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New platelet glycoprotein polymorphisms causing maternal immunization and neonatal alloimmune thrombocytopenia

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Abstract

BACKGROUND—Maternal immunization against low-frequency, platelet (PLT)-specific antigens is being recognized with increasing frequency as a cause of neonatal alloimmune thrombocytopenia (NAIT).

STUDY DESIGN AND METHODS—Serologic and molecular studies were performed on PLTs and DNA from two families in which an infant was born with severe thrombocytopenia not attributable to maternal immunization against known PLT-specific alloantigens.

RESULTS—Antibodies reactive only with paternal PLTs were identified in each mother using flow cytometry and solid-phase assays. Unique mutations encoding amino acid substitutions K164T in glycoprotein (GP)IIb (Case 1) and R622W in GPIIIa (Case 2) were identified in paternal DNA and in DNA from the affected infants. Each maternal antibody recognized recombinant GPIIb/IIIa mutated to contain the polymorphisms identified in the corresponding father. None of 100 unselected normal subjects possessed these paternal mutations.

CONCLUSIONS—Severe NAIT observed in the affected infants was caused by maternal immunization against previously unrecognized, low-frequency antigens created by amino acid substitutions in GPIIb/IIIa (α_{IIb}/β_3 integrin). A search should be conducted for novel paternal antigens in cases of apparent NAIT not explained on the basis of maternal-fetal incompatibility for known human PLT antigens.

Neonatal alloimmune thrombocytopenia (NAIT), caused by maternal antibodies produced against fetal platelet (PLT) alloantigens inherited from the father of an affected infant, occurs about once in 1000 live births and can result in intracranial hemorrhage leading to death or disability.[1–4] It is important that a serologic diagnosis be made even in mild cases of NAIT because infants subsequently born to the same mother can be more severely affected.[1,5] The most common antigen implicated as a trigger for NAIT in Western countries is human PLT antigen (HPA)-1a, determined by a leucine/proline polymorphism at Position 33 in the β-subunit of the PLT glycoprotein (GP)IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin).[6] However, multiple cases of NAIT caused by antibodies reactive with antigens designated HPA-2a/b carried on GPIb, HPA-3a/b on GPIIb, HPA-4a/b on GPIIIa, HPA-5a/b on GPIa, and HPA-15a/b on CD109 have been described.[7] In recent years, sporadic reports have

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CONFLICT OF INTEREST
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appeared of infants with NAIT caused by maternal immunization against a group of low-frequency PLT antigens created by amino acid polymorphisms on PLT GP complexes IIb/IIIa, Ib/IX, and Ia/IIa.[7–10] In this report, we describe two recently encountered cases of severe NAIT caused by maternal immunization against novel, low-frequency PLT-specific antigens, one carried on PLT GPIIb and the other on GPIIIa.

CASE REPORTS

Case 1 (HPA-22bw, Sey)

The first child, a girl, born to a 31-year-old mother by a spontaneous vaginal delivery, was found to have scattered petechial hemorrhages at birth. The PLT count was 13×10^9/L. Other hematologic indices were normal. A random-donor PLT transfusion and intravenous immune globulin (IVIG) 1.0 g/kg body weight were administered, after which the PLT count increased to 80×10^9/L. During the next 11 days, the child received two additional PLT transfusions and two IVIG infusions. After each transfusion, the PLT count increased to the range of 50×10^9 to 80×10^9/L but subsequently declined. On Day 9, the PLT count decreased to 22×10^9/L and bloody stools were observed. A PLT transfusion and IVIG were again administered. PLTs rose to 65×10^9/L and increased steadily thereafter. The child was discharged on Day 15 with a normal PLT count.

Case 2 (HPA-23bw, Hug)

The second child, a boy, born to a 22-year-old woman by spontaneous vaginal delivery developed widespread petechial and subconjunctival hemorrhages soon after birth and was found to have a PLT level of 13×10^9/L. Other hematologic findings were unremarkable except for a weakly positive direct antiglobulin test thought to be a consequence of maternal-fetal incompatibility for blood group B. At 1 day of age, the infant was transferred to Vanderbilt University Medical Center where it was found to have resolving petechial hemorrhages and a PLT level 27×10^9/L. IVIG, 1 g/kg body weight, was administered. The PLT counts were 38×10^9/L the next day and 102×10^9/L 2 days later. The child was discharged on Day 5.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies (MoAb) AP2 specific for the GPIIb/IIIa complex and AP3 specific for GPIIIa were described previously.[10] MoAbs 314.5, 142.11, and 202.2 specific for the GPIIb calf-2 domain, GPIb/IX, and GPIa/IIa, respectively, were produced in the Monoclonal Antibody Laboratory of the Blood Research Institute. MoAb Tab specific for GPIIb calf-2 was a gift from R. McEver (University of Oklahoma, Oklahoma City, OK). Alloantibodies specific for HPA-1a (Pl^{A1}) and HPA-3a (Bak^a) were from the Platelet and Neutrophil Immunology Laboratory of the Blood-Center of Wisconsin.

Serologic studies

Maternal samples from suspected NAIT cases were tested against paternal PLTs and PLTs from group O donors of known phenotypes using flow cytometry as previously described.[10–12] Antibodies reactive with GPIIb/IIIa, GPIb/IX, GPIa/IIa, and GPIV were detected by direct and modified antigen-capture enzyme-linked immunosorbent assay (ELISA, ACE, and MACE).[10]

Genotyping and frequency analysis

PLT genotyping for HPA-1-6a/b, -9a/b, and -15a/b was performed as described previously.[10] Genotyping for known single-nucleotide polymorphisms (SNPs) encoding low-frequency antigens of the HPA-7 to -14 and -16 to -21, Swi[13] and Cab2[14] was performed using a custom-designed genotyping system (Taqman OpenArray, Applied Biosystems, Foster City, CA).[15–17] Full-length sequencing of GPIIb and GPIIIa was performed as described previously.[10] Genotyping of 100 normal donor DNA samples for newly identified mutations was performed with an allelic discrimination assay using quantitative polymerase chain reaction (PerfeCTa, qPCR SuperMix, UNG, Low ROX, Quanta Biosciences, Gaithersburg, MD) and a real-time PCR system (Model 7500, Applied Biosystems).

Chinese hamster ovary cell lines expressing mutant versions of GPIIb/IIIa

Throughout this report, nucleotide (nt) 1 refers to A of the ATG translation start codon of human GPIIb or GPIIIa. Point mutations were generated using a site-directed mutagenesis kit (QuickChange IIXL, Stratagene, La Jolla, CA) and were verified by direct sequencing. Full-length human ITGA2B (GPIIb) cDNA in mammalian expression vector pCDNA3.1 Neo (Invitrogen, Carlsbad, CA) was modified at nt 584 from an “A” to a “C” to encode GPIIb K164T. Full-length ITGB3 (GPIIIa) in mammalian expression vector pcDNA3.1 Zeo (Invitrogen) was mutated at nt 1942 from a “C” to a “T” resulting in GPIIIa R622W. GPIIb K164T with GPIIIa, GPIIb with GPIIIa R622W, and GPIIb with GPIIIa plasmids were cotransfected into Chinese hamster ovary (CHO)-K1 cells as previously described.[18] Stable cell lines expressing recombinant forms of GPIIb and GPIIIa were identified after multiple sorts (FACStar Plus, Becton Dickinson, Franklin Lakes, NJ) for high integrin expression using MoAb AP2[10] and cloning at limiting dilution. Stable CHO-K1 cell lines were maintained as described.[10]

Human research

Patient samples were from suspected NAIT cases referred for routine testing to the Platelet & Neutrophil Immunology Laboratory, BloodCenter of Wisconsin. All studies involving human subjects were approved by the institutional research review board of BloodCenter of Wisconsin.

RESULTS

Serologic findings

Each mother had an IgG antibody that reacted strongly with paternal PLTs in flow cytometry but not with PLTs from normal group O donors carrying the HPA alloantigens HPA-1a/b, -2a/b, 3a/b, 4a, -5a/b, -6a, and -15a/b. Neither serum contained Class I HLA antibodies. When tested in MACE using monoclonal AP2 for capture, each mother was found to have an antibody that recognized GPIIb/IIIa on paternal PLTs. Blood types of fathers were A_1 (Sey) and A_2B (Hug). Because high-titer IgG maternal antibodies specific for blood groups A or B can cause neonatal thrombocytopenia in infants who possess a genetic trait that causes PLTs to express many times the normal levels of A or B antigens,[12,19] the maternal sera were absorbed with washed group A_1 and B red blood cells. This treatment had no effect on the reactions with paternal PLTs. All serologic findings are summarized in Table 1.

PLT typing

Genotyping of parental DNA for antigens of the HPA-1-6, -9, and -15 systems showed that there was potential maternal-fetal incompatibility for HPA-1b in Case 1 and HPA-2b, -3b,

and -15a in Case 2. However, reactions of maternal sera with normal PLTs carrying one or more of these markers were completely negative (Table 1). DNA from each father was also typed for the recognized low-frequency alleles HPA-4b, 6-14bw, 16-21bw, Swi,[13] and Cab2,[14] but none were detected.

Mutations predicting novel amino acid changes in GPIIb (HPA-22bw, Sey) and GPIIIa (HPA-23bw, Hug) were identified in DNA from the fathers and the affected infants

The strong reactions of maternal serum with paternal GPIIb/IIIa together with failure to identify one of the known common or low-frequency HPA antigens as a cause of maternal immunization suggested that the maternal antibodies might be specific for previously non-described PLT-specific antigens. Therefore, paternal DNA encoding the extracellular domains of GPIIb and GPIIIa was sequenced to detect mutations predicting amino acid polymorphisms in these proteins. In Case 1 (Sey), sequencing of paternal ITGA2b (GPIIb) Exon 5 revealed an A- to C- SNP at nt 584 that predicted a lysine-to-threonine switch at Position 164 (Fig. 1). In Case 2 (Hug), sequencing of paternal ITGb3 (GPIIIa) Exon 12 revealed a C-to-T SNP at nt 1942 that predicted an arginine-to-tryptophan switch at Position 622 (Fig. 1). The same novel mutations were found in DNA obtained by buccal swab from the affected infants but not in DNA from their mothers (data not shown). Neither mutation was detected in any of 100 unselected normal individuals.

Maternal antibodies recognized GPIIb/IIIa mutated to contain the appropriate paternal mutation

To confirm that the maternal antibodies were specific for the mutations identified in the two fathers, stable CHO cell lines expressing GPIIb/IIIa mutated to contain threonine 164 in GPIIb (Sey) and tryptophan 622 in GPIIIa (Hug) were generated and used as targets for maternal antibody. As shown in Fig. 2, each antibody recognized the appropriate paternal mutant, but not nonmutated GPIIb/IIIa using both flow cytometric and MACE assays for antibody detection.

DISCUSSION

These observations demonstrate novel mutations predicting amino acid substitutions in GPIIb (K164T) and GPIIIa (R622W) in fathers of Cases 1 and 2, respectively, and in the affected infants. In each case, maternal antibodies were detected that reacted only with paternal PLTs and with recombinant GPIIb/IIIa mutated to contain the identified paternal mutation. Together, the findings indicate that maternal immunization against the newly identified antigens was the cause of severe NAIT experienced by each of the infants.

Location of the HPA-22bw (Sey) and HPA-23bw (Hug) antigens in the PLT GPIIb/IIIa complex is illustrated in Fig. 3. HPA-22bw is the first alloantigen to be localized to the β propeller domain of GPIIb; HPA-23bw is the fifth antigen identified in the GPIIIa cystatin domain, located immediately adjacent to the cell membrane. Locations of other recognized low-frequency antigens produced by amino acid polymorphisms on GPIIb/IIIa are also shown in Fig. 3. It is apparent that there is clustering of mutations in the βA and cystatin domains of GPIIIa. Recent structural studies show that, in its resting state, GPIIb/IIIa (α_{IIb}/β_3 integrin) is folded in such a way that the βA and cystatin domains are adjacent to each other,[20] bringing all 9 of the sites where these mutations occur into close proximity. As would be expected, each of the sites is predicted to be solvent exposed.[20] The HPA-22bw mutation in the GPIIb β propeller domain is very close to the RGD recognition site that is critical for binding of fibrinogen to activated GPIIb/IIIa.[21–23] It is conceivable that interference of the HPA-22bw antibody with this function was in part responsible for the severe bleeding experienced by the infant although, in a heterozygote, half of the GPIIb/IIIa

receptors would be unaffected by antibody and its father had no history of spontaneous bleeding.

Low-frequency PLT alloantigens shown to cause maternal immunization leading to NAIT include those now designated HPA-4b;[24,25] HPA-6bw;[26,27] HPA-7bw;[28] HPA-8bw;[29] HPA-9bw;[30] HPA-10bw;[31] HPA-11bw;[32] HPA-12bw;[33] HPA-13bw;[34] HPA-14bw;[35] HPA-16bw;[36] HPA-17bw;[37] HPA-18bw;[38] HPA-19bw, -20bw, and -21bw;[10] HPA-22bw;[39] Swi;[13] and Cab2.[14] The two new antigens described here bring the total to 21. The most important of these antigens from a clinical standpoint appears to be HPA-9bw, found on PLT GPIIb of approximately 1 in 400 persons of Northern European ancestry.[18,30,40] Fourteen cases of NAIT caused by HPA-9bw antibodies have now been reported, many of which were severe and five of which were complicated by intracranial hemorrhage.[18,30,40] Other reports of NAIT caused by maternal immunization against low-frequency antigens involved single cases with the exception of HPA-4b,[24,25] HPA-6bw,[26,27,41] HPA-8bw,[29,41] HPA-10bw,[31,41] HPA-11bw,[9,32] and HPA-13bw.[9,34,42]

Between 65[11] and 80%[41] of suspected NAIT cases are unresolved on the basis of maternal-fetal incompatibility for “common” antigens of the HPA-1, -2, -3, and -5 systems. The number of low-frequency PLT antigens that appears to be capable of triggering NAIT is steadily growing and it is important to define the extent to which maternal immunization against these markers contributes to the mix of NAIT cases. Ghevaert and coworkers[41] typed about 1000 paternal DNA samples from fathers (mainly Caucasians) of suspected NAIT cases unresolved by standard testing for 11 low-frequency antigens. They found eight fathers who possessed the antigens HPA-6b, -10bw, -11bw, or -12bw and concluded that NAIT caused by sensitization against low-frequency HPA antigens is rare in Caucasian populations. However, typing for HPA-9bw, clearly an important trigger for NAIT, and other, more recently described antigens, was not performed. Recent studies indicate that two low-frequency antigens, HPA-4b and HPA-6bw, are much more prevalent in Asian populations than in Caucasians.[43,44] Further studies are needed to determine whether other low-frequency antigens are relatively common in certain racial groups and may therefore be important causes of NAIT in those populations.

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JAP, BC, JM, and RHA each contributed to research design, oversight of laboratory studies, interpretation of results, and manuscript preparation. SP, MG, and AK performed laboratory studies, contributed to interpretation of findings made, and helped with manuscript preparation. JR and VK identified the affected patients, provided clinical details, and obtained blood and buccal swab samples.

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ABBREVIATIONS

CHO	Chinese hamster ovary
GP	glycoprotein
MACE	modified antigen-capture enzyme-linked immunosorbent assay
NAIT	neonatal alloimmune thrombocytopenia
nt	nucleotide (when followed by a number)

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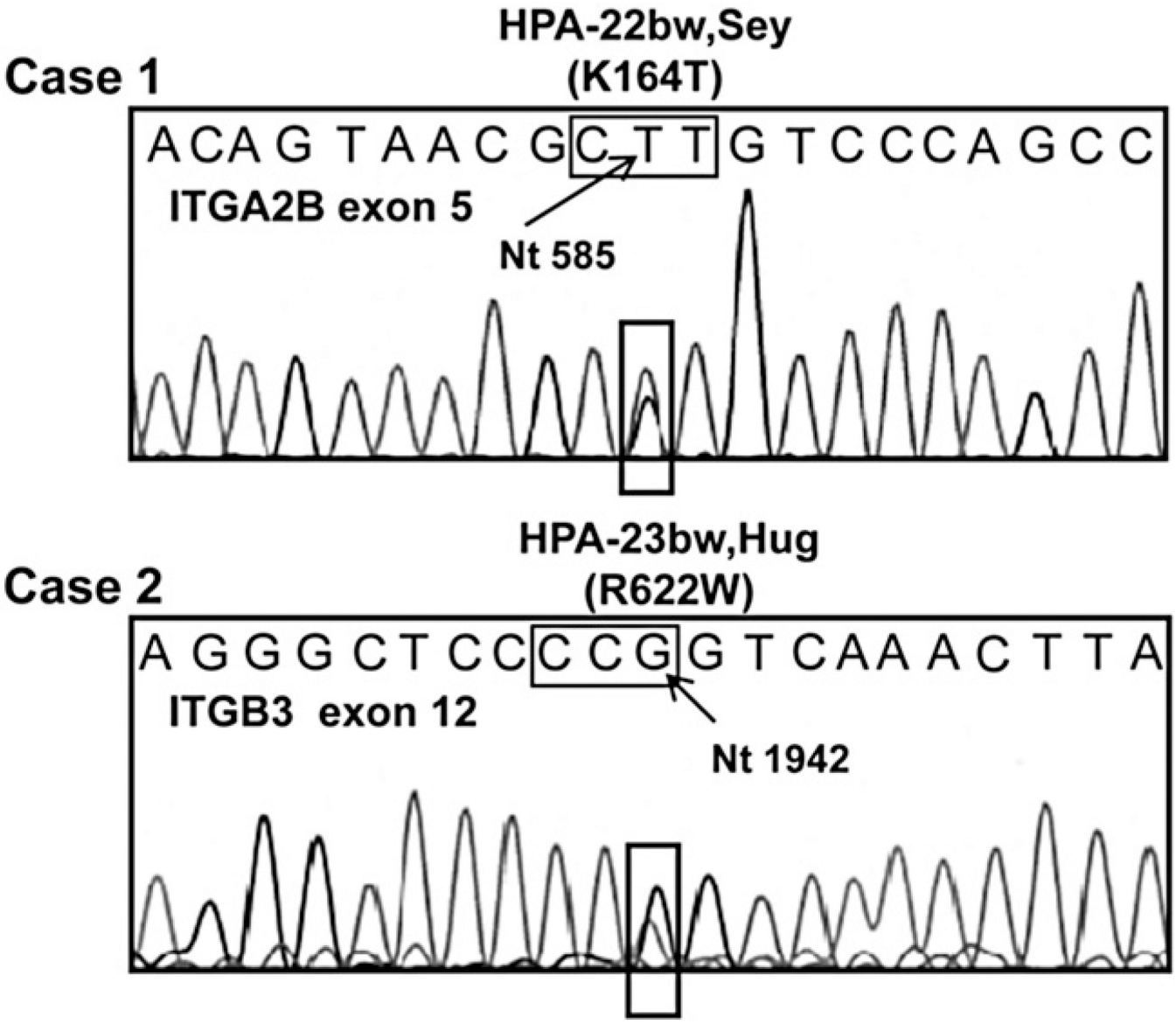

Fig. 1.

Novel mutations predicting amino acid substitutions in GPIIb and GPIIIa. Case 1—Sequencing of GPIIb revealed an A-to-C substitution at nt 584 resulting in a lysine-to-threonine switch at Position 164. Case 2—A C-to-T substitution at nt 1942 predicting an arginine-to-tryptophan switch at Position 622 in GPIIIa was identified. Results shown were obtained with antisense primers. Corresponding results were obtained with sense primers (data not shown). Nucleotides coding for the mutant amino acid are boxed.

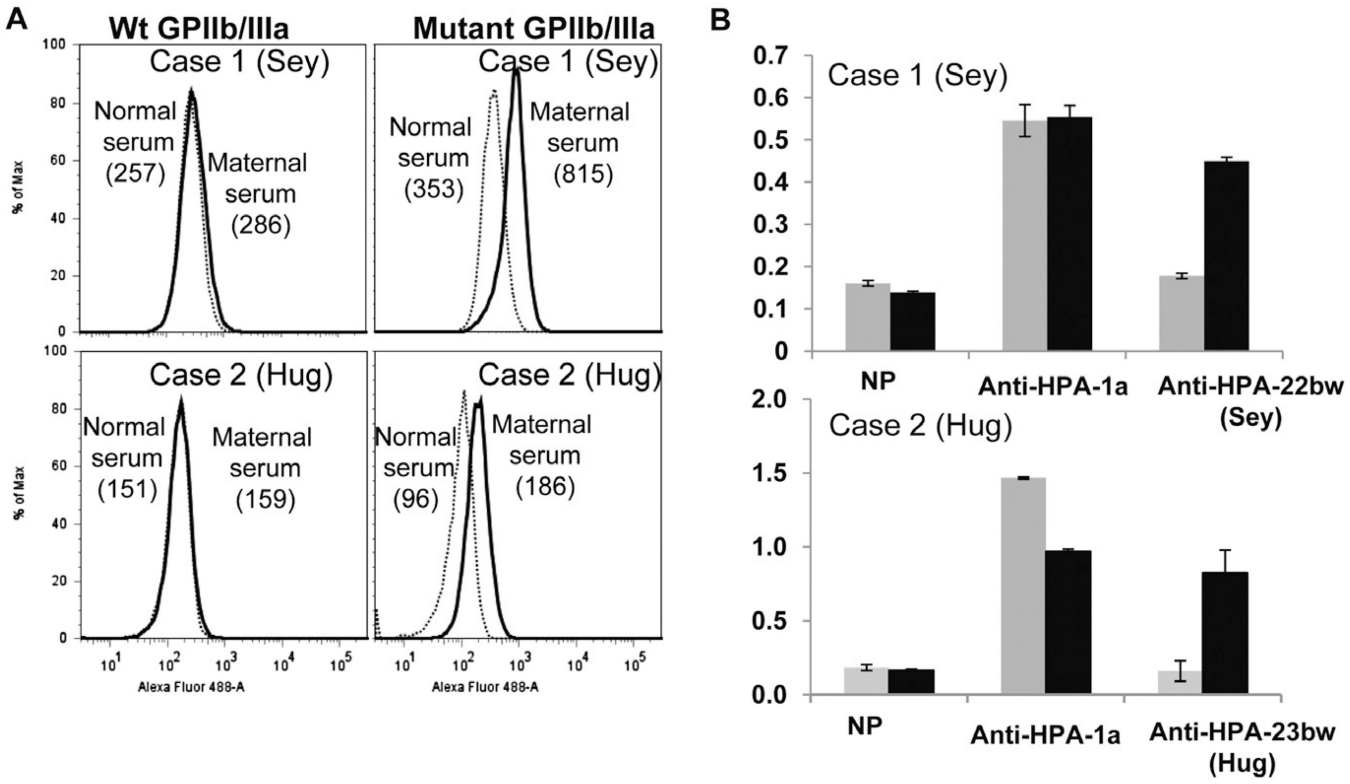

Fig. 2.

Reactions of maternal sera with CHO cell lines stably expressing recombinant wild type (Wt) GPIIb/IIIa and recombinant mutated GPIIb/IIIa. (A) Reactions of Case 1 and Case 2 maternal sera with transfected CHO cell lines (flow cytometry). Serum from Case 1 (Sey), top, and Case 2 (Hug), bottom, recognized GPIIb/IIIa mutated to contain the amino acid change identified in paternal DNA (right) but not wild-type GPIIb/IIIa (left). Numbers shown are median fluorescence intensity values. (B) Reactions of maternal sera with transfected CHO cell lines (MACE).Maternal antibody from Case 1 (top) and Case 2 (bottom) precipitated GPIIb/IIIa containing the mutation identified in paternal DNA (■) but not wild-type GPIIb/IIIa (■). Anti-HPA-1a recognized both constructs and normal serum (NP) recognized neither. Brackets denote mean of triplicate determinations ±1 SD.

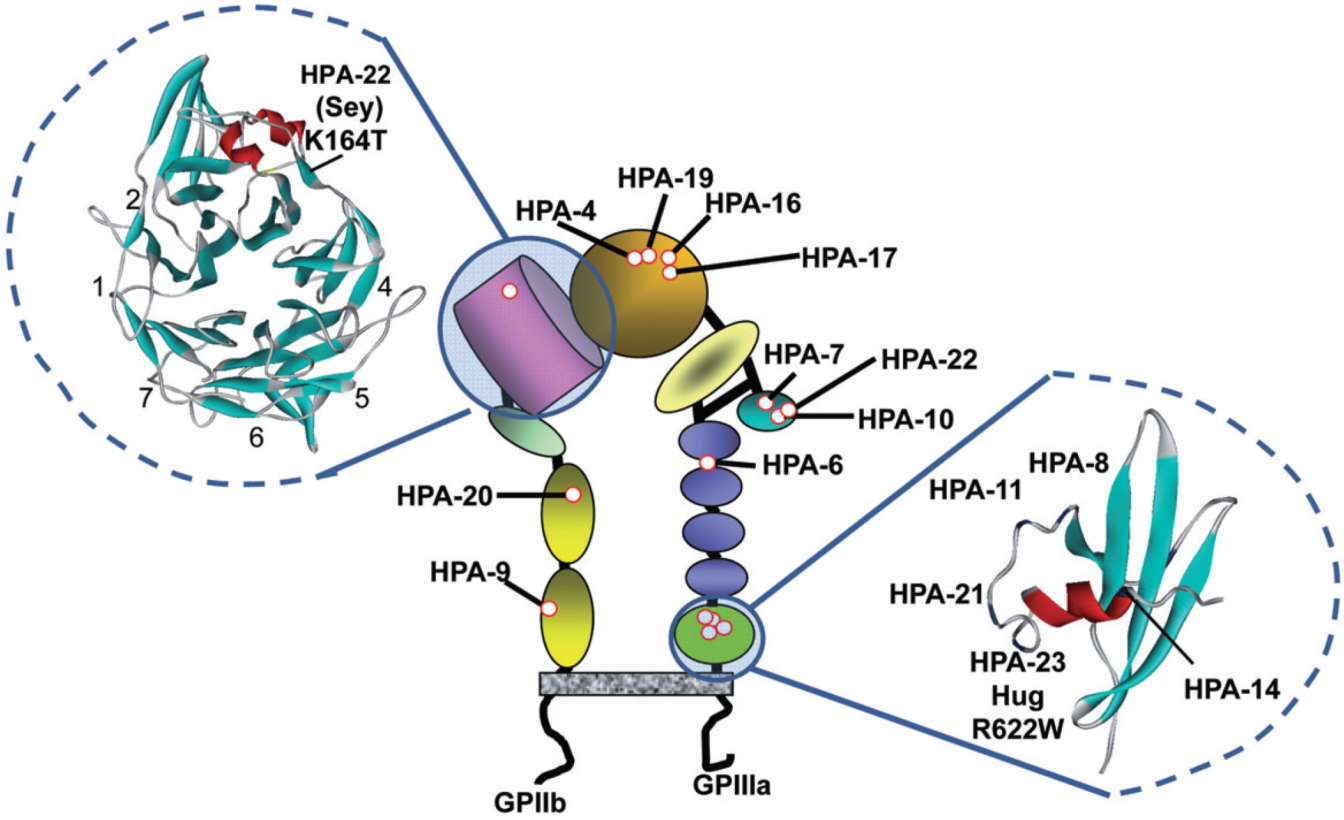

Fig. 3.

Location of HPA-22bw (Sey), HPA-23bw (Hug), and other low-frequency HPA antigens in the GPIIb/IIIa complex. Ribbon diagrams of the β propeller domain of GPIIb (left) and the β3 cystatin domain (right) were generated using crystal coordinates reported by Zhu et al.[20]

TABLE 1

Summary of findings

Molecular and serologic characterization	Case 1 (HPA-22 [Sey])	Case 2 (HPA-23 [Hug])
Maternal serum versus healthy donor GPIIb/IIIa (HPA-1a/a, -3a/a)		
Flow cytometric analysis	IgG−/IgM−	IgG−/IgM−
MACE (AP2 capture of GPIIb/IIIa)	−	−
Maternal serum versus healthy donor GPIIb/IIIa (HPA-1b/b, -2a/a, -3b/b, -5a/b, -15a/b)		
Flow cytometric analysis	IgG−/IgM−	IgG−/IgM−
MACE (AP2 capture of GPIIb/IIIa)	−	−
Maternal serum versus paternal GPIIb/IIIa		
Flow cytometric analysis	IgG+/IgM+	IgG+/IgM−
(AP2 capture of GPIIb/IIIa)	−	−
MACE (AP2 capture of GPIIb/IIIa)	+	+
Mother/Father blood type	O/A1	O/A2B
MACE reactivity after absorption of anti-A or anti-B	+	+
Molecular characterization		
Exon/nucleotide change	5/A584C	12/C1942T
Amino acid change	K164T	R622W
Protein	GPIIb	GPIIIa
NCBI dsSNP accession number	rs142811900	rs139166528
Maternal serum vs. recombinant GPIIb/IIIa mutated to express paternal SNP		
Flow cytometric analysis with transfected CHO cell lines	+	+
MACE with mutated GPIIb/IIIa (AP2/Tab capture)	+	+