

Paper I

Characterization of a Human Platelet Antigen-1a–Specific Monoclonal Antibody Derived from a B Cell from a Woman Alloimmunized in Pregnancy

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Human platelet Ag (HPA)-1a, located on integrin $\beta 3$, is the main target for alloantibodies responsible for fetal and neonatal alloimmune thrombocytopenia (FNAIT) in the white population. There are ongoing efforts to develop an Ab prophylaxis and therapy to prevent or treat FNAIT. In this study, an mAb specific for HPA-1a, named 26.4, was derived from an immortalized B cell from an alloimmunized woman who had an infant affected by FNAIT. It is the only HPA-1a–specific human mAb with naturally paired H and L chains. Specific binding of mAb 26.4, both native and recombinant forms, to platelets and to purified integrins $\alpha \text{IIb}\beta 3$ (from platelets) and $\alpha \text{V}\beta 3$ (from trophoblasts) from HPA-1a⁺ donors was demonstrated by flow cytometry and surface plasmon resonance technology, respectively. No binding to HPA-1a[–] platelets or integrins was detected. Moreover, the Ab binds with higher affinity to integrin $\alpha \text{V}\beta 3$ compared with a second HPA-1a–specific human mAb, B2G1. Further in vitro experimentation demonstrated that mAb 26.4 can opsonize HPA-1a⁺ platelets for enhanced phagocytosis by monocytes, inhibit binding of maternal polyclonal anti-HPA-1a Abs, and weakly inhibit aggregation of HPA-1a–heterozygous platelets, the latter with no predicted clinical relevance. Thus, mAb 26.4 is highly specific for HPA-1a and could potentially be explored for use as a prophylactic or therapeutic reagent for FNAIT intervention and as a phenotyping reagent to identify women at risk for immunization. *The Journal of Immunology*, 2015, 194: 5751–5760.

Human platelet Ag (HPA)-1 is associated with a single amino acid polymorphism in integrin $\beta 3$ at position 33 with leucine (HPA-1a) and proline (HPA-1b) as the most common variants (1). In populations of European descent, the HPA-1a and HPA-1b allele frequencies are ~0.85 and 0.15, respectively, and ~2% of this population is HPA-1b–homozygous. In other ethnic groups, the HPA-1b allele is less common (2). Integrin $\beta 3$ forms part of the fibrinogen receptor ($\alpha \text{IIb}\beta 3$), which is the most abundant integrin on platelets. It is also part of the vitronectin receptor ($\alpha \text{V}\beta 3$) expressed on various cell types, for

example, trophoblasts. Allogeneic mismatch between fetal and maternal integrin $\beta 3$ in pregnancy, with HPA-1b–homozygous mother and HPA-1a⁺ fetus, may lead to maternal immunization with the production of anti-HPA-1a IgG Abs. These Abs cross the placenta, bind fetal platelets, and may cause platelet destruction and fetal and neonatal alloimmune thrombocytopenia (FNAIT). FNAIT occurs at a rate of ~1 in 1200 pregnancies (3, 4) and is associated with increased risk for intracranial bleeding before and after delivery, which in severe cases may cause brain damage or death (5). Anti-HPA-1a Abs account for most (85–90%) of severe FNAIT cases (6).

Currently, there is no safe and effective strategy to treat or prevent FNAIT. Furthermore, the condition is usually not recognized until after delivery of a severely thrombocytopenic child with symptoms of hemorrhage at birth. Thus, efficient management of FNAIT will depend on introduction of general antenatal screening to identify at-risk pregnancies and development of prophylaxis or new treatment approaches.

Potentially, anti-HPA-1a Abs could serve as efficient tools for each of these tasks. Already, HPA-1a–reactive mAbs have been used in laboratory tests to identify HPA-1a[–] individuals who are at risk for alloimmunization against HPA-1a (7, 8). A prophylactic strategy to prevent FNAIT occurrence using anti-HPA-1a Abs derived from pooled donor plasma has been proposed (9). Such a strategy has been proved to prevent thrombocytopenia in newborn pups in a murine model of FNAIT (10).

An attractive source of anti-HPA-1a Abs for eventual FNAIT prophylaxis or therapy would be human mAbs. In contrast with IgG preparations extracted from donor plasma, mAbs may be produced in virtually limitless amounts, the specificity and function of mAbs can be characterized in detail, and a therapeutic dose can be determined accurately providing more reproducibility in treatment.

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Abbreviations used in this article: CMFDA, 5-chloromethyl fluorescein diacetate; FNAIT, fetal and neonatal alloimmune thrombocytopenia; FR, framework region; HPA, human platelet Ag; IMGT, ImMunoGeneTics; MAIPA, mAb immobilization of platelet Ags; RT, room temperature; RU, response unit; SPR, surface plasmon resonance; TRAP, thrombin receptor activating peptide.

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Furthermore, mAbs can be functionally tailored for specific therapeutic interventions using recombinant technology (11–14).

Arguably, Abs that have been naturally selected in human immune responses would be optimal for *in vivo* administration. Natural selection will likely drive toward minimal autoreactivity by deletion of self-reactive B cell clones. This may be a particularly important issue with anti-HPA-1a Abs because there is only a single amino acid residue difference between the allogeneic and autologous epitopes, and therefore the potential for cross-reactivity is significant.

In this study, we aimed to develop a human mAb highly specific for HPA-1a that would be suitable for prophylactic, therapeutic, and screening purposes. To accomplish this, we immortalized Ag-specific memory B cells from an HPA-1a⁻ woman who had developed anti-HPA-1a Abs upon immunization in connection with pregnancy. The rationale was that the specificity of an Ab naturally selected in an individual alloimmunized in connection with pregnancy would likely be optimal in terms of Ag specificity and minimal autoreactivity.

Materials and Methods

This study was approved by the Regional Committee for Medical Research Ethics, North-Norway (approval no. 2009/1585 and 2013/126/REK). All volunteers donated blood samples after signing a written, informed consent (Blood Bank, University Hospital of North Norway).

Donor material

Peripheral blood was donated by a woman who was HPA-1a immunized in connection with pregnancy. She gave birth to two HPA-1a⁺ siblings with severe thrombocytopenia and s.c. hemorrhages at birth. Blood was donated 4 wk after delivery of the second child. Plasma anti-HPA-1a Ab level was 150 IU/ml as measured by modified quantitative mAb immobilization of platelet Ags (MAIPA) assay (15, 16). PBMCs were isolated by centrifugation over 1077 g/ml density gradient medium (Lymphoprep; Axis-Shield) (17). PBMCs were frozen in aliquots of 5×10^7 cells and stored in liquid nitrogen. Frozen cells were thawed the day before an experiment and cultured overnight in complete medium (IMDM, 10% FBS and 100 U/ml penicillin, 100 U/ml streptomycin).

Isolation of memory B lymphocytes

Memory B cells were isolated as described previously (18) with minor modifications. In brief, Ab-labeled CD22⁺ cells were isolated using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), incubated with FITC-conjugated goat anti-human IgD, IgM, and IgA Abs (Dako, Glostrup, Denmark). The CD22⁺IgD⁻IgM⁻IgA⁻ cell population, IgG⁺ memory B cells, was identified and isolated by FACS (FACSAria; Becton Dickinson, Franklin Lakes, NJ). Flow-cytometry data were analyzed by FlowJo software (Tree Star, Ashland, OR).

EBV transformation

Isolated memory B cells were centrifuged 4 min at $400 \times g$ and supernatant discarded. Cell pellet was loosened by gentle agitation and suspended in 1 ml EBV-containing supernatant from the marmoset lymphoblast cell line B95.8 (ATCC VR-1492; generated by culturing B95.8 cells in complete medium) and incubated 1 h at 37°C. Cells were then diluted in complete medium containing 0.6 µg/ml phosphorothioated CpG ODN2006 (19) (Integrated DNA Technologies, Leuven, Belgium), seeded at 400 cells/well in 96 U-bottom cell culture plates and cultured at 37°C, in a 7.5% CO₂ humidified atmosphere. After 2 wk, culture supernatants were tested for the presence of HPA-1a-specific IgG.

Selection of HPA-1a-specific B lymphoblasts

HPA-1a⁺ platelets were prepared from platelet-rich plasma (by pelleting) and labeled with 0.5 µg/ml CFSE according to the manufacturer's recommended procedure (Invitrogen). Cells from B lymphoblast cultures secreting anti-HPA-1a IgG were stained with PerCP-conjugated anti-CD45 Ab (Caltag, Burlingame, CA) and incubated with CFSE-labeled platelets. B lymphoblasts binding HPA-1a⁺ platelets were sorted one cell per well into 96-well U-bottom tissue culture plates by FACS, as described earlier, and cultured in the presence of gamma-irradiated allogeneic PBMCs (10,000 cells/well).

Generation and detection of anti-HPA-1a IgG secreting hybridomas

Clonal B lymphoblasts were fused to a nonsecreting mouse-human heteromyeloma cell line K6H6/B5 (ATCC CRL-1823) at a 1:10 ratio using stirring method with polyethylene glycol (HybriMax; Sigma-Aldrich, St. Louis, MO). Fused cells were seeded into the wells of a 48-well plate and cultured in complete medium. Hypoxanthine, aminopterin, and thymidine (Sigma-Aldrich) selection was initiated 24 h after cell fusion and continued for 7 d. Hybridoma supernatants were screened for anti-HPA-1a IgG Abs by MAIPA (16) and flow cytometry. For MAIPA (detailed later), we used 50 µl culture supernatant; for the flow cytometry assay, 2×10^7 HPA-1a⁺ platelets were incubated with 50 µl cell culture supernatant, washed, and stained with FITC-conjugated rabbit anti-human IgG Abs (Dako). Positive cultures were further subcloned three times to isolate stable anti-HPA-1a Ab-secreting hybridomas. The IgG subclass of mAb was tested by ELISA. Goat anti-human Ig Abs (Caltag) were used to coat the ELISA plate (MaxiSorp; NUNC, Roskilde, Denmark), and biotin-conjugated mouse anti-human IgG1, IgG2, IgG3, and IgG4 mAbs were used as detection Abs (clones HP6069, HP6002, HP6047, and HP6025, respectively; Invitrogen).

MAIPA assay

We used the MAIPA technique described in detail by Killie et al. (16). In brief, washed platelets were incubated with human serum or human mAb followed by a mouse monoclonal anti-integrin β3 Ab, clone Y2/51 (Dako). Platelets were then lysed and supernatant added to a microplate precoated with anti-mouse IgG. Human Abs bound to αIIbβ3 were detected with labeled anti-human IgG and a suitable substrate. National Institute of Biological Standards and Control polyclonal anti-HPA-1a standard (20) was used to create a linear standard curve for quantitative MAIPA. Levels of specific Abs in the samples were calculated using a linear regression equation.

Purification of IgG from cell culture supernatants

The IgG fraction of cell culture supernatant was isolated by 40% saturated ammonium sulfate precipitation followed by protein G affinity chromatography (Protein G Sepharose 4 FastFlow; GE Healthcare, Little Chalfont, U.K.). Eluted IgG was dialyzed against PBS and concentrated using Microcon centrifugal filter devices (Ultracel YM-50; Millipore, Billerica, MA).

Amplification and sequencing of Ig V region genes

Total RNA was isolated from clonal B lymphoblasts using RNeasy Mini Spin kit (QIAGEN, Hilden, Germany), and cDNA was synthesized via reverse transcription using primers specific for human IgG constant regions (21). Resulting cDNA was used as a template for PCR to amplify IgG variable H and L chain region genes (VH, Vλ, and Vκ). The genes were amplified in separate PCRs for individual H and L chain gene families, using sense primer specific for one of the leader regions, and antisense primer to the H and L chains constant regions (21). The PCR products were identified using 1.5% agarose gel electrophoresis and cloned into pCR2.1-TOPO vectors (TOPO TA cloning kit; Invitrogen) followed by sequencing of plasmid minipreps (Miniprep kit; QIAGEN). Sequencing reactions were precipitated and run on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at the sequencing core facility at the Faculty of Health Sciences, University of Tromsø - The Arctic University of Norway.

Analysis of Ig V region genes and mutations

Nucleotide sequences were analyzed in the international ImMunoGeneTics (IMGT) database of human germline genes using IMGT/V-QUEST program available online at <http://www.imgt.org> (22). Affinity maturation process (Ag-selective pressure) leads to clustering of replacement (R) mutations as opposed to silent (S) mutations within CDRs, which bind the Ag. Framework regions (FRs) maintain Ab structure and accumulate S as opposed to R mutations. To analyze the Ag-driven selection in the CDRs and FRs, we used a focused selection test described previously (23, 24). The online tool is presently available at <http://clip.med.yale.edu/selection> (25).

Generation of recombinant anti-HPA-1a IgG1 and IgG3

Synthesis of the 26.4 H and L chain genes. H and L chain V region genes coding for Ab 26.4 were synthesized by GenScript (Piscataway, NJ) optimizing the codon usage in the synthesized genes for high-level Ab expression in human cells. Two variants of the 26.4 H chain gene were synthesized using the γ1 and γ3 H chain constant regions. Restriction enzyme recognition sites Esp3I and EcoRI were inserted into the flanks of the synthesized genes, for subsequent use in cloning of the genes into a modified pLNO vector (26).

Cloning of Ig genes. Each of the 26.4 genes was supplied in the pUC57 vector (GenScript). Vector pUC57 containing the synthesized gene was digested with restriction enzymes Esp3I and EcoRI (Fermentas, Burlington, ON), and DNA fragment corresponding to the size of heavy or L chain was isolated by agarose gel electrophoresis using Gelelute kit (QIAGEN). The cloning vector was processed in the same way by digestion with restriction enzymes Esp3I and EcoRI, and subsequent isolation of digested vector by agarose gel electrophoresis. Digested genes were ligated into linearized vector using T4 DNA ligase (New England BioLabs, Ipswich, MA) and then transformed into XL-10 GOLD competent cells (Stratagene, La Jolla, CA). Transformed cells were selected on ampicillin containing growth agar. Bacterial colonies were selected by growing 14 h in ampicillin containing liquid media, and vector DNA was isolated using plasmid minipreps. Vector DNA was verified to contain the correct insert by restriction enzyme analysis.

Transient transfection of HEK293E cells for expression of Ab 26.4. Five million HEK293E cells were added to 25 ml DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS and 4 mM L-glutamine. Cell-containing medium was transferred to a standard medium cell culture flask (T75) and incubated for 18 h in humidified atmosphere at 37°C, 5% CO₂. A transfection mixture was prepared by adding 5 µg vector DNA (0.1 µg/ml) expressing 26.4 L chain, 5 µg vector DNA (0.1 µg/ml) expressing desired 26.4 H chain (γ1 or γ3) and 375 µl RPMI-1640 into a test tube. The mixture was preheated to 80°C and cooled to 4°C. Polyethylenimine Max (PEI Max, 2 mg/ml; Polysciences, Warrington, PA) was heated simultaneously but cooled to room temperature (RT) to prevent precipitation. Sixty-five microliters PEI solution was added to the transfection mixture before the tube was left to incubate at RT for 8 min, and 3375 µl DMEM (10% FBS, 4 mM L-glutamine) was then added to the test tube. Medium from the cell culture flask with HEK293E adherent cells was removed and replaced with the reaction mixture. The reaction mixture was allowed to cover cells for 2 h before adding 25 ml DMEM supplemented with 10% FBS and 4 mM L-glutamine. Transfected cells were allowed to grow for 2–5 d before supernatant was harvested and tested for production of IgG. Concentration of human IgG1 and IgG3 in samples was quantitated by ELISA, with goat anti-human IgG Fc (Sigma-Aldrich) as coating and alkaline phosphatase-conjugated goat anti-human IgG Fc (Sigma-Aldrich) as detection Abs. Purified human myeloma plasma IgG1 and IgG3 (Sigma-Aldrich) were used as internal standards.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) technology was used to assess binding properties of mAbs (Biacore T200 instrument; Biacore AB, Uppsala, Sweden). Integrin αIIbβ3 was isolated from HPA-1aa and -1bb platelets by affinity chromatography as described previously (27), using a Sepharose (CNBr-activated Sepharose 4 Fast Flow; GE Healthcare) column coupled with mouse anti-human β3 mAb (hybridoma AP3; ATCC HB-242). Integrin αVβ3 was purified from human placenta by affinity chromatography using immobilized mAbs to αVβ3 integrin (Millipore). Purified αIIbβ3 integrins, HPA-1a and HPA-1b Ags, and αVβ3 were immobilized to the surface of a CM5 sensor chip on three different flow cells at a density of 400, 340, and 480 response units (RU), respectively, using standard amine coupling chemistry. A flow cell treated with the same chemicals but without protein was used as a reference surface. Various concentrations of mAb samples were injected over the chip surface at a flow rate of 30 µl/min. An association step of 120 s was followed by a dissociation step of 120 s. Regeneration of the sensor chip surface was accomplished using 10 mM glycine-HCl (pH 1.5). Experiments were performed at 25°C. Collected data were analyzed using BiaEvaluation 2.0.1 software. Amount of the immobilized β3 integrin available for Ab binding was measured by injection of anti-β3 mAb (clone SZ21; Dako) at a concentration of 20 µg/ml. Around 80 RUs on the αIIbβ3-immobilized chip (Fig. 4B) and 25 RUs on the αVβ3-immobilized chip (data not shown) have been generated. All chemicals for the Biacore experiment were purchased from GE Healthcare.

Flow-cytometric Ab binding inhibition assay

The capacity of mAbs 26.4 to inhibit binding of mAb SZ21 to the HPA-1a epitope was evaluated using beads indirectly coupled with β3 integrin and compared with a previously described recombinant HPA-1a Ab B2G1 (8, 28). First, Dynabeads M-270 Epoxy (Life Technologies) were coupled with an anti-β3 Ab (clone EPR2417Y, specific for C-terminal part of β3-integrin; Abcam, Cambridge, U.K.) according to the manufacturer's instructions. Next, beads were incubated with cell lysate from a trophoblast cell line expressing β3-integrin (TCL-1 [29], kindly provided by Dr. S. Takeda, Department of Obstetrics and Gynecology, Faculty of Medicine, Juntendo University, Tokyo, Japan; genotyped HPA-1aa) or platelet lysate from HPA-1a⁺ platelets overnight at 4°C, to bind β3 integrin from cell lysates. Beads were washed with radio-immunoprecipitation assay buffer

(Sigma-Aldrich) and incubated with various concentrations of 26.4 and B2G1 in radio-immunoprecipitation assay buffer for 15 min at RT. After a washing step, beads were incubated with 5 µl FITC-conjugated mAb SZ21 in 200 µl bead suspension for 15 min at RT in the dark. After a washing step, beads were resuspended in PBS and analyzed by flow cytometry. GraphPad Prism 5 software (San Diego, CA) was used to analyze and present the data.

MAIPA inhibition assay

mAb 26.4 F(ab')₂ fragment was prepared using Pierce F(ab')₂ Preparation Kit (Pierce, Appleton, WI). The purified F(ab')₂ fragment concentration (0.7 mg/ml) was determined by spectrophotometry from the absorbance at 280 nm using an extinction coefficient of $1.4 \text{ L} \times \text{g}^{-1} \times \text{cm}^{-1}$. The ability of 26.4 to block binding of polyclonal maternal anti-HPA-1a IgG Abs was evaluated by a modified adaptation of the MAIPA technique (28). In brief, HPA-1a-homozygous fresh platelets (2×10^7) were incubated with 50 µl of 26.4 F(ab')₂ for 1 h at RT before adding 100 µl diluted 1:10 serum samples for 15 min. Further, the MAIPA assay was performed as described previously (15, 16). We tested a panel of 10 donor serum samples with anti-HPA-1a activity ranging from 10 to 150 IU/ml as measured by quantitative MAIPA (16). GraphPad Prism 5 software (San Diego, CA) was used to analyze and present the data.

Platelet aggregometry

Impedance platelet aggregometry was used to assess the effect of mAb 26.4 on platelet aggregation (Multiplate analyzer; Roche Diagnostics, Basel, Switzerland). Study participants ($n = 3$ of each HPA-1 genotype) were healthy volunteers with known HPA-1 genotype who did not take any medications affecting platelet function 10 d before blood collection. Whole blood samples were drawn by peripheral venipuncture into 3-ml tubes, containing recombinant hirudin as anticoagulant. Blood samples were kept at RT and measurements performed within 2 h from blood collection. Volumes of 480 µl blood were incubated with various mAb concentrations (20 µl volume) for 5 min before the addition of platelet activator, thrombin receptor activating peptide-6 (TRAP-6). Blood samples with addition of 20 µl PBS buffer were used to determine the individual platelet function triggered by TRAP-6. To test the effect of 26.4 on platelet aggregation without platelet activator, we used 0.9% sodium chloride solution instead of TRAP-6. Aggregation was continuously recorded over 6 min in two independent measuring units per test. Increase of impedance due to attachment of platelets to electrodes was detected and converted into arbitrary aggregation units plotted against time. Aggregation was quantified as the area under the curve in aggregation units (aggregation units × min). Platelet count in blood samples was measured using Sysmex XN-1000 Hematology analyzer (Sysmex, Kobe, Japan). Sigma Plot 12.5 software (San Jose, CA) was used to analyze and present the data.

Anti-HPA-1a Ab-mediated platelet phagocytosis by monocytes assay

Monocytes were isolated from PBMCs using RosetteSep Human Monocyte Enrichment Cocktail (STEMCELL Technologies, Vancouver, BC) as described previously (30) and adjusted to 2×10^6 cells/ml in 10% FBS-IMDM.

In 1-ml volume, 10^8 platelets were labeled with CellTracker Green CMFDA (5-chloromethyl fluorescein diacetate; Invitrogen, Carlsbad, CA) at 0.25 µM final concentration according to the manufacturer's instructions. CMFDA-stained platelets were adjusted to 2×10^8 /ml in 0.2% PBS supplemented with BSA 0.3% EDTA, and 50 µl was incubated with different concentrations of human monoclonal anti-HPA-1a IgG 20 min at RT. After a washing step, 50 µl monocytes was added resulting in a total volume of 100 µl and platelet-to-monocyte ratio of 100:1 in duplicate tubes and incubated 2 h at 37°C, in a 7.5% CO₂ humidified atmosphere. Monocytes were pelleted by centrifugation at $300 \times g$ and incubated with 0.25% trypsin/EDTA solution (Invitrogen) for 2 min at 37°C to remove extracellular adherent platelets. After a washing step, cells were stained with PE-conjugated anti-CD14 Ab (Invitrogen) and analyzed by flow cytometry. Human IgG1 and IgG3 of irrelevant specificities were used as assay negative controls. Sigma Plot 12.5 software was used to analyze and present the data.

Results

Isolation and immortalization of a B cell-producing anti-HPA-1a-specific IgG

We reasoned that B cells carrying anti-HPA-1a-specific IgG may be present at increased numbers in circulation in women who have given birth to a child affected by FNAIT, and that each of these

could cause production of mAbs. We isolated such cells from blood drawn from an HPA-1a–alloimmunized woman 4 wk after delivery of an FNAIT-affected child: from 40 million PBMCs, $\sim 3 \times 10^6$ CD22⁺ cells were isolated by MACS followed by isolation of cells negative for IgM, IgA, and IgD by FACS. The IgM[−]IgA[−]IgD[−] cells ($\sim 10^5$ cells), amounting to 5.6% of the CD22⁺ cells (Fig. 1A), were likely enriched for IgG⁺ cells; in a separate experiment, the IgM[−]IgA[−]IgD[−] population of CD22⁺ cells was shown to consist of mostly IgG⁺ cells (data not shown). Isolated cells were treated with EBV to induce transformation and divided in 240 wells (~ 400 cells/well) on microtiter plates for outgrowth of transformed cells. After 2 wk, 27 B lymphoblast cultures containing HPA-1a–specific Abs were identified by MAIPA. After 7 additional days in culture, only half of the B lymphoblast cultures retained production of specific Abs. To isolate single anti-HPA-1a Ab–producing lymphoblasts from these polyclonal cultures, which we assumed would express surface IgG in addition to the secreted form, we incubated cells from the culture containing the highest amount of anti-HPA-1a IgG in the supernatant with CFSE-stained HPA-1a⁺ platelets. Lymphoblasts able to bind fluorescent platelets were then isolated individually by FACS (Fig. 1B) and expanded in culture. Notably, in a parallel experiment, we observed much nonspecific adherence of HPA-1a[−] platelets to B lymphoblasts; this negative control contained almost the same frequency of CFSE⁺ lymphoblasts (data not shown). After 3 wk of expansion, 1 clonal B lymphoblast culture (D18BL26.4) secreting HPA-1a–specific IgG was identified (out of 120 sorted cells, only 1 gave rise to a clonal culture secreting anti-HPA-1a IgG). The Ab secreted by the D18BL26.4 cells (hereafter referred to as mAb 26.4) bound specifically to HPA-1a⁺ platelets (Fig. 2A, 2B). To stabilize the Ab production (31), we generated a hybridoma cell line D18BL26.4H secreting mAb 26.4 by fusing D18BL26.4 cells to a heteromyeloma cell line. The secreted IgG subclass was found to be IgG3 by ELISA.

Ig V region gene sequence analysis

Expression of various H and L chain V region genes in D18BL26.4 cells was examined by RT-PCR. Only two PCR products for VH6 and VK3 gene families were amplified (data not shown), suggesting that the cell line was clonal. The PCR products were sequenced and the closest known germline genes, and the V, D, and J gene segments used during somatic recombination, were identified (Fig. 3,

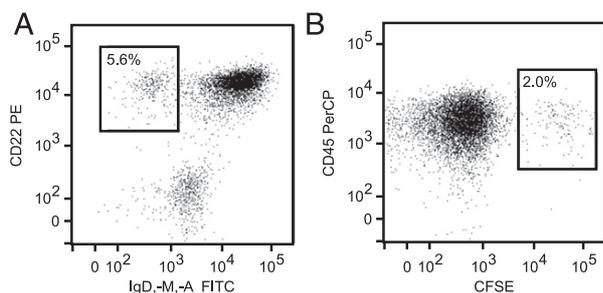


FIGURE 1. Isolation of mAb 26.4. **(A)** CD22⁺ PBMCs were isolated by MACS from an HPA-1a–alloimmunized woman and subsequently labeled with FITC-conjugated anti-human IgM, IgA, and IgD (IgMAD) Abs. The CD22⁺IgMAD[−] cells (gated population; enriched for IgG⁺ B cells) were isolated by FACS and cultured with EBV. **(B)** EBV-transformed B cells from a culture identified as producing HPA-1a–specific Abs to the culture supernatant by MAIPA (data not shown) were incubated with CFSE-labeled platelets and fluorochrome-conjugated anti-CD45 Abs. Platelet-binding CD45⁺ lymphoblasts (gated population) were isolated individually by FACS into 96-well U-bottom microplates. A clonal B cell culture secreting anti-HPA-1a mAb 26.4 was generated from one of these wells.

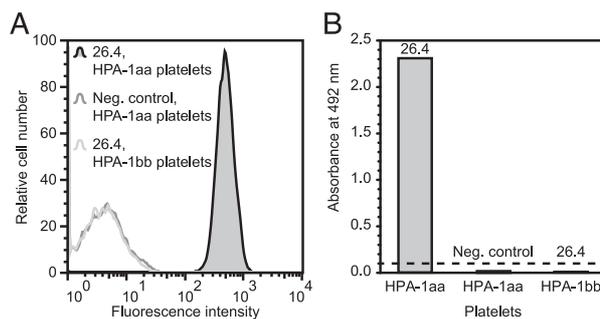


FIGURE 2. mAb 26.4 binds specifically to HPA-1a⁺ platelets. **(A)** Binding of 26.4 to HPA-1aa and HPA-1bb platelets was analyzed by flow cytometry. Platelets were incubated with 26.4 cell culture supernatant, or medium as a negative control, and subsequently with FITC-conjugated anti-human IgG. Platelets were identified by forward and side scatter and gated to exclude debris and lymphocytes (data not shown). **(B)** mAb 26.4 was assayed for binding to HPA-1aa and HPA-1bb platelets by MAIPA. Normal serum was used as a negative control. Samples were run in duplicates and data are presented as average absorbance values after background subtraction. Dashed line represents negative cutoff. Results are representative of at least three independent experiments. mAb 26.4 derived from B lymphoblast, hybridoma, and recombinant 26.4 IgG1 and IgG3 gave similar results in both assay systems (data not shown).

Table I). For the H chain, IGHV6-1*01, IGHD6-13*01, and IGHJ6*02 gene segments were identified, and IGKV3-11*01 and IGKJ4*01 for the L chain.

Comparison of the gene sequences coding for mAb 26.4 variable regions with those of two human anti-HPA-1a Abs also derived from women alloimmunized in connection with pregnancy demonstrated that 26.4 is unique (Table I). Low numbers of silent and replacements mutations in CDRs and FRs, as well as Ag-driven selection analysis (25), suggest a low level of Ag-driven affinity maturation for all three Abs (Table I).

Generation of rAbs

To facilitate exploration of mAb 26.4 function with different Ig isotypes, we combined the gene encoding the Ig H chain V region in D18BL26.4 cells with IgG1 and IgG3 constant domains in different expression constructs. The L chain V region gene was combined with a κ 1 constant domain in a third construct. One H chain and L chain constructs were expressed in HEK293E cells after transient transfection. Typically, transfected cultures produced recombinant 26.4 IgG1 and IgG3 variants to supernatants at concentrations of 20–50 and 5–20 μ g/ml, respectively, as measured by ELISA. Identical to the native 26.4, rIgG1 and rIgG3 variants bound specifically to HPA-1a⁺ intact platelets when assayed by flow cytometry and MAIPA (Fig. 2A, 2B). No binding to HPA-1a[−] platelets was observed. All the experiments from this point on were performed with the recombinant mAb 26.4 IgG1 variant unless otherwise noted.

mAb 26.4 is highly specific for the HPA-1a Ag

For more sensitive assessment of Ag specificity, 26.4 binding to purified platelet integrin α Ib β 3 was measured by SPR. mAb 26.4 bound exclusively to α Ib β 3 from HPA-1a⁺ individuals; there was no measurable binding to HPA-1a[−] α Ib β 3 (Fig. 4A). Rapid association and slow dissociation indicate that 26.4 binds strongly to HPA-1a.

Direct comparison of mAb 26.4 and B2G1, another human HPA-1a–specific mAb (28), gave similar association and dissociation curves indicating that affinities of the two mAbs are in the same range (Fig. 5A). mAb B2G1 was generated by phage display

Heavy chain

	<-----FR1----->
26.4 IGHV	Q V Q L Q Q S G P G L V K P S Q T L S L T C A cag gta cag ttg cag cag tca ggt cca gga ctg gtg aag ccc tcg cag acc ctg tca ctc acc tgt gcc
IGHV6-1*01	-----c-----
	>-----CDR1-----<-----FR2----->
26.4 IGHV	I S G D S V S S N S A A W N W I R Q S P S R G atc tcc ggg gac agt gtc agc agc aac agt gct gct tgg aac tgg atc agg cag tcc cca tcg aga ggc
IGHV6-1*01	-----tct-----
	>-----CDR2-----<-----FR3----->
26.4 IGHV	L E W L G R T Y F R S N W Y N D Y A A S V K S ctt gag tgg ctg gga agg aca tac ttc agg tcc aac tgg tac aat gat tat gca gca tct gtg aaa agt
IGHV6-1*01	-----Y-----K-----V-----
	>-----FR3-----<-----FR3----->
26.4 IGHV	R I T I N Q D T S K N Q L S L Q L N S V T P E cga ata acc atc aac caa gac aca tcc aag aac cag ctg tcc ctg cag ctg aac tct gtg act ccc gag
IGHV6-1*01	-----P-----F-----
	>-----CDR3-----<-----FR3----->
26.4 IGHV	D T A M Y Y C A R D G A W G G S S W W P G L P gac acg gct atg tat tac tgt gca aga gat ggg gcc tgg ggt ggc agc agc tgg tgg cca ggc ctt cct
IGHV6-1*01	-----V-----g-----
	>-----FR4-----<-----FR4----->
26.4 IGHV	H H Y Y S G M D V W G Q G T T V T V S S cac cac tac tac tct ggt atg gac gtc tgg ggc cag ggg acc acg gtc acc gtc tcc tca
IGHV6-1*01	-----
Light chain	
	<-----FR1----->
26.4 IGKV	E I V L T Q S P A T L S L S P G E R A T L S C gaa att gtg ttg aca cag tct cca gcc acc ctg tca ttg tct cca ggg gaa aga gcc acc ctc tcc tgc
IGKV3-11*01	-----t-----
	>-----CDR1-----<-----FR2----->
26.4 IGKV	R A S Q S V S S Y L A W Y Q Q K P G Q A P R L agg gcc agt cag agt gtt agc agc tac tta gcc tgg tac caa cag aag cct ggc cag gct ccc agg ctc
IGKV3-11*01	-----a-----
	>-----CDR2-----<-----FR3----->
26.4 IGKV	L I Y D A S K R A T G I P A R F S G S G S G T ctc atc tat gat gca tcc aaa agg gcc act ggc atc cca gcc agg ttc agt ggc agt ggg tct ggg aca
IGKV3-11*01	-----N-----c-----
	>-----CDR3-----<-----FR3----->
26.4 IGKV	D F S L T I R S L E P E D F A V Y Y C Q Q R S gac ttc agt ctc acc atc aga agc ctc gag cct gaa gat ttt gca gtt tat tac tgt caa cag cgt agc
IGKV3-11*01	-----T-----S-----a-----g-----
	>-----FR4-----<-----FR4----->
26.4 IGKV	D W Q G L T F G G G T K V E I K gac tgg cag gga ctc act ttc ggc gga ggg acc aag gtg gag atc aaa
IGKV3-11*01	-----N-----P-----ct cc

FIGURE 3. Nucleotide and amino acid sequence of mAb 26.4. H and L chain V regions compared with the most homologous germline sequences. Analyzed by IMGT/V-QUEST (22).

technology from B cells isolated from a woman alloimmunized in pregnancy (28).

Binding affinity of the B2G1 to the recombinant α IIB β 3 was measured previously, $K_D = 6 \times 10^{-8}$ (32). In contrast with 26.4 and B2G1, the murine mAb SZ21 (33) bound both HPA-1a⁺ and HPA-1a⁻ integrin α IIB β 3; however, it displayed a considerably higher affinity for HPA-1a as it associated slower and dissociated faster from the HPA-1a⁻ integrin (Fig. 4B).

mAb 26.4 displays a unique binding pattern to integrin α V β 3

Because integrin β 3 is also part of the vitronectin receptor (α V β 3), we examined the relative binding efficiencies of HPA-1a-specific mAbs 26.4 and B2G1 to purified α V β 3 compared with the

binding to purified α IIB β 3. Both mAbs bound to the sensor chip surface coupled with α IIB β 3 (HPA-1a), as well as the surface coupled with α V β 3 (Fig. 5A, 5B). Interestingly, whereas 26.4 and B2G1 generated near similar binding responses to α IIB β 3 (Fig. 5A, Table II), mAb 26.4 generated a 40% higher binding response to α V β 3 compared with B2G1 (Fig. 5B, Table II). Also, whereas both mAbs dissociated from α IIB β 3 with nearly identical rates (Fig. 5A, Table II), B2G1 dissociated >50% faster from α V β 3 compared with 26.4; only 35.1% of 26.4 dissociated compared with 70.5% of B2G1 by the end of the dissociation phase (Fig. 5B, Table II).

The observed difference in binding to α V β 3 cannot be attributed to any loss of Ag because the B2G1 samples were run before the

Table I. Analysis of Ig variable gene sequences and mutations of human HPA-1a-specific mAbs

Clone	VH Gene				VL Gene			
	VDJ Gene Segments	CDR-H3 Length	FR R/S	CDR R/S	VJ Gene Segments	CDR-L3 Length	FR R/S	CDR R/S
26.4	V6/D6/J6	25	4/2	4/2	KV3/KJ4	10	3/3	0/0
B2G1 ^a	V1/D3/J6	15	6/7	2/4	—	—	—	—
ML1 ^b	V4/D6/J3	20	7/3	3/1	—	—	—	—

^aThe scFv fragment isolated by phage display from a woman HPA-1a immunized in connection with pregnancy (28).

^bThe scFv fragment was isolated from a woman HPA-1a immunized in connection with pregnancy who has given birth to a child with FNAIT (T.S. Kickler, personal communication, October 2013) (39).

—, for these two mAbs, the H chain was isolated without an L chain; R, replacement mutations; S, silent mutations.

26.4 samples over both α IIB β 3 (Fig. 5A) and α V β 3 (Fig. 5B) surfaces. Furthermore, the results were produced with various Ab concentrations (only the highest is shown), and similar results have been obtained using a sensor chip coupled with higher amounts of integrins (data not shown).

Because of the observed difference in binding to α V β 3, we decided to examine the relative efficiencies of 26.4 and B2G1 at inhibiting the binding of a third anti-HPA-1a mAb, SZ21, to α IIB β 3 and α V β 3 (Fig. 5C, 5D). In this set of experiments, mAb 26.4 was more efficient than B2G1 at inhibiting binding of SZ21 to beads coupled with α V β 3 from trophoblasts (Fig. 5D). In comparison, there was little difference in the efficiency of the two human mAbs at inhibiting SZ21-binding to beads coupled with α IIB β 3 from platelets (Fig. 5C). Therefore, although mAbs 26.4 and B2G1 appear to bind similarly to HPA-1a on integrin α IIB β 3, they differ in binding efficiency to integrin α V β 3. Despite this

difference, both mAbs appeared to bind with similar intensity to cells from the HPA-1a-expressing TCL-1 trophoblast cell line when analyzed by flow cytometry (data not shown).

mAb 26.4 inhibits binding of polyclonal anti-HPA-1a IgG to platelets

One potential therapeutic use of mAb 26.4 would involve blocking access of pathogenic anti-HPA-1a Abs to fetal platelets. Therefore, we tested the capacity of 26.4 to inhibit binding of maternal polyclonal anti-HPA-1a IgG using the MAIPA technique. Binding to HPA-1a-homozygous platelets in 10 of 10 samples was considerably inhibited after preincubation of platelets with 26.4 F(ab')₂ fragment. The inhibition ranged from 65 to 100% at a highest fragment concentration of 35 μ g in 50 μ l volume (Fig. 6).

mAb 26.4 has an inhibitory effect on platelet aggregation

Because the integrin heterodimer α IIB β 3 is a fibrinogen receptor on platelets, we assessed whether 26.4 affects platelet aggregation (Fig. 7). mAb 26.4 inhibited HPA-1aa platelet aggregation in a concentration-dependent manner: 15, 35, and 80% inhibition at concentrations of 1, 6, and 12 μ g/ml, respectively. At the lowest mAb concentration, inhibition of aggregation of HPA-1ab platelets was similar to HPA-1aa. Six and 12 μ g/ml mAb equally inhibited aggregation of HPA-1ab platelets by 20–28%. Importantly, 26.4 did not affect HPA-1bb platelet aggregation. mAb 26.4 did not affect platelet function when aggregation was measured in samples without platelet activator (data not shown). Similar results were generated in parallel experiments with mAb B2G1 (data not shown). Platelet count in samples with mAb added in different concentrations did not differ from control samples without mAb for each participant. Decrease of platelet aggregation was therefore attributed solely to inhibition of platelet function.

mAb 26.4 is potent in inducing platelet phagocytosis

One possible mechanism of Ab-induced prophylaxis is removal of sensitized platelets by phagocytosis. To assess whether 26.4 can induce platelet phagocytosis by monocytes, we incubated freshly isolated monocytes with 26.4-sensitized CFSE-labeled platelets and measured the portion of monocytes with ingested platelets by flow cytometry. mAb 26.4, both IgG1 and IgG3 variants, induced phagocytosis of HPA-1a-homozygous platelets in a concentration-dependent manner (Fig. 8A, 8B). At concentrations of 10, 1, and 0.1 μ g/ml, the Abs induced around 90, 70, and 30% phagocytic activity, respectively. At mAb concentration 0.01 μ g/ml, the phagocytic activity was at the same level as the negative controls. The phagocytic activity when using HPA-1ab platelets was ~20% lower at each concentration compared with HPA-1aa platelets (Fig. 8B). Preincubation of HPA-1a⁻ platelets with 26.4 did not induce phagocytosis (data not shown). No synergistic effect was observed when a 1:1 mixture of 26.4 IgG1 and IgG3 variants was tested (data not shown).

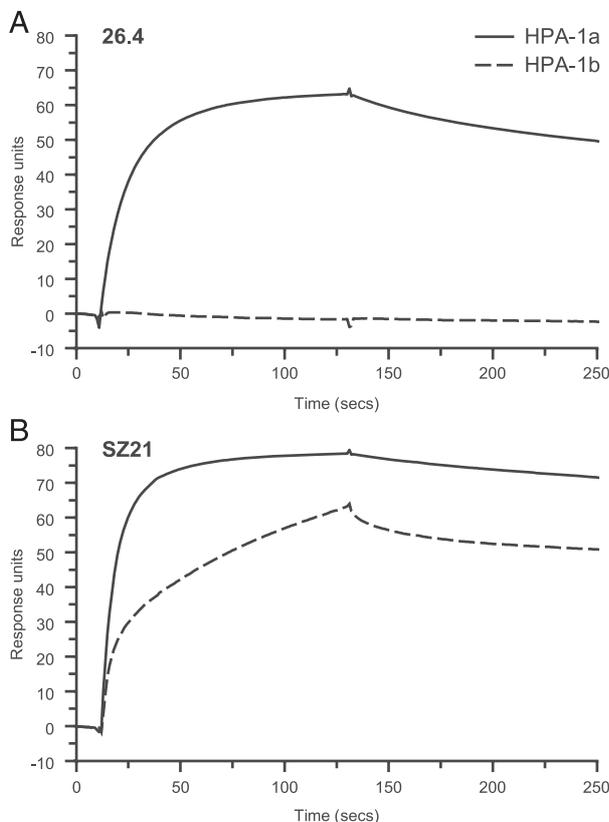


FIGURE 4. SPR analysis of mAb binding to HPA-1 Ags. Sensorgrams generated by binding of mAb 26.4 (**A**) and the murine mAb SZ21 (**B**) to purified α IIB β 3 from the HPA-1a (solid line) or HPA-1b (dashed line) individuals were measured by SPR technology.

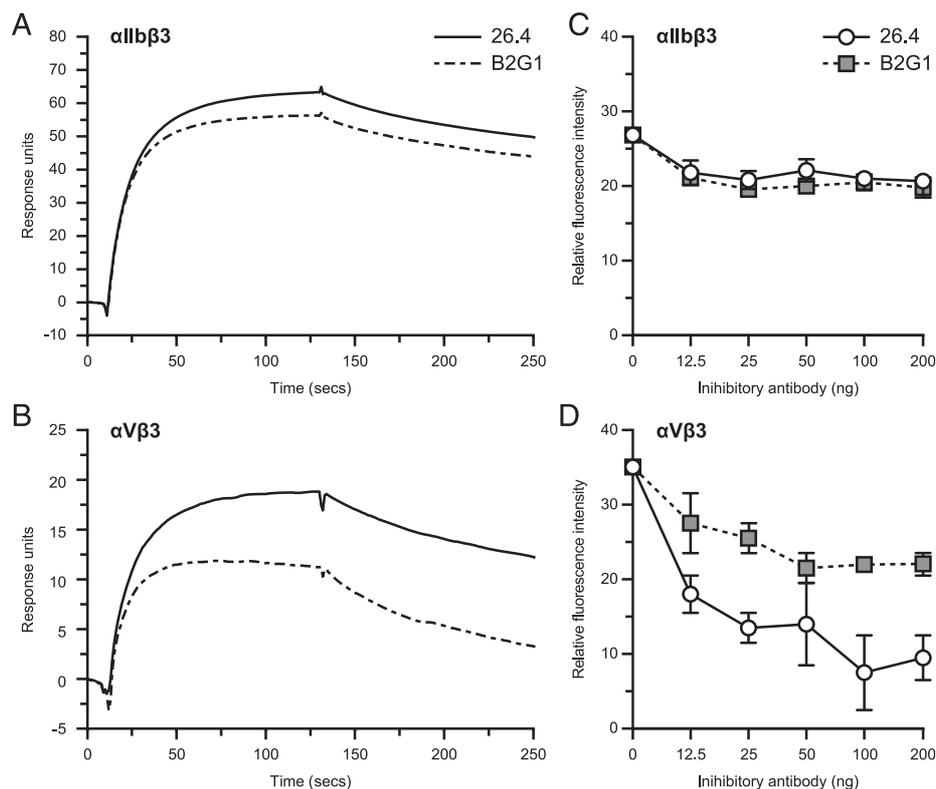


FIGURE 5. mAb 26.4 and the HPA-1a-specific human mAb B2G1 bind with relatively similar efficiency to $\alpha\text{IIb}\beta\text{3}$ but with different efficiency to $\alpha\text{V}\beta\text{3}$. SPR sensograms were generated by binding of 26.4 (solid line) and B2G1 (dashed) to HPA-1a on $\alpha\text{IIb}\beta\text{3}$ (A) and $\alpha\text{V}\beta\text{3}$ (B) immobilized to the sensor chip surface. mAb samples were used in three different concentrations; the highest concentration is shown. Results are representative of the two independent experiments. To compare the capacity of 26.4 and B2G1 to inhibit binding of mAb SZ21 to HPA-1a Ag, we preincubated beads coupled with β3 integrin with various concentrations of 26.4 or B2G1, and subsequent binding of FITC-conjugated SZ21 to HPA-1a Ag was evaluated by flow cytometry (C and D). Relative fluorescence intensity = mean fluorescence intensity of each sample (mean \pm SEM) – mean fluorescence intensity of beads coupled with β3 integrin from HPA-1bb platelet lysate. Every sample was run in duplicate. The presented graphs are representative for four independent experiments using beads coupled with β3 integrin from platelet lysate (C) or from trophoblast cell lysate (D).

Discussion

In this study, a mAb specific for HPA-1a was derived from a single memory B cell. This B cell was isolated from a woman known to be HPA-1a immunized in connection with pregnancy. We succeeded in expressing this Ab, mAb 26.4, recombinantly by transient transfection of human cells. We found that 26.4 binds strongly to HPA-1a and is highly specific; no reactivity with the HPA-1b allotype was detected. It binds both $\alpha\text{IIb}\beta\text{3}$ and $\alpha\text{V}\beta\text{3}$, and binds the latter with relatively high affinity compared with other HPA-1a-specific human mAb, B2G1 (8, 28). Furthermore, 26.4 can inhibit binding of human polyclonal anti-HPA-1a IgG Abs to platelets and can opsonize platelets for enhanced monocyte phagocytosis. Thus, 26.4 holds potential for FNAIT prophylaxis, therapy, and HPA-1a phenotyping.

Currently, no specific treatment is available to prevent FNAIT; nor is there any specific therapy. However, a strategy to prevent alloimmune thrombocytopenia by Ab prophylaxis has been proved successful in mice (10), and clinical trials (<http://www.profnait.eu>)

are under way to test the potential of hyperimmune anti-HPA-1a IgG in preventing HPA-1a immunization in connection with pregnancy (9). In principle, HPA-1a-specific mAbs may be as efficient as polyclonal antisera in preventing alloimmunization and could hold important advantages, not the least in terms of tailorability, for enhanced function and as a limitless source of such Abs. Because the HPA-1a epitope is formed by a single amino acid difference between mother and child, all conceivable recognition by maternal Abs that can bind the HPA-1a epitope likely overlap with the allogeneic residue. Therefore, a single mAb may be sufficient to sterically hinder activation of HPA-1a-specific maternal B cell clones, a potential mechanism of Ab prophylaxis.

Ab prophylaxis and Ab therapy are in principle different strategies relying on different modes of action. The aim of prophylactic treatment with mAb 26.4 is to inhibit formation of maternal anti-HPA-1a Abs in connection with pregnancy. This treatment principle is similar to prevention of the alloimmune

Table II. SPR analysis of mAb 26.4 and B2G1 binding to immobilized $\alpha\text{IIb}\beta\text{3}$ and $\alpha\text{V}\beta\text{3}$

Integrin Complex	26.4			B2G1		
	Bound (RU)	Bound after Dissociation (RU)	% Dissociated	Bound (RU)	Bound after Dissociation (RU)	% Dissociated
$\alpha\text{IIb}\beta\text{3}$	63.1	49.6	21.4	56.1	43.8	22
$\alpha\text{V}\beta\text{3}$	18.8	12.2	35.1	11.2	3.3	70.5

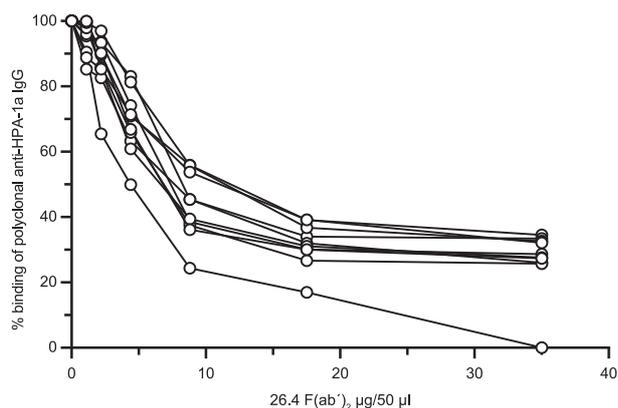


FIGURE 6. mAb 26.4 can inhibit binding of polyclonal anti-HPA-1a IgG to HPA-1a-homozygous platelets. HPA-1aa platelets were reacted with various concentrations of 26.4 F(ab')₂ fragment before adding polyclonal anti-HPA-1a IgG samples. Binding of anti-HPA-1a IgG to platelets was measured by MAIPA. Uninhibited binding of polyclonal Abs was taken as maximum or 100% binding. Binding in the presence of 26.4 F(ab')₂ fragment is presented as a percentage of maximum binding. Circles connected by black lines represent binding of donor samples.

condition hemolytic disease of the fetus and newborn. In hemolytic disease of the fetus and newborn, maternal alloimmunization toward fetal erythrocytes (most commonly anti-D Abs) may lead to fetal/neonatal anemia. Administration of anti-D Abs to an RhD⁻ mother shortly after delivery of an RhD⁺ child is well-known to efficiently prevent maternal anti-D immunization. Similarly, we envision that administered mAb 26.4 to an HPA-1a⁻ mother shortly after delivery of an HPA-1a⁺ child will bind fetal platelet- and placenta-derived HPA-1a, and thereby prevent formation of anti-HPA-1a Abs in the mother. Proposed mechanisms of this prophylaxis are to inhibit access of the maternal immune system to alloantigens of fetal origin, either by rapid removal or by masking the alloantigen, or by engagement of inhibitory FcRs, and thereby prevent alloimmunization and formation of maternal anti-HPA-1a Abs (34). In support of the feasibility of Ab prophylaxis with HPA-1a-specific mAbs, we have demonstrated in vitro that both IgG1 and IgG3 subclasses of recombinantly

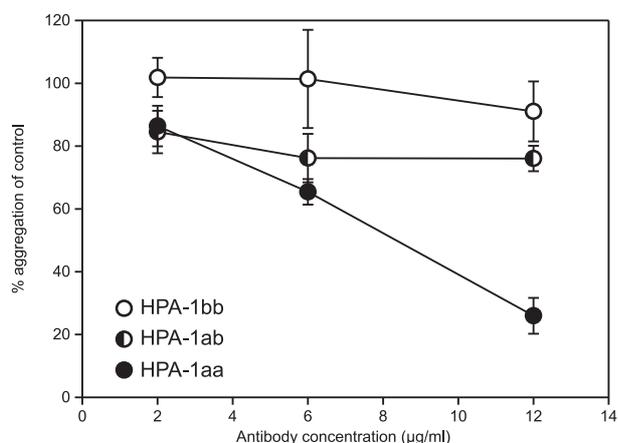


FIGURE 7. The effect of mAb 26.4 on platelet aggregation. Blood samples from HPA-1-genotyped donors ($n = 3$ of each HPA-1 genotype) were preincubated with various concentrations of 26.4 before platelet activator was added to induce platelet aggregation. Aggregation data for blood samples preincubated with 26.4 are presented as percentage of platelet aggregation without mAb.

expressed mAb 26.4 are able to induce phagocytosis of HPA-1a⁺ platelets. Also, rapid clearance of autologous HPA-1ab platelets that were sensitized ex vivo with the HPA-1a-specific human mAb B2G1 was demonstrated in a recent study (35). Furthermore, our demonstration that mAb 26.4 can efficiently block maternal polyclonal HPA-1a-specific IgG from various donors from binding platelets suggests that the mAb could also interfere with binding to receptors on HPA-1a-specific B cell clones in women susceptible to immunization.

In contrast with prophylaxis, the aim of therapeutic treatment with mAb 26.4 will be to inhibit fetal thrombocytopenia in pregnancies where anti-HPA-1a Abs have already been formed. This may be achieved by protecting fetal tissues and platelets from potentially damaging anti-HPA-1a Abs with Abs that compete for binding to HPA-1a but lack the ability to activate immune effector functions. This is not a new concept and has been proved to function in principle with HPA-1a-specific mAbs both in a murine model and in human volunteers (35, 36). The stable binding of mAb 26.4 to both platelet-derived and trophoblast-derived HPA-1a, as demonstrated in this study, could be an additional advantage for therapeutic purposes because anti-HPA-1a Abs have been reported to have possible negative effects on fetal growth, in addition to causing thrombocytopenia in the fetus (5, 37).

To introduce mAb 26.4 to the clinic, the functionality of both strategies described earlier will be tested in a murine model of FNAIT that is currently under development in our laboratory. In this model, mice express the human MHC class II molecule HLA-DRA/DRB3*01:01, which is strongly associated with HPA-1a alloimmunization and FNAIT occurrence. This will allow monitoring of T cell responses in parallel with anti-HPA-1a Ab formation, and thereby elucidation of the biological effect of prophylaxis. An important question to address is whether T cell responses that drive anti-HPA-1a Ab formation are activated by prophylaxis and which consequences these may have for therapy.

In addition to testing in animals, the principle functionality and safety of anti-HPA-1a Ab prophylaxis will soon be tested in a large multicenter clinical trial using IgG preparations from HPA-1a-immunized donors as the prophylactic drug (<http://www.profnait.eu>). In this upcoming trial, pregnant women identified as HPA-1a⁻ and who have the MHC class II allele DRB3*01:01 will, in connection with delivery, be offered prophylactic treatment with hyperimmune anti-HPA-1a polyclonal Ab preparations (9). The treated women will be monitored for development of anti-HPA-1a Abs after delivery to assess the effect of the prophylactic treatment. Arguably, findings from this trial may to a large extent pave the way for similar treatment with mAb 26.4, being of similar specificity. Modifications may be introduced by recombinant technology to enhance or dampen specific function to make mAb 26.4 suitable for these different purposes (11, 12, 14, 38).

mAb 26.4 is not the only mAb that can distinguish between HPA-1a and -1b. However, it is the only HPA-1a-specific human mAb derived with naturally intact H and L chains from a single B cell. Two other HPA-1a-specific human mAbs, B2G1 (28) and ML1 (39), were derived by phage display technology from HPA-1a-alloimmunized individuals. Comparison of V-region sequences of 26.4 with B2G1 and ML1 demonstrated that the gene segments used in 26.4 are different and that there is no preference among these Abs for particular V-, D-, or J-segments. Thus, 26.4 is unique. Two additional HPA-1a-reactive mAbs were developed by conventional hybridoma technology after immunization of mice with human platelets (33, 40). One of these, clone LK-4, differentiates HPA-1a from HPA-1b on platelet extracts but not intact platelets (40). The second, mAb SZ21, binds HPA-1a on intact platelets, but is also reactive with HPA-1a⁻ platelets when used at

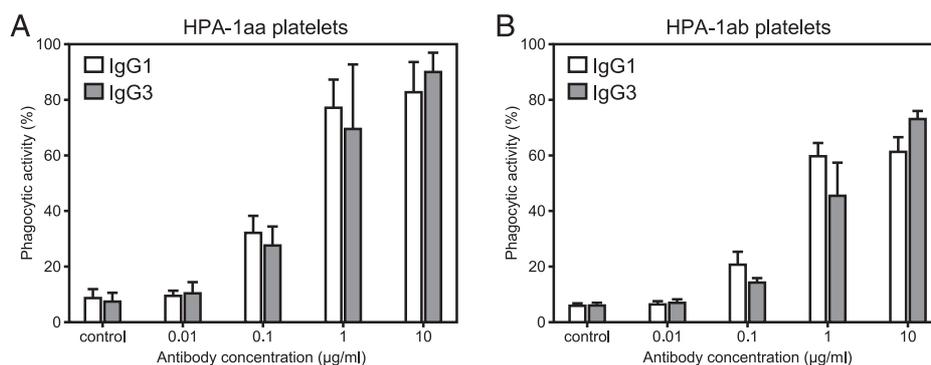


FIGURE 8. Monocyte phagocytosis of platelets is enhanced by mAb 26.4. Platelets from donors with known HPA-1 genotype ($n = 3$ of each HPA-1 genotype) were CMFDA labeled, sensitized with various concentrations of 26.4 IgG1 or IgG3, and incubated with autologous monocytes. After washing and removal surface adherent platelets, monocytes were stained with PE-conjugated anti-CD14 Ab and analyzed by flow cytometry. The monocyte population was identified by forward and side scatter and gated (data not shown). The percentage of CD14⁺ and CMFDA⁺ monocytes was defined as phagocytic activity (%). Data presented is average phagocytic activity of monocytes from HPA-1a-homozygous (**A**) and HPA-1ab (**B**) donors.

increased Ab concentrations (33). Still, it can distinguish the HPA-1a allotype due to relatively lower binding affinity to the HPA-1b allotype (33, 41), and it is successfully used as an HPA-1 phenotyping reagent (7). Also, the B2G1 mAb has been proved useful as an *in vitro* HPA-1a phenotyping reagent (8). Direct comparison of the human mAbs 26.4 and B2G1 demonstrated that they are quite similar in their capacity to bind to HPA-1a on platelet-derived integrin α IIB β 3. However, one notable difference between these mAbs, as demonstrated in this study, is that 26.4 binds more stably to trophoblast-derived α V β 3 and is more efficient at inhibiting binding of anti-HPA-1a Abs (SZ21) to α V β 3. The molecular basis of the binding difference remains unclear and requires further investigation. In terms of prophylactic and therapeutic potential, stable binding to HPA-1a on trophoblasts may be an advantageous property. It has been speculated that HPA-1a on α V β 3 expressed on trophoblast cells could initiate an alloimmune response in the mother (42, 43). One could envision that stable binding of 26.4 to α V β 3 derived from placenta could prevent alloimmunization by blocking activation of Ag-specific B cells and by accelerated removal of cells and material expressing this Ag from the maternal circulation.

When contemplating use of HPA-1a-specific mAbs for prophylaxis, a particularly relevant consideration is the risk for cross-reactivity with recipient (maternal) tissues; because there is only a single amino acid residue difference between the allogeneic (HPA-1a) and autologous (HPA-1b) epitopes, the potential for cross-reactivity is significant. Arguably, Abs that have been naturally selected in human immune responses would be optimal for *in vivo* administration; natural selection will likely drive toward minimal autoreactivity by deletion of self-reactive B cell clones. Indeed, it was demonstrated in this study by sensitive binding assays that there is no measurable cross-reactivity of mAb 26.4 with the native HPA-1b allotype. In contrast, when anti-HPA-1a Ab responses are formed after immunization of mice, a similar pressure to select for minimal cross-reactivity with HPA-1b will be lacking because of differences in integrin β 3. This line of thinking is consistent with the observations in this study of considerable cross-reactivity of the murine mAb SZ21 with HPA-1b, whereas none was detectable with the human mAbs 26.4 and B2G1.

Another important consideration with Abs that bind to HPA-1a on platelets is the risk for altering platelet function and especially platelet aggregation. Integrin α IIB β 3, which harbors HPA-1a, is the fibrinogen receptor on platelets and plays a critical role in platelet aggregation, which is central in hemostasis and thrombosis. Relatively high concentrations of mAb 26.4 inhibited ag-

gregation of HPA-1ab platelets, representing the genotype of the fetus, by 20–28%. Anti-HPA-1a Abs can bind only 50% of α IIB β 3 on HPA-1ab platelets. There exists a parallel to this situation in Glanzmann thrombasthenia carriers, who have 40–50% reduced expression of α IIB β 3 molecules on the platelet surface; still, Glanzmann thrombasthenia carriers do not have hemorrhagic symptoms (44). Therefore, blocking of up to 50% of α IIB β 3 receptors on fetal platelets in the context of conceivable therapeutic intervention with anti-HPA-1a Abs should similarly be of no clinical relevance. It has been hypothesized that anti-HPA-1a IgG Abs sterically hinder access to the RGD binding site on α IIB β 3 and thereby inhibit platelet aggregation (45). A third concern is whether anti-HPA-1a Abs may activate platelets. In a study on the effect of a HPA-1a-specific human mAb on platelet function, no activating effect on platelets was observed (45), suggesting that platelets are not activated by direct binding to the HPA-1a Ag. However, anti-platelet Abs can activate platelets indirectly via Ab-mediated complement activation and by interaction with the platelet receptor Fc γ RIIa (46). For clinical applications, it may therefore be necessary to modify the Fc portion of 26.4 to avoid platelet activation.

In conclusion, we have developed a novel HPA-1a-specific Ab derived from a single B cell of a woman HPA-1a alloimmunized in connection with pregnancy. The Ab has no detectable cross-reactivity with the HPA-1b allotype and binds stably to HPA-1a on both α IIB β 3 and α V β 3. The recombinant version of this Ab can potentially be developed into a diagnostic reagent to identify individuals at risk for HPA-1a immunization, as well as a drug to prevent or treat FNAIT.

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Disclosures

M.K., T.E.M., A.H., and B.S. are current employees of Prophylix Pharma AS and/or holders of stock and/or stock options in the company. M.E., H.T., T.E.M., Ø.I., A.H., B.S., and T.B.S. are named coinventors on a patent application relating to this work. The other authors have no financial conflicts of interest.

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