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 IIIa/IIIb-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 IIIa. 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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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 antirabbit 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 CaCl₂, 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 (Elab Oy, Helsinki, Finland). The following MoAbs against different epitopes on the GP Ib/IIa complex were used: MoAbs Gi 5 (PA3), and P2 (anti-GP Ib/IIa complex), MoAb SZ21 (anti-GP IIa), and MoAb SZ22 (anti-GP IIb). MoAb Gi 14 reacts with the GP Ia/IIa complex, and MoAb A-1AS with GP IIa, the common β chain of GP Ia/IIa, IC/IIa, and IC'/IIa heterdimers. MoAb FMC 25 recognizes GP IX, tightly complexed to GP IIb; and MoAb w6.32 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, SZ21, and SZ22 were purchased from Dianova; and FMC 25 was a gift of Dr H. Zola, Adelaide, Australia.

Radioimmunoprecipitation was done with 125I-labeled platelet counts as recently described. In brief, 5 x 10⁵ labeled platelets were incubated with 100 µL 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 µL 0.01 mmol/mL 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 µL of a 10% suspension of staphylococcus aureus, Cowan I strain (Pharmacia, Freiburg, West Germany), which was preincubated with 100 µL rabbit antihuman IgG (dilution 1:10; Dako, Hamburg, West Germany). The bacteria were washed first with 500 µL 0.01 mol/L Tris, 0.1% Nonidet P-40, and 0.5% LiCl, pH 8.0, then with 500 µL 0.01 mol/L Tris and 0.1% Nonidet P-40, pH 8.0, and finally with 500 µL 0.01 mol/L Tris, pH 8.0. After resuspension in 100 µL 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, 125I-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.

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 semidry technique of Kyh~e-Anderson.

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 µL of eluates of the human sera diluted in 3 mL of 1% BSA/PBS. After three washings, 3 mL 125I-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.

Aliquots of 3 x 10⁷ platelets were incubated with 60 to 300 µL 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 IIb/IIIa-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 IIb/IIIa complex. Antigens of the Zw and Bak system of panel platelets 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. OD, optical density.](image-url)
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). 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 Ila, whereas no fragment could be precipitated from the supernatants after enzymatic treatment. Lane 1: 14C molecular weight standard.

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*.

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 Ila (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 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(a+b-) than with Zw(a-) individuals. Therefore the reactivity of anti-Zw* with Sr(a+) and Sr(a-) members of family Sr was determined in the MAIPA assay. If Sr* were the third allele of the Zw antigen system, one would expect only half the reactivity of anti-Zw* with platelets from Sr(a+), 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
Table 1. Platelet Alloantigens, Blood Groups, and HLA Antigens of Family Sr

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<tr>
<th>Family Member</th>
<th>Zw</th>
<th>Bak</th>
<th>Yrk</th>
<th>ABO</th>
<th>Rh</th>
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<th>B</th>
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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...
PRIVATE PLATELET ALLOANTIGEN Sr

values equivalent to unrelated homozygous Zw\(^\ddagger\)Zw\(^\ddagger\) individuals, indicating that all family members are Zw\(^\ddagger\)Zw\(^\ddagger\) homozygous.

Immunoochemical studies provided clear evidence that the Sr\(^\ddagger\) antigen resides on GP IIa. The antigen could only be immobilized in the MAIPA assay by MoAbs directed against different epitopes of the GP IIb/IIIa complex, e.g., MoAb Gi 5 (Fig 1). In radioimmunoprecipitation, anti-Sr\(^\ddagger\) produced a band of 89-Kd, similar to anti-Zw\(^\ddagger\), 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\(^\ddagger\) and the Zw\(^\ddagger\) antigens were still detectable on a 68- and a 74-Kd fragment of GP IIa, respectively. The 68-Kd fragment after chymotrypsin treatment probably corresponds to the 66-Kd fragment described by Kornecki et al\(^2\), or the 55- to 65-Kd band of Dancis et al\(^3\) 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\(^3\).

In the nonreduced immunoblot, anti-Zw\(^\ddagger\) labeled the same GP IIIa band as anti-Sr\(^\ddagger\), which under reducing conditions disappeared, again in agreement with previous observations of Kunicki et al\(^2\) with regard to the Zw\(^\ddagger\) antigen.

Taken together, our immunogenetic and immunoochemical data indicate that the Sr\(^\ddagger\) antigen is a rare (private) antigen on GP IIa 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\(^\ddagger\). 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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