDNA sequence abolishes a cleavage site for the restriction endonuclease BstYI, which cleaves at 5'-CCN(7)GG-3' but not at 5'-CCN(7)GA-3' sequences. The digestion of the PCR products (primers 6A and 6B) yielded fragments of expected size for Max+ and Max- individuals (data not shown).

Studies on genomic DNA. The detected substitution, localized at base number 13,940 of the GPIIb genomic sequence, eliminates a cleavage site for the restriction enzyme BstNI, which cuts at 5'-CCAGG-3' but not at 5'-CCAGA-3' sequences (Fig 4A). Therefore, a 448-bp fragment from genomic DNA of the father, three Max+ individuals, and one Max- donor was amplified (primers Bak1 and Bak2)
and digested with BstNI. As shown in Fig 4B, the G → A mutation was present in all Max++ individuals. Furthermore, we concluded that all Max++ subjects were found to be heterozygous for the new polymorphism (Fig 4B). Phenotyping and genotyping of 50 Max- unrelated donors was in full agreement.

Correlation Between Max and HPA-3 Polymorphism

The G → T difference responsible for the HPA-3 alloantigen system resides 19 bp downstream of the base substitution underlying the Max polymorphism. Therefore, the 448-bp fragment encompassing the Max and HPA-3 polymorphic sites were subcloned and tested for HPA-3 and Max-specific restriction sites by Fok I and BstNI digestion, respectively. As shown in Fig 5, the Max allele was associated with a HPA-3b allele. In the father, who is HPA-3b homozygous but Max heterozygous, Max+/HPA-3b clones were also found. By PCR, all HPA-3a individuals studied so far were found to be Max++.

Max Genotyping by ASPA

As the new polymorphism can be responsible for other cases of NATP, we developed an ASPA for the genotyping of the parents in cases of unresolved NATP. The ASPA strategy is illustrated in Fig 6A: the combination of the primers Bak1 and Max-A did not give a 123-bp product in Max- subjects, but did in Max++ individuals. The primer set Bak1/Max-G amplified the expected product in both Max++ and Max- subjects because all of them, including the father, were heterozygous for the Max polymorphism (Fig 6B). In all lanes, the control product of 371 bp is present.

The genotypes determined by ASPA were identical to those established by PCR-ASRA and with the observed phenotypes detected by PIFT and by MAIPA.

**DISCUSSION**

We report the identification of a new platelet-specific alloantigen, Max+, that was responsible for a case of neonatal alloimmune thrombocytopenia in a baby born from a healthy mother after an uneventful first pregnancy.

The antibody was detected in the maternal serum when it was tested with the paternal platelets. Reactions with platelets from selected donors with all the known platelet antigens were negative. The possibility that the NATP resulted from autoantibodies was excluded when we found negative reactions on the platelets from the mother after incubation with her own serum.

Because of positive reactions with MoAbs against the GPIIb-IIIa complex in the MAIPA assay, the antigen was located on the GPIIb-IIIa complex. All anti-GPIIb-IIIa MoAbs gave positive reactions except for the MoAb MB9, which recognizes an epitope on GPIIb. It was shown that this MoAb inhibits anti-Max antibody binding to Max++ platelets, as did another MoAb against GPIIb, 11A. However, this antibody was only very weakly inhibitory in the MAIPA. For this discrepant finding, we do not have an explanation. However, because of these findings we reasoned that the Max epitope was close to the GPIIb region in which the epitopes recognized by MB9 and 11A are located, and thus that Max was on GPIIb.

Max+ expression on GPIIb was also studied in immunoprecipitation and immunoblot experiments, but no platelet membrane glycoprotein was detected, neither with the maternal serum nor with an eluate from platelets sensitized with maternal serum. Thus, it appears that anti-Max antibodies had a too low-affinity and/or a too low-concentration to precipitate the GPIIb-IIIa complex or to bind to blotted GPIIb. Failure to react in the immunoblot could also be due to loss of the Max+ epitope by denaturation of GPIIb in this assay.

Anti-Max+ antibodies reacted with platelets of the father and three additional members of the paternal family. The inheritance pattern indicated an autosomal codominant inheritance. Furthermore, platelets from 500 donors were tested in the MAIPA with maternal serum. Because only three of them were found to carry the Max++ antigen, it appears to be a low-frequency platelet alloantigen with a phenotype...
The aggregation of platelets of Max⁺⁺ individuals was normal in response to all agonists tested; therefore, we excluded that the Max⁺ antigen affects the function of the GPIIb-IIIa complex. Until now, only anti-HPA-1a and anti-HPA-4a alloantibodies have been shown to inhibit platelet aggregation of HPA-1a⁺ and HPA-4a⁺ platelets, respectively. A weak inhibition of aggregation of Max⁺⁺ platelets was found when tested in the presence of anti-Max⁺ plasma. However, because Max⁺⁻ control platelets were weakly inhibited, it was probably an aspecific effect of the anti-Max⁺ plasma.

To elucidate the molecular basis of the Max⁺ antigen, we analyzed the nucleotide sequence of GPIIb cDNA after PCR amplification and subcloning. In the father we detected a G→A substitution at base 2603, predicting a valine→methionine difference at residue 837, close to the C terminus of the GPIIb heavy chain and near the HPA-3 polymorphism (amino acid position 843).²³

The detected substitution was confirmed by ASRA on cDNA from the father. Furthermore, full concordance between Max-phenotyping and Max-genotyping was shown by ASRA on genomic DNA obtained from the father and from all three Max⁺⁺ donors. All of them were found to be heterozygous for this polymorphism, as they showed the patterns derived from the digestion of both Max⁺⁻ and Max⁺⁺ alleles.

Because the G→A mutation was the only one detected in the entire GPIIb cDNA and full agreement was found between phenotyping and genotyping, it is likely that the detected substitution is indeed responsible for the Max⁺ epitope. We analyzed whether Max⁺ and HPA-3b epitopes cosegregate, because the responsible base substitution is sep-
GPllb MUTATION RESULTING IN MAX\(^*\) ANTIGEN

**A**

Max specific ASPA

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<th>exon 26</th>
</tr>
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<tbody>
<tr>
<td>Bak1</td>
<td>Max-G</td>
</tr>
<tr>
<td>Bak1</td>
<td>no product</td>
</tr>
<tr>
<td>Bak1</td>
<td>no product</td>
</tr>
<tr>
<td>Bak1</td>
<td>123 bp</td>
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</tbody>
</table>

GP IIb gene

- Bak1 Max-G
- Bak1 Max-A
- Max-A
- Max-G
- Max-a-
- Max-a+

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**B**

371 bp

1 2 3 4 M 5 6 7 8

123 bp

**Fig 6.** (A) Schematic representation of the region of the GPllb gene containing the Max polymorphism and strategy for the ASPA to distinguish between Max\(^+\) and Max\(^-\) alleles. The combination of primers Bak1 and Max-G amplifies a 123-bp product from a Max\(^+\) allele. The combination of primers Bak1 and Max-A amplifies a 123-bp fragment only from a Max\(^+\) allele. PCR products are not expected when Bak1 and Max-G are used for Max\(^-\) alleles or when Bak1 and Max-A are used for Max\(^-\) alleles. (B) Lanes 1 to 4, primers Bak1 + Max-G and primers 2,221 + 2,222; lanes 5 to 8, primers Bak1 + Max-A and primers 2,221 + 2,222; lanes 1, 2, 5, and 6, Max\(^+\) template; lanes 3, 4, 7, and 8, Max\(^-\) template. The 100-bp standard markers are in lane M. The 371-bp fragment amplified as an internal control with primers 2,221 and 2,222 is present in all lanes.

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The combination of primers Bak1 and Max-G amplifies a 123 bp fragment only from a Max\(^+\) allele. The combination of primers Bak1 and Max-A amplifies a 123 bp fragment only from a Max\(^+\) allele. PCR products are not expected when Bak1 and Max-G are used for Max\(^-\) alleles or when Bak1 and Max-A are used for Max\(^-\) alleles. When Bak1 and Max-G are used for Max\(^+\) alleles or when Bak1 and Max-A are used for Max\(^+\) alleles, the combination of primers Bak1 + Max-G and primers 2,221 + 2,222 amplifies a 123 bp product. When Bak1 and Max-A are used for Max\(^+\) alleles or when Bak1 and Max-G are used for Max\(^+\) alleles, the combination of primers Bak1 + Max-A and primers 2,221 + 2,222 amplifies a 123 bp product. Lanes 1 to 4, primers Bak1 + Max-G and primers 2,221 + 2,222; lanes 5 to 8, primers Bak1 + Max-A and primers 2,221 + 2,222; lanes 1, 2, 5, and 6, Max\(^+\) template; lanes 3, 4, 7, and 8, Max\(^-\) template. The 100-bp standard markers are in lane M. The 371-bp fragment amplified as an internal control with primers 2,221 and 2,222 is present in all lanes.

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**REFERENCES**


Max(a), a new low-frequency platelet-specific antigen localized on glycophosphatidylinositol IIb, is associated with neonatal alloimmune thrombocytopenia

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