# Low-avidity HPA-1a alloantibodies in severe neonatal alloimmune thrombocytopenia are detectable with surface plasmon resonance technology

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**BACKGROUND:** Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is mostly caused by maternal alloantibodies directed against the human platelet alloantigen (HPA)-1a. Currently, the serologic diagnosis of FNAIT is based on the characterization of the HPA alloantibodies in monoclonal antibody–based antigen-capture assays (e.g., MAIPA assay). Accumulated current evidence indicated that such assays may overlook some HPA-1a antibodies.

**STUDY DESIGN AND METHODS:** This study employed surface plasmon resonance (SPR) technology using immunoaffinity-purified glycoprotein IIb/IIIa isoforms immobilized on biosensor chips to study the binding kinetics of HPA-1a alloantibodies from different FNAIT cases in real time.

**RESULTS:** Analysis of HPA-1a alloantibodies from FNAIT cases (n = 9) in SPR showed a moderate relative response (22.2-69.7 resonance units [RU]) and slow antibody dissociation. After the dissociation phase, varying amounts of bound antibodies (41%-79%) remained on the chip. In contrast in HPA-1a alloantibodies from a patient suffering from posttransfusion purpura, a high relative response (~490 RU) was observed at the end of the association phase and no dissociation of antibody binding was detectable. Of particular relevance, by the use of this SPR technique, HPA-1a alloantibodies were detected in two severe FNAIT cases that had determined as false negative by MAIPA assay. In SPR, these HPA-1a alloantibodies showed low-avidity nature characterized by gradual dissociation of antibody during the association phase and complete detachment of antibody binding after the dissociation phase. This high "off-rate" character of low-avidity HPA-1a alloantibodies indicates that such antibody binding is easily detachable by the extensive washing procedure of the MAIPA.

**CONCLUSIONS:** Our results demonstrated that the SPR method can facilitate the diagnosis of clinically relevant low-avidity HPA-1a antibodies.

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is induced by maternal immunization against a fetal human platelet alloantigen (HPA).[1] In Caucasians, FNAIT has an incidence of 1:1000 to 1:2000 births.[2,3] Approximately 75 percent of serologically verified cases of alloantibodies against HPA-1a are responsible for the disease.[4]

Antibodies against HPA-1a react with the Leu33 but not Pro33 isoform of platelet (PLT) glycoprotein (GP)IIIa or integrin β3 subunit.[5] Recently, the three-dimensional structure of the β3 integrin has been elucidated;[6,7] however, the precise binding site(s) of HPA-1a alloantibodies is not known. A previous study suggested that the amino terminal PSI domain containing the polymorphic residue 33 is sufficient to form HPA-1a epitopes.[8] However, various studies have shown that other residues located in the hybrid and epidermal growth factor domains of the β3

**ABBREVIATIONS:** D-PBS = Dulbecco's phosphate-buffered saline; FNAIT = fetal and neonatal alloimmune thrombocytopenia; GP(s) = glycoprotein(s); MAIPA assay = monoclonal antibody–based antigen-capture assay; PAIFT = platelet adhesion immunofluorescence test; PTP = posttransfusion purpura; RU = resonance units; SPR = surface plasmon resonance.

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subunit are necessary for the binding of some HPA-1a antibodies.[9,10] Recently, Stafford and colleagues[11] found that recombinant β3 without an A domain (ΔβA) contains the most reliable region for HPA-1a antibody binding.

Nowadays, monoclonal antibody–based antigen-capture assays (e.g., MAIPA assay) represent the standard technique for the characterization of PLT antibodies.[12] However, recent evidence has indicated that this assay may overlook some HPA-1a alloantibodies.[13]

In the past few years, surface plasmon resonance (SPR) technology has gained widespread use in the study of molecular interactions between different biomolecules (e.g., antigen and antibody) in real time, without any labeling or washing procedure.[14] The technology involves the immobilization of one interaction partner on the sensor surface (ligand; e.g., antigen) and passing a sample over it containing the other interaction partner (analyte; e.g., antibody). Binding of analyte to the sensor surface generates a response that is proportional to the bound mass. Recently, we showed that SPR is a valuable method to study the binding kinetics of monoclonal antibodies (MoAbs) against PLT receptors and human recombinant MoAbs against HPA-1a alloantibodies.[15]

In this study, we established this SPR method to analyze the binding nature of HPA-1 antibodies by the use of immobilized purified HPA-1a or -1b ligand from PLTs. By this method we were able to identify low-avidity HPA-1a alloantibodies in cases of clinically severe FNAIT, which were not detectable by the standard MAIPA assay.

## MATERIALS AND METHODS

### Case reports

#### *Case 1*

The 23-year-old mother delivered her first child after 41 weeks' gestation (2900 × *g*, Apgar 9/10/10). On Day 2 the child showed signs of tremor and petechiae on the palate (PLT count, $4 \times 10^9$/L). Treatment with a random PLT concentrate and intravenous immune globulin (IVIG) failed. After transfusion of maternal PLTs on Day 4, the PLT count increased to $273 \times 10^9$ per L. Sonography on Day 5 and computed tomography revealed signs of previous older parietooccipital bleeding. Two additional transfusions with maternal PLTs led to a normal PLT count after more than 4 weeks. Only a HPA-1a mismatch, but no maternal antibody, could be detected at that time (Serum Sample 1.1). Six years later the mother was pregnant again. Upon serologic examination at 34 weeks' gestation, the MAIPA assay was negative, but anti-HPA-1a was detected in the adhesion immunofluorescence test. Since the parents did not agree to fetal blood sampling, elective cesarean section was performed at 34 weeks 5 days' gestation (2960 × *g*, Apgar 8/9/9). The PLT count of the newborn was $3 \times 10^9$ per L. The child was immediately transfused with 30 mL of HPA-1a–negative apheresis PLTs and the PLT count increased to $265 \times 10^9$ per L. Only distinct signs of cutaneous bleeding, but no intracranial hemorrhage, were observed. On Days 2 and 3, IVIG was administered. After the PLT count had decreased to $65 \times 10^9$ per L on Day 5, a second transfusion with compatible donor PLTs was given before the PLT count had recovered. A blood sample (Serum Sample 1.2) was obtained a couple of weeks after delivery.

#### *Case 2*

The 29-year-old mother delivered her first child at 40 weeks' gestation (3245, Apgar 9/10). During the first day, multiple petechiae developed and a PLT count of $2 \times 10^9$ per L was determined. IVIG and random-donor PLTs did not lead to a sustained increase of the PLT count. Maternal and paternal typing analysis documented HPA-1a incompatibility (mother HPA-1b1b; father HPA-1a1a).

The child received 3 HPA-1a–negative apheresis PLT concentrates on Days 2, 8, and 14 and corticosteroids. After 3 weeks the PLT count increased steadily (Serum Sample 2.1). Two years later the mother was pregnant again. A blood sample was obtained after 23 weeks' gestation (Serum Sample 2.2).

### Sera

Sera containing anti-HPA-1a (n = 9), anti-HPA-1b (n = 1), and anti-HPA-5b plus HLA Class I alloantibodies (n = 1) were obtained from mothers who delivered babies with FNAIT. One serum sample from a posttransfusion purpura (PTP) patient containing HPA-1a alloantibody was included. Normal human serum from a healthy blood donor (blood group AB) was used as control. Maternal sera from two homozygous HPA-1bb mothers, who delivered healthy heterozygous HPA-1a–positive neonates without clinical signs of thrombocytopenia, were provided by Dr Vagner (Campinas, Brazil). These samples were selected from a large cohort of a prospective study to determine the prevalence of genotypic mismatches in unselected newborns as a risk factor for thrombocytopenia.[16] All samples were screened for antibody specificity using the PLT adhesion immunofluorescence test (PAIFT), flow cytometry, and the antigen-capture immunoassay (MAIPA assay) as described below.

### MoAbs

MoAb Gi5 specific for the PLT GPIIb/IIIa complex and MoAb Gi16 against the GPIIb subunit were generated and characterized in our laboratory.[17] MoAbs P2, SZ21, and SZ22 specific for the GPIIb/IIIa complex and GPIIIa and GPIIb subunits, respectively, were purchased from Immunotech (Marseille, France). MoAb MBC 132.1 against the GPIIb subunit was provided by Dr J. Peterson (Blood Research Institute, Milwaukee, WI).

### Antibody binding tests

For flow cytometry analysis, PLTs of HPA-1a1a– or -1b1b–phenotyped individuals were isolated from acid-citrate-dextrose–anticoagulated blood by differential centrifugation. PLTs were washed in Tyrode buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L $NaHCO_3$, 0.14 mmol/L $NaHPO_4$, 0.35% bovine serum albumin, and 0.1% glucose, pH 7.4) and adjusted to a final concentration of $3 \times 10^5$ per µL. Aliquots of $6 \times 10^6$ PLTs were incubated with 20 µL of undiluted human sera for 30 minutes at 37°C, washed, stained with 50 µL of fluorescein-conjugated rabbit anti-human immunoglobulin G (IgG; 1:50; Dako, Hamburg, Germany), and immediately subjected to flow cytometry analysis (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

PAIFT was performed as previously described.[18] MAIPA was performed according to the standard procedure.[19] MoAbs Gi5, Gi16, and P2 were used as capture alloantibodies.

### Isolation of IgG fractions from human sera

IgG was isolated from 50 µL of serum or plasma samples using a gel IgG spin purification kit and the manufacturer's recommended conditions (Melon, Pierce, Munich, Germany). Plasma was recalcificated with 25 mmol per L $CaCl_2$ (Dade Behring, Marburg, Germany) before IgG isolation. After purification, samples were dialyzed against running buffer (Biacore AB, Uppsala, Sweden). Protein concentrations were measured using the bicinchoninic acid protein assay (Pierce). The purity of our IgG preparations was verified by silver staining.

### Purification of HPA-1a and -1b proteins from PLTs

Outdated PLT concentrates from HPA-1a and -1b homozygous blood group O donors were used as a PLT source. PLTs were washed three times in 10 mmol per L Tris, 3 mmol per L ethylenediaminetetraacetate, 150 mmol per L NaCl for 15 minutes at 4000 × *g*, and then solubilized in a 1 percent Triton X-100 (Sigma, Munich, Germany), 20 mmol per L Tris-HCl buffer containing 3.3 percent protease inhibitor cocktail (Sigma), and 1 percent phenylmethylsulfonyl fluoride (Sigma) at 4°C for 1 hour. The PLT lysate was centrifuged at 17,000 × *g* for 45 minutes at 4°C and subjected to immunoaffinity column purification as previously described.[20] Purified HPA-1a and -1b isoforms were dialyzed in Dulbecco's phosphate-buffered saline (D-PBS, Invitrogen, Karlsruhe, Germany) overnight at 4°C and their protein concentrations were determined as described above. Purified antigens were visualized by silver staining and stained by immunoblotting using MoAbs against GPIIb or GPIIIa or human antiserum against HPA-1a or HPA-1b.

### Analysis of HPA-1a alloantibodies binding by SPR

Real-time analysis of antigen-antibody interactions was performed by SPR on an automated SPR device (Biacore 2000, GE Healthcare, Freiburg, Germany) as previously described.[15] Purified HPA-1a and -1b antigens were immobilized covalently to the surface of a CM5 sensor chip by an amine-coupling procedure as recommended by the manufacturer (Biacore). An aliquot of 200 µL of antigen (100 µg/mL) in 10 mmol per L acetate buffer (pH 4.0) was injected into the buffer stream (D-PBS, Invitrogen) and passed through the flow cells at 5 µL per minute. Residual, active ester groups on the sensor surface were deactivated by injecting 50 µL of 1 mol per L ethanolamine (Biacore). In addition, noncovalently bound protein was removed from the sensor surface by passing

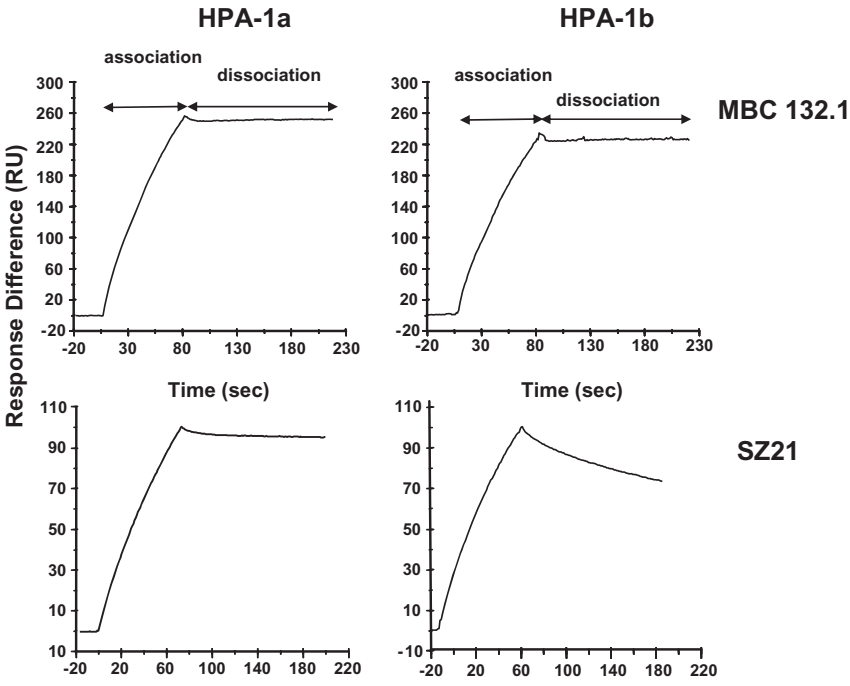


**Fig. 1. Analysis of antigen-antibody binding of GPIIb/IIIa-specific MoAbs to immobilized purified HPA-1 antigens in real time by SPR. Purified MoAbs MBC 132.1 and SZ21 (250 ng) specific for GPIIb and GPIIIa, respectively, were serially injected over HPA-1aa– or HPA-1bb–coupled sensor chips at a flow rate of 20 µL per minute. After the association phase, running buffer was injected to start the dissociation phase. Binding kinetics (association and dissociation) was recorded as sensorgrams: RU against time (sec).**

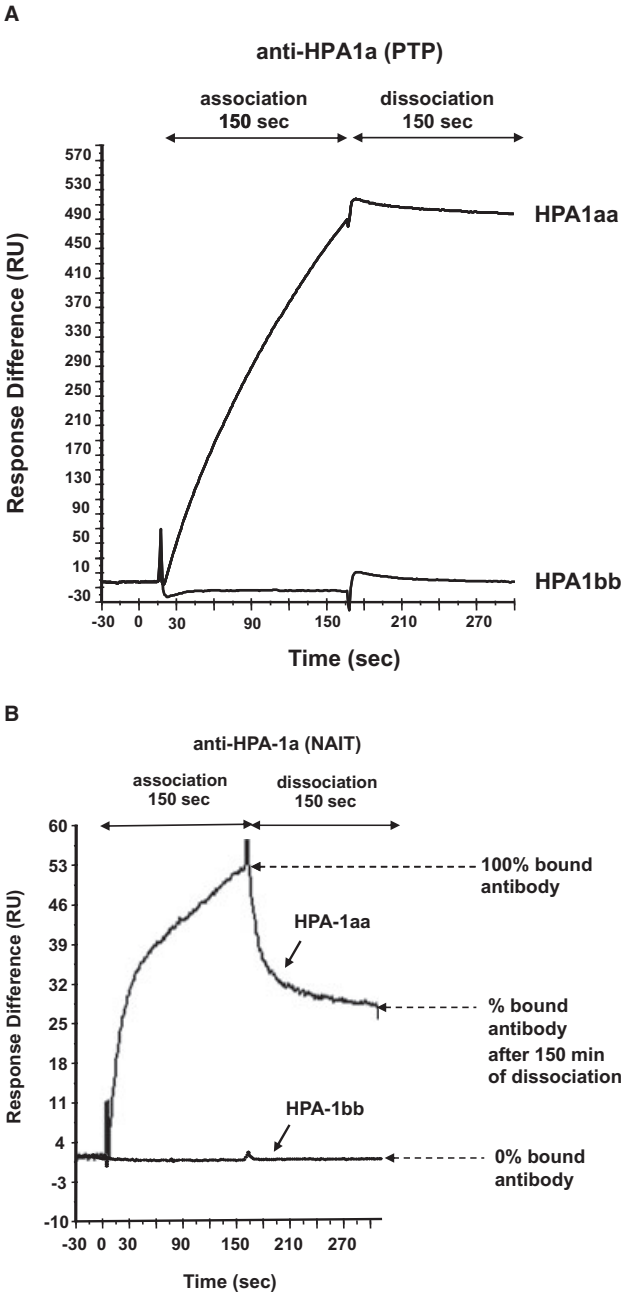


**Fig. 2. Analysis of antigen-antibody binding of different anti-HPA-1a alloantibodies to purified HPA-1 antigens in real time by SPR. Purified IgG fraction alloantibodies (20 µg) from PTP serum (Serum Sample 12) containing HPA-1a alloantibody (A) and from one FNAIT sera (B) containing anti-HPA-1a (Sample 11) alloantibodies were serially injected over HPA-1aa– or HPA-1bb–coupled sensor chip at a flow rate of 10 µL per minute. After the association phase of 150 seconds, running buffer was injected to start the dissociation phase and followed for an additional 150 seconds. Binding kinetics (association and dissociation) were recorded as sensorgrams: RU against time (sec). The remaining bound antibodies were calculated as the percentage difference of relative response at the beginning and at the end of the dissociation phase.**

D-PBS buffer containing 0.005 percent surfactant P20 (Biacore) through the flow cells until a stable baseline was achieved. Aliquots of 25 µL of MoAbs (250 ng) or purified human IgG (25-50 µg) in D-PBS (dilution 1:3) were serially injected using the Biacore software (KINJECT) at a flow rate of 20 or 10 µL per minute, respectively. The sample was replaced by D-PBS after the association phase of 150 seconds. The dissociation phase was then recorded for 150 seconds. The alloantibodies remaining bound were removed with 25 mmol per L NaOH after each run. Subsequently, the sensor chip was washed at a high flow rate (100 µL/min) to remove residual NaOH. The sensor chip coated with 20 µg of bovine serum albumin (Pierce) was used as reference, and the background signal from the reference was subtracted from every data set. Data were analyzed using the supplied computer software (BIAevaluation, Version 3.2, Biacore).

## RESULTS

### SPR analysis of HPA-1a and -1b antibodies

To study the binding characteristics of HPA-1 alloantibodies, we purified the GPIIb/IIIa complex from homozygous HPA-1a and -1b PLT donors on an affinity-chromatography column coupled to MoAb Gi5 that is directed against the GPIIb/IIIa complex. Silver staining and immunoblotting analyses confirmed the purity and activity of the isolated antigens.[20] Purified HPA-1a and -1b native antigens were immobilized onto the Biacore sensor surface to reach a final signal response of 9000 resonance units (RU) and verified by the use of MoAb MBC 132.1 (anti-GPIIb) and MoAb SZ21 (anti-GPIIIa). When MoAb MBC 132.1 was injected at a concentration of 60 ng over the sensor chip coated with HPA-1a or HPA-1b antigen, similar interaction responses (approx. 250 RU) during the association phase were observed (Fig. 1). No dissociation of MoAb MBC 132.1 bound to both sensor chips was detected during the dissociation phase. Similar results were obtained with MoAb P2 directed against the GPIIb/IIIa complex (data not shown). These results demonstrated that HPA-1a and HPA-1b proteins immobilized on sensor chips exhibited similar binding properties.

Previous studies had demonstrated that MoAb SZ21 (anti-GPIIIa) bound markedly less to HPA-1b in comparison to HPA-1a PLTs[21] and could, therefore, be used for rapid PLT phenotyping of the HPA-1 system.[22] When we injected MoAb SZ21 over HPA-1a or HPA-1b sensor chips, similar interaction responses (approx. 100 RU) were detected during the association phase (Fig. 1). During the dissociation phase, however, MoAb SZ21 steadily dissociated from HPA-1b antigens whereas it remained bound to HPA-1a. This observation indicated that the capability of

**TABLE 1. Clinical data on nine FNAIT patients due to anti-HPA-1a (Patients 3-11), anti-HPA-5b (Patient 13), one PTP (Patient 12), and two control sera (Patients 14 and 15) from HPA-1bb–phenotyped mothers, who delivered healthy heterozygous HPA-1ab–positive neonates without clinical signs of thrombocytopenia and one control serum (Patient 16) from a healthy blood donor***

| Patient | Case | Antibody specificity | Clinical outcome | PLT count ($\times 10^9$/L) | Association phase (RU) | Dissociation phase (RU) | Bound IgG after dissociation (%) |
|---|---|---|---|---|---|---|---|
| 3 | FNAIT | HPA-1a | MC, ICH | 45 | 32.1 | 24.6 | 76.6 |
| 4 | FNAIT | HPA-1a | MC, ICH | 31 | 22.7 | 15.7 | 69.2 |
| 5 | FNAIT | HPA-1a | MC | 9 | 28.8 | 15.9 | 55.2 |
| 6 | FNAIT | HPA-1a | MC, ICH | 10 | 48.6 | 19.7 | 40.5 |
| 7 | FNAIT | HPA-1a | MC | 16 | 57.2 | 44.9 | 78.5 |
| 8 | FNAIT | HPA-1a | No bleeding | 26 | 32.4 | 18.9 | 58.3 |
| 9 | FNAIT | HPA-1a | MC, GB | 20 | 24.0 | 16.8 | 70.0 |
| 10 | FNAIT | HPA-1a | MC, ICH | NK | 29.7 | 18.2 | 61.3 |
| 11 | FNAIT | HPA-1a | MC, ICH | 20 | 49.7 | 25.0 | 50.3 |
| 12 | PTP | HPA-1a | MC | 9 | 490.8 | 503.3 | 100 |
| 13 | FNAIT | HPA-5b, HLA Class I | No bleeding | 28 | 1.2 | 1.3 | 0 |
| 14 | † | None | No bleeding | 173 | 0 | 0 | 0 |
| 15 | † | None | No bleeding | 230 | 0.8 | 0.7 | 0 |
| 16 | ‡ | None | No bleeding | NK | 0 | 0 | 0 |

* Bleeding symptoms are recorded as mucocutaneous (MC), gastrointestinal (GB), or intracranial (ICH).
† Healthy heterozygous HPA-1ab–positive neonates without clinical signs of thrombocytopenia.
‡ Healthy blood donor.
NK = not known.

MoAb SZ21 in distinguishing between HPA-1a and -1b is most probably due to rapid dissociation of this antibody from, rather than the binding affinity toward, HPA-1b. This observation demonstrated the capability of SPR for the analysis of PLT antibody binding characteristics.

After thorough characterization of our HPA-1 biosensor chips and SPR conditions, we analyzed the binding characteristics of human alloantibodies directed against HPA-1a. In this experimental SPR setting, we only used purified IgG to exclude possible "unspecific interactions" between soluble ligands of GPIIb/IIIa present in human sera (e.g., fibrinogen). When 20 µg of purified IgG from a PTP patient (Serum Sample 12) was injected onto the sensor surface with immobilized HPA-1a antigen, a relatively high response (approx. 490 RU) was observed during the association phase. In contrast, no response was detected with immobilized HPA-1b antigen (Fig. 2A). In a control experiment, purified IgG from a serum containing HPA-5b alloantibodies and strong HLA alloantibodies (Serum Sample 13) and a normal human serum (Serum Sample 16) did not show any interaction (data not shown). It was remarkable that the interaction between HPA-1a antigen and the PTP-derived antibodies occurred rapidly as indicated by the steep slope of the association phase. In addition, strong antibody binding was observed, as at the end of the dissociation phase almost all the HPA-1a alloantibodies were still bound to HPA-1a antigen on the chip. The binding characteristics were reflection of high-concentration and high-avidity of HPA-1a alloantibodies in PTP patients, which have been postulated in this syndrome.

Next, we examined the binding characteristics of HPA-1a and HPA-1b alloantibodies from 10 FNAIT cases (Serum Samples 3-11; see Table 1) that had been serologically verified by having positive reactions in the MAIPA assay. The SPR sensorgram of a representative anti-HPA-1a immunoreactivity (Serum Sample 11) is presented in Fig. 2B. As expected, purified anti-HPA-1a alloantibodies reacted solely with immobilized HPA-1a antigens and not with HPA-1b antigens. In the control experiment, anti-HPA-1b bound only sensor chip coated with HPA-1b (data not shown). In comparison to HPA-1a activity derived from the PTP serum, the interaction between HPA-1a antigen and alloantibodies from FNAIT sera occurred more slowly and were characterized by moderate association responses (22.7-57.2 RU; see Table 1). When the percentage of bound HPA-1a remaining alloantibodies was calculated at the end of the dissociation phase, a varying amount (41%-79%) was detected to indicate a diversity of HPA-1a–binding avidity in FNAIT cases.

### Analysis of two cases of severe FNAIT determined as false negative by the MAIPA assay

We observed severe thrombocytopenia in two newborns from HPA-1a–negative mothers (Cases 1 and 2) without detectable HPA-1a alloantibodies by MAIPA assay in the first pregnancy (see case reports and Table 2). Screening of maternal sera (Samples 1.1 and 2.1) in PAIFT indicated that weak activity HPA-1a alloantibodies may exist in these sera (data not shown). Since flow cytometry has been described to be advantageous in the detection of labile PLT alloantibodies,[23] we then analyzed both maternal sera using freshly isolated HPA-1–phenotyped homozygous PLTs by flow cytometry. Only weak reactions were observed with HPA-1a1a PLTs, which could not

**TABLE 2. Clinical data on two cases of suspected FNAIT (Cases 1 and 2)***

| Patient | Year of pregnancy | Sample | Case | Antibody specificity | Clinical outcome | PLT count ($\times 10^9$/L) | Association phase (RU) | Dissociation phase (RU) | Bound IgG after dissociation (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1998† | 1.1 | FNAIT | | MC, ICH | 4 | 32.7 | 0 | 0 |
| | 2004‡ | 1.2 | FNAIT | HPA-1a | MC | 3 | 76.7 | 0 | 0 |
| 2 | 2002† | 2.1 | FNAIT | | MC | 2 | 31.3 | 0.5 | 0 |
| | 2004‡ | 2.2 | FNAIT | HPA-1a | MC | NK | 69.7 | 13.4 | 19 |

* Antibodies from the first pregnancy are not detectable in the MAIPA assay. Bleeding symptoms are recorded as mucocutaneous (MC), gastrointestinal (GB), or intracranial (ICH).
† First pregnancies.
‡ Second pregnancy.
NK = not known.

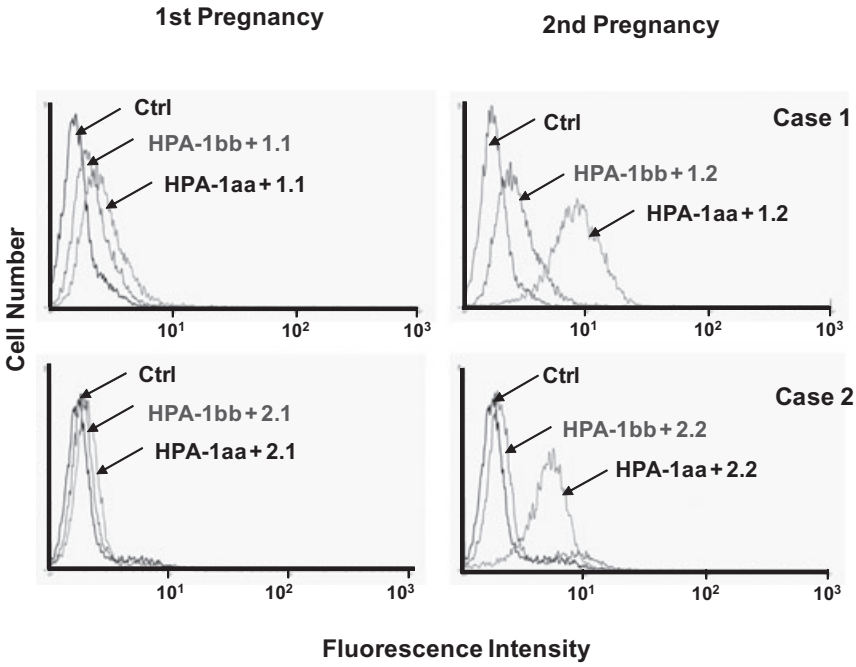


**Fig. 3. Analysis of FNAIT sera in flow cytometry. Washed PLTs derived from homozygous HPA-1aa and HPA-1bb individuals were incubated with sera from two FNAIT mothers (Cases 1 and 2) as indicated. After being washed bound PLT-reactive antibodies were detected with fluorescein-conjugated secondary IgG antibody and analyzed by flow cytometry. Left panel shows the reactivity of the sera from the first pregnancy (Serum Sample 1.1 and 2.1), and right panel from the second pregnancy (Serum Samples 1.2 and 2.2). Ctrl = control (reaction between AB sera and normal PLTs). Note the right shift of fluorescence intensity when sera from the second pregnancies (Serum Samples 1.2 and 2.2) were tested with HPA-1aa in comparison to HPA-1bb PLTs.**

definitively be distinguished from the reaction with HPA-1b1b PLTs (Fig. 3).

When maternal sera from the first pregnancy were tested with HPA-1a1a PLTs in the MAIPA assay using MoAb Gi5 (anti-GPIIb/IIIa) as capture antibody, negative reactions were observed in both cases (Fig. 4). Similar results were obtained with purified IgG from these sera (data not shown). MoAbs directed against different epitopes on the GPIIb/IIIa complex (MoAb P2) or against GPIIb subunit (MoAb Gi16) were also applied in the MAIPA assay to exclude competition between MoAb and human alloantibodies. Again, no reactions were detectable (Fig. 4). The presence of other PLT antibodies against GPIa/IIa, GPIb/IX, CD109, and HLA antigens could be excluded by the use of a panel of PLT MoAbs (data not shown).

In contrast, in maternal sera obtained during the subsequent pregnancy (Serum Samples 1.2 and 2.2) strong reactions of HPA-1a alloreactivity in flow cytometry (Fig. 3) as well as in MAIPA were documented (Fig. 4). This observation strongly indicated the existence of low-avidity HPA-1a alloantibodies in the first pregnancy, which changed to high avidity during the second pregnancy.

From this observation, we assume that weak anti-HPA-1a activity in the first pregnancy may dissociate due to extensive washing procedure in the MAIPA assay, leading to negative results. Weak, positive reactions in PAIFT could be ascribed to the mild washing steps of this method.

To substantiate this hypothesis, we sought to employ the SPR technique to detect the presence of HPA-1a alloantibodies (MAIPA negative) from the first pregnancy. Interestingly, HPA-1a alloantibodies derived from Serum Samples 1.1 and 2.1 could be specifically detected by the SPR method (Fig. 5). In comparison to the other HPA-1a alloantibodies (MAIPA-positive), it is remarkable that these HPA-1a alloantibodies reflected a low-avidity nature as characterized by gradual dissociation of antibody during the association phase and complete detachment of antibody after the dissociation phase. Analysis of sera from both mothers after the second pregnancy (Serum Samples 1.2 and 2.2)

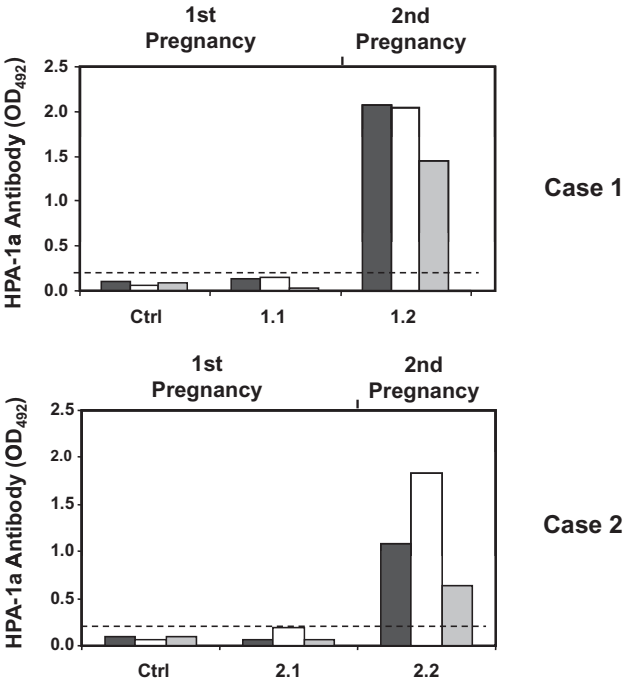


**Fig. 4. Analysis of FNAIT sera with HPA-1aa PLTs using three different capture MoAbs against different epitopes on GPIIb/IIIa in MAIPA assay. Washed PLTs derived from a homozygous HPA-1aa individual were incubated with serum from two NAIT mothers (Cases 1 and 2) as indicated. After being washed, PLTs were incubated with either MoAb Gi5 (anti-GPIIb/IIIa; ■) P2 (anti-GPIIIa; □), or Gi16 (anti-GPIIb; □) and then lysed and subjected to the MAIPA assay as described under Materials and Methods. Bound antibodies were measured by the use of horseradish-labeled anti-human IgG antibodies and substrate system in a microtiter reader. Ctrl = control (reaction between AB sera and normal PLTs). $OD_{492}$ = optical density at 492 nm.**

showed an increasing amount of bound HPA-1a alloantibodies and absence of the gradual dissociation of antibody during the association phase. This development may result in a higher antibody-binding capacity that could then be detected in the MAIPA assay.

In control experiments, in analyses of two sera derived from HPA-1b homozygous mothers (Serum Samples 14 and 15), who gave birth to healthy HPA-1a–positive newborns, no specific antibody binding to the immobilized HPA-1a antigen was observed, indicating the absence of low-avidity HPA-1a alloantibodies in these mothers (Table 1).

## DISCUSSION

The specific and sensitive detection of PLT-specific antibodies is essential for the adequate treatment of patients suffering from FNAIT. With the availability of well-defined MoAbs against PLT GPs, the antigen-capture assay became "state of the art" for the detection of PLT alloantibodies.[19] Current evidence, however, indicates that this method does not appear to detect all clinically relevant HPA-1a alloantibodies.

Recently, we developed a rapid HPA-1a alloantibodies assay termed gel antigen-specific assay (GASA). This assay is based on the use of purified antigens directly coupled to gel without MoAbs or any washing steps. During evaluation of the procedure, we observed that some HPA-1a sera with moderate reactivity in MAIPA, in contrast, reacted strongly in gel antigen-specific assay.[20] This phenomenon may be attributed to the fact that 1) GPIIb/IIIa-specific MoAbs used in the MAIPA assay compete with the binding site(s) of some anti-HPA-1a alloantibodies and/or 2) an excessive washing procedure in the MAIPA assay leads to the dissociation of weak antigen-antibody binding. Competition between human antibodies and MoAbs could be minimized by the correct choice of MoAb or by the use of MoAbs recognizing different epitopes.[24] Recent evidence, however, has indicated that human PLT antibodies are heterogeneous in their specific recognition sites,[15,25] a fact that may complicate the right selection of MoAbs. Furthermore, all methods used currently for the detection of PLT antibodies include washing steps, which could lead to dissociation of low-avidity antibodies.

To avoid these problems, we devised a SPR method allowing direct analysis of HPA-1a antibody binding without any wash step, the use of capture MoAbs, or a labeling step. Additionally, this method allowed us to study the antibody-binding characteristics (antibody concentration, antibody avidity) more precisely by monitoring the antigen-antibody interaction in real time. Our previous experience with analyzing MoAbs has indicated that the SPR method is a potential tool for determining PLT antigen-antibody interactions.[15]

Here, we have been able to show by studying HPA-1a alloantibodies from a PTP patient and different FNAIT patients that the SPR method was able to identify different binding characteristics. Some HPA-1a alloantibodies reflected high concentration and high avidity, while some were of low concentration and low avidity, indicating a diversity of HPA-1a antibody-binding characteristics. This fact may affect the sensitivity of PLT antibody testing especially in assays, which involve a need for excessive washing procedures.

Of particular relevance, we could identify two severe FNAIT cases (Table 2) in the first pregnancy caused by HPA-1a antibody using the SPR technique, which were initially undetectable by the MAIPA test. Although several MoAbs reacting against different epitopes on GPIIb/IIIa were applied to exclude the possibility of competitive inhibition by HPA-1a alloantibodies, negative results were

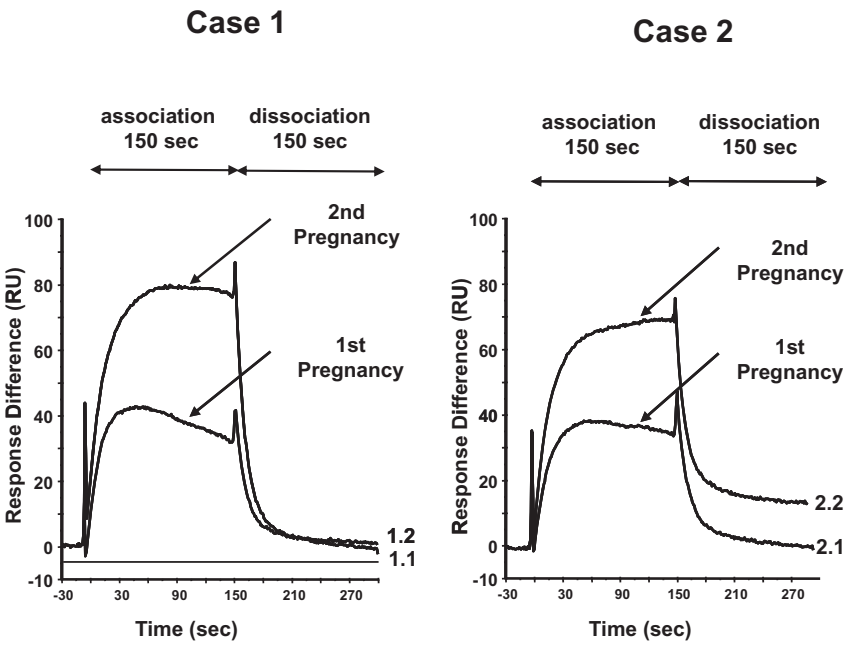


**Fig. 5. Analysis of antigen-antibody binding with FNAIT Cases 1 and 2 on immobilized purified HPA-1aa antigen in real time by SPR. Purified IgG fraction antibodies (20 μg) derived from maternal sera of Case 1 (Samples 1.1 and 1.2; first and second pregnancies, respectively) and from Case 2 (Samples 2.1 and 2.2; first and second pregnancies, respectively) were analyzed by SPR technique as described in the legend to Fig. 2. All sera-specific reactions with immobilized HPA-1aa were recorded. Note that bound HPA-1a antibodies from the first pregnancy dissociated gradually during the association phase and dissociated rapidly and completely during the dissociation phase.**

found. By the use of SPR, however, we were able to detect the presence of low-avidity HPA-1a alloantibodies in these two cases. In comparison to high avidity, the HPA-1a low-avidity alloantibodies showed 1) gradual dissociation of antibody during the association phase and 2) complete detachment of antibody after the dissociation phase. This may indicate a high "off-rate" character of low-avidity HPA-1a alloantibodies. It is conceivable that such antibody binding is easily detachable by the extensive washing procedure in the MAIPA assay. In the in vivo situation, however, antigen and antibody react to an equilibrated state, in which antibodies can steadily associate and dissociate. In the case of low-avidity antibodies, this process may occur rapidly in comparison to high-avidity antibodies. However, this process may be sufficient for the reticuloendothelial system to recognize and subsequently to clear PLT-bound antibody, especially for high-density antigens such as GPIIb/IIIa complex.

The avidity of antibodies for their cognate antigens increases during immune responses.[26] Interestingly, during the second pregnancy HPA-1a alloantibodies were detectable by the MAIPA assay, which may reflect antibody maturation during the course of the FNAIT disease state. In both FNAIT cases, the maturation process of HPA-1a alloantibodies during the time course of pregnancy (Serum Sample 1.1 vs. Serum Sample 1.2 or Serum Sample 2.1 vs. Serum Sample 2.2) was correlated with the increased antibody concentration and antibody avidity in SPR.

Recently, several groups have focused on the level of maternal HPA-1a antibodies as a predictive parameter for the severity of thrombocytopenia in the newborn. Discrepancies were observed; some could find a positive correlation and some not.[27-29] Technical differences could be one explanation. Some groups used the enzyme-linked immunoassay method, whereas other groups used the MAIPA assay.[27,28] Both techniques, however, differ significantly in respect to the intensity and frequency of the wash procedure. It would be interesting to compare whether SPR is a more appropriate method for the analysis of antibody level as a predictive parameter for thrombocytopenia.

Although our SPR method showed some advantages over the antigen-capture assays, there are still a few drawbacks. First, only purified IgG antibodies can be applied, because several ligands of the GPIIb/IIIa receptor present in the serum could also react in this assay leading to false-positive results. Second, the purity and stability of the antigen(s) used are of prime importance. The question as to whether this technique is also applicable to other human PLT antigens, especially for "labile" HPAs (HPA-2, -3, and -15), remains.[23,30] In this respect, the use of recombinant PLT alloantigens may help in the further development of the SPR technique for the detection of PLT antibodies.[11,31]

In conclusion, our results showed that the SPR method can facilitate the diagnosis of clinically relevant low-avidity HPA-1a antibodies. Further studies are required to establish the impact of this new method in determining the binding characteristics of maternal antibodies as predictive parameter for the clinical severity of NAIT.

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