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Research Article

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.

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

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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 20×10^9 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 20×10^9/l), IVIG therapy does not achieve a safe fetal
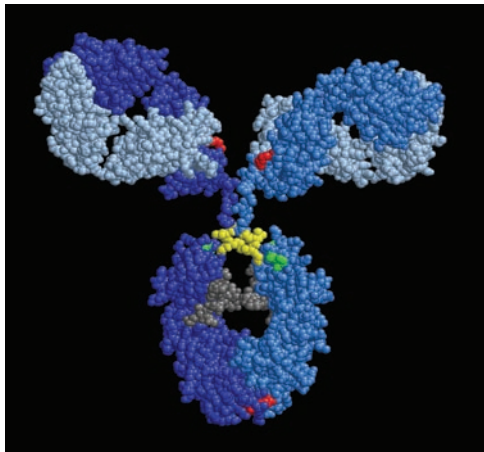
platelet count (19–23). Although IVIG therapy may reduce the incidence of ICH in this high-risk group without a rise in platelet count (24), ICHs still occur in some cases (21, 25, 26). The most common side effects of IVIG therapy, such as headaches, myalgia, and allergic reactions, can be easily treated (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 FcRn (35), removing the need for hazardous intrauterine administration. The precise mechanism of platelet destruction is assumed to involve the high-affinity Fcγ

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 Δa 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 Δb mutation. The image was generated from the PDB file of an IgG1 model (59) using RasMol V2.7.3.

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 × 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–FcγRIII and complement C1q. Residues of IgG1 responsible for the immunogenic 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 β3-deficient (β3$^{-/-}$ mice) against a β3$^{+/-}$ fetus (41). We therefore generated β3$^{-/-}$ mice transplanted with littermate bone marrow transduced with a lentivirus vector containing the human β3 gene *ITGB3* encoding either

Leu33 (HPA-1a) or Pro33 (HPA-1b). Transplanted mice express a hybrid murine/human αIIbβ3 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 2, A 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 2, A and B) but were above that observed with the control antibody VAZO-5 or F(ab′)$_2$ B2G1 (Figure 2, A 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 sample 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
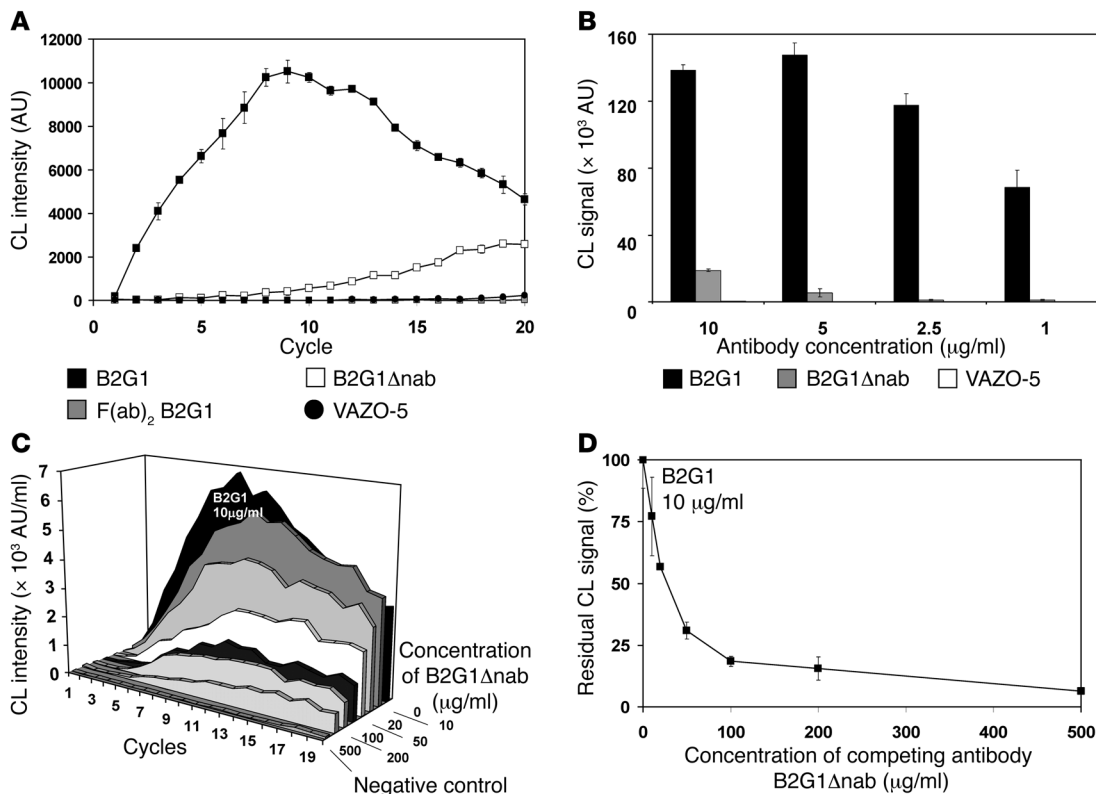
Figure 2
Monocyte CL response to recombinant HPA-1a antibodies. (**A**) CL response to HPA-1a1b platelets sensitized with 10 μg/ml of B2G1, B2G1Δnab, F(ab')₂ B2G1, and a negative control antibody VAZO-5. CL intensity was recorded over 20 cycles of 2.35 minutes each. (**B**) The CL signal corresponding to the sum of the CL intensity for the 20 cycles (surface under the curve) recorded with decreasing concentrations of B2G1, B2G1Δnab, and VAZO-5. (**C**) Competition study in the CL assay. HPA-1a1b platelets were sensitized with 10 μg/ml B2G1 and decreasing concentrations of B2G1Δnab. (**D**) Competition curve generated from the results in **C**. The CL signal obtained with 10 μg/ml B2G1 in the absence of the competing antibody B2G1Δnab corresponds to 100% residual CL signal on the graph. Data are the mean ± SD of 3 separate experiments.

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 (Fig-

ure 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).

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

UPN	Platelet count at birth (×10⁹/l)	ICH	Potency (AU/ml)	CL signal (AU/ml)	Maximum inhibition in MAIPA, % (dilution factor)	Conc. of F(ab')₂ B2G1 at maximum MAIPA inhibition (μg/ml)	Maximum inhibition in CL (%)	Conc. of B2G1Δnab at maximum CL inhibition (μg/ml)
1	262	No	<0.5	2	NDᴬ	NDᴬ	NDᴮ	NDᴮ
2	2	Yes	1.7	5	NDᶜ	NDᶜ	72	6
3	3	Yes	1.7	16	97 (1/2)	800	91	400
4	12	Yes	7.1	18	72 (1/4)	36	88	400
5	19	No	7.5	17	80 (1/4)	800	87	3,200
6	5	No	13	24	95 (1/8)	800	91	80
7	211ᴰ	No	14	11	80 (1/8)	800	82	3,200
8ᴱ	19	No	15	19	86 (1/8)	4,000	55	400
9ᴱ	5	No	23	10	89 (1/8)	4,000	65	40
10	101	No	31	0.3	70 (1/8)	2,000	NDᴮ	NDᴮ
11	84	No	31	22	82 (1/8)	800	87	3,200
12	6	Yes	33	24	78 (1/8)	4,000	87	3,200
13	7	No	33	19	70 (1/8)	2,000	75	3,200
14	8	Yes	98	30	77 (1/8)	2,000	80	3,200
15	2	No	99	26	87 (1/8)	2,000	93	3,200
16	17	Yes	100	23	95 (1/8)	4,000	87	3,200
17	8	No	193	32	86 (1/8)	200	92	3,200
18	32	No	NDᶠ	28	85 (1/8)	2,000	94	3,200
19	234	No	NDᶠ	4	82 (1/4)	800	NDᴮ	NDᴮ
20	10	Yes	NDᶠ	6	97 (1/4)	2,000	80	80

ᴬPotency too weak for competition studies. ᴮCL signal too weak for competition studies. ᶜBinding to HPA-1a1b platelets increased in the presence of F(ab')₂ B2G1 by 3-fold. ᴰIUTs. ᴱSera containing both HPA-1a and HPA-5b antibodies. ᶠNon-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.

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. In calculating the amount of antibody necessary to achieve thrombocytopenia, 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 FcγR profile is different from that in humans (43), using the B2G1Δnab construct whose constant region has been tailored to abrogate binding to human FcγR 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 FcγR 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
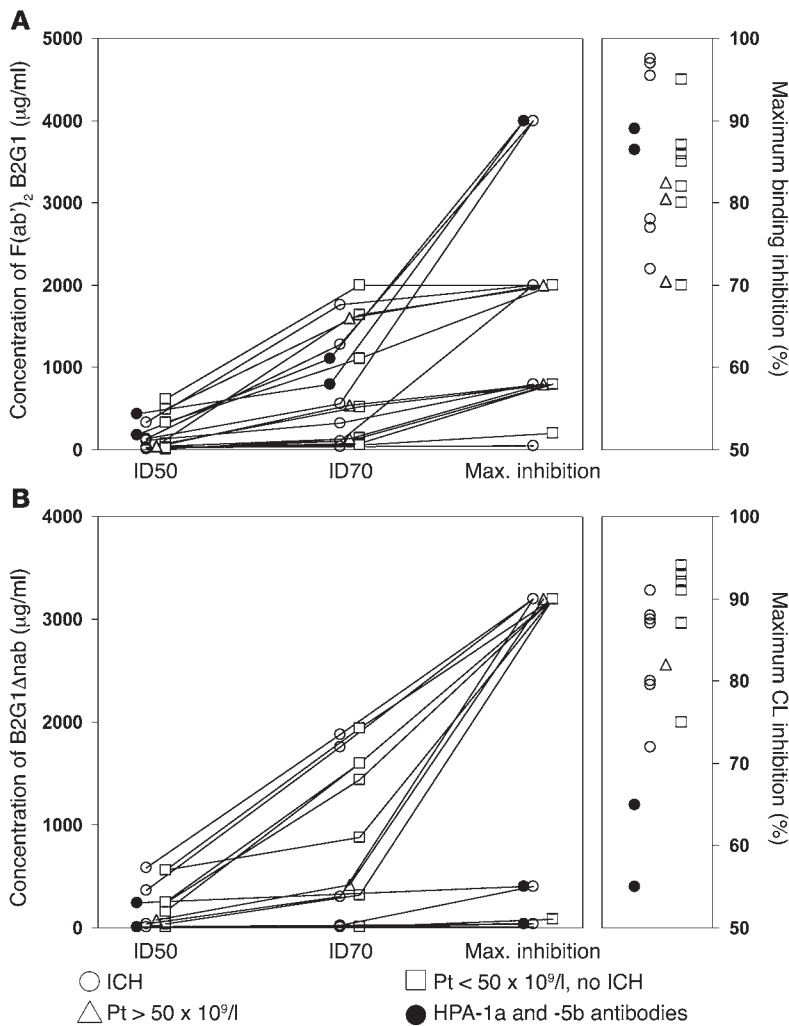
Figure 3
Competition studies with 20 clinical sera containing HPA-1a antibodies. (**A**) Concentrations of F(ab')$_2$ B2G1 (μg/ml) necessary to inhibit binding in the MAIPA assay by 50% and 70% and to achieve maximum inhibition for each clinical sample. The maximum inhibition achieved (%) is shown in the right panel. In UPN 1, the maternal HPA-1a antibody was too weak to establish an inhibition curve, and in UPN 2 the F(ab')$_2$ B2G1 increased platelet binding of maternal HPA-1a antibodies by 3-fold. This particular serum was found by ELISA to contain anti-F(ab')$_2$ antibodies. (**B**) Concentrations of B2G1Δnab (μg/ml) necessary to inhibit CL response by 50% and 70% and to achieve maximum inhibition for each clinical sample. The maximum inhibition achieved (%) is shown in the right panel. In 3 cases (UPNs 1, 10, and 19), the CL signal was too weak to establish an inhibition curve. For the 2 samples containing HPA-5b as well as -1a antibodies (UPNs 8 and 9), maximum inhibition failed to reach 70%.

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).
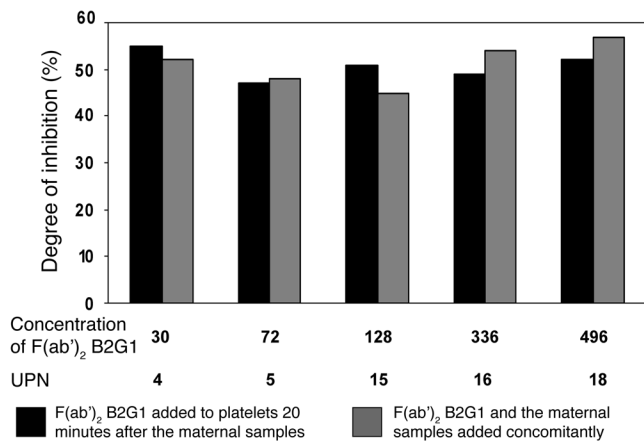
Two mice (Huβ3 Pt chimerism of 26% and 21%) were injected i.p. with 200 μl CAM00E (~7 IU/ml in circulation). In both mice, platelet destruction was observed, with Huβ3 Pt falling to 45%–55% of baseline after 4 hours (Figure 7C). No effect was seen in HPA-1b or β3$^{-/-}$ mice (data not shown). Competition 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')$_2$ B2G1 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 ($K_d = 6 \times 10^{-8}$ M) (37) and the polyclonal sera, as there is good evidence that even high-affinity polyclonal antibodies have at most a K_d of 0.3×10^{-9} to 1×10^{-9} M (44). HPA-1a polyclonal sera have different footprints on

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.

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 (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
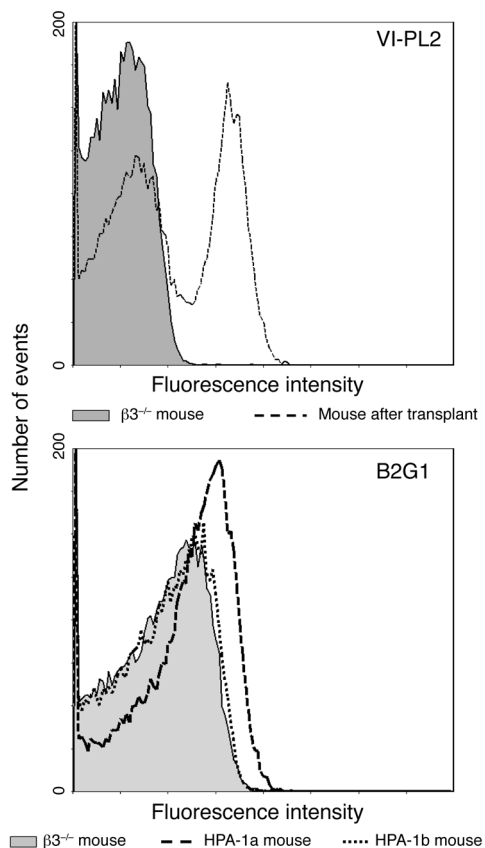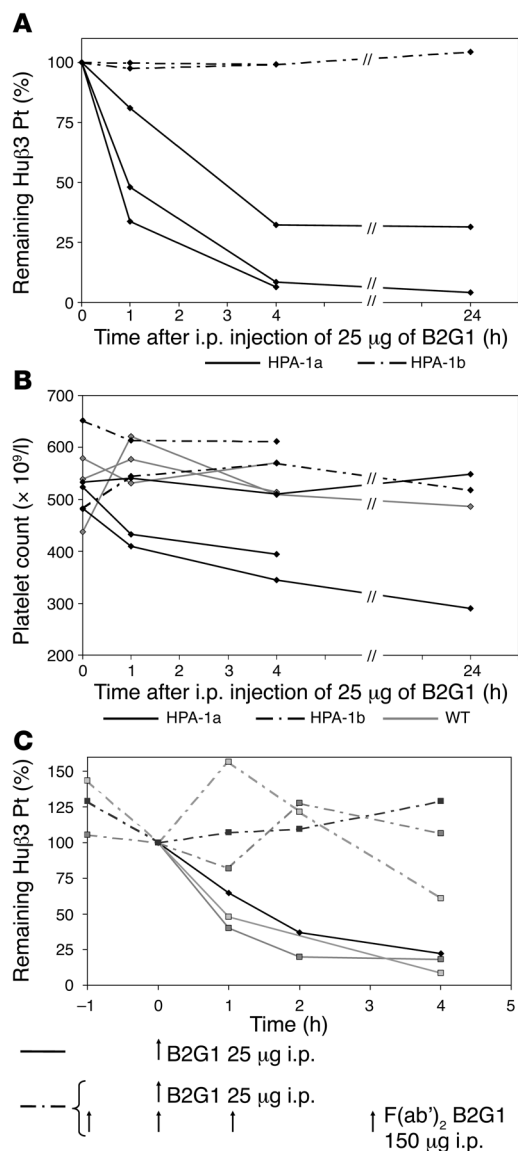
was notably less (55% and 65%), as the platelets used in the experiment were from a heterozygous HPA-5a5b donor. Since the epitopes defining the HPA-5 antigens are on platelet glycoprotein Ia (α2 integrin), these findings are consistent with the inhibitory effect of the B2 mutated monoclonal antibodies 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 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 Huβ3 Pt 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.

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 Huβ3 Pt in 3 HPA-1a mice (solid lines) and 2 HPA-1b mice (dotted lines) after i.p. injection of 25 μg B2G1. Huβ3 Pt chimerism for each mouse at time 0 (49%, 21%, and 12%) corresponds to 100% Huβ3 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 Huβ3 Pt in 3 HPA-1a mice (baseline Huβ3 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.

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 $20 \times 10^9/l$ (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 $50 \times 10^9/l$. 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 between maternal and fetal plasma, to reach a concentration of antibody capable of inhibiting 70% of binding and CL response as was achieved here would require between 30 mg and 6 g of recombinant antibody. Such doses can be generated by recombinant technology, as shown by the dose of monoclonal anti-CD20 (rituximab) used for lymphoma therapy (55). Depending on the

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 allotypic 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
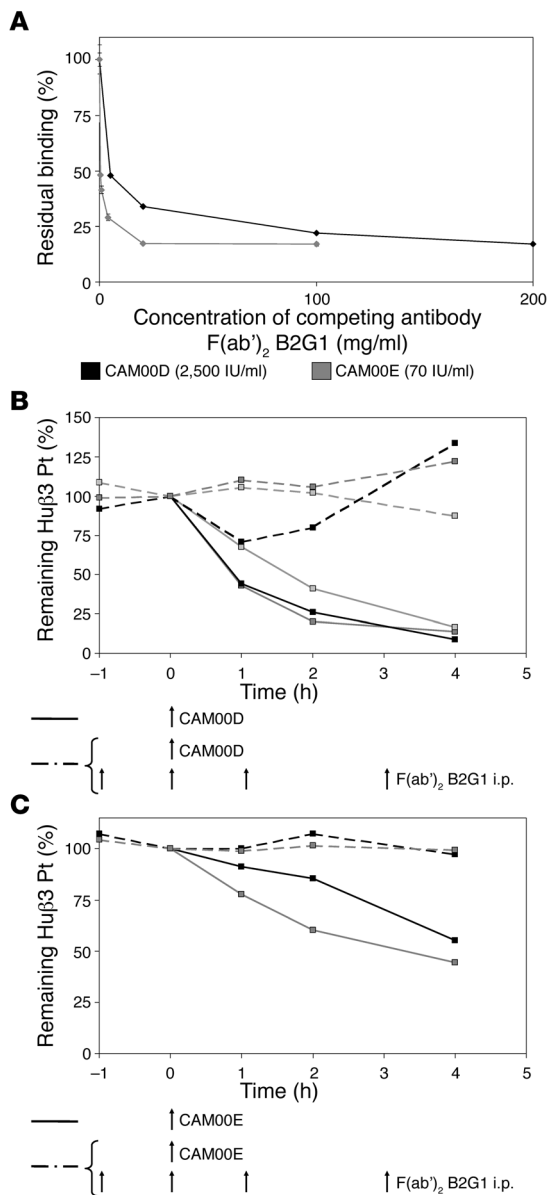
A

B

C

Figure 7

Platelet destruction studies in the murine model with polyclonal HPA-1a antibodies and F(ab')₂ B2G1. (**A**) Binding of the human polyclonal Ig anti–HPA-1a preparations CAM00D and CAM00E to HPA-1a heterozygous platelets by MAIPA in the presence of increasing concentrations of the F(ab')₂ fragment of B2G1. (**B**) The proportion of Huβ3 Pt in 3 HPA-1a mice (baseline Huβ3 Pt chimerism of 49%, 60%, and 34%) after i.p. injection of CAM00D alone (100 μl for the first 2 mice and 10 μl for the last one) (solid lines) and the proportion of Huβ3 Pt in 3 HPA-1a mice (baseline Huβ3 Pt chimerism of 67%, 83%, and 22%) after CAM00D (100 μl for the first 2 mice; 10 μl for the last one) plus i.p. boluses of F(ab')₂ B2G1 (260, 130, 130, and 130 μg for the first 2 mice; 50, 25, 12.5, and 12.5 μg for the last one) (dotted lines) at the times indicated. (**C**) The proportion of Huβ3 Pt in 2 HPA-1a mice (baseline Huβ3 Pt chimerism of 26% and 21%) after i.p. injection of 200 μl CAM00E alone (solid lines) and after CAM00E plus i.p. boluses of 100, 50, 25, and 25 μg F(ab')₂ B2G1 (dotted lines) at times shown.

Methods

Production of recombinant HPA-1a antibodies. The generation of B2G1, a human IgG1λ version of an anti–HPA-1a single-chain Fv, has been previously described (38), as has the vector containing the modified IgG1 gene, pSVgptFog1VHHuIgG1Δab (39). Briefly, IgG2 residues from positions 233–236 (Δb) were substituted together with IgG4 residues 327, 330, and 331 (Δa) into IgG1 to generate G1Δab. The null allotype (Δn) 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 IgG1Δnab constant region gene which was cloned as a *Bam*HI-*Not*I fragment into pSVgptB2VHHuIgG2 (38) to yield the vector pSVgptB2VHHuIgG1Δnab. Thus the 2 full-length HPA-1a antibodies generated for this study had the same B2 variable region and were designated "B2G1" and "B2G1Δnab."

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 × 10⁹/l without ICH (n = 7); (c) anti–HPA-1a alone and platelet count of greater than 100 × 10⁹/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 × 10⁹ and 19 × 10⁹/l, respectively (n = 2).

Serum samples from 2 FMAIT cases with anti–HPA-1a and neonatal platelet counts of 2 × 10⁹ and 13 × 10⁹/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 Na₂HPO₄, 7 mM NaH₂PO₄, pH 2.8, and

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.

neutralization with 214 mM TRIS, 22 mM Na_2HPO_4, 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 50×10^9/l. Platelet suspension (50 μl) were 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 Fcγ 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 Fcγ (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-1a antibodies B2G1 and B2G1Δnab or polyclonal anti–HPA-1a 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-1a 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) (3×10^8/l), and left to incubate in white flat-bottomed 96-well plates (Optiplate-TM 96; PerkinElmer) for 2 hours at 37°C in a humidified atmosphere of 5% CO_2. 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 (200×10^9/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) (53).

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.

Mice 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 αIIb/human β3 in β3$^{-/-}$ 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 2×10^4 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 FcγR profile (43), F(ab′)$_2$ B2G1 (which lacks a constant region altogether and therefore does not bind to either human or mouse FcγR), rather than B2G1Δnab (whose constant region has been tailored to abrogate binding to human FcγR), 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.

Acknowledgments

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1. Burrows, R.F., and Kelton, J.G. 1993. Fetal thrombocytopenia and its relation to maternal thrombocytopenia. *N. Engl. J. Med.* **329**:1463–1466.
2. Mueller-Eckhardt, C., et al. 1989. 348 cases of suspected neonatal alloimmune thrombocytopenia. *Lancet.* **1**:363–366.
3. Berry, J.E., et al. 2000. Detection of Gov system antibodies by MAIPA reveals an immunogenicity similar to the HPA-5 alloantigens. *Br. J. Haematol.* **110**:735–742.
4. Davoren, A., Curtis, B.R., Aster, R.H., and McFarland, J.G. 2004. Human platelet antigen-specific alloantibodies implicated in 1162 cases of neonatal alloimmune thrombocytopenia. *Transfusion.* **44**:1220–1225.
5. Williamson, L.M., et al. 1998. The natural history

of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (PlA1, Zwa) as determined by antenatal screening. *Blood.* **92**:2280–2287.

6. Jaegtvik, S., et al. 2000. Neonatal alloimmune thrombocytopenia due to anti-HPA 1a antibodies; the level of maternal antibodies predicts the severity of thrombocytopenia in the newborn. *BJOG.* **107**:691–694.

7. Durand-Zaleski, I., et al. 1996. Screening primiparous women and newborns for fetal/neonatal alloimmune thrombocytopenia: a prospective comparison of effectiveness and costs. Immune Thrombocytopenia Working Group. *Am. J. Perinatol.* **13**:423–431.

8. Dreyfus, M., et al. 1997. Frequency of immune thrombocytopenia in newborns: a prospective study. Immune Thrombocytopenia Working Group. *Blood.* **89**:4402–4406.

9. Turner, M.L., et al. 2005. Prospective epidemiologic study of the outcome and cost-effectiveness of antenatal screening to detect neonatal alloimmune thrombocytopenia due to anti–HPA-1a. *Transfusion.* **45**:1945–1956.

10. Kjeldsen-Kragh, J., et al. 2007. A screening and intervention program aimed to reduce mortality and serious morbidity associated with severe neonatal alloimmune thrombocytopenia. *Blood.* **110**:833–839.

11. Giovangrandi, Y., et al. 1990. Very early intracranial haemorrhage in alloimmune fetal thrombocytopenia. *Lancet.* **336**:310.

12. Murphy, M.F., et al. 1994. Antenatal management of fetomaternal alloimmune thrombocytopenia--report of 15 affected pregnancies. *Transfus. Med.* **4**:281–292.

13. Engelfriet, C.P., et al. 2003. Prenatal management of alloimmune thrombocytopenia of the fetus. *Vox. Sang.* **84**:142–149.

14. Ranasinghe, E., et al. 2001. Provision of platelet support for fetuses and neonates affected by severe fetomaternal alloimmune thrombocytopenia. *Br. J. Haematol.* **113**:40–42.

15. Overton, T.G., Duncan, K.R., Jolly, M., Letsky, E., and Fisk, N.M. 2002. Serial aggressive platelet transfusion for fetal alloimmune thrombocytopenia: platelet dynamics and perinatal outcome. *Am. J. Obstet. Gynecol.* **186**:826–831.

16. Buscaglia, M., et al. 1996. Percutaneous umbilical blood sampling: indication changes and procedure loss rate in a nine years' experience. *Fetal Diagn.Ther.* **11**:106–113.

17. Paidas, M.J., et al. 1995. Alloimmune thrombocytopenia: fetal and neonatal losses related to cordocentesis. *Am. J. Obstet. Gynecol.* **172**:475–479.

18. Ghevaert, C., et al. 2007. Management and outcome of 200 cases of fetomaternal alloimmune thrombocytopenia. *Transfusion.* **47**:901–910.

19. Bussel, J.B., et al. 1996. Antenatal management of alloimmune thrombocytopenia with intravenous gamma-globulin: a randomized trial of the addition of low-dose steroid to intravenous gamma-globulin. *Am. J. Obstet. Gynecol.* **174**:1414–1423.

20. Berkowitz, R.L., et al. 2006. Parallel randomized trials of risk-based therapy for fetal alloimmune thrombocytopenia. *Obstet. Gynecol.* **107**:91–96.

21. Kaplan, C., Murphy, M.F., Kroll, H., and Waters, A.H. 1998. Feto-maternal alloimmune thrombocytopenia: antenatal therapy with IvIgG and steroids--more questions than answers. European Working Group on FMAIT. *Br. J. Haematol.* **100**:62–65.

22. Birchall, J.E., Murphy, M.F., Kaplan, C., and Kroll, H. 2003. European collaborative study of the antenatal management of feto-maternal alloimmune

thrombocytopenia. *Br. J. Haematol.* **122**:275–288.

23. Radder, C.M., Brand, A., and Kanhai, H.H. 2001. A less invasive treatment strategy to prevent intracranial hemorrhage in fetal and neonatal alloimmune thrombocytopenia. *Am. J. Obstet. Gynecol.* **185**:683–688.

24. Kanhai, H.H., van den Akker, E.S., Walther, F.J., and Brand, A. 2006. Intravenous immunoglobulins without initial and follow-up cordocentesis in alloimmune fetal and neonatal thrombocytopenia at high risk for intracranial hemorrhage. *Fetal Diagn. Ther.* **21**:55–60.

25. Kroll, H., et al. 1994. Maternal intravenous immunoglobulin treatment does not prevent intracranial haemorrhage in fetal alloimmune thrombocytopenia. *Transfus. Med.* **4**:293–296.

26. Berkowitz, R.L., Bussel, J.B., and McFarland, J.G. 2006. Alloimmune thrombocytopenia: state of the art 2006. *Am. J. Obstet. Gynecol.* **195**:907–913.

27. El Shanawany, T., and Jolles, S. 2007. Intravenous immunoglobulin and autoimmune disease. *Ann. N.Y. Acad. Sci.* **1110**:507–515.

28. Power, J.P., et al. 1995. Hepatitis C infection from anti-D immunoglobulin. *Lancet.* **346**:372–373.

29. Smith, D.B., et al. 1999. A second outbreak of hepatitis C virus infection from anti-D immunoglobulin in Ireland. *Vox Sang.* **76**:175–180.

30. Ironside, J.W. 2006. Variant Creutzfeldt-Jakob disease: risk of transmission by blood transfusion and blood therapies. *Haemophilia.* **12**(Suppl. 1):8–15.

31. Pendergrast, J.M., Sher, G.D., and Callum, J.L. 2005. Changes in intravenous immunoglobulin prescribing patterns during a period of severe product shortages, 1995–2000. *Vox Sang.* **89**:150–160.

32. Bayry, J., Kazatchkine, M.D., and Kaveri, S.V. 2007. Shortage of human intravenous immunoglobulin — reasons and possible solutions. *Nat. Clin. Pract. Neurol.* **3**:120–121.

33. Goldberger, A., Kolodziej, M., Poncz, M., Bennett, J.S., and Newman, P.J. 1991. Effect of single amino acid substitutions on the formation of the PlA and Bak alloantigenic epitopes. *Blood.* **78**:681–687.

34. Greenwood, J., and Clark, M.R. 1993. *Protein engineering of antibody molecules for prophylactic and therapeutic applications in man.* Academic Titles. Nottingham, United Kingdom. 85–100.

35. Firan, M., et al. 2001. The MHC class I-related receptor, FcRn, plays an essential role in the maternofetal transfer of gamma-globulin in humans. *Int. Immunol.* **13**:993–1002.

36. Griffin, H.M., and Ouwehand, W.H. 1995. A human monoclonal antibody specific for the leucine-33 (P1A1, HPA-1a) form of platelet glycoprotein IIIa from a V gene phage display library. *Blood.* **86**:4430–4436.

37. Santoso, S., et al. 2006. A naturally occurring Leu-Val mutation in beta3-integrin impairs the HPA-1a epitope: the third allele of HPA-1. *Transfusion.* **46**:790–799.

38. Garner, S.F., et al. 2000. A rapid one-stage whole-blood HPA-1a phenotyping assay using a recombinant monoclonal IgG1 anti–HPA-1a. *Br. J. Haematol.* **108**:440–447.

39. Armour, K.L., Clark, M.R., Hadley, A.G., and Williamson, L.M. 1999. Recombinant human IgG molecules lacking Fcgamma receptor I binding and monocyte triggering activities. *Eur. J. Immunol.* **29**:2613–2624.

40. Turner, C.P., and Hadley, A.G. 2003. The role of P-selectin in the immune destruction of platelets. *Br. J. Haematol.* **121**:623–631.

41. Ni, H., et al. 2006. A novel murine model of fetal and

neonatal alloimmune thrombocytopenia: response to intravenous IgG therapy. *Blood.* **107**:2976–2983.

42. Fang, J., et al. 2005. Therapeutic expression of the platelet-specific integrin, alphaIIbbeta3, in a murine model for Glanzmann thrombasthenia. *Blood.* **106**:2671–2679.

43. Mestas, J., and Hughes, C.C. 2004. Of mice and not men: differences between mouse and human immunology. *J. Immunol.* **172**:2731–2738.

44. Bye, J.M., et al. 1992. Germline variable region gene segment derivation of human monoclonal anti-Rh(D) antibodies. Evidence for affinity maturation by somatic hypermutation and repertoire shift. *J. Clin. Invest.* **90**:2481–2490.

45. Valentin, N., Visentin, G.P., and Newman, P.J. 1995. Involvement of the cysteine-rich domain of glycoprotein IIIa in the expression of the human platelet alloantigen, PlA1: evidence for heterogeneity in the humoral response. *Blood.* **85**:3028–3033.

46. Stafford, P., et al. 2008. Immunologic and structural analysis of eight novel domain-deletion beta3 integrin peptides designed for detection of HPA-1 antibodies. *J. Thromb. Haemost.* **6**:366–375.

47. Armour, K.L., van de Winkel, J.G., Williamson, L.M., and Clark, M.R. 2003. Differential binding to human FcgammaRIIa and FcgammaRIIb receptors by human IgG wildtype and mutant antibodies. *Mol. Immunol.* **40**:585–593.

48. Hadley, A.G., et al. 1998. The ability of the chemiluminescence test to predict clinical outcome and the necessity for amniocenteses in pregnancies at risk of haemolytic disease of the newborn. *Br. J. Obstet. Gynaecol.* **105**:231–234.

49. Isaacs, J.D., Clark, M.R., Greenwood, J., and Waldmann, H. 1992. Therapy with monoclonal antibodies. An in vivo model for the assessment of therapeutic potential. *J. Immunol.* **148**:3062–3071.

50. Joutsi-Korhonen, L., et al. 2004. The effect of recombinant IgG antibodies against the leucine-33 form of the platelet beta3 integrin (HPA-1a) on platelet function. *Thromb. Haemost.* **91**:743–754.

51. Radder, C.M., Beekhuizen, H., Kanhai, H.H., and Brand, A. 2004. Effect of maternal anti–HPA-1a antibodies and polyclonal IVIG on the activation status of vascular endothelial cells. *Clin. Exp. Immunol.* **137**:216–222.

52. Armour, K.L., et al. 2006. Intravascular survival of red cells coated with a mutated human anti-D antibody engineered to lack destructive activity. *Blood.* **107**:2619–2626.

53. Ghevaert, C., et al. 2007. HPA-1a antibody potency and bioactivity do not predict severity of fetomaternal alloimmune thrombocytopenia. *Transfusion.* **47**:1296–1305.

54. Armstrong-Fisher, S., et al. 2004. In vitro maternofetal transfer of native and Fc mutated recombinant antibodies. *Vox Sang.* **87**(Suppl. 3):37.

55. Fanale, M.A., and Younes, A. 2007. Monoclonal antibodies in the treatment of non-Hodgkin's lymphoma. *Drugs.* **67**:333–350.

56. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**:680–685.

57. Bugert, P., et al. 2005. Microarray-based genotyping for blood groups: comparison of gene array and 5′-nuclease assay techniques with human platelet antigen as a model. *Transfusion.* **45**:654–659.

58. Allen, D., et al. 2005. Collaborative study to establish the first international standard for quantitation of anti–HPA-1a. *Vox Sang.* **89**:100–104.

59. Clark, M.R. 1997. IgG effector mechanisms. *Chem. Immunol.* **65**:88–110.