Sr*, a Private Platelet Antigen on Glycoprotein IIIa Associated With Neonatal Alloimmune Thrombocytopenia

By Hartmut Kroll, Volker Kiefel, Sentot Santoso, and Christian Mueller-Eckhardt

A new platelet alloantigen, Sr*, is described that was defined by an alloantibody detected in the serum of a healthy mother who delivered a child with typical clinical signs of neonatal alloimmune thrombocytopenia (NAIT). The antibody reacted strongly with the child’s and father’s platelets, but not with platelets of the mother or with those of a highly selected panel representing all known platelet alloantigens. Platelets from 300 unselected normal blood donors also tested negative, suggesting a phenotype frequency in the German population of less than 0.01. The antigen was present in 9 of 20 members within three generations of the paternal family, indicating autosomal codominant inheritance. By immunochemical analysis using a glycoprotein (GP)-specific immunoassay and a variety of GP Ib/IIa-specific monoclonal antibodies for antigen immobilization (MAIPA assay), radioimmunoassay, and Western blotting, we could show that the antigen resides on a 68-Kd proteolytic fragment of GP IIa. Immunogenetic data and gene dosage studies revealed that the Sr* antigen is not related to any of the other known platelet alloantigens. In accordance with established criteria, the Sr* antigen represents the first example of a “private” platelet alloantigen that bears significance in rare instances of NAIT.

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Neonatal alloimmune thrombocytopenia (NAIT) is a bleeding disorder of newborns with increased platelet destruction caused by transplacentally transferred maternal platelet-specific alloantibodies. The most important antigen responsible for maternal immunization is Zw* (PI*A),2,3 while the newly discovered Br* antigen4 is the second most frequent antigen involved in NAIT in the European population. All other antibody specificities, ie, anti-Bak*, anti-Bakb, anti-Zw*, anti-Ko*, have only rarely been associated with fetomaternal platelet incompatibility. In our recent survey of 349 cases of clinically suspected NAIT, 78% of serologically verified cases were due to anti-Zw*, and 19% to anti-Br*; all other specificities accounted for less than 5% of NAIT cases.5 The situation is different in the Oriental population, in which anti-Yuk6 appears to play the prominent role.7

In typical NAIT cases where the mother is Zw(a+) and the maternal serum does not show any reactivity with a selected platelet panel carrying all known platelet antigens, it is important to assess the maternal serum against paternal platelets (crossmatch) to avoid missing any unknown or rare platelet alloantibodies. Using this approach we have discovered a new, rare platelet alloantigen, Sr*, that is involved in NAIT and will be described in detail here.

CASE REPORT

In 1986, a 24-year-old white primigravida gave birth to a mature girl (2,670 g, 47 cm, Apgar 9/10/10) after an uneventful pregnancy. Delivery was by cesarean section because of breech presentation. Two hours later the neonate developed generalized petechiae and thrombocytopenia (platelet count postpartum was 23 × 10^9/L, nadir 16 × 10^9/L on day 1). Because the child showed no clinical or laboratory signs of sepsis or intrauterine infection, NAIT was suspected and the child was treated with daily doses of 1 g intravenous immunoglobulin for 3 days. This led to rapid, complete remission (platelet count on day 12 was 146 × 10^9/L). Sonographic examination revealed no signs of cerebral hemorrhage. Since then the child has developed normally. The platelet counts of both parents were normal.

In 1988 the mother became pregnant again. To evaluate the risk of intrauterine bleeding, fetal blood sampling was performed at 35 weeks of gestation. The fetal platelet count was normal (167 × 10^9/L) and the platelets did not react with maternal serum. In June 1989, a healthy child was delivered with no signs of bleeding tendency and with a normal platelet count of 200 × 10^9/L.

Materials and Methods

Antisera, test platelets, and HLA typing. Blood samples of the mother and the first child were referred to us shortly after delivery in 1986 because of suspected NAIT. After identification of the antibody with paternal platelets in 1988, blood samples of all available paternal family members were collected. Serum was kept frozen at −20°C. Platelets were isolated from EDTA-anticoagulated blood by differential centrifugation and stored at 4°C in isosonic saline containing 0.1% NaN3 (final concentration). Panel platelets were selected from a large number of blood donors with known platelet antigens Zw(a,b), Bak(a,b), Br(a,b), Yuk(a,b), HLA, and ABO blood group antigens. For typing of paternal alloantigens of family members and donors, the following typing reagents were used: Anti-Zw* and anti-Bak* from patients with posttransfusion purpura (PTP); anti-Zw* and anti-Br* from polytransfused patients; and anti-Bak* and anti-Br* from mothers of children with NAIT. Anti-Yuk* and anti-Yuk* were gifts from Dr Y. Shibata, Tokyo.

Lymphocytes of patients were harvested from ACD-anticoagulated blood and typed for HLA antigens using the immunomagnetic bead technique.8

Serological and immunohematological techniques. Maternal serum was screened for lymphocytotoxic HLA antibodies in the National Institutes of Health standard method of the lymphocytotoxicity test (LCTT)9 and for platelet-specific antibodies in the platelet adhesion immunofluorescence test (PAIFT).10 Special studies were performed using a glycoprotein (GP)-specific immunoassay based on immobilization of platelet antigens with monoclonal antibodies (MoAbs, MAIPA assay), by radioimmunoassay, and by immunoblotting. The MAIPA assay was carried out as described by Kiefel et al.5; 5 × 10^9 platelets were incubated with 40 µL human serum and 0.2 to 0.8 µg of a GP-specific MoAb...
for 30 minutes at 37°C, washed and solubilized in 0.01 mol/L Tris, 0.5% Nonidet P-40, and 0.154 mol/L NaCl. The lysates were centrifuged (13,000 g for 30 minutes at 4°C) and the supernatants transferred to a microtiter tray coated with a goat antimouse polyclonal antibody (Dianova, Hamburg, West Germany). After incubation for 90 minutes at 4°C the microtiter tray was washed four times with 0.01 mol/L Tris, 0.5% Nonidet P-40, 0.05% Tween 20, 0.5 mmol/L CaCl$_2$, and 0.154 mol/L NaCl (washing buffer). During this step, the GPs recognized by the mouse MoAb were fixed by washing of the wells and addition of p-nitrophenyl phosphate substrate solution (Sigma, Munich, West Germany). Optical density was measured in a Titertek Multiscan photometer (E llenby O y, Helsinki, Finland). The following MoAbs against different epitopes on the GP Ib/IIa complex were used: MoAbs Gi 5 (PA3)$^{18}$ and P2$^{20}$ (anti-GP Ib/IIa complex), MoAbs ZZ21 (anti-GP IIa),$^{19}$ and MoAbs ZZ22 (anti-GP IIb).$^{19}$ MoAb Gi 14$^{20}$ reacts with the GP Ia/IIa complex, and MoAb A-1A5$^{51}$ with GP IIa, the common $\beta$ chain of GP Ia/IIa, Ic/IIa, and Ic'/IIa heterodimers. MoAb FMC 25$^{25}$ recognizes GP IX, tightly complexed to GP Ib; and MoAb w6.32$^{2}$ is specific for a monomorphic epitope on the heavy chain of HLA class I molecules. MoAbs Gi 5 and Gi 14 were raised in our own laboratory; MoAbs P2, ZZ21, and ZZ22 were purchased from Dianova; and FMC 25 was a gift of Dr H. Zola, Adelaide, Australia.

Radioimmunoprecipitation was done with $^{125}$I-labeled platelets as recently described.$^{20}$ In brief, 5 x $10^4$ labeled platelets were incubated with 100 $\mu$L human serum for 30 minutes at 37°C, then washed two times with TENA buffer (0.01 mol/L Tris, 5 mmol/L EDTA, and 0.154 NaCl, pH 7.4) solubilized in 100 $\mu$L 0.01 mol/L Tris, 1% leupeptin (Sigma), 1% Nonidet P-40, and sonicated for 3 minutes. After centrifugation of the lysates (13,000 g for 30 minutes) the supernatants were incubated for 30 minutes with 100 $\mu$L of a 10% suspension of staphylococcus aureus, Cowan I strain (Pharmaicia, Freiburg, West Germany), which was preincubated with 100 $\mu$L rabbit antihuman IgG (dilution 1:10; Dako, Hamburg, West Germany). The bacteria were washed first with 500 $\mu$L 0.01 mol/L Tris, 0.1% Nonidet P-40, and 0.5% LiCl, pH 8.0, then with 500 $\mu$L 0.01 mol/L Tris and 0.1% Nonidet P-40, pH 8.0, and finally with 500 $\mu$L 0.01 mol/L Tris, pH 8.0. After resuspension in 100 $\mu$L sample buffer containing 2% sodium dodecyl sulfate (SDS) and boiling for 5 minutes, the bacteria were spun down (13,000g for 5 minutes) and the supernatant containing the platelet GPs separated in 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). The gel was dried and autoradiographed. For proteolytic treatment, $^{125}$I-labeled platelets were incubated for 225 minutes at 37°C with chymotrypsin or trypsin (Sigma) at a final concentration of 1 mg/mL. The enzymatic reaction was terminated by addition of phenylmethylsulfonylfluoride or trypsin inhibitor, respectively (final concentration 1 mg/mL; Sigma).

The immunoblotting procedure was modified from Towbin et al.$^{24}$ Either fresh or stored platelets were washed and solubilized in 0.01 mol/L Tris, 1% leupeptin, and 1% Nonidet P-40, and sonicated for 3 minutes. The platelet proteins were separated on 7.5% SDS-PAGE under nonreducing conditions and electrophoretically transferred to nitrocellulose sheets by the semidyne technique of Kyhse-Anderson.$^{25}$ After blocking with phosphate-buffered saline with 3% bovine serum albumin (3% BSA/PBS) for 60 minutes at 37°C, the nitrocellulose strips were washed three times with 3 mL PBS containing 0.1% Tween 20 (washing buffer) and incubated overnight with 100 to 200 $\mu$L of eluates of the human sera diluted in 3 mL of 1% BSA/PBS. After three washings, 3 mL $^{125}$I-labeled sheep antihuman IgG (100,000 cpm; Amersham Buchler, Braunschweig, West Germany) per strip were added for 60 minutes. The nitrocellulose strips were washed three times again, dried, and autoradiographed.

The preparation of eluates for immunoblotting was adapted from the acid elution technique described by Hotchkiss et al.$^{26}$ Aliquots of 3 x $10^8$ platelets were incubated with 60 to 300 $\mu$L human serum for 30 minutes at 37°C, washed three times in isotonic saline, and acidified to a pH of 2.8 by addition of 150 mmol/L acetic acid in 0.1 mol/L NaCl containing 1.5% BSA for 10 minutes. After centrifugation, the pH of the supernatant was adjusted to 7.2 with 2.5 mol/L Tris base.

For removal of blood group A antibodies, 1 mL of serum containing anti-Sr$^+$ was absorbed three times (for 60 minutes at 37°C) with 2 mL of packed red blood cells (RBCs) from a blood group A, donor.

RESULTS

The serum containing anti-Sr$^+$ did not react with a panel of nine donor cells in the LCTT, indicating the absence of HLA-specific lymphocytotoxic antibodies. When tested in the PAIFT using a platelet panel of five typed donors, the reactions were negative; strong fluorescence was observed with paternal platelets. This reactivity was still discernible when serum Sr was exhaustively preabsorbed with blood group A, RBCs. In the MAIPA assay, serum Sr was strongly positive with paternal platelets using the GP Ib/IIa-specific MoAb Gi 5 for antigen immobilization, whereas the platelets of 28 typed panel donors, including those of a

[Fig 1. Reactivity of anti-Sr$^+$ (■) tested in MAIPA assay with five panel platelets and paternal platelets using MoAb Gi 5 for immobilization of the GP Ib/IIa complex. Antigens of the Zw and Bak system of platelet GPs are indicated. Paternal platelets tested without antibody (□) as negative and Zw(a+) platelets with a strong anti-Zw$^+$ (□) as positive control. Anti-Sr$^+$ reacted with paternal platelets but not with panel platelets carrying all known alloantigens of the Zw or Bak systems on the GP Ib/IIa complex. OD, optical density.]
Yuk(a+b+) individual (kindly made available to us by Dr Shibata, Tokyo) did not react with anti-Sr*. The results of a representative experiment are illustrated in Fig 1. Identical results with serum Sr were obtained when other MoAbs directed against the GP IIb/IIIa complex (MoAbs P2, SZ21, and SZ22) were used for immobilization of this GP complex. Serum Sr failed to react if MoAbs specific for GPs Ia/IIa, Ic/IIa, Ic'/IIa, Ib/IX, and HLA class I were used (data not shown). These data indicate that the antibody in serum Sr has specificity for an epitope on the GP IIb/IIIa complex, which is different from all other known platelet alloantigens on this complex.

To further localize the antigen, serum Sr was investigated by immunoprecipitation and immunoblotting. Figure 2 shows the results of immunoprecipitation by anti-Sr* and anti-Zw* in the presence of 3 and 5 mmol/L EDTA. Anti-Sr* precipitated predominantly GP IIIa with a molecular mass of 89-Kd (lanes 3 and 5), much like anti-Zw* (lanes 2 and 4). The slight coprecipitation of GP IIb was abrogated in the presence of 5 mmol/L EDTA (lanes 4 and 5).27 In immunoblotting experiments using Sr(a+), Zw(a+), and Bak(a+) platelets and antibody eluates (Fig 3), anti-Zw* and anti-Sr* reacted with GP IIIa (lanes 2 and 3), clearly distinct from anti-Bak*, which bound to GP IIb (lane 4). Under reducing conditions both the Zw* antigen and the Sr* antigen were no longer discernible.

When immunoprecipitation was performed after proteolytic digestion of platelets with chymotrypsin and trypsin (Fig 4), anti-Sr* and anti-Zw* precipitated fragments with a molecular weight of 68 and 74 Kd after cleavage with chymotrypsin (lanes 2 and 3) or trypsin treatment (lanes 4 and 5). No fragments were precipitable from the platelet supernatants after enzymatic degradation.

The inheritance pattern of the Sr* antigen in family Sr is presented in Fig 5. Table 1 compiles immunogenetic data of
Fig 4. A 7.5% SDS-PAGE of ¹²⁵I-labeled GP fragments after proteolytic digestion of platelets with chymotrypsin (lanes 2, 3, 6, and 7) and trypsin (lanes 4, 5, 8, and 9). GP fragments were immunoprecipitated with anti-Zw⁺ (lanes 2, 4, 6, and 8) and anti-Sr⁺ (lanes 3, 5, 7, and 9) from platelets (PL) and supernatants (SN) in the presence of 5 mmol/L EDTA. Anti-Zw⁺ and anti-Sr⁺ immunoprecipitated the platelet-bound 68- and 74-Kd fragments of GP IIIa, whereas no fragment could be precipitated from the supernatants after enzymatic treatment. Lane 1: ¹⁴C molecular weight standard.

all family members tested with regard to ABO and Rhesus blood groups, platelet antigens, and HLA-A, B, C, and DR phenotypes and haplotypes. It is evident that the Sr⁺ antigen segregates as an autosomal-codominant trait. Because the offspring of three Sr⁺(a+) fathers within two generations were Sr⁺(a+) as well as Sr⁺(a-), all Sr⁺(a+) individuals must be heterozygous for this character. Because both Sr⁺ and Zw⁺ are apparently localized on the same proteolytic fragment of GP IIIa (Figs 3 and 4) and all family members were Zw⁺(a+b-) (Table 1), the possibility had to be precluded that Sr⁺ represents a third rare allele of the Zw antigen system. For this purpose, we used the known gene dose effect for the Zw⁺ alloantigen, which had already been described by Shulman et al.¹ They showed that anti-Zw⁺ reacts more strongly with platelets from Zw⁺Zw⁺(a+b-) individuals than with Sr⁺(a-), Zw⁺(a+b-) platelets. As illustrated in Fig 6, Sr⁺(a+) as well as Sr⁺(a-) individuals showed approximately the same strength of reaction with anti-Zw⁺, not different from Zw⁺ homozygous controls. In contrast, platelets from Zw⁺(a+b-) heterozygous, Sr⁺(a-) unrelated donors bound only half the amount of Zw⁺ antibodies.

The frequency of the Sr⁺ antigen in the German population was estimated by typing of platelets from 300 unrelated blood donors with anti-Sr⁺ in the MAIPA assay. No positive individual was found suggesting an antigen frequency of less than 0.01.

DISCUSSION

We here report a new, rare platelet alloantigen. The corresponding antibody was found in the serum of a healthy white mother who gave birth to her first child. Although the neonate presented with the typical clinical features of NAIT, to our surprise no platelet antibodies were detectable on first investigation in maternal serum using a selected platelet panel possessing all known platelet antigens. The observation that the child responded well to IgG therapy further favored the clinical diagnosis. Only on retrospective analysis using the original maternal serum and paternal platelets (cross-match) was it possible to verify the NAIT serologically. This finding clearly emphasizes the necessity to perform crossmatches. Whereas this is common practice in red cell serology, it is seldom applied with platelets. This is particularly true because most reference laboratories practice one or another modification of the immunofluorescence assay for screening, which requires fresh platelets not attainable from

Fig 5. Pedigree of family Sr. Black symbols represent Sr⁺(a+) and open symbols Sr⁺(a-) individuals. The arrow indicates the child with NAIT caused by anti-Sr⁺.
Table 1. Platelet Alloantigens, Blood Groups, and HLA Antigens of Family Sr

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Abbreviations: HT, haplotype; NT, not tested.

*Refer to Fig 5.

referred samples. Hence, in patients with clinically suspected NAIT where none of the already established alloantibody specificities can be found with a well-typed platelet panel, techniques must be used that reliably function with stored, refrigerated platelets, ie, the MAIPA assay or similarly adapted binding tests.

Antibody Sr reacted with platelets of the affected infant, his father, and seven additional members of the paternal family (Fig 5) indicating autosomal-codominant inheritance. Because none out of more than 300 unselected blood donors had this antigen, the phenotype frequency of Sr* is less than 0.01 and the chance to detect an Sr* homozygous individual is extremely low. Incidentally, all members of family Sr typed Zw(a+b-). Theoretically, the possibility existed that Sr* would constitute a rare, as yet unknown third allele of the Zw system. This possibility was precluded by determination of the Zw* gene dosage of Zw(a+b-) individuals of family Sr who were either positive or negative for the Sr* determinant as compared with unrelated Sr(a-), but homozygous or heterozygous Zw* individuals. As illustrated in Fig 6, there was no significant difference of anti-Zw* binding between Sr(a+) and Sr(a-) family members, with relative binding
values equivalent to unrelated homozygous Zw'Zw' individuals, indicating that all family members are Zw'Zw' homozygous.

Immunochemochemical studies provided clear evidence that the Sr' antigen resides on GP IIIa. The antigen could only be immobilized in the MAIPA assay by MoAbs directed against different epitopes of the GP IIIa/IIIa complex, e.g., MoAb Gi 5 (Fig 1). In radioimmunoprecipitation, anti-Sr' produced a band of 89-Kd, similar to anti-Zw', consistent with GP IIIa in the presence of high EDTA concentrations (Fig 2). After treatment of Sr(a+), Zw(a+) platelets with chymotrypsin or trypsin, the Sr and the Zw antigens were still detectable on a 68- and a 74-Kd fragment of GP IIIa, respectively. The 68-Kd fragment after chymotrypsin treatment probably corresponds to the 66-Kd fragment described by Kornecki et al20 or the 55- to 65-Kd band of Dancis et al21, whereas the 74-Kd band after tryptic cleavage confirms findings of Newman et al.20 The smaller fragments of 17- and 23-Kd masses reported by Newman et al22 and van der Schoot et al23 were not reliably traced. This was most likely due to the enzymatic treatment of purified GPs by the former authors instead of the use of whole platelets, as in our study. A similar discordance has already been noted by Dancis et al.25 In the nonreduced immunoblot, anti-Zw' labeled the same GP IIIa band as anti-Sr', which under reducing conditions disappeared, again in agreement with previous observations of Kunic et al22 with regard to the Zw' antigen.

Taken together, our immunogenetic and immunochemochemical data indicate that the Sr' antigen is a rare (private) antigen on GP IIIa that is not included in any other known platelet alloantigen system on the GP IIb/IIIa complex (ie, Zw, Yuk, or Bak).

Of interest was our observation that the immunized mother Sr carries the HLA antigen DR3, which is known to be found in 70% to 90% of individuals immunized against Zw.33 Although only a single observation, our case strengthens the view that HLA-DR3 plays a role as an immune response marker for immunization against platelet alloantigens on the GP IIb/IIIa complex.

ACKNOWLEDGMENT

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Sra, a private platelet antigen on glycoprotein IIIa associated with neonatal alloimmune thrombocytopenia

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