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Developing recombinant HPA-1a–specific antibodies with abrogated Fcγ receptor binding for the treatment of fetomaternal alloimmune thrombocytopenia

Cedric Ghevaert,1,2 David A. Wilcox,3,4,6 Juan Fang,3,4 Kathryn L. Armour,6 Mike R. Clark,6 Willem H. Ouwehand,1,2 and Lorna M. Williamson1,2

Fetomaternal alloimmune thrombocytopenia (FMAIT) is caused by maternal generation of antibodies specific for paternal platelet antigens and can lead to fetal intracranial hemorrhage. A SNP in the gene encoding integrin β3 causes a clinically important maternal-paternal antigenic difference; Leu33 generates the human platelet antigen 1a (HPA-1a), whereas Pro33 generates HPA-1b. As a potential treatment to prevent fetal intracranial hemorrhage in HPA-1a alloimmunized pregnancies, we generated an antibody that blocks the binding of maternal HPA-1a–specific antibodies to fetal HPA-1a1b platelets by combining a high-affinity human HPA-1a–specific scFv (B2) with an IgG1 constant region modified to minimize Fcγ receptor–dependent platelet destruction (G1Δnab). B2G1Δnab saturated HPA-1a+ platelets and substantially inhibited binding of clinical HPA-1a–specific sera to HPA-1a+ platelets. The response of monocytes to B2G1Δnab-sensitized platelets was substantially less than their response to unmodified B2G1, as measured by chemiluminescence. In addition, B2G1Δnab inhibited chemiluminescence induced by B2G1 and HPA-1a–specific sera. In a chimeric mouse model, B2G1 and polyclonal Ig preparations from clinical HPA-1a–specific sera reduced circulating HPA-1a+ platelets, concomitant with transient thrombocytopenia. As the Δnab constant region is uninformative in mice, F(ab′)2 B2G1 was used as a proof of principle blocking antibody and prevented the in vivo platelet destruction seen with B2G1 and polyclonal HPA-1a–specific antibodies. These results provide rationale for human clinical studies.

Introduction

Fetomaternal alloimmunization to paternal human platelet antigens (HPAs) is the most common cause of severe thrombocytopenia in term neonates (1), with 75% of cases due to alloantibodies against HPA-1a (2–5). One in 4 babies born to HPA-1a–immunized mothers have fewer than 20 × 109 platelets/l (5–10), which leads to intracranial hemorrhage (ICH) in 10% to 20% of all cases with fetomaternal alloimmune thrombocytopenia (FMAIT), from 16 weeks of pregnancy through the postnatal period (8, 11, 12). There is no consensus on the most effective antenatal therapy for FMAIT (13). Intrauterine transfusions (IUTs) of HPA-1a+ platelets are a logistical challenge (14), and the risk of fetal loss is up to 15% (15–18). Several trials have shown the benefit of intravenous immunoglobulin (IVIG) therapy, but in 50% of patients with severe disease (ICH in the previous pregnancy or an initial fetal platelet count of fewer than 20 × 109/l), IVIG therapy does not achieve a safe fetal platelet count (19–23). Although IVIG therapy may reduce the incidence of ICH in this high-risk group without a rise in platelet count (24), ICHs still occur in some cases (21, 25, 26). The most common side effects of IVIG therapy, such as headaches, myalgia, and allergic reactions, can be easily treated (27). However, the infectious risk associated with pooled blood products such as IVIG cannot be dismissed, as a previous outbreak of hepatitis C associated with plasma-derived anti-D immunoglobulin has shown (28, 29), and there is concern over emerging infectious agents such as prions, for which donors are not screened and which are resistant to heat treatment (30). IVIG therapy is expensive and its chronic worldwide shortage well documented (31, 32). Therefore, a safe and effective recombinant alternative for antenatal treatment of FMAIT would be useful.

It has been shown that the binding site for polyclonal HPA-1a antibodies is limited to a finite number of epitopes on the β3 integrin, with Leu33 being a critical residue in the antibody binding site (33). We reasoned that it should be possible to generate a recombinant nondestructive blocking HPA-1a antibody of sufficiently high affinity to block binding of maternal polyclonal HPA-1a antibodies to fetal HPA-1a1b platelets. A potential therapeutic antibody would require an Fc portion to maintain the long half-life of IgG (34) and to mediate placental transport via FeRn (35), removing the need for hazardous intravenous administration. The precise mechanism of platelet destruction is assumed to involve the high-affinity Fcγ
relied on isotypic antibodies generated in recombinant antibodies. Ni and colleagues developed a murine chemiluminescence (CL) in an interaction mediated by Fc anti–HPA-1a described above have been shown to trigger monocyte from this scFv was sufficiently specific for HPA-1a to permit its IgG1 CH2 domain involved in binding to Fc receptors (FcγRs) on effector cells. The therapeutic antibody Fc portion would therefore have to be modified to prevent binding to FcγRs, particularly the high-affinity FcγRI (CD64).

We previously generated a human single-chain variable domain antibody fragment (scFv) of nanomolar affinity ($K_d = 6 \times 10^{-8} M$) for HPA-1a from the maternal B cells of a case of FMAIT by phage display (36, 37). The recombinant IgG1 antibody (B2G1) derived from this scFv was sufficiently specific for HPA-1a to permit its use as a routine phenotyping reagent (38).

To generate a complete antibody lacking destructive activity, residues from IgG2 and IgG4 were substituted into regions of the IgG1 CH2 domain involved in binding to FcγRI–FcγRII and complement C1q. Residues of IgG1 responsible for the immunoglobulin Gm allotype were replaced by the non-immunogenic residues from IgG2 (Figure 1) (39). Platelets sensitized with the wild-type IgG1 anti–HPA-1a described above have been shown to trigger monocyte chemiluminescence (CL) in an interaction mediated by FcγRI and FcγRII (40). It is therefore logical to hypothesize that the modified recombinant HPA-1a antibody (B2G1Δnab) could block binding of maternal HPA-1a antibodies to platelets, reduce monocyte activation, and ultimately prevent platelet destruction.

The biallelic HPA-1 system only exists in humans, and there is no satisfactory animal model to investigate the possible use of these recombinant antibodies. Ni and colleagues developed a murine model to look at the efficacy of IVIG therapy, but their study relied on isotypic antibodies generated in $\beta^3$-deficient ($\beta^3{\sim}$ mice) against a $\beta^3{\sim}$ fetus (41). We therefore generated $\beta^3{\sim}$ mice transplanted with littermate bone marrow transduced with a lentivirus vector containing the human $\beta_3$ gene ITGB3 encoding either Leu33 (HPA-1a) or Pro33 (HPA-1b). Transplanted mice express a hybrid murine/human $\alpha_{IIb}\beta_3$ complex on the platelet surface as previously described (42) but crucially, this complex bears the corresponding HPA-1a or -1b antigen, making the mice suitable for use in studying the effects of natural and recombinant human HPA-1a antibodies on platelet survival in vivo.

This study demonstrates that the modified HPA-1a antibody B2G1Δnab inhibits binding of maternal HPA-1a antibodies from FMAIT cases to platelets and abrogates monocyte CL responses to anti–HPA-1a–coated platelets and that its F(ab')2 fragment prevents removal from murine circulation of HPA-1a–expressing platelets by destructive HPA-1a antibodies.

Results

Binding of IgG1 and mutated recombinant HPA-1a antibodies to HPA-1a1b platelets. We assessed the binding characteristics of the parent IgG1 (B2G1) and modified antibody (B2G1Δnab) to the HPA-1a antigen on the surface of HPA-1a1b platelets (matching the fetal phenotype). In flow cytometry and the monoclonal antibody immobilization of platelet antigen (MAIPA) assay, dilutions of B2G1 and B2G1Δnab demonstrated identical levels of binding to HPA-1a1b platelets, with saturation at 5 μg/ml (data not shown). There was no binding to homozygous HPA-1b1b platelets (data not shown).

Competition studies were carried out in the MAIPA using F(ab')2 B2G1 as a blocking antibody. Binding of 10 μg/ml B2G1 and B2G1Δnab to platelets was inhibited to the same extent by F(ab')2 B2G1, requiring 3, 8, and 50 μg/ml to achieve 50%, 70%, and maximum (98%) inhibition (data not shown).

Monocyte CL responses to IgG1 and mutated recombinant HPA-1a antibodies. We assessed the effect on monocyte activation of the mutations introduced in the constant region of the modified antibody B2G1Δnab. The monocyte CL response to HPA-1a1b platelets sensitized with the parent IgG1 B2G1 reached a maximum at 10 μg/ml (Figure 2A and B). The CL responses to B2G1Δnab-sensitized HPA-1a1b platelets were reduced to less than 15% of B2G1 values ($P < 0.001$) across the antibody concentration range (Figure 2A and B) but were above that observed with the control antibody VAZO-5 or F(ab')2 B2G1 (Figure 2A and B). No CL response was obtained with HPA-1b1b platelets (data not shown).

In competition studies, increasing concentrations of B2G1Δnab inhibited the CL response to 10 μg/ml B2G1 (Figure 2C), requiring 26, 49, and more than 500 μg/ml to achieve 50%, 70%, and maximum (85%) inhibition, respectively (Figure 2D).

Inhibition of platelet binding and CL responses to maternal polyclonal anti–HPA-1a by recombinant HPA-1a antibodies. Assays were performed to determine whether F(ab')2 B2G1 could inhibit binding of maternal polyclonal HPA-1a antibodies from 20 FMAIT cases and whether B2G1Δnab could reduce monocyte responses to the same sera. The anti–HPA-1a potencies of the maternal samples ranged from 0.01 to 193 IU/ml, and the monocyte CL signal induced by each sample ranged from 0.3 to 31.2 CL units (Table 1). Each patient was assigned a unique patient number (UPN).

For all maternal sera, we established inhibition curves in the MAIPA assay using F(ab')2 B2G1 as described above, and the concentrations were calculated for F(ab')2 B2G1, which was found to inhibit binding by 50% (50% inhibition of binding [ID50]), 70% (ID70), and by the maximum amount achievable.

For 18 sera, the ID50 values ranged from 4.8 to 616 μg/ml F(ab')2 B2G1 and ID70 values from 36 to 2,000 μg/ml F(ab')2 B2G1 (Figure 3A). The maximum achievable inhibition of binding ranged from

**Figure 1**

Model of human IgG1 indicating the positions of the mutated residues in the modified nondestructive G1Δnab constant region. The immunoglobulin IgG heavy chains are shown in light blue and the immunoglobulin light chains and Fc-associated carbohydrate in dark gray. The red residues were altered by the Δn mutation in order to replace the IgG1 G1m(1,17) allotypic residues Lys214, Asp356, and Leu358 with the corresponding IgG2 residues Thr, Glu, and Met. The blue amino acids are IgG1 residues Ala327, Ala330, and Pro331 changed to the IgG4 residues Gly, Ser, and Ser by the Δn mutation. The green residues of IgG1 (Glu233, Leu234, Leu235, and Gly236) were substituted with the corresponding amino acids of IgG2 (Pro, Val, Ala, and a deleted residue) by the Δγ mutation. The immunochemistry of the modified nondestructive G1Δnab constant region. The immunochemistry of the modified nondestructive G1Δnab constant region. The immunochemistry of the modified nondestructive G1Δnab constant region.
70% to 97% (Figure 3A), with a F(ab′)2 B2G1 concentration ranging from 20 to 4,000 μg/ml (Figure 3A). In one case the maternal HPA-1a antibody was too weak to establish an inhibition curve, and in another the F(ab′)2 B2G1 increased the OD by 3-fold. This particular serum was found by ELISA to contain anti-F(ab′)2 antibodies (data not shown). In a further experiment, F(ab′)2 B2G1 was added 20 minutes after pre-sensitization of the platelets with each of 5 maternal sera, at a concentration previously shown to inhibit binding by 50%. The degree of inhibition was comparable with that obtained when both were added concomitantly (Figure 4). This result proves the ability of the recombinant antibodies to displace maternal polyclonal antibodies already bound to platelets. There was, however, no correlation between maternal antibody potency and concentrations of mutant antibody required for inhibition of binding (Table 1).

Inhibition curves were generated in the CL assay for all clinical samples using increasing concentrations of B2G1Δnab mixed with a fixed amount of polyclonal serum. The concentrations of B2G1Δnab that inhibited the CL response to each polyclonal serum by 50%, 70%, and the maximum amount achievable were calculated. In 17 cases, the ID50 values ranged from 6 to 560 μg/ml B2G1Δnab and the ID70 values from 10 to 1,940 μg/ml B2G1Δnab (Figure 3B). The maximum inhibition achieved in the CL assay ranged from 72% to 95% for the 15 pure anti–HPA-1a sera (Figure 3B, right hand-side panel), requiring 6–3,200 μg/ml B2G1Δnab (Figure 3B). For 2 sera known to contain a mixture of HPA-1a and -5b antibodies, maximum inhibition was 55% and 65%, respectively (the platelets used in the assay were from a HPA-5a5b heterozygous donor). In 3 cases the CL signal was too weak to establish an inhibition curve. There was no correlation between concentrations of inhibitory antibody required in the CL studies and maternal antibody potency, nor between concentrations required for the same degree of inhibition in MAIPA and CL.

A murine model to study inhibition of HPA-1a platelet destruction by blocking antibodies in vivo. Bone marrow from β3–/– mice was transduced with a viral construct containing the human ITGB3 cDNA encoding either leucine or proline at position 33 (HPA-1a or -1b, respectively), and the transduced bone marrow was transplanted into lethally irradiated β3–/– mice. Upon bone marrow recovery, the transplanted mice had a chimeric platelet population, containing platelets generated from the original β3–/– megakaryocytes and platelets expressing the human β3 (Huβ3 Pt) and the corresponding HPA-1a or -1b epitope (Figure 5). Huβ3 Pt chimerism levels (the fraction of Huβ3 Pt present in circulation expressed as a percentage of the total platelet population) varied from 10% to 60% in the HPA-1a mice (n = 10) and from 54% to 81% in the HPA-1b mice (n = 4). Specific binding of FITC-B2G1 confirmed the expression of the HPA-1a antigen on platelets circulating within HPA-1a mice. HPA-1a was not detected on the platelets of HPA-1b or β3–/– mice (Figure 5).
We first assessed the in vivo destructive effect of the recombinant IgG1 HPA-1a antibody B2G1 on the Huβ3 Pt by i.p. injection. To be able to compare the different animals entered into the experiment, the remaining fraction of Huβ3 Pt present in circulation after i.p. injection was expressed as a percentage of the baseline Huβ3 Pt chimerism for each mouse. We assumed complete absorption from the i.p. injection into a 2-ml circulatory blood volume. i.p. injection of 25 μg B2G1 (i.e., ~12.5 μg/ml in circulation) in 3 HPA-1a mice (baseline Huβ3 Pt chimerisms of 49%, 21%, and 12%) resulted in a drop in Huβ3 Pt levels of 18%–67% at 1 hour, reaching maximum reduction of 67%–93% at 4 hours (Figure 6A). In the 2 highest expressors, this was reflected by a decrease in platelet count proportional to their baseline Huβ3 Pt chimerism (Figure 6B). There was no effect of B2G1 on Huβ3 Pt or platelet count in HPA-1b (Figure 6, A and B) or in β3–/– mice (Figure 6B). Huβ3 Pt levels returned to baseline in all HPA-1a mice 7–10 days after the experiment.

Since the mouse FcyR profile is different from that in humans (43), using the B2G1Δnab construct whose constant region has been tailored to abrogate binding to human FcyR would be uninformative. Therefore, we opted to use a F(ab′)2 construct of B2G1 that lacks a constant region altogether and therefore does not bind to either human or mouse FcyR as a proof of principle protective antibody. To assess the ability of a F(ab′)2 fragment of B2G1 to inhibit B2G1-induced platelet clearance, competition studies were performed in 3 HPA-1a mice (Huβ3 Pt chimerism of 53%, 21%, and 10%). Platelet destruction studies were first performed as described above using 25 μg B2G1 i.p. injection, and the clearance of Huβ3 Pt was confirmed (Figure 6C, solid lines). After the peripheral platelet counts returned to normal, the mice were entered into the competition study. Given the shorter half-life of a F(ab′)2 fragment compared with a full-length antibody in vivo, the mice had a loading dose of 150 μg F(ab′)2 B2G1 i.p. 1 hour prior to injecting 25 μg B2G1 i.p., followed by 150 μg F(ab′)2 B2G1 i.p. at 0, 1, and 3 hours (Figure 6C). In all 3 mice, the proportion of Huβ3 Pt remaining in circulation after injection of B2G1 in the presence of F(ab′)2 B2G1 was close to baseline values, supporting its protective effect (Figure 6C, dotted lines).

To carry out equivalent in vivo studies using polyclonal FMAIT sera, we generated 2 polyclonal IgG preparations (CAM00D and CAM00E) from 2 FMAIT cases. CAM00D and CAM00E anti-HPA-1a potency was measured by MAIPA as 2,500 and 70 IU/ml, respectively (high concentrations were deliberately generated to take into account the dilution in the mice’s circulatory volumes). Competition studies of binding were carried out using MAIPA with F(ab′)2 B2G1 as described above, using dilutions of 1/200 (12.5 IU/ml) and 1/40 (1.75 IU/ml) for CAM00D and CAM00E, respectively. Results corrected for the dilution factor are shown in Figure 7A. ID50 and ID70 were reached at 5 and 20 mg F(ab′)2 B2G1, respectively, for CAM00D and at 0.4 and 2.4 mg F(ab′)2 B2G1, respectively, for CAM00E. Maximum inhibition was 82% for CAM00D with 200 mg F(ab′)2 B2G1 and 83% for CAM00E with 20 mg F(ab′)2 B2G1. These high values reflect the high concentration of HPA-1a antibodies in these 2 preparations.

Two mice (Huβ3 Pt chimerism of 49% and 60%, respectively) were injected i.p. with 100 μl CAM00D (i.e., ~125 IU/ml in circulation) and 1 mouse (Huβ3 Pt chimerism of 34%) with 10 μl (i.e., ~12.5 IU/ml in circulation). In all 3 cases, significant platelet destruction was observed, with Huβ3 Pt falling to 9%–17% of

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**Table 1**

Clinical details, HPA-1a antibody potency, CL signal, and inhibition data

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^A Potency too weak for competition studies. ^B CL signal too weak for competition studies. ^C Binding to HPA-1a/β3 platelets increased in the presence of F(ab′)2 B2G1 by 3-fold. ^DNU. ^E Sera containing both HPA-1a and HPA-5b antibodies. ^F Non-parallel lines with the proposed anti-HPA-1a standard due to the presence of anti-F(ab′)2 antibodies in the patient’s serum. Conc., concentration; ND, not determined.
baseline after 4 hours (Figure 7B). No effect was seen in HPA-1b or β3−/− mice (data not shown). Competition studies were carried out in 3 mice with Huβ3 Pt chimerism equivalent to that of the mice used in the destructive studies (Huβ3 Pt chimerism of 67%, 83%, and 22%, respectively). The first 2 received 100 μl CAM00D and F(ab′)2 B2G1 boluses of 260, 130, 130, and 130 μg at –1, 0, 1, and 3 hours, and the last mouse received 10 μl CAM00D and F(ab′)2 B2G1 boluses of 50, 25, 12.5, and 12.5 μg at the same times. In all 3 cases, platelet destruction was prevented by the competing antibody (Figure 7B).

Two mice (Huβ3 Pt chimerism of 26% and 21%) were injected i.p. with 200 μl CAM00E (~7 IU/ml in circulation). In both mice, platelet destruction was observed, with Huβ3 Pt falling to 45%–55% of baseline after 4 hours (Figure 7C). No effect was seen in HPA-1b or β3−/− mice (data not shown). Competition studies were carried out in the same mice with F(ab′)2 B2G1 boluses of 100, 50, 25, and 25 μg given at –1, 0, 1, and 3 hours. In both mice, platelet destruction was completely prevented by the competing antibody (Figure 7C).

Discussion

The studies reported here confirm that it is possible to inhibit the binding of human polyclonal HPA-1a antibodies to HPA-1a1b platelets with a single human recombinant HPA-1a antibody (B2G1), and that the addition of a modified constant region (B2G1Δnab) abrogates monocyte CL responses to anti–HPA-1a sensitized platelets. Furthermore, we show in a unique murine model that HPA-1a antibody-induced platelet clearance is prevented in vivo by F(ab′)2 B2G1.

In competition studies, 70%–95% inhibition of anti–HPA-1a binding to platelets was seen with all but one clinical sera tested. Anti-F(ab′)2 antibodies were later found in this sample (data not shown), which may explain this discrepant result. The concentration of F(ab′)2 necessary to inhibit platelet binding was variable but showed no correlation with maternal anti–HPA-1a potency. This may be because the potency of each polyclonal serum depends on both the concentration and affinity of each of its constituent antibody clones with variation in on/off rates for antigen binding. There were sera for which complete inhibition of binding could be achieved at low concentrations of inhibitor antibody (800 μg/ml) (UPNs 3 and 6), and in contrast, there were also sera that showed more than 20% residual binding (UPNs 4, 10, 12, 13, and 14), even with 4,000 μg/ml of F(ab′)2 B2G1 (Table 1). This cannot be explained by major differences in affinity between B2G1 (Kd = 6 × 10⁻⁸ M) (37) and the polyclonal sera, as there is good evidence that even high-affinity polyclonal antibodies have at most a Kd of 0.3 × 10⁻⁹ to 1 × 10⁻⁹ M (44). HPA-1a polyclonal sera have different footprints on
the β3 integrin (45, 46): type I antibodies, which bind to the plexin semaphorin integrin (PSI) domain (e.g., the first 54 residues of β3), and type II antibodies, which require residues outside the PSI domain. The competition results may therefore also reflect the differences between the antibody footprints covered by the maternal polyclonal antibodies and that covered by our monoclonal antibody (which belongs to the type II category).

Monocyte CL responses to platelets sensitized with the modified HPA-1a antibody were reduced by 85% when compared with the IgG1 parent antibody. These results are in keeping with the previous observation that the Δnab mutations reduced the binding of a human RhD IgG1Δnab antibody to FcγRI by 104-fold (39) and to the stimulatory receptor FcγRIIa by 10-fold (47). The mutated RhD antibodies, when coated onto RhD-positive red cells, also failed to stimulate monocyte CL responses, also failed to stimulate monocyte CL responses (39), a parameter previously shown to correlate with the severity of hemolytic disease of the newborn (48). However, in contrast to the complete absence of monocyte CL responses observed in the red cell studies, B2G1Δnab elicited a residual response in excess of that seen with a F(ab′)2 anti–HPA-1a antibody that lacks a constant region altogether. This difference may be an in vitro artefact explained by P-selectin–mediated direct adhesion of platelets to monocytes, which enhances both the rate and, at low antibody concentrations, the magnitude of the monocyte CL response to anti–HPA-1a-sensitized platelets (40).

In keeping with the effect on platelet binding, we have shown that monocyte CL responses induced by heterozygous platelets sensitized with maternal HPA-1a antibodies could be inhibited by more than 70% in all cases in which anti–HPA-1a was present alone. For the 2 sera that also contained anti–HPA-5b, maximum inhibition was notably less (55% and 65%), as the platelets used in the experiment were from a heterozygous HPA-5a5b donor. Since the epitopes defining the HPA-5 antigens are on platelet glycoprotein 1a (α2 integrin), these findings are consistent with the inhibitory effect of the B2 mutated monoclonal antibodies being blockade of the interaction between maternal anti–HPA-1a and its epitope.

The murine studies confirmed the effectiveness of using a blocking HPA-1a antibody with reduced binding to FcγR to prevent platelet destruction. The B2G1Δnab construct in mice competition studies would have been uninformative because human IgG2 and IgG4 antibodies from which the mutations were derived would interact efficiently with murine FcγR (43, 49). Instead, as proof of principle, we opted to use a F(ab′)2 construct that lacks an Fc domain altogether and therefore does not bind to either human or mouse FcRs. Having demonstrated that the recombinant antibody B2G1 and 2 anti–HPA-1a polyclonal IgG preparations caused clearance only of platelets that expressed the HPA-1a alloantigen, we were able to show that administration of the protective competing antibody F(ab′)2 B2G1 prevented platelet destruction in all 8 mice studied. Interestingly, the dose of F(ab′)2

**Figure 4**

Competition studies with 5 clinical sera containing HPA-1a antibodies with concomitant and postponed addition of F(ab′)2 B2G1. To assess the ability of the recombinant antibodies to displace polyclonal antibodies already bound to platelets, F(ab′)2 B2G1 was added 20 minutes after pre-sensitization of the platelets with each of 5 maternal sera, at a concentration previously shown to inhibit binding by 50% and inhibition compared with that obtained when both were added concomitantly.

**Figure 5**

Platelet chimerism in β3−/− transplanted mice and specific expression of the HPA-1a epitope. Lethally irradiated β3−/− mice were transplanted with bone marrow from β3−/− mice transduced with a viral construct containing the human ITGB3 cDNA encoding either leucine or proline at position 33 (HPA-1a or -1b, respectively). Upon bone marrow recovery, the transplanted mice had a chimeric platelet population, containing platelets generated from the original β3−/− megakaryocytes and HuJ3Pt as shown in the top panel, where platelets were stained with the PE-labeled human β3–specific antibody VI-PL2. The bottom panel shows specific binding of the FITC-labeled HPA-1a recombinant antibody B2G1 to platelets in mice transplanted with bone marrow transduced with the Leu33 human β3.
B2G1 necessary to achieve this correlated with the polyclonal anti–HPA-1a potencies and was consistent with the results obtained in the in vitro competition studies.

These results would support studies in human subjects to address safety, efficacy, and dose. In terms of safety, we have already shown that the B2 series of antibodies have no significant adverse effects on platelet activation and function (50). HPA-1a antibodies also bind to endothelial cells αvβ3, but there is published evidence that B2G1 does not alter endothelial cell growth and activation status (51). Moreover, we have shown that ligand binding to αvβ3 is unaffected in the presence of B2G1 (C. Ghevaert, unpublished observations). Finally, we have minimized the potential immunogenicity by the use of a fully human antibody and removal of the Gm alloantic residues.

With regard to efficacy, we have previously demonstrated improved intravascular survival in RhD-positive healthy volunteers of autologous red cells sensitized with an RhD antibody containing the same Δnab constant region compared with red cells sensitized with the parent IgG1 RhD antibody (52), showing that the encouraging results demonstrated in vitro in the CL assay translate to an in vivo protective effect in humans. Whether these results also apply to platelets sensitized with the modified anti–HPA-1a will be assessed in a forthcoming human volunteer study.

With regard to dosing, there is good evidence that in cases affected with anti–HPA-1a antibodies, ICH tends to occur only in fetuses with a platelet concentration of less than 20 × 10^9/l (18). Therefore, rather than restoring a normal platelet count, our aim with antenatal therapy should be to bring the fetal count into the absolutely safe zone of more than 50 × 10^9/l. In hemolytic disease of the newborn, clinically significant hemolysis is associated with CL responses of more than 30% of the maximum response obtained with a control antibody (48). With HPA-1a antibodies, there appears to be no correlation between either in vitro potency or monocyte CL responses to maternal anti–HPA-1a and fetal platelet count (53). Therefore, at this stage it would be difficult to estimate by how much maternal antibodies would have to be blocked to generate a clinically significant effect. This particular question will have to be answered in clinical trials, where fetal blood sampling will be necessary to assess dose response. It is, however, possible to calculate the concentration of therapeutic antibody required to achieve a given degree of inhibition for each patient from the results of an in vitro competition assay, as demonstrated in the murine study. Whether the 15% intrinsic activity of B2G1Δnab will limit clinical efficacy is also unknown.

It is conceivable that the recombinant antibody could initially be assessed in patients undergoing therapy with serial IUTs, in whom the antibody could be administered straight into the fetal circulation, along with a platelet transfusion. The effect on the fetal platelet count and the requirement for further IUTs could then be assessed by serial fetal blood sampling in a similar fashion to current ongoing IVIG trials (26). The long-term aim, however, is to remove the risk of cordocentesis altogether by administering the antibody to the mother. We have already demonstrated that our Fc modifications do not abrogate placental transport (54) and binding to the placental transport receptor FcRn is unaffected.

Our in vitro data give some clues as to what concentration and total dose of antibody might be required for maternal administration. Assuming an adult plasma volume of 3 l and equilibrium distribution of 53%, 21%, and 10%) after i.p. injection of 25 μg B2G1 i.p. (C) The proportion of H uj3 Pt in 3 HPA-1a mice (baseline H uj3 Pt chimerism of 53%, 21%, and 10%) after i.p. injection of 25 μg B2G1 alone (solid lines) and after 25 μg B2G1 plus i.p. boluses of 150 μg F(ab')2 B2G1 (dotted lines) at times shown.

**Figure 6**
Platelet destruction studies with HPA-1a recombinant antibodies in the mice. β3−/− mice were transplanted with murine bone marrow transduced with the human β3 integrin encoding with either Leu33 (HPA-1a) or Pro33 (HPA-1b). (A) The proportion of H uj3 Pt in 3 HPA-1a mice (solid lines) and 2 HPA-1b mice (dotted lines) after i.p. injection of 25 μg B2G1. H uj3 Pt chimerism for each mouse at time 0 (49%, 21%, and 12%) corresponds to 100% H uj3 Pt on the graph. (B) Platelet counts in the 3 HPA-1a (solid lines), 2 HPA-1b (dotted lines), and 3 β3−/− (gray lines) mice after 25 μg B2G1 i.p. (C) The proportion of H uj3 Pt in 3 HPA-1a mice (baseline H uj3 Pt chimerism of 53%, 21%, and 10%) after i.p. injection of 25 μg B2G1 alone (solid lines) and after 25 μg B2G1 plus i.p. boluses of 150 μg F(ab')2 B2G1 (dotted lines) at times shown.
results of pharmacokinetic studies, multiple boluses of antibody may have to be administered repeatedly to protect from ICH from 16 weeks gestation until delivery. In conclusion, this study has shown for what we believe to be the first time that it is possible to prevent binding of polyclonal sera to the HPA-1a epitope using a single recombinant high-affinity human antibody. We have shown that the modifications introduced in the constant region aimed at reducing binding to FcγRs substantially decrease monocyte response to platelets sensitized with the modified antibody and, furthermore, that monocyte responses to polyclonal HPA-1a antibodies can be abrogated by the nondestructive antibody. Finally, using a unique murine model, we have shown in proof of principle studies that in vivo platelet destruction by polyclonal anti-HPA-1a can be prevented by a blocking antibody lacking a destructive constant region. Although the program of clinical evaluations will require extensive discussion with obstetricians and regulators, these results would support progression to human studies.

Methods

Production of recombinant HPA-1a antibodies. The generation of B2G1, a human IgG1A version of an anti-HPA-1a single-chain Fv, has been previously described (38), as has the vector containing the modified IgG1 gene, pSVgptFog1VHHuIgG1 (39). Briefly, IgG2 residues from positions 233–236 (Ab) were substituted together with IgG4 residues 327, 330, and 331 (Δa) into IgG1 to generate G1ab. The null allotype (An) mutations (Lys214 to Thr, Asp356 to Glu, and Leu358 to Met) were introduced in this template by sequential overlap extension PCR, using Pwo DNA polymerase (Boehringer Mannheim) to generate the IgG1Anab constant region gene which was cloned as a BamHI-HindIII fragment into pSVgptB2VHHuG1 (38) to yield the vector pSVgptB2VHHuG1Anab. Thus the 2 full-length HPA-1a antibodies generated for this study had the same B2 variable region and were designated “B2G1” and “B2G1Anab.”

For each antibody, the heavy-chain vector was cotransfected with the B2 λ-chain expression vector (38) into a rat myeloma cell line, YB2/0, and stable transfectants secreting the highest levels of antibody were isolated as previously described (39). Cell culture supernatant containing monoclonal antibody IgG was passed through a 0.22-μm filter and IgG purified using a 5-ml Protein G Sepharose 4 Fast Flow column (Pharmacia). Protein G-bound material was eluted by using 0.1 M glycine, pH 2.7, then mixed with 12 μl/ml 5 M NaCl and 30 μl/ml 1 M Tris buffer, pH 9, to ensure isotonicity at pH 7.0. Purity of IgG was confirmed using a 3%–5% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis under nonreducing conditions as previously described (56).

Patients’ sera. Twenty maternal sera taken at delivery from FMAIT cases and shown by the MAIPA assay to contain HPA-1a antibodies were retrieved from the serum bank at the Cambridge Blood Centre, meeting national requirements for patient consent. The sera were categorized by neonatal outcome (Table 1): (a) Anti–HPA-1a alone and ICH (n = 7); (b) anti–HPA-1a alone and neonatal platelet count of less than 50 × 10^9/l without ICH (n = 7); (c) anti–HPA-1a alone and platelet count of greater than 100 × 10^9/l at birth with a previously affected pregnancy and a homozygous partner (n = 4); and (d) anti–HPA-1a and anti–HPA-5b with platelet counts of 5 × 10^9/l and 19 × 10^9/l, respectively (n = 2).

Serum samples from 2 FMAIT cases with anti–HPA-1a and neonatal platelet counts of 2 × 10^9/l and 13 × 10^9/l were used to generate 2 polyclonal IgG preparations (CAM00D and CAM00E) for the mouse studies. The HPA-1a antibodies in the maternal serum samples were isolated by alloabsorption onto platelets from an apheresis HPA-1a homozygote donation (NHS Blood and Transplant) washed in PBS containing 0.2% BSA (Sigma-Aldrich) 5 mM EDTA (PBS/BSA/EDTA). Following elution with 76 mM citric acid, 93 mM NaCl, 7 mM Na3HPO4, 7 mM NaH2PO4, pH 2.8, and
neutralization with 214 mM TRIS, 22 mM Na₂HPO₄, each sample was dialyzed in PBS using a 12- to 14-kDa membrane (Medicell International Ltd.). The IgG fraction was purified using a protein G column as described above, concentrated by centrifugation in 10-kDa Vivaspin tubes (Sartorius) and passed through a 0.2-μm filter.

Platelet immunofluorescence assay. Cryopreserved HPA-1a1a and -1b1b genotyped platelets (57) were thawed and resuspended in PBS/BSA/EDTA at a concentration of 50 × 10⁶/L. Platelet suspension (50 μl) was sensitized with each recombinant antibody, washed, and incubated with a FITC-labeled rabbit anti-human Ig antibody (Dako). Data were analyzed using a Beckman Coulter XL-MCL flow cytometer.

MAIPA assay. The MAIPA assay was performed as described before (53) using cryopreserved HPA-1a1b and HPA-1b1b platelets, the murine capture antibody NBS-PAB-1 (International Blood Group Reference Laboratory), and microtiter plates coated with a goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Bound human IgG was detected with a HRP-conjugated goat anti-human Fc antibody (Jackson ImmunoResearch Laboratories). Each assay was performed in duplicate, and an average of the absorbance at 490 nm (OD) was read on an MRX plate reader (Dynex Technologies).

The potency of each clinical sample and both polyclonal IgG preparations was measured using the international anti–HPA-1a potency standard 03/152 (100 IU/ml) (National Institute for Biological Standards and Control) (53, 58).

In the MAIPA assay, the F(ab′)2 fragment of B2G1 (IBGRL) did not react with the detecting HRP-conjugated goat anti-human Fcy (data not shown) and therefore could be used as a blocking antibody in competition studies in which increasing concentrations of F(ab′)2; B2G1 were added to a fixed concentration of either the full-length recombinant HPA-1a1b antibodies B2G1 and B2G1Δnab or polyclonal anti–HPA-1a1b pre-diluted so as to be on the linear part of the standard curve. The decrease in OD seen in these competition studies was presumed to be proportional to the reduction of the amount of full-length antibodies bound to the platelets. F(ab′)2; B2G1 in increasing concentrations and full-length anti–HPA-1a1b antibodies or maternal sera were added to the platelets concomitantly and the MAIPA assay performed as above. The concentrations of F(ab′)2; B2G1 that inhibited binding by 50%, 70%, and the maximum amount achievable were calculated. The values obtained were then multiplied by the dilution factor to be able to compare each maternal serum undiluted.

Monocyte CL. The monocyte CL assay has been described before (53). Briefly, monocytes were prepared from pooled whole blood samples from 6 random donors (40), resuspended in HBSS, 20% RPMI (Sigma-Aldrich), 2% FCS (HBSS/RPMI/FCS) (3 × 10⁹/ml), and left to incubate in white flat-bottomed 96-well plates (Optitiate-TM 96; PerkinElmer) for 2 hours at 37°C in a humidified atmosphere of 5% CO₂. Cryopreserved HPA-1a1b platelets were thawed and sensitized with the recombinant antibodies or the clinical samples. After washing the excess antibody, 50 μl of sensitized platelets (200 × 10⁶/l) and 50 μl of pre-warmed 4-mM luminol (37°C) were added to each well and CL was recorded at 37°C using a PolarstarGalaxy (BMG), taking 1-s measurements every 2.35 min for a total of 20 cycles (47 min). Platelets incubated with a human IgG1 anti-varicella zoster (VAZO-5; IBGRL) were used as a negative control. The CL signal for each patient was calculated as previously described as the total CL response (area under the curve) obtained over the 20 cycles expressed as a percentage of the response obtained with a positive control (B2G1 10 μg/ml).

For the competition studies, platelets were sensitized with patients’ sera or B2G1 10 μg/ml and increasing concentrations of B2G1Δnab and the CL assay performed as described above.

Mouse studies. Animal studies complied with institutional guidelines and were approved by the Animal Care and Use Committee of the Medical College of Wisconsin’s American Association for the Accreditation of Laboratory Animal Care-approved Biomedical Resource Center. The expression of the complex murine eIlb/human β3 in B3–/– mice has been described previously (42). Expression of human β3 by murine platelets was measured by flow cytometry in a whole blood assay using the PE-labeled human β3–specific antibody VI-PL2 (BD Biosciences) and the HPA phenotype assessed using FITC-labeled B2G1 (IBGRL). Mice were anesthetized with an inhalation anesthetic, and blood (25 μl) was collected by tail-vein bleed into a microtube containing 1.0 ml of Tyrode’s buffer with 0.13 M sodium citrate anticoagulant and 1 μg of prostaglandin E1 (Sigma-Aldrich), similar to a previously described protocol (42). Samples were then incubated for 30 min with PE- and/or FITC-conjugated antibodies, diluted with 750 μl of buffer, and analyzed on a FACScan flow cytometer (Becton Dickinson). The data were analyzed with Win MDI software. A minimum of 2 × 10⁶ events were collected from entities exhibiting forward- and side-scattering properties of murine blood platelets. A marker was set on the platelets from β3–/– mice, and the percentage of platelets giving a positive signal above this marker was considered to be the percentage of Huβ3 Pt.

To assess the effect of recombinant anti–HPA-1a or polyclonal sera on platelet survival, mice were injected i.p. with the given dose of antibodies and tail bleeds were performed at the times indicated in the Figure 3 legend. Circulating platelet levels were measured from whole blood with an Animal Blood Counter (Oxford Science), and flow cytometry was used to calculate the proportion of Huβ3 Pt present in circulation. Given the difference between mouse and human FcyR profile (43), F(ab′)2; B2G1 (which lacks a constant region altogether and therefore does not bind to either human or mouse FcyR), rather than B2G1Δnab (whose constant region has been tailored to abrogate binding to human FcyR), was used as a proof of principle protective antibody in competition studies. The F(ab′)2; was injected i.p. using different dosing schedules as described above.

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