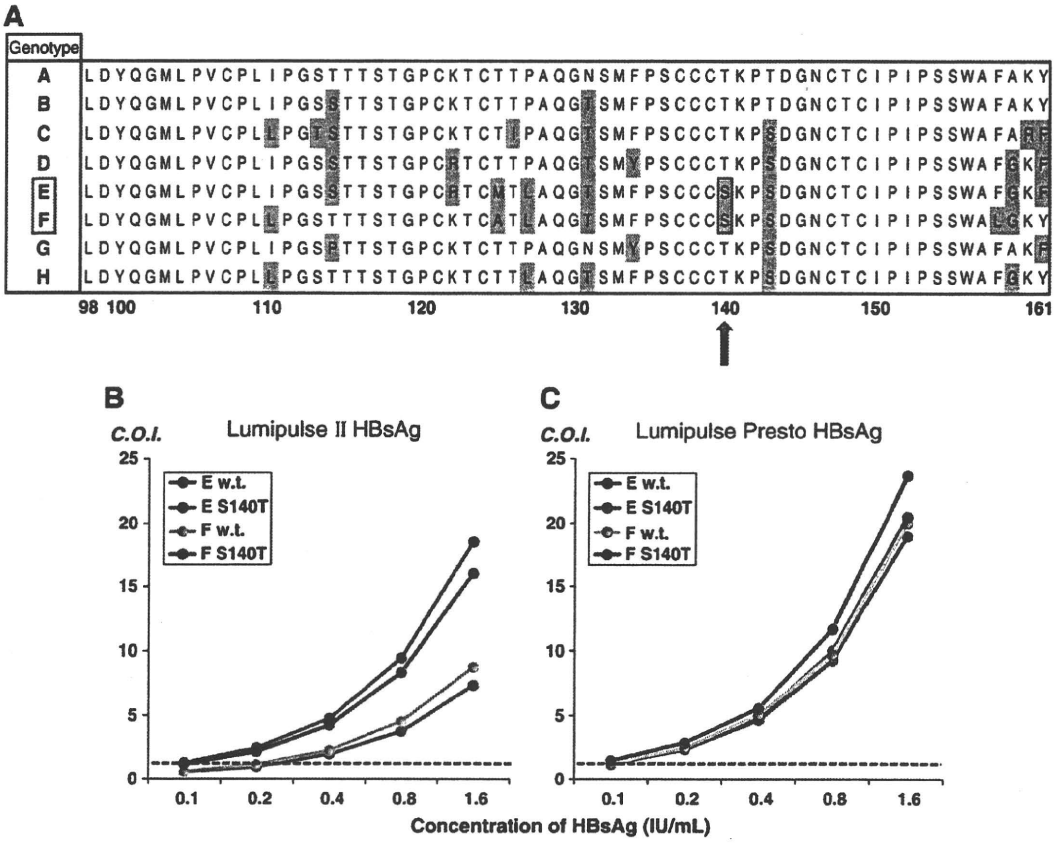

Fig. 1. A Comparison of amino acid sequences of HBsAg "a" determinants of various HBV genotypes. Accession numbers for amino acid sequences of genotypes A to H shown in the figure are listed below. Genotype A (AY902775, http://www.ncbi.nlm.nih.gov/nuccore/59802797); genotype B (AY293309, http://www.ncbi.nlm.nih.gov/nuccore/38147024); genotype C (AY205125, http://www.ncbi.nlm.nih.gov/nuccore/60279615); genotype D (AY796032, http://www.ncbi.nlm.nih.gov/nuccore/56090033); genotype E (DQ060829, http://www.ncbi.nlm.nih.gov/nuccore/70794948); genotype F (AY090459, http://www.ncbi.nlm.nih.gov/nuccore/22135721); genotype G (AB056516, http://www.ncbi.nlm.nih.gov/nuccore/15425700); genotype H (AB179747, http://www.ncbi.nlm.nih.gov/nuccore/60115422). B: Detection of recombinant HBsAg derived from genotypes E (E w.t.) and F (F w.t.) and their amino acid-substituted counterparts (ES140T and FS140T) by Lumipulse II HBsAg and C: Lumipulse Presto HBsAg kits. Each point indicates the mean of triplicate results. Error bars are too short to be indicated. Dashed lines indicate COI = 1.0.

References

[1] Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059–73.
[2] Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994;198:489–503.
[3] Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69 (Pt 10):2575–83.
[4] Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67–74.
[5] Mizuochi T, Okada Y, Umemori K, Mizusawa S, Yamaguchi K. Evaluation of 10 commercial diagnostic kits for in vitro expressed hepatitis B virus (HBV) surface antigens encoded by HBV of genotypes A to H. J Virol Methods 2006;136:254–6.
[6] Coleman PF, Chen YC, Mushahwar IK. Immunoassay detection of hepatitis B surface antigen mutants. J Med Virol 1999;59:19–24.
[7] Ly TD, Servant-Delmas A, Bagot S, et al. Sensitivities of four new commercial hepatitis B virus surface antigen (HBsAg) assays in detection of HBsAg mutant forms. J Clin Microbiol 2006;44:2321–6.
[8] Moerman B, Moons V, Sommer H, Schmitt Y, Stetter M. Evaluation of sensitivity for wild type and mutant forms of hepatitis B surface antigen by four commercial HBsAg assays. Clin Lab 2004;50:159–62.
[9] Mohsan S, Suzuki R, Kondo M, et al. Evaluation of hepatitis C virus core antigen assays in detecting recombinant viral antigens of various genotypes. J Clin Microbiol 2009;47:4141–3.

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SHORT COMMUNICATION

Establishment of a cell line panel as an alternative source of platelet antigens for a screening assay of anti-human platelet antibodies

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SUMMARY

Background: A panel of platelets expressing various human platelet antigens (HPAs) for a platelet antibody screening assay is difficult to prepare because some antigens are rarely expressed. Therefore, an alternative method without using platelets would be helpful in detecting HPA antibodies. This study describes the establishment of cell lines that stably express specific HPAs and their application for detecting specific antibodies.

Methods: Wild-type β3, HPA-1b, -6b, -7b and -7 variant cDNA as well as wild-type αIIb and HPA-3b cDNA were individually co-transduced with wild-type αIIb and β3 cDNA in the K562 cell line. We performed an immunobead monoclonal antibody immobilisation of platelet antigens (MAIPA) assay to evaluate this cell line panel for antibody detection using identified sera containing HPA antibodies, whose specificities had been determined by the mixed passive haemagglutination test.

Results and Conclusion: Of the 12 sera containing HPA-1a ($n = 2$), HPA-3a ($n = 6$), HPA-6b ($n = 3$) or HPA-7 variant ($n = 1$) antibodies, all antibodies were detected and determined by our new method, except for two HPA-3a antibodies. One of the two antibodies was also negative for conventional platelet MAIPA, suggesting that the cell line panel might be used as an alternative source of platelet antigens in the MAIPA assay.

Key words: cell line panel, HPA antibodies, immunobead MAIPA, integrin αIIbβ3.

The surface membranes of platelets contain a variety of molecules, including ABH antigens (Ogasawara *et al.*, 1993), human leucocyte antigens (HLAs), human platelet antigens (HPAs) (Metcalfe *et al.*, 2003) and the Nak^a antigen localised in CD36 (Ikeda *et al.*, 1989). Antibodies to these molecules are postulated to be the cause of reactions elicited by the transfusion of platelets such as refractoriness, post-transfusion purpura, neonatal alloimmune thrombocytopaenia and idiopathic thrombocytopaenic purpura (Metcalfe *et al.*, 2003). Therefore, the detection of these antibodies is important to diagnose and prevent these disorders. To date, many methods have been developed to detect antibodies against HPAs. The monoclonal antibody immobilisation of platelet antigens (MAIPA) assay is a highly sensitive and specific assay that uses specific antibodies against platelet glycoproteins that is considered as the gold standard technique in platelet immunology (Kaplan *et al.*, 2007).

A variety of HPAs have been found to result from the substitution of a single amino acid (18 cases) or an amino acid deletion in platelet glycoproteins (Santoso *et al.*, 2002, 2006; Metcalfe *et al.*, 2003; Stafford *et al.*, 2008a; Koh *et al.*, 2010). Fourteen HPAs – HPA-1, HPA-3, HPA-4, HPA-6, HPA-7, HPA-8, HPA-9, HPA-10, HPA-11, HPA-14, HPA-16, HPA-17, HPA-1 variant and HPA-7 variant – are associated with integrin αIIb and β3, which form a complex on the surface of the platelet. According to the Immuno Polymorphism Database (IPD) website (www.ebi.ac.uk/ipd/hpa/), the frequency of HPA phenotypes differ significantly among ethnic groups. Although the preparation of a well-characterised panel of platelets is necessary for the determination of HPA antibodies, the frequency of some HPAs is extremely low. Therefore, from a practical standpoint, it is difficult

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to prepare such a panel. A panel of cell lines that stably express specific HPAs would be useful for the detection of HPA antibodies regardless of ethnic origin. Therefore, we attempted to establish a cell line panel that expresses HPAs associated with αIIbβ3, including HPA-1, HPA-3, HPA-6 and HPA-7.

The K562 cell line, which is a non-adherent cell line derived from human erythroleukaemia, does not express HLA, human neutrophil antigens (Yasui *et al.*, 2007), HPAs or CD36 (Hayashi *et al.*, 2009). This study describes a method for the establishment of K562 cell lines that stably express specific HPAs and their use in the detection of HPA-1a, 3a, 6b and 7 variant antibodies with a modified immunobead MAIPA assay.

MATERIALS AND METHODS

Reagents

Puromycin and G418 were purchased from Nacalai Tesque (Kyoto, Japan). Phosphate-buffered saline (PBS) and anti-mouse IgG-horseradish peroxidase were purchased from Sigma-Aldrich (Tokyo, Japan) and Promega Corporation (Madison, WI, USA), respectively. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD41a and FITC-conjugated mouse IgG were purchased from BD Biosciences (Tokyo, Japan). pQCXIP, pQCXIN and packaging cells were purchased from Takara Bio (Shiga, Japan). Dynabeads M280 conjugated with sheep anti-mouse IgG, a site-directed mutagenesis system, Platinum Taq DNA polymerase and pCR2·1-TOPO were purchased from Invitrogen (Carlsbad, CA, USA). HIP8 (anti-αIIb) and B7 (anti-β3) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). P2 (anti-αIIbβ3 complex antibody) was purchased from Acris Antibodies GmbH (Herford, Germany).

Vector construction

As shown in Table 1, primers were designed and constructed on the basis of published αIIb and β3 cDNA sequences (Metcalfe *et al.*, 2003; Koh *et al.*, 2010). GFP with green fluorescent protein-fusion αIIb and DsRed-fusion β3 cDNA (Hayashi *et al.*, 2005) were used as templates for the polymerase chain reaction (PCR). Full-length cDNAs of αIIb and β3 were amplified by PCR using the following primer sets: αIIb for TOPO and β3 for TOPO (Table 1). PCR products were cloned into the TOPO TA cloning vector (Invitrogen). TOPO vectors containing the cDNA of HPA-1b, HPA-6b, HPA-7b and the HPA-7 variant and HPA-3b were prepared using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) with Platinum Taq DNA polymerase and the appropriate primer pairs (Table 1). All procedures were carried out according to the manufacturer's instructions. Each cDNA was then cloned into the retrovirus vectors pQCXIN and pQCXIP, respectively, using the In-Fusion 2·0 PCR Cloning Kit (Takara Bio) with *Eco*RI restriction endonuclease, β3 for pQ and αIIb for pQ primer pairs (Table 1).

Gene transduction

The transfection and infection experiments were performed according to the previously described method (Yasui *et al.*, 2007). For infections, K562 cells were incubated with culture supernatants from the packaging cell line containing retroviral particles carrying both the αIIb and β3 cDNAs. Mock transfection was carried out using empty vectors. Transfected K562 cells were cloned by the limiting dilution method in the cultivate medium (RPMI1640 supplemented with 10% foetal bovine serum and 0·5–1·0 $\mu g\ mL^{-1}$ puromycin and 0·4–0·8 $mg\ mL^{-1}$ G418). The surface expressions of αIIb were determined using an FITC-labelled CD41a and a flow cytometer (FACSCalibur, BD Biosciences). Several clones were selected for the cell line panel on the basis of high αIIb expression. The clones were termed HP-αIIbβ3 (wild-type), HP-1b, HP-3b, HP-6b, HP-7b and HP-7 variant based on the genes they expressed. HP-αIIbβ3 expressed HPA-1a, HPA-3a, HPA-6a, HPA-7a and HP-1b expressed HPA-1b, HPA-3a, HPA-6a and HPA-7a. The phenotypes of Hayashi's platelet-associated (HP) cell lines are summarised in Table 2. Each cell was maintained in the cultivate medium for 1 year.

Immunoprecipitation and Western blotting

Immunoprecipitation assay of αIIbβ3 on the HP cell clones was carried out using anti-αIIb (clone: HIP8) and M280 Dynabeads according to the manufacturer's instructions. Immunoprecipitants were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Electrophoresed proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was first incubated with monoclonal antibody against β3 (B7) as the primary antibody and then treated with horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Promega Corporation) as the secondary antibody. Immunoreactive bands were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Waltham, MA, USA) and ChemiDoc™ (Bio-Rad Laboratories, Hercules, CA, USA).

Immunobead MAIPA assay

The MAIPA assay was performed according to the method described by Crossley *et al.* (1997) with minor

Table 1. Primer sequences for vector construction

Primer name	Forward primer (5′–3′)	Reverse primer (5′–3′)
β3 for TOPO	attgacgcaaatgggcggtaggc	gagtggaattccttaagtgccccggtacgtgatattggtg
αIIb for TOPO	attgacgcaaatgggcggtaggc	gcagaattcgtcactcccccctcttcatcatcttcttc
HPA-1b	tctgatgaggccctgcctccgggctcacctcgct	gaggcagggcctcatcagagcaccaggcaca
HPA-3b	tggactgggggctgcccagccccagcccctcccc	tgggcagcccccagtccaccttgagagggtt
HPA-6b	caggacgaatgcagcccccaggagggtcagcccg	gggggctgcattcgtcctgctgggaagggcgata
HPA-7b	aggccaaggtgcgaggctgtgcccaggagaaggag	acagcctcgcaccttggcctcaatgctgaa
HPA-7 variant	aggccaaggtgcgaggctgttcccaggagaaggag	acagcctcgcaccttggcctcaatgctgaa
β3 for pQ	aacggatccggaattgaattcgagctcggtacccggggat	gagaggggcggaattccttaagtgccccggtacg
αIIb for pQ	aacggatccggaattgaattcgggaggaagatggccagagc	gagaggggcggaattgaattcgtcactcccccctcttcatc

Table 2. HPA system on HP cell lines

HPA system number	HP cell line						
	Mock	αIIbβ3	1b	3b	6b	7b	7 variant
1	—	a	b	a	a	a	a
3	—	a	a	b	a	a	a
6	—	a	a	a	b	a	a
7	—	a	a	a	a	b	Variant

Lower-case letters indicate notation of HPA type.

modifications. Samples from the HP cell panel ($1{\cdot}0 \times 10^6$ cells) were suspended in 50 μL tris-buffered saline supplemented with 0·2% BSA (TBS/BSA) and incubated with 25 μL of either test or control serum samples at 37 °C for 30 min. Serum was removed by washing with TBS/BSA and the cell pellet was incubated with anti-αIIbβ3 complex antibody P2 at 37 °C for 30 min. The cells were washed three times and then solubilised with 250 μL PBS (containing 0·5% Triton X-100) for 15 min. The lysates were centrifuged to exclude debris and 200 μL of the supernatants were incubated with 10 μL M280 Dynabeads for 40 min. The beads were washed five times with TBS containing 0·05% Tween-20 and 0·5% Nonidet P-40 (T-TBS/NP). Captured human IgG in the test sample was detected by the addition of 100 μL peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After 30-min incubation, the beads were washed five times with T-TBS/NP. Staining was performed by adding a peroxidase substrate (KPL, Inc., Gaithersburg, MD, USA) for 15 min and was followed by adding a stop solution (KPL). The absorbance was measured at 450 nm in a microplate reader (MTP-120; Hitachi, Tokyo, Japan).

Serum samples

Normal serum samples were obtained from healthy personnels at our blood centre. Serum samples containing HPA antibodies were obtained from patients showing non-haemolytic transfusion reactions, patients who were refractory to platelet transfusion and patients with neonatal alloimmune thrombocytopaenia. One of the HPA-1a antibodies was purchased from Olympus (Tokyo, Japan). The specificity of each serum was determined by the mixed passive haemagglutination (MPHA) test using an anti-HPA MPHA panel kit (Olympus) (Shibata *et al.*, 1986; Ohto *et al.*, 2004). The presence of HLA antibodies was tested by LabScreen (OneLambda, Canoga Park, CA, USA). The conventional MAIPA assay using platelet panel was performed according to Campbell *et al.* (2007) on three serum samples containing HPA-3a antibodies. The characteristics of all sera tested are summarised in Table 3.

RESULTS

Establishment of the cell line panel

We observed stable αIIbβ3 expression in all the transduced K562 cells for 1 year (Fig. 1a). To examine whether the transduced HP cells expressed heterodimeric αIIb/β3 proteins, an immunoprecipitation assay was performed (Fig. 1b). αIIb and β3 from the HP-αIIbβ3, HP-1b, HP-3b, HP-6b, HP-7b and HP-7 variant cells were co-immunoprecipitated. We did not detect the expression of αIIb or β3 in the HP-mock cells. The densities of the αIIb and β3 protein bands from the HP-6b cells were higher compared to the other cells, whereas those from the HP-αIIbβ3 cells were lower (Fig. 1b).

Detection of HPA antibodies using the HP cell line panel by immunobead MAIPA

We determined background nonspecific binding using the HP cell line panel and five normal serum samples (data not shown). We calculated the ratio for the absorbance of the signals with panel cells (HP-αIIbβ3, HP-1b, HP-3b, HP-6b, HP-7b and HP-7 variant cells) to noise signals with HP-mock cells. The mean ± 2 SD for the ratio of signal to noise was 1·2 ± 0·6 (between 0·6 and 1·7). On the basis of these results, we defined

Table 3. Results of the immunobead MAIPA assay using HP cell line panels

Sample number	Antibody specificity	HLA antibody	Condition	HP cell line						Specificity determined by	
				αIIbβ3	1b	3b	6b	7b	7 variant	HP cell line immunobeads MAIPA	Platelet MAIPA[1]
1	HPA-1a[2]	ND	COM	6·9 (++)	1·5 (−)	9·3 (++)	13·0 (++)	7·3 (++)	8·7 (++)	HPA-1a	ND
2	HPA-1a[3]	+	NAIT	3·7 (++)	1·2 (−)	3·7 (++)	5·2 (++)	3·5 (++)	4·1 (++)	HPA-1a	ND
3	HPA-3a[3]	+	PTR	1·1 (−)	1·1 (−)	1·0 (−)	1·3 (−)	1·4 (−)	1·0 (−)	—	—
4	HPA-3a[3]	+	PTR	2·3 (++)	2·1 (++)	1·1 (−)	2·8 (++)	2·8 (++)	2·2 (++)	HPA-3a	ND
5	HPA-3a[3]	−	PTR	1·4 (−)	1·3 (−)	0·8 (−)	1·2 (−)	1·5 (−)	1·1 (−)	—	ND
6	HPA-3a[3]	+	PTR	3·0 (++)	3·7 (++)	1·1 (−)	6·5 (++)	4·4 (++)	3·7 (++)	HPA-3a	HPA-3a
7	HPA-3a[3]	+	PTR	2·2 (++)	2·5 (++)	1·4 (−)	5·1 (++)	3·1 (++)	1·9 (+)	HPA-3a	ND
8	HPA-3a[3]	−	NAIT	2·0 (+)	2·2 (++)	1·5 (−)	2·6 (++)	2·9 (++)	1·9 (+)	HPA-3a	HPA-3a
9	HPA-6b[3]	+	PTR	1·7 (−)	1·2 (−)	1·1 (−)	3·1 (++)	1·0 (−)	1·1 (−)	HPA-6b	ND
10	HPA-6b[3]	−	NAIT	1·2 (−)	1·0 (−)	1·1 (−)	4·3 (++)	1·1 (−)	1·1 (−)	HPA-6b	ND
11	HPA-6b[3]	−	NAIT	1·3 (−)	1·1 (−)	1·2 (−)	3·7 (++)	1·4 (−)	1·2 (−)	HPA-6b	ND
12	HPA-7 variant[3]	+	NAIT	1·0 (−)	0·9 (−)	1·0 (−)	0·8 (−)	1·5 (−)	2·3 (++)	HPA-7 variant	ND

COM, commercial (purchased from Olympus); NAIT, neonatal alloimmune thrombocytopaenia; ND, not done; PTR, platelet transfusion refractoriness.

All results were analysed using P2 antibody as the capturing antibody. Antibody reactivity was considered positive (++), weakly positive (+) and negative (− and filled with grey) when the ratio of HP and HP-mock cells was greater than 2·0, between 1·8 and 2·0 and less than 1·8, respectively.

[1]Classification of antibody reactivity in the conventional platelet MAIPA assay was as follows: greater than 0·2, positive; less than 0·2, negative.

[2]Anti-serum specificity was determined using the MPHA test.

[3]Antibody specificities were determined by MPHA and LabScreen (OneLambda) in our laboratory. The conventional MAIPA assay was performed using a platelet panel in our laboratory (serum samples 3, 6 and 8).

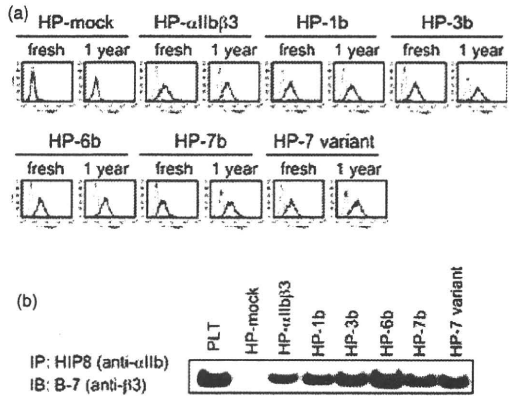

Fig. 1. Stable expression of heterodimeric αIIbβ3 on the surface of the HP cell line panel. (a) HP-mock cells, HP-αIIbβ3, HP-1b, HP-3b, HP-6b, HP-7b and HP-7 variant cells were maintained in continuous culture for more than 1 year and compared to freshly established cultures. HP cells were incubated with FITC-labelled anti-CD41a (bold lines) or isotype control antibodies (thin lines) and washed with PBS. A flow cytometry analysis was then performed.
(b) Immunoprecipitates were obtained by using monoclonal anti-αIIb (HIP8) antibodies from $1{\cdot}0 \times 10^6$ platelets (PLT) and $5{\cdot}0 \times 10^5$ HP cells. Each sample was subjected to SDS–PAGE, and the electrophoresed proteins were transferred to a PVDF membrane. Protein bands were visualised using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and detected using ChemiDoc™ (Bio-Rad Laboratories).

the reactivity of antibodies with a signal to noise ratio greater than 2·0 as positive, 1·8–2·0 as weakly positive and less than 1·8 as negative. We then tested a total of 12 well-characterised serum samples, including 2 HPA-1a, 6 HPA-3a, 3 HPA-6b and 1 HPA-7 variant antibodies (Table 3). The antibody reactivity in commercial serum sample 1 was positive for HP-αIIbβ3, HP-3a, HP-6b, HP-7b and HP-7 variant cells, whereas it was negative for HP-1b cells. These results indicate that the antibody in serum sample 1 recognised HPA-1a. To determine antibody specificity, this method is quite similar to that applied in the conventional MAIPA assay using platelets. The pattern of results obtained with antibodies from serum samples 2–12 indicated that antibodies of HPA-1a, HPA-6b and HPA-7 variant could be identified using this cell panel. However, we were only able to detect and determine the specificity for four of the six HPA-3a antibodies and the others were not detectable under the present experimental conditions.

DISCUSSION

Campbell *et al.* (2007) pointed out the potential risk of false-positive cases by the MAIPA assay due to the association of the co-immunoprecipitation of αIIb and/or β3 with HLA class I molecules. As the parental cell line K562, and hence our cells as well, does not express HLA class I molecules (Yasui *et al.*, 2007), the use of these cells in the MAIPA assay would exclude the risk of false-positive results. In fact, the HP cell line panel established in the present study detected HPA-specific antibodies, regardless of the presence of HLA antibodies (Table 3).

Interestingly, the HPA-3a antibodies in samples 3 and 5 did not react with the entire HP cell line panel and generated false-negative results. However, this may not be surprising considering the findings of Kataoka *et al.* (2004), Socher *et al.* (2008) and Barba *et al.* (2010) who concluded that HPA-3a alloantibodies can be divided into several groups based on the heterogeneity in reactivity. Among the results of these previous studies, it was demonstrated that certain HPA-3a alloantibody can be detected by a certain assay but not by another test, while a different HPA-3a alloantibody can be detected by the latter, but not by the former. We found similar heterogeneity in our result. Notably, the conventional MAIPA assay using platelets revealed a result consistent with that of our new MAIPA assay. The conventional MAIPA assay using platelets revealed that serum samples 6 and 8 were anti-HPA-3a positive, whereas serum sample 3 was negative, although only three samples could be used to compare the conventional MAIPA assay. Thus, HPA-3a samples we used appear heterogeneous in nature. Otherwise, the MAIPA assay performed in the present study and possibly the conventional MAIPA assay are not as sensitive as the MPHA test. The issues of sensitivity, specificity and heterogeneity of HPA-3a antibodies remain to be solved in a future study. Taken together, these results indicate that our cell line panel might be used as an alternative source of platelet antigens, at least, when the MAIPA assay is performed to detect HPA antibodies.

Stafford *et al.* (2008b) have recently published unique methods to detect αIIbβ3-associated HPA antibodies using recombinant β3 peptides that contained seven β3-bw antigens, termed SuperRare peptide. They demonstrated that the SuperRare peptide is suitable for the detection of HPA antibodies, which suggest that the additional cell line panel composed of a prototype with 'b' haplotype in all HPAs and its derivatives except each one of HPAs would make it easier to determine the specificity of HPA alloantibodies. In addition, cell lines expressing HPAs other than HPA-1, HPA-3, HPA-6 and HPA-7 need to be established. We plan to establish such a cell line panel in future.

The technologies to detect HPA antibodies using substitutions for platelets could not detect all HPA

antibodies until now, and hence, HPA antibodies should be determined by several methods on the basis of their properties. In the present study, we could use only a limited number of identified serum samples. Our findings suggest that the established cell line panel permit the detection of some antibodies against HPAs. However, it is necessary to further validate and characterise our cell line panel by using additional samples containing normal serum. Hereafter, a collaborative study will be conducted as a means to resolving these issues, as many samples were difficult to obtain in a single laboratory. Recently, several vendors began to distribute high-throughput immunoprecipitation systems using magnetic immunobeads. Therefore, automated immunobeads MAIPA might be more frequently utilised in the future.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

REFERENCES

Barba, P., Pallarés, P., Nogués, N., Canals, C., Gracia, M., Vinyets, I. & Muñiz-Diaz, E. (2010) Post-transfusion purpura caused by anti-HPA-3a antibodies that are only detectable using whole platelets in the platelet immunofluorescence test. *Transfusion Medicine*, **20,** 200–202.

Campbell, K., Rishi, K., Howkins, G. *et al.* (2007) A modified rapid monoclonal antibody-specific immobilization of platelet antigen assay for the detection of human platelet antigen (HPA) antibodies: a multicentre evaluation. *Vox Sanguinis*, **93,** 289–297.

Crossley, A., Calvert, J.E., Taylor, P.R. & Dickinson, A.M. (1997) A comparison of monoclonal antibody immobilization of platelet antigen (MAIPA) and immunobead methods for detection of GPIIb/IIIa antiplatelet antibodies in immune thrombocytopenic purpura. *Transfusion Medicine*, **7,** 127–134.

Hayashi, T., Tanaka, S., Hori, Y., Terada, C., Ueda, Y. & Tani, Y. (2005) Insertion of a C in the exon 28 of integrin alphaIIb gene leading to a frameshift mutation is responsible for Glanzmann thrombasthenia in a Japanese case. *Journal of Thrombosis and Haemostasis*, **3,** 489–496.

Hayashi, T., Yasui, K., Matsuyama, N. *et al.* (2009) Establishment of a novel method for detecting Nak antibodies by using a panel cell line. *Transfusion*, **49,** 390–392.

Ikeda, H., Mitani, T., Ohnuma, M., Haga, H., Ohtzuka, S., Kato, T., Nakase, T. & Sekiguchi, S. (1989) A new platelet-specific antigen, Naka, involved in the refractoriness of HLA-matched platelet transfusion. *Vox Sanguinis*, **57,** 213–217.

Kaplan, C., Freedman, J., Foxcroft, Z. *et al.* (2007) Working Party on Platelet Immunology: monoclonal platelet antigen capture assays (MAIPA) and reagents: a statement. *Vox Sanguinis*, **93,** 298–299.

Kataoka, S., Kobayashi, H., Chiba, K., Nakamura, M., Shinada, S., Morita, S., Lin, M. & Shibata, Y. (2004) Neonatal alloimmune thrombocytopenia due to an antibody against a labile component of human platelet antigen-3b (Bak b). *Transfusion Medicine*, **14,** 419–423.

Koh, Y., Taniue, A., Ishii, H. *et al.* (2010) Neonatal alloimmune thrombocytopenia caused by an antibody specific for a newly identified allele of human platelet antigen-7. *Transfusion*, **50,** 1276–1284.

Metcalfe, P., Watkins, N.A., Ouwehand, W.H. *et al.* (2003) Nomenclature of human platelet antigens. *Vox Sanguinis*, **85,** 240–245.

Ogasawara, K., Ueki, J., Takenaka, M. & Furihata, K. (1993) Study on the expression of ABH antigens on platelets. *Blood*, **82,** 993–999.

Ohto, H., Miura, S., Ariga, H., Ishii, T., Fujimori, K. & Morita, S., Collaborative Study Group. (2004) The natural history of maternal immunization against foetal platelet alloantigens. *Transfusion Medicine*, **14,** 399–408.

Santoso, S., Kiefel, V. & Richter, I.G. (2002) A functional platelet fibrinogen receptor with a deletion in the cysteine-rich repeat region of the beta(3) integrin: the Oe(a) alloantigen in neonatal alloimmune thrombocytopenia. *Blood*, **99,** 1205–1214.

Santoso, S., Kroll, H., Andrei-Selmer, C.L., Socher, I., Rankin, A., Kretzschmar, E., Watkins, N.A. & Ouwehand, W.H. (2006) A naturally occurring LeuVal mutation in beta3-integrin impairs the HPA-1a epitope: the third allele of HPA-1. *Transfusion*, **46,** 790–799.

Shibata, Y., Miyaji, T., Ichikawa, Y. & Matsuda, I. (1986) A new platelet antigen system Yuk^a/Yuk^b. *Vox Sanguinis*, **51,** 334–336.

Socher, I., Zwingel, C., Santoso, S. & Kroll, H. (2008) Heterogeneity of HPA-3 alloantibodies: consequences for the diagnosis of alloimmune thrombocytopenic syndromes. *Transfusion*, **48,** 463–472.

Stafford, P., Garner, S.F., Rankin, A., Kekomaki, R., Watkins, N.A. & Ouwehand, W.H. (2008a) A single-nucleotide polymorphism in the human ITGB3 gene is associated with the platelet-specific alloantigen Va (HPA-17bw) involved in fetal maternal alloimmune thrombocytopenia. *Transfusion*, **48,** 1432–1438.

Stafford, P., Garner, S.F., Huiskes, E. *et al.* (2008b) Three novel beta3 domain-deletion peptides for the sensitive and specific detection of HPA-4 and six low frequency beta3-HPA antibodies. *Journal of Thrombosis and Haemostasis*, **6,** 376–383.

Yasui, K., Miyazaki, T., Matsuyama, N. *et al.* (2007) Establishment of cell lines stably expressing HNA-1a, -1b, and -2a antigen with low background reactivity in flow cytometric analysis. *Transfusion*, **47,** 478–485.

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RETROVIROLOGY

SHORT REPORT Open Access

An Endogenous Murine Leukemia Viral Genome Contaminant in a Commercial RT-PCR Kit is Amplified Using Standard Primers for XMRV

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Abstract

During pilot studies to investigate the presence of viral RNA of xenotropic murine leukemia virus (MLV)-related virus (XMRV) infection in sera from chronic fatigue syndrome (CFS) patients in Japan, a positive band was frequently detected at the expected product size in negative control samples when detecting a partial *gag* region of XMRV using a one-step RT-PCR kit. We suspected that the kit itself might have been contaminated with small traces of endogenous MLV genome or XMRV and attempted to evaluate the quality of the kit in two independent laboratories. We purchased four one-step RT-PCR kits from Invitrogen, TaKaRa, Promega and QIAGEN in Japan. To amplify the partial *gag* gene of XMRV or other MLV-related viruses, primer sets (419F and 1154R, and GAG-I-F and GAG-I-R) which have been widely used in XMRV studies were employed. The nucleotide sequences of the amplicons were determined and compared with deposited sequences of a polytropic endogenous MLV (PmERV), XMRV and endogenous MLV-related viruses derived from CFS patients. We found that the enzyme mixtures of the one-step RT-PCR kit from Invitrogen were contaminated with RNA derived from PmERV. The nucleotide sequence of a partial *gag* region of the contaminant amplified by RT-PCR was nearly identical (99.4% identity) to a PmERV on chromosome 7 and highly similar (96.9 to 97.6%) to recently identified MLV-like viruses derived from CFS patients. We also determined the nucleotide sequence of a partial *env* region of the contaminant and found that it was almost identical (99.6%) to the PmERV. In the investigation of XMRV infection in patients of CFS and prostate cancer, researchers should prudently evaluate the test kits for the presence of endogenous MLV as well as XMRV genomes prior to PCR and RT-PCR tests.

Findings

Xenotropic murine leukemia virus (MLV)-related virus (XMRV), which resembles endogenous MLV, was discovered in prostate cancer patients in 2006 [1,2]. In 2009, a high incidence of XMRV infection was also documented in chronic fatigue syndrome (CFS) patients in the United States [3]. Since then, surveys on XMRV infection of CFS patients have been conducted in several countries [4-9]; however, there is a vigorous debate over conflicting results in CFS patients [10-12]. Moreover, recently, Lo et al. detected MLV-related viruses which are distinct from XMRV but resemble polytropic endogenous MLVs in CFS patients and healthy blood donors [13].

In studies investigating XMRV infection, a PCR approach to detect proviral DNA and/or a RT-PCR approach to detect viral RNA have been commonly employed [1,3-6,8,13-15]. We (the Japanese Red Cross [JRC]) have been studying the prevalence of XMRV infection in Japanese patients with prostate cancer and CFS as well as healthy blood donors. To study the presence of XMRV RNA in plasma from CFS patients, we selected a commercial one-step RT-PCR kit. In the pilot study, we encountered a puzzling result. A positive band was frequently detected at the expected product size in the negative control (water) using primer sets to detect a partial *gag* region of XMRV. We suspected that the test kit itself might have been contaminated with small traces of endogenous MLV genome or XMRV and attempted to evaluate the quality of the kit in two independent laboratories, in JRC and Institute for Virus Research (IVR), Kyoto University (Kyoto, Japan).

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We used the following RT-PCR kits which were purchased in Japan: SuperScript®III One-Step RT-PCR System with the Platinum® Taq High Fidelity Kit (Cat. no. 12574-030) (Invitrogen, Carlsbad, CA, USA) (abbreviated as Kit I); AccessQuick™RT-PCR Sysytem (Cat. no. A1701) (Promega, Madison, WI, USA) (abbreviated as Kit P); One Step RT-PCR Kit (Cat. no. PRO24A) (TaKaRa, Ohtsu, Shiga, Japan) (Abbreviated as Kit T); One Step RT-PCR Kit (Cat. no. 210210) (QIAGEN GmbH, Hilden, Germany) (Abbreviated as Kit Q).

To amplify the partial *gag* gene of XMRV or other MLV-related viruses, primers 419F (5'-ATCAGTT AACCTACCCGAGTCGGAC-3') and 1154R (5'-GCC GCCTCTTCTTCATTGTTCTC-3') [3], and GAG-I-F (5'-TCTCGAGATCATGGGACAGA-3') and GAG-I-R (5'-AGAGGGTAAGGGCAGGGTAA-3') [1] were used. To amplify the partial *env* gene of polytropic endogenous MLV, primers p-env1f (5'-AGAAGGTCCAGCGTTCT-CAA-3'), p-env1r (5'-TTGCCACAGTAGCCCTCTCT-3'), p-env3f (5'-GATGAGACTGGACTCGGGTG-3') and p-env5r (5'-GTGGAGGCCTGGGGAGCATGATC-3') were designed based on the sequence of a polytropic endogenous MLV (PmERV) present in mouse (*Mus musculus*) chromosome (chr) 7 [GenBank: AC167978]. To enhance one-step RT-PCR reactions, 2.5 μl of 1 μg/μl carrier RNA from QIAamp UltraSens™ Virus Kit (Cat. no. 53704) (QIAGEN) was added to the reaction mixtures of the one-step RT-PCR reactions as indicated in Figure 1. To examine whether the contaminant was RNA, 2 μl of 10 μg/ml RNaseA (Cat. no. 19101) (QIAGEN) were added in the one-step RT-PCR reaction mixture as indicated in Figure 1C. The RT-PCR was conducted in 25 μl (Kit I, Kit T, and Kit Q) or 25.5 μl (Kit P) of reaction mixture according to manufacturers' instructions.

By adding carrier RNA in the samples to enhance the RT-PCR reaction, we consistently detected a positive band using Kit I in negative controls using two primer sets (419F and 1154R, and GAG-I-F and GAG-I-R) which are widely used to amplify XMRV (Figure 1A). These results were confirmed by two independent laboratories (JRC and IVR) under the same experimental conditions. The positive reaction was observed in all four batches (derived from four different lots) of the kit tested. To exclude the possibility that water, the carrier RNA or the primers used were contaminated with an XMRV-like genome, we tested additional one-step RT-PCR kits, termed Kit P and Kit T, from two different manufacturers. Consequently, we could not detect positive bands utilizing these kits (Figure 1B) strongly suggesting that the component(s) of Kit I contained XMRV-like viral genomes. Most of the contaminants appeared to be RNA because the positive bands disappeared after adding RNaseA in the reaction mixture from Kit I (Figure 1C).

To further investigate the contaminant in Kit I, nucleic acids purified from the enzymes (a mixture of reverse transcriptase and *Taq* DNA polymerase) and the buffer contained in the kit were tested by adding the individual components to three different one-step RT-PCR kits (Kit T, Kit P, and Kit Q) (Figure 2A-C). As a result, we detected positive bands when the nucleic acids purified from the enzymes of Kit I were added to RT-PCR Kit T, Kit P or Kit Q using two primer sets (419F and 1154R, and GAG-I-F and GAG-I-R). On the contrary, we could not detect the presence of MLV genomes in the buffer of Kit I. These data indicated that the enzyme mixture of Kit I was contaminated with XMRV-like viral RNA.

PCR products amplified using primers 419F and 1154R were cloned into pCR4Blunt -TOPO (Invitrogen) and sequenced for both strands. Three clones (two clones at JRC and one clone at IVR) were sequenced and found to be nearly identical (one nucleotide difference between one another). These sequences have a 9 nucleotide deletion observed in some endogenous polytropic MLVs in place of the XMRV-specific 24 nucleotide deletion in the 5' *gag* leader region and are nearly identical to polytropic endogenous MLVs encoded in multiple chromosomal locations of the C57BL/6J mouse genome. The nucleotide sequences of the representative clone [GenBank: AB597300] were aligned with sequences deposited in GenBank as follows: MLV-like virus from CFS patients types 1, 2 and 3 [GenBank: HM630562, HM630558, and HM630559] [13], XMRV strain VP62 [GenBank: NC_007815] [2] and one representative PmERV on chr 7 [GenBank: AC167978; nt 65,391-64,647] (Figure 3). The contaminant was nearly identical (99.4% identity) to the PmERV chr 7 with only 4 nucleotide differences in the sequenced region. In addition, the contaminant was quite similar (96.9-97.6% identity) to the MLV-like viral sequences (CFS types 1, 2, and 3) derived from CFS patients.

To further characterize the contaminant, we conducted additional RT-PCRs (Figure 1D) amplifying partial *env* regions with two primer sets (p-env1f and p-env1r, and p-env3f and p-env5r) based on the sequence of the PmERV chr 7, and then sequenced the amplicons directly. We determined 674 bp of the N-terminal *env* region [GenBank: AB597301] and found that the contaminant was nearly identical (99.6% identity) to the PmERV chr 7 [GenBank: AC167978; nt 59,992-59,319] (Figure 4).

It should be noted that Kit I contains an anti-DNA polymerase monoclonal antibody to accomplish hot start-PCR and to reduce non-specific amplification.

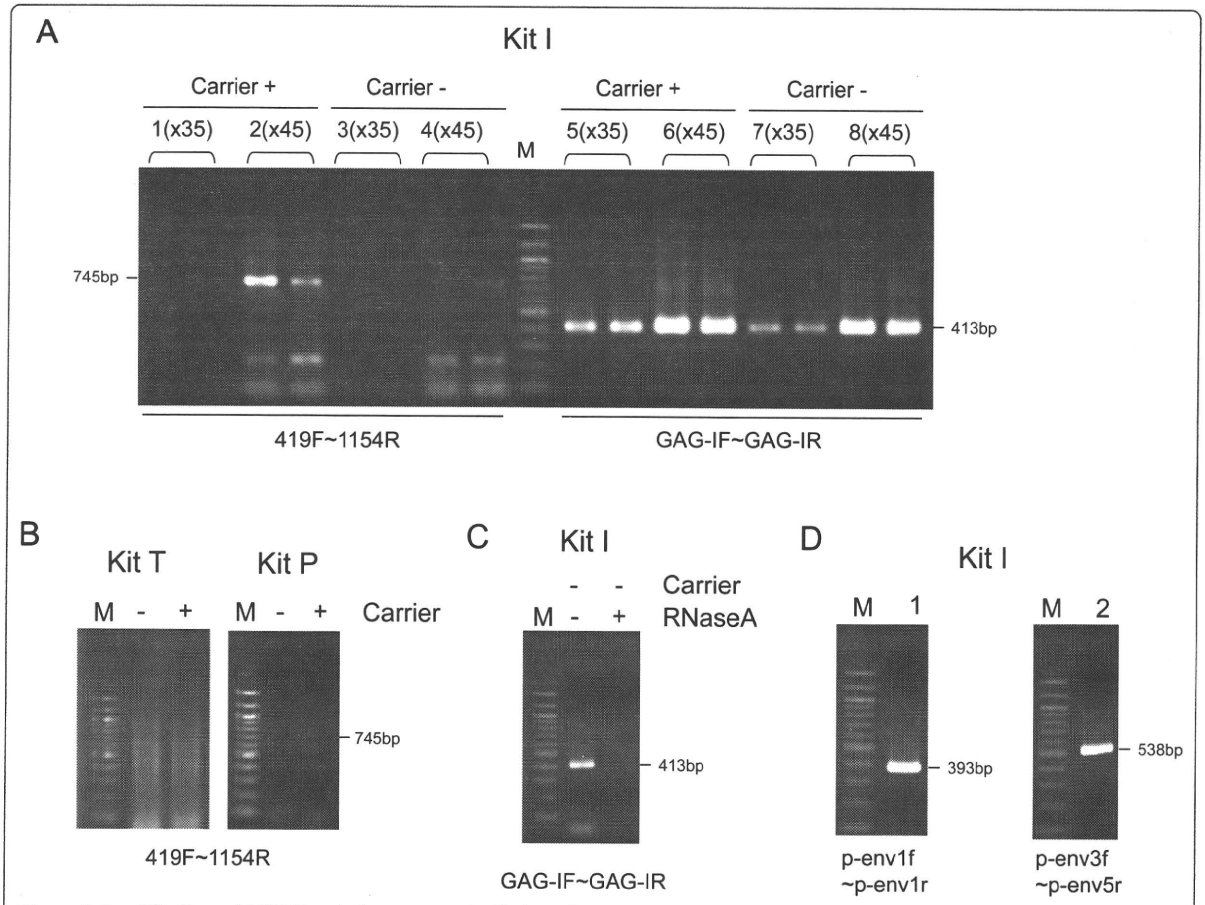

Figure 1 Amplification of MLV-like viral sequences in Kit I. (A) One-step RT-PCR was conducted using Kit I with the indicated primer sets. The RT-PCR conditions were as follows: reverse transcription at 55°C for 30 minutes; activation at 94°C for 2 minutes; 35 (lanes 1, 3, 5 and 7) or 45 cycles (lanes 2, 4, 6 and 8) of the following steps: 94°C for 15 s, 57°C for 30 s, and 68°C for 1 minute; and a final extension at 68°C for 3 minutes. Lanes 1, 2, 5 and 6: one-step RT-PCR with carrier RNA; Lanes 3, 4, 7 and 8: one-step RT-PCR without carrier RNA. Each reaction was carried out in duplicate. (B) One-step RT-PCR was conducted using Kit T (left panel) and Kit P (right panel) with primers 419F and 1154R with or without carrier RNA. The RT-PCR conditions using Kit T were as follows: reverse transcription at 50°C for 30 minutes; activation at 94°C for 2 minutes; 45 cycles of the following steps: 94°C for 30 s, 57°C for 30 s, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. The RT-PCR conditions using Kit P were as follows: reverse transcription at 45°C for 45 minutes; activation at 95°C for 2 minutes; 45 cycles of the following steps: 95°C for 30 s, 57°C for 30 s, and 70°C for 45 s; and a final extension at 70°C for 5 minutes. (C) One-step RT-PCR was conducted with primers GAG-I-F and GAG-I-R using Kit I with or without RNaseA. Carrier RNA was not added to the reaction mixtures. The RT-PCR conditions were as follows: reverse transcription at 55°C for 30 minutes; activation at 94°C for 2 minutes; 45 cycles of the following steps: 94°C for 15 s, 57°C for 30 s, and 68°C for 1 minute; and a final extension at 68°C for 3 minutes. (D) One-step RT-PCR was conducted using Kit I to amplify *env* region of the contaminants. One-step RT-PCR was carried out using two primer sets p-env1f and p-env1r (lane 1), and p-env3f and p-env5r (lane 2). The RT-PCR conditions were the same as in Figure 1C with the exception of the number of PCR cycles (60 cycles instead of 45 cycles). M: DNA size marker.

Mice have enormous copy numbers of endogenous retroviruses in their genomes; and hybridomas, for manufacturing monoclonal antibodies, have been found to produce high amounts of retroviral particles [16]. Therefore, we suspect that the *Taq* DNA polymerase in Kit I was contaminated with the endogenous MLVs. This possibility has been also pointed out by others [17,18]. Because the reverse transcriptase (SuperScriptIII) and the *Taq* DNA polymerase (Platinum Taq) in Kit I can be purchased separately from the manufacturer, we attempted to detect the MLV genome in the Platinum Taq polymerase using the same protocol as the one performed in Figure 2A-B. As a result, we detected MLV genomes in the Platinum Taq DNA polymerase using the RT-PCR Kit P and Kit T (Figure 2D for Kit P and data not shown for Kit T).

Surveys have been conducted by several research groups on XMRV infection in CFS patients, but the

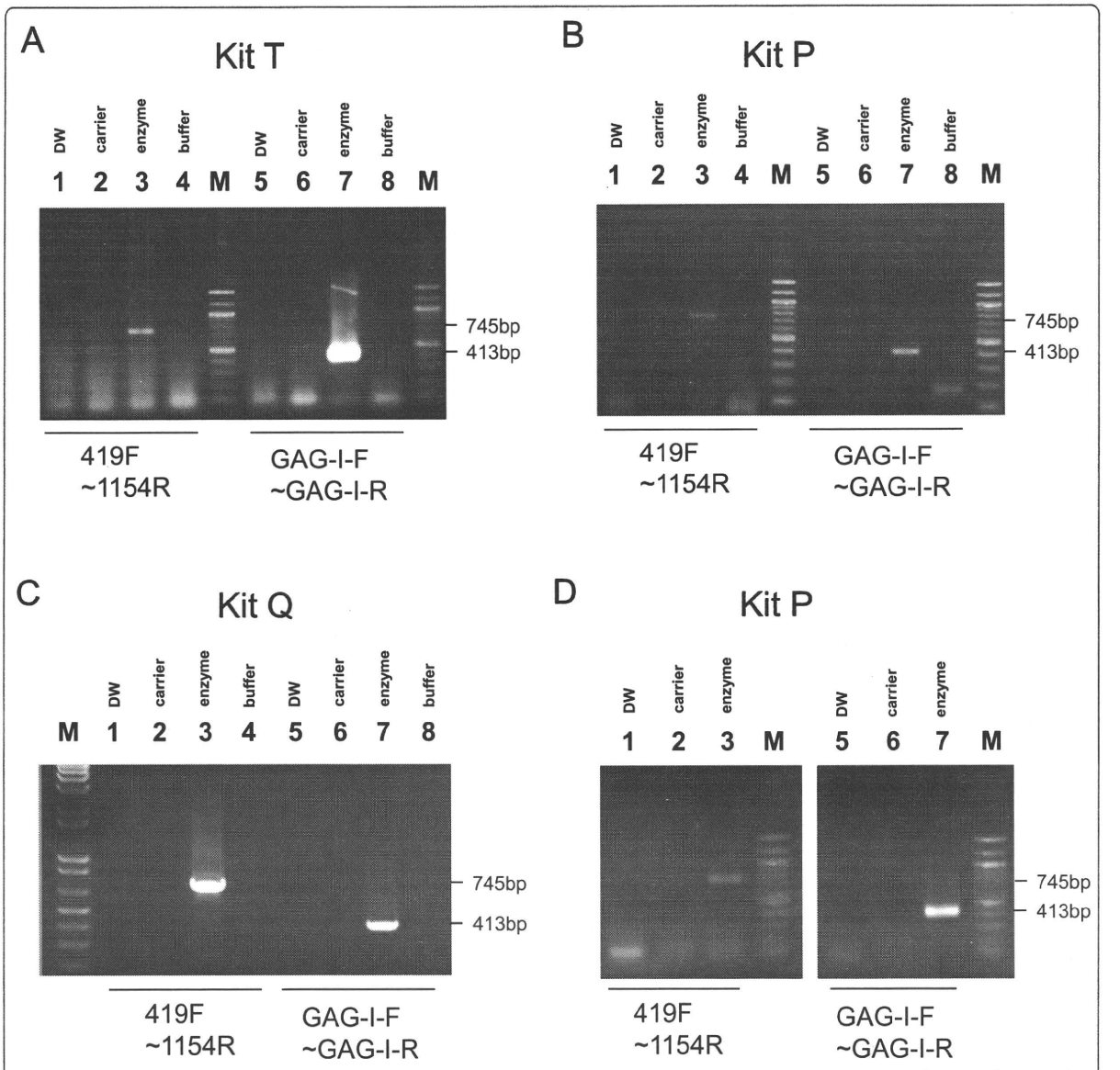

Figure 2 One-step RT-PCR for identification of contaminants in Kit I and Platinum Taq. (A-C) One-step RT-PCR for identification of a contaminated component in Kit I. The experiments were conducted in two independent laboratories, IVR and JRC. In IVR, nucleic acids were extracted from 50 μl of the enzyme mix of the RT-PCR Kit I using an RNA purification column (QIAamp viral RNA mini kit [Cat. no. 52904] [QIAGEN]) and the presence of polytropic endogenous MLV was examined by using the RT-PCR Kit T (A) and Kit P (B). In JRC, nucleic acids were extracted from 75 μl of the enzyme mix of RT-PCR Kit I using an RNA/DNA purification column (PureLink™ Viral RNA/DNA Kit [Cat. no. 12280-050] [Invitrogen]), and the presence of polytropic endogenous MLV was examined using Kit Q (C). Five μl of test samples were examined with primers indicated below the corresponding lanes. The RT-PCR conditions for Kit T and Kit P were the same as in Figure 1B. The RT-PCR conditions for Kit Q were as follows: reverse transcription at 50°C for 30 minutes; activation at 95°C for 15 minutes; 45 cycles of the following steps: 94°C for 30 s, 57°C for 30 s, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. Lanes 1 and 5, DW; lanes 2 and 6, column-purified carrier RNA (carrier); lanes 3 and 7, column-purified nucleic acids from enzyme mix (enzyme) of the Kit I; lanes 4 and 8, 1 μl buffer of the Kit I plus 4 μl DW (buffer). (D) One-step RT-PCR for the detection of MLV RNA in Platinum Taq. Nucleic acids were extracted from 50 μl of the Platinum Taq using an RNA purification column (QIAamp viral RNA mini kit [QIAGEN]) and the presence of MLV RNA was examined by using the RT-PCR Kit P. Five μl of test samples were examined with primers indicated below the corresponding lanes. The RT-PCR condition was the same as in Figure 1B with the exception of the PCR cycles (60 cycles instead of 45 cycles). Abbreviation; DW: distilled water. M: DNA size marker.

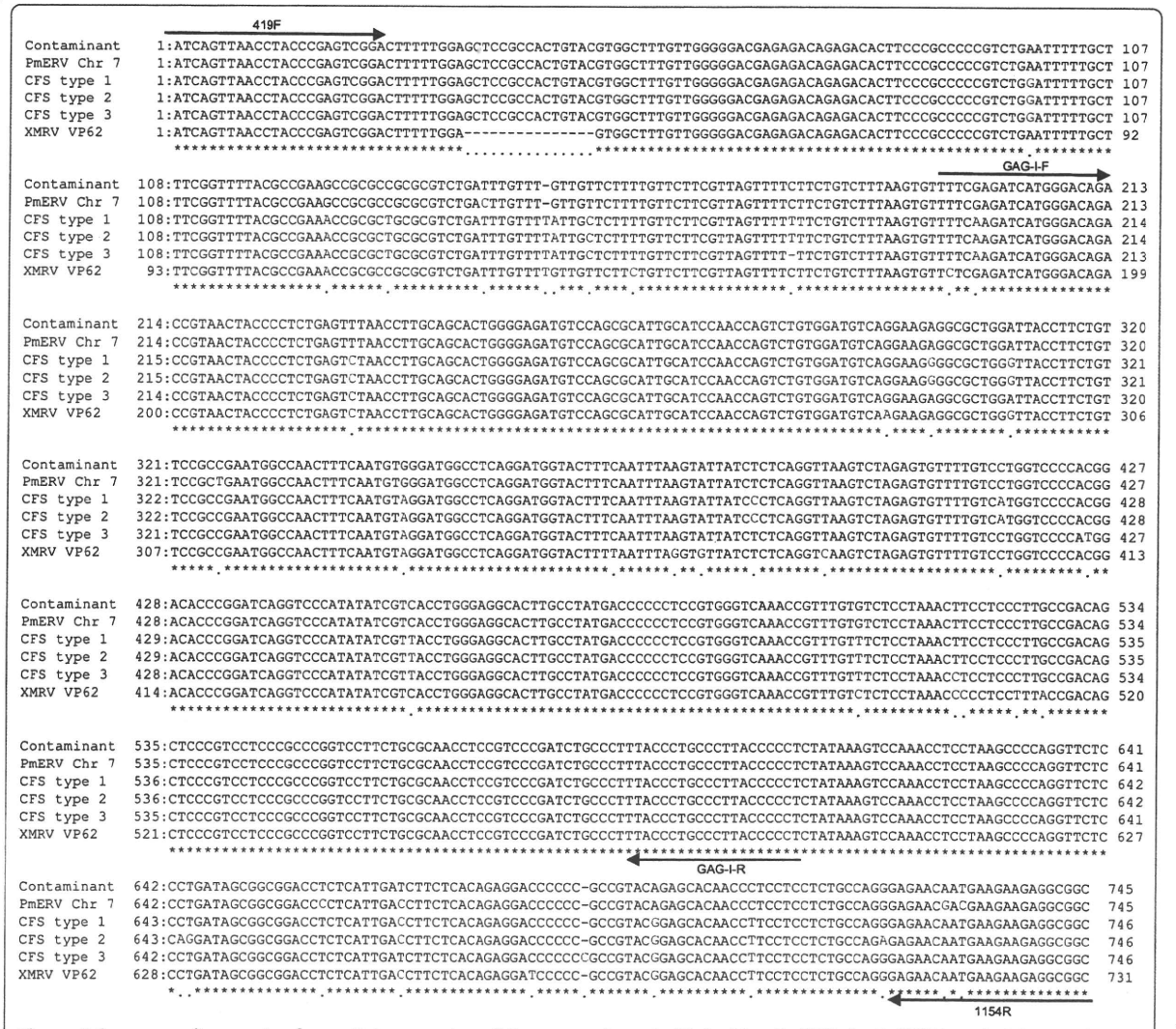

Figure 3 Sequence alignments of a partial *gag* region of the contaminant in Kit I with a PmERV chr 7, XMRV strain VP62, and MLV-like sequences derived from CFS patients (CFS types 1 to 3). Origins of the sequences used for the alignment are described in the Findings. Sequence alignments were performed using GENETYX Win ver. 6 (GENETYX, Shibuya, Tokyo, Japan).

results have been inconsistent. Although all research groups carefully performed their experiments to test XMRV infection by PCR and/or RT-PCR, it is still difficult to conclude that the positive results linking XMRV with CFS are not laboratory artifacts. Xenotropic (or polytropic) MLVs are widespread, and there may be many opportunities for samples to get contaminated with such ubiquitous viruses in laboratories when conducting biological or medical research [17]. In this study, we evaluated several one-step RT-PCR kits and a *Taq* DNA polymerase for the contamination of MLV-related genomes and found that the test kit and the *Taq* DNA polymerase from Invitrogen were contaminated with MLV-related genomes.

The findings in the present study indicate that contaminating nucleic acids in the test kits can potentially produce false-positive PCR results in studies of XMRV and other MLV-related viruses. In particular, our results raise the possibility that the PCR products described by Lo et al. [13] were derived from contaminating MLV RNA and/or DNA. It should be noted, however, that in contrast to our data which shows MLV contamination even in water controls, their report demonstrated that polytropic MLV sequences

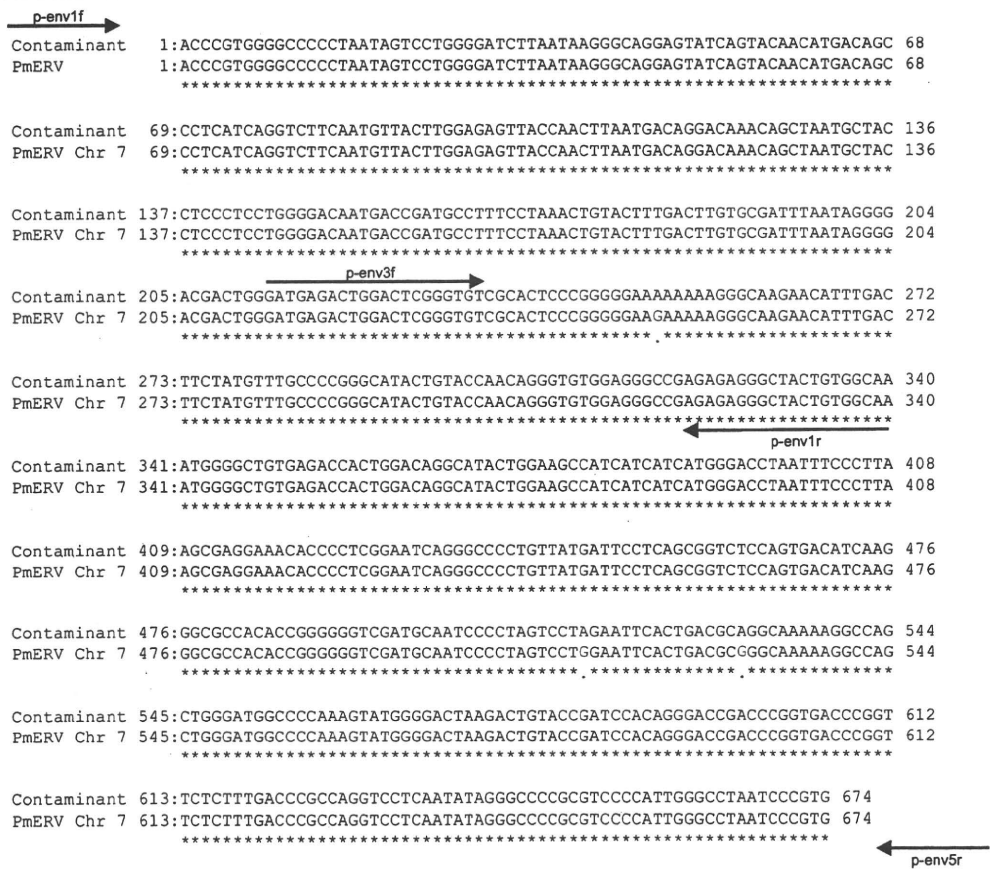

Figure 4 Sequence alignments of a partial *env* region of the contaminant in Kit I with a PmERV chr 7. Origins of the sequences used for the alignment are described in the Findings. Sequence alignments were performed using GENETYX Win ver. 6 (GENETYX, Shibuya, Tokyo, Japan).

were found more frequently in CFS patients than in healthy controls and not at all in water controls. Nonetheless, Lo et al. mentioned in the report that Platinum Taq from Invitrogen gave them the best results among *Taq* polymerases from several suppliers and was used to test the patient samples [13]. They also used Invitrogen's Superscript II RT and Platinum Taq for RT-PCR, albeit in a two-step cDNA synthesis and PCR amplification procedure [13]. As pointed out by Erlwein et al. in the comments [19] responding to the report by Lo et al. [13], assurance that control samples were assayed simultaneously with the positively identified ones in a blinded, randomized way was missing in their study, unfortunately.

The requirement for quality control to avoid contamination of endogenous retroviral genomes in test kits may differ depending on the intended purpose. However, in XMRV studies, many researchers conduct ultrasensitive PCR or RT-PCR to detect extremely small amounts of XMRV. Therefore, in the investigation of XMRV infection, researchers should prudently evaluate test kits for the presence of genomes of endogenous MLV as well as XMRV.

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Authors' contributions
RAF, ES, and TM designed the experiments. RAF and ES performed the experiments. TM wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References

1. Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, Klein EA, Malathi K, Magi-Galluzzi C, Tubbs RR, Ganem D, Silverman RH, DeRisi JL: **Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant.** *PLoS Pathog* 2006, **2**:e25.
2. Dong B, Kim S, Hong S, Das Gupta J, Malathi K, Klein EA, Ganem D, Derisi JL, Chow SA, Silverman RH: **An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors.** *Proc Natl Acad Sci USA* 2007, **104**:1655-1660.
3. Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL, Ruscetti SK, Bagni RK, Petrow-Sadowski C, Gold B, Dean M, Silverman RH, Mikovits JA: **Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome.** *Science* 2009, **326**:585-589.
4. Erlwein O, Kaye S, McClure MO, Weber J, Wills G, Collier D, Wessely S, Cleare A: **Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome.** *PLoS One* 2010, **5**:e8519.
5. Groom HC, Boucherit VC, Makinson K, Randal E, Baptista S, Hagan S, Gow JW, Mattes FM, Breuer J, Kerr JR, Stoye JP, Bishop KN: **Absence of xenotropic murine leukaemia virus-related virus in UK patients with chronic fatigue syndrome.** *Retrovirology* 2010, **7**:10.
6. Hong P, Li J, Li Y: **Failure to detect Xenotropic murine leukaemia virus-related virus in Chinese patients with chronic fatigue syndrome.** *Virol J* 2010, **7**:224.
7. McClure M, Wessely S: **Chronic fatigue syndrome and human retrovirus XMRV.** *BMJ* 2010, **340**:c1099.
8. Switzer WM, Jia H, Hohn O, Zheng H, Tang S, Shankar A, Bannert N, Simmons G, Hendry RM, Falkenberg VR, Reeves WC, Heneine W: **Absence of evidence of xenotropic murine leukemia virus-related virus infection in persons with chronic fatigue syndrome and healthy controls in the United States.** *Retrovirology* 2010, **7**:57.
9. van Kuppeveld FJ, de Jong AS, Lanke KH, Verhaegh GW, Melchers WJ, Swanink CM, Bleijenberg G, Netea MG, Galama JM, van der Meer JW: **Prevalence of xenotropic murine leukaemia virus-related virus in patients with chronic fatigue syndrome in the Netherlands: retrospective analysis of samples from an established cohort.** *BMJ* 2010, **340**:c1018.
10. Dolgin E: **Chronic controversy continues over mysterious XMRV virus.** *Nat Med* 2010, **16**:832.
11. Enserink M: **Chronic fatigue syndrome. Conflicting papers on hold as XMRV frenzy reaches new heights.** *Science* 2010, **329**:18-19.
12. Enserink M: **Chronic fatigue syndrome. New XMRV paper looks good, skeptics admit–yet doubts linger.** *Science* 2010, **329**:1000.
13. Lo SC, Pripuzova N, Li B, Komaroff AL, Hung GC, Wang R, Alter HJ: **Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors.** *Proc Natl Acad Sci USA* 2010, **107**:15874-15879.
14. Barnes E, Flanagan P, Brown A, Robinson N, Brown H, McClure M, Oxenius A, Collier J, Weber J, Günthard HF, Hirschel B, Fidler S, Phillips R, Frater J: **Failure to detect xenotropic murine leukemia virus-related virus in blood of individuals at high risk of blood-borne viral infections.** *J Infect Dis* 2010, **202**:1482-1485.
15. Cornelissen M, Zorgdrager F, Blom P, Jurriaans S, Repping S, van Leeuwen E, Bakker M, Berkhout B, van der Kuyl AC: **Lack of detection of XMRV in seminal plasma from HIV-1 infected men in The Netherlands.** *PLoS One* 2010, **5**:e12040.
16. Shepherd AJ, Wilson NJ, Smith KT: **Characterisation of endogenous retrovirus in rodent cell lines used for production of biologicals.** *Biologicals* 2003, **31**:251-260.
17. Weiss RA: **A cautionary tale of virus and disease.** *BMC Biol* 2010, **8**:124.
18. Silverman RH, Nguyen C, Weight CJ, Klein EA: **The human retrovirus XMRV in prostate cancer and chronic fatigue syndrome.** *Nat Rev Urol* 2010, **7**:392-402.
19. Kaye S, Robinson M, McClure M: **Chronic fatigue syndrome: xenotropic murine leukemia virus-related virus, murine leukemia virus, both, or neither?** *Proc Natl Acad Sci USA* 2010, **107**:E161.

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Detection of antibodies against human platelet antigens 15a and 15b by using a cell line panel

The platelet surface membrane contains a variety of molecules, including ABO blood type antigens (Curtis *et al*, 2000), human leucocyte antigens (HLAs), human platelet antigens (HPAs) and Nak[a] antigen. Antibodies to these molecules have been regarded as the principal causes of various reactions elicited by platelet transfusion, such as refractoriness and post-transfusion purpura. These antibodies are also thought to cause neonatal alloimmune thrombocytopenia and idiopathic thrombocytopenic purpura (Smith *et al*, 1995; Bordin *et al*, 1997).

The HPA-15 (Gov) alloantigen system is localized on the CD109 protein, a glycosylphosphatidylinositol-linked glycoprotein of 175 kDa that is found on several tissues and also on a subset of haematopoietic stem cells, progenitor cells and activated platelets and T cells. HPA-15 alleles differ by an A/C single nucleotide polymorphism at position 2108 of the coding region of *CD109* cDNA, resulting in a Tyr/Ser substitution at CD109 amino acid 682 (Schuh *et al*, 2002). Despite differences between races, the genotypic frequencies of HPA-15a and HPA-15b antigens are roughly equal (Cardone *et al*, 2004).

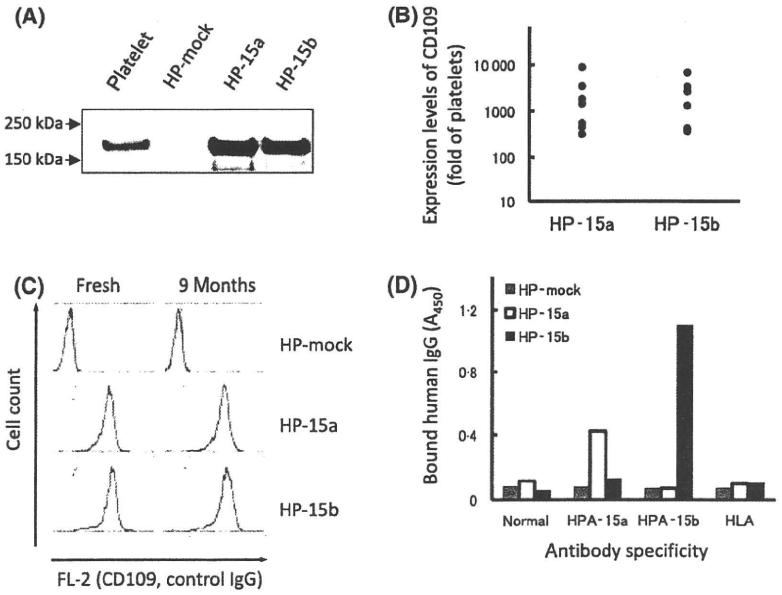

Fig 1. Expression of CD109 on HP-15 cell lines. (A) Immunoprecipitation and Western blot analyses of CD109 in the established cell lines. 1×10^9 human platelets and 5×10^5 each of HP-mock cells (pQCXIP-infected K562 cell), HP-15a cells and HP-15b cells were incubated with 2·5 μg of TEA 2/16 monoclonal anti-CD109 mouse antibody. After washing, cells were lysed with 1 ml of RIPA buffer (Thermo Scientific, Yokohama, Japan). The lysates were precipitated by 20 μl of anti-mouse IgG-conjugated M280 beads and subjected to Western blot analyses. Protein aliquots were subjected to 7·5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The electrophoresed proteins were transferred to a polyvinylidene fluoride membrane, and anti-CD109 sheep antibody (R&D system, MN, USA) and anti-sheep HRP-IgG (Rockland Immunochemicals, Inc., PA, USA) were used as the primary and secondary antibodies, respectively. Protein bands were visualized by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA, USA) and detected by ChemiDoc™ (Bio-Rad Laboratories, Hercules, CA, USA). (B) Expression levels of CD109 in HP-15a and HP-15b cell lines. Immunoprecipitation and Western blot analyses were carried out as described in (A). Then, the densities of the CD109 bands in the HP cells were calculated and compared with those of platelets derived from eight individuals. The densities were determined using ChemiDoc software (Bio-Rad Laboratories). (C) Stable expression of CD109 in HP-15 cell lines. HP-mock, HP-15a and HP-15b cells were cultured for 9 months in the presence of 0·5 μg/ml puromycin for the maintenance of transgene expression. Their reactivities with the isotype control antibody (thin lines) and CD109 monoclonal antibody (bold lines) were analysed by flow cytometry immediately (fresh) and 9 months after their establishment. (D) Detection of HPA-15 antibodies using HP-15 cell line panel. To evaluate the cell line panel, well-characterized sera were used for MAIPA using the cell line panel. MAIPA was performed according to the methods described by Berry (Berry *et al*, 2000) and Campbell (Campbell *et al*, 2007) with 3×10^5 panel cells substituting for platelets. Representative data are shown.

The immunogenicity for HPA-15 alloantigens appears to be similar to that of the antigens of the HPA-5 system, and the HPA-15b alloantigen appears to be more immunogenic than the HPA-15a alloantigen (Berry *et al*, 2000). The amount of CD109 molecules on the platelet is very low, is variable among individuals and is decreased by cooling and freezing (Berry *et al*, 2000). Therefore, preparing a feasible panel of platelets for detecting HPA-15 antibodies is difficult. Consequently, we attempted to establish cell lines that stably expressed larger amounts of CD109 compared to those in platelets for detecting HPA-15a and HPA-15b antibodies.

Recently, we reported a new method for the detection of Nak^a antibodies. This method was not affected by the presence of HLA antibodies (Hayashi *et al*, 2009). In this method, the target genes were introduced into K562 cells that did not express HLA molecules or other human neutrophil antigens. As K562 cells do not express HPAs, we adopted this method to establish cell lines stably expressing HPA-15a and HPA-15b. Briefly, full-length *CD109* cDNA provided Dr. Kawai (Wakunaga Pharmaceutical Co., Ltd., Hiroshima, Japan) were individually ligated into pQCXIP (Takara, Shiga, Japan) vectors. Subsequently, the vectors were packed into viral particles and used to infect K562 cells. Clones were selected based on CD109 expression, which was flow-cytometrically examined using the CD109 monoclonal antibody TEA 2/16 (BD Biosciences, Tokyo, Japan) (data not shown). Two clones that expressed HPA-15a and HPA-15b were selected and named HP-15a and HP-15b, respectively. Immunoprecipitation and Western blot analyses revealed that the established HP-15a and HP-15b cells expressed CD109 molecules that had the same molecular weight as those in platelets (Fig 1A). Densitometric analysis on the blotted molecules showed that HP-15 cells expressed 300–9000-fold more CD109 than platelets (Fig 1B). Such strong CD109 expression in HP-15a and HP-15b cells was observed continuously for at least 9 months (Fig 1C).

To examine whether our HP-15 cell line panel could be applied for clinical diagnosis, we analysed its properties by using monoclonal antibody-specific immobilization of platelet antigens (MAIPA) and well-characterized serum samples (Fig 1D). We tested two serum samples containing HPA-15a and HPA-15b antibodies that had been characterized by one of the authors. The result showed that HPA-15a and HPA-15b antibodies were unambiguously detected by the HP-15 cell panel. In contrast, the HP-15 cell line panel showed low background reactivity to 23 random control sera and six sera containing HLA antibodies that were characterized by LABScreen® (One Lambda, Canoga Park, CA, USA; data not shown) at the Japanese Red Cross Osaka Blood Center. The sensitivity of our HP-15 panel cell system to detect the CD109 antibody was determined using a monoclonal anti-human CD109 antibody derived from sheep (R&D system, Minneapolis, MN, USA) and horseradish peroxidase (HRP)-conjugated anti-sheep IgG (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA). We found that our assay system could detect the CD109 antibody at a concentration as low as 0·5 μg/ml (data not shown).

These results suggest that the new HP-15 cell panel system enables the detection of HPA-15 antigen-reactive platelet antibodies in human serum samples. However, it will be necessary to further validate and characterize our cell line panel by using additional samples containing normal serum.

Conflict of interest

The authors have no conflict of interests to declare.

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References

Berry, J.E., Murphy, C.M., Smith, G.A., Ranasinghe, E., Finberg, R., Walton, J., Brown, J., Navarrete, C., Metcalfe, P. & Ouwehand, W.H. (2000) Detection of Gov system antibodies by MAIPA reveals an immunogenicity similar to the HPA-5 alloantigens. *British Journal of Haematology*, **110**, 735–742.

Bordin, J.O., Kelton, J.G., Warner, M.N., Smith, J.W., Denomme, G.A., Warkentin, T.E., McGrath, K., Minchinton, R. & Hayward, C.P. (1997) Maternal immunization to Gov system alloantigens on human platelets. *Transfusion*, **37**, 823–828.

Campbell, K., Rishi, K., Howkins, G., Gilby, D., Mushens, R., Ghevaert, C., Metcalfe, P., Ouwehand, W.H. & Lucas, G. (2007) A modified rapid monoclonal antibody-specific immobilization of platelet antigen assay for the detection of human platelet antigen (HPA) antibodies: a multicentre evaluation. *Vox Sanguinis*, **93**, 289–297.

Cardone, J.D.B., Chiba, A.K., Boturão-Neto, E., Vieira-Filho, J.P.B. & Bordin, J.O. (2004) Gene frequencies of the HPA-15 (Gov) platelet alloantigen system in Brazilians. *Transfusion Medicine*, **14**, 433–437.

Curtis, B.R., Edwards, J.T., Hessner, M.J., Klein, J.P. & Aster, R.H. (2000) Blood group A and B antigens are strongly expressed on platelets of some individuals. *Blood*, **96**, 1574–1581.

Hayashi, T., Yasui, K., Matsuyama, N., Furuta, R.A., Hori, Y., Tanaka, S., Hirayama, F., Tani, Y., Shibata, H. & Inoue, M. (2009) Establishment of a novel method for detecting Nak antibodies by using a panel cell line. *Transfusion*, **49**, 390–392.

Schuh, A.C., Watkins, N.A., Nguyen, Q., Harmer, N.J., Lin, M., Prosper, J.Y., Campbell, K., Sutherland, D.R., Metcalfe, P., Horsfall, W. & Ouwehand, W.H. (2002) A tyrosine703serine polymorphism of CD109 defines the Gov platelet alloantigens. *Blood*, **99**, 1692–1698.

Smith, J.W., Hayward, C.P., Horsewood, P., Warkentin, T.E., Denomme, G.A. & Kelton, J.G. (1995) Characterization and localization of the Gova/b alloantigens to the glycosylphosphatidylinositol-anchored protein CDw109 on human platelets. *Blood*, **86**, 2807–2814.

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NOTCH1 PEST domain mutation is an adverse prognostic factor in B-CLL

B-cell chronic lymphocytic leukaemia (B-CLL) is a heterogeneous disease with a highly variable clinical course (Chiorazzi *et al*, 2005) and several factors at diagnosis predict prognosis and help guide treatment decisions (Seiler *et al*, 2006). Unmutated immunoglobulin heavy variable (*IGHV*) genes and high expression of zeta-chain-associated protein kinase 70 (ZAP-70) are well-known factors for poor prognosis (Rassenti *et al*, 2004). When they are discordant other cytogenetic and molecular biological factors such as 17p-, *IGHV3-21* or microRNA signature appear helpful (Gribben, 2008). NOTCH signalling activation was recently implicated in B-CLL cell survival and apoptosis resistance (Rosati *et al*, 2009) and in an attempt to identify the underlying mechanism we found a *NOTCH1* PEST domain mutation in a minority of B-CLL patients (Di Ianni *et al*, 2009). As these findings led us to hypothesize that this *NOTCH1* mutation might be a novel prognostic marker in B-CLL, we extended a DNA-based sequencing screening strategy to a consecutive series of 133 unselected B-CLL patients and, when possible, correlated the results with *IGHV* gene status, ZAP-70 expression and clinical outcome. Given the marked structural similarities between NOTCH1 and NOTCH2 and the recent demonstration of *NOTCH2* gain-of-function mutations in lymphomas (Lee *et al*, 2009) we also screened for the presence of *NOTCH2* mutations in a sub-group of these B-CLL patients.

NOTCH1 mutational status was correlated with *IGHV* gene status, ZAP-70 expression and clinical outcome in 111 (69 males, 42 females) of the 133 patients who were analysed. Median age at diagnosis was 60 years (range 40–84 years). According to the Binet staging system, 85/111 (76·5%) patients were in stage A, 19 (17·1%) patients were in stage B, and 7 (6·3%) patients in stage C. *IGHV* was unmutated in 45/111 (40·5%) patients and ZAP70 expression was >20% in 66/111(59·4%). Peripheral blood samples were collected at diagnosis, before disease progression or before any treatment. Mutations of *NOTCH1* exons 26, 27 and 34 were investigated by DNA-based polymerase chain reaction as previously described (Di Ianni *et al*, 2009). The mutation was confirmed by independent amplicon amplification.

To evaluate the clinical significance of *NOTCH1* mutation we selected time from diagnosis to initial treatment (TTT) as the primary end-point. Our first step in cohort validation was to analyse TTT in relation to *IGHV* status and ZAP70 expression. Median TTT was significantly shorter in patients with unmutated *IGHV* genes (36 vs. 77 months; $P = 0{\cdot}0028$) and in patients with ZAP70 expression >20% (45 vs. 77 months; $P = 0{\cdot}0135$).

A *NOTCH1* PEST domain mutation was found in 7/133 (5·3%) B-CLL patients, and extended our previous findings to a much larger cohort. Neither *NOTCH2* heterodimerization nor PEST domains were mutated in the sub-group of 73 patients, thus excluding a *NOTCH2* gene mutation from involvement in altered NOTCH signalling in B-CLL. As we found a *NOTCH1* mutation only in a small minority of patients, it seems other mechanisms come into play since both NOTCH1 and NOTCH2 are constitutively activated in all B-CLL patients (Rosati *et al*, 2009). Consequently a *NOTCH1* PEST mutation in this subgroup of B-CLL patients is therefore only one of the possible mechanisms implicated in the NOTCH signalling activation.

Six of the seven patients bearing *NOTCH1* activating mutation had unmutated *IGHV* genes and ZAP70 positivity. The other had mutated *IGHV* and ZAP70 positivity. Strikingly, median TTT was significantly shorter in all seven patients with *NOTCH1* mutation than in the 104 with wild-type *NOTCH1* (24·5 vs. 63 months, $P = 0{\cdot}0053$) (Fig 1A) and than in the 39 patients with unmutated *IGHV* genes and wild-type *NOTCH1* (24·5 vs. 48 months, $P = 0{\cdot}0481$) (Fig 1B). Interestingly, as patients carrying the *NOTCH1* mutation showed poorer prognosis than patients with unmutated *IGHV*, the *NOTCH1* mutation emerged as a potentially factor for poor prognosis in B-CLL.

The analysis of CD38 expression in the seven *NOTCH1* mutated patients did not reveal any correlation between these

Neonatal alloimmune thrombocytopenia caused by an antibody specific for a newly identified allele of human platelet antigen-7

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BACKGROUND: Neonatal alloimmune thrombocytopenia (NAIT) is a neonatal disorder characterized by maternal alloimmunization against fetal platelet (PLT) antigens inherited from the father. A healthy 30-year-old Japanese woman (Hit) gave birth to her second child after an uneventful pregnancy. Nine hours after birth, the infant presented with severe petechiae and a PLT count of 6×10^9/L.

STUDY DESIGN AND METHODS: To elucidate the maternal cause of NAIT in the infant, serologic and genetic studies, including PLT genotyping and sequence-based analysis, were conducted. Additionally, serologic screening for the new PLT antigen was performed.

RESULTS: Serum from the NAIT infant's mother contained antibodies directed against a human PLT antigen (HPA) of the newborn. Using five-cell-lineage flow cytometry, we localized the antigen to a PLT glycoprotein (GP). Subsequent monoclonal antibody immobilization of PLT antigen assay and PLT immunofluorescence inhibition experiments localized the antigen to the GPIIIa subunit of the GPIIb/IIIa complex. GPIIIa localization was confirmed by sequence-based typing studies, which identified a 1297C>T (407proline>serine substitution) mutation on the ninth exon of the GPIIIa gene. This mutation identified the third allele of HPA-7. Anti-Hit^a reacted with mutated GPIIIa-transfected cells but not with stable transfectants expressing wild-type GPIIIa. Serologic screening for Hit^a in the Japanese population revealed a phenotypic frequency of approximately 0.0015.

CONCLUSIONS: We identified a new third allele of HPA-7, which is characterized by a 1297C>T mutation in the GPIIIa gene. This 1297C>T allele was found in 0.15% of the Japanese population. An antibody against this antigen could be the cause of severe NAIT.

Antibodies against human platelet (PLT) antigens (HPAs) induce severe clinical problems, including neonatal alloimmune thrombocytopenia (NAIT), posttransfusion purpura, and refractoriness to PLT transfusion. To date, 24 HPAs have been identified using immune sera, 12 of which are grouped in six biallelic systems (HPA-1, -2, -3, -4, -5, and -15). Of the remaining 12, alloantibodies against the thetical, but not the antithetical, antigen have been observed.[1] The 6 major HPAs (HPA-1 to -6) and HPA-15 have been shown to be polymorphic in Japan, whereas polymorphism in the low-frequency HPA antigens (HPA-7 to -14 and -16) is extremely low or zero.[1,2] Alloantigenic determinants (epitopes) of all of these HPAs have been localized on PLT membrane glycoproteins (GP): GPIa, GPIb, GPIIb, and GPIIIa. With the exception of HPA-14, all of these alloantigens have been found to result from single-point mutations in the encoding genes, which lead to single-amino-acid substitutions.[1,2] The HPA-14b antigen is generated by three nucleotide deletions in the GPIIIa gene.[3] The GPIIb/IIIa protein complex is the PLT fibrinogen receptor αIIbβ3, which carries the majority of these HPAs. HPA-1, -4, -6, -7, -8, -10, -11, -14, and -16 are located on GPIIIa, whereas HPA-3 and -9 are located on GPIIb. Another integrin, GPIa/IIa (α2β1),

ABBREVIATIONS: FCM = flow cytometry; GP = glycoprotein; MAIPA assay = monoclonal antibody-specific immobilization of PLT antigens assay; MPHA = mixed passive hemagglutination; NAIT = neonatal alloimmune thrombocytopenia; np = nucleotide position (when followed by a number).

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is a mediator of PLT adhesion to both fibrillar and nonfibrillar collagen. HPA-5 and -13 are located on the GPIa protein.[1,2] The other complex, GPIb-a/Ib-b/IX, is composed of three polypeptides, namely, GPIbα, GPIbβ, and GPIX. GPIb functions as a receptor for von Willebrand factor and thrombin. HPA-2 and HPA-12 are located on GPIbα and GPIbβ, respectively.[1,2]

The phenotypic frequencies of the HPA system are known to vary among populations.[2] Moreover, using newly developed genotyping techniques, it has been demonstrated that gene frequencies also exhibit variation among different populations.[2] Notably, several remarkable differences have been observed between the gene frequencies of HPAs in Caucasian and non-Caucasian (Asian) populations.[2] In Caucasians, the gene frequency of HPA-1b is calculated to be 0.10 to 0.15, whereas in Asian populations, the frequency of this gene is extremely low,[2,4] and that of HPA-1a is almost 1.0.

NAIT is a severe bleeding disorder of the fetus caused by the reaction of maternal alloantibodies against fetal blood antigens, which arises due to blood group incompatibility between the mother and fetus. Intracranial hemorrhage leading to death or permanent neurologic disability may occur in the fetus. NAIT is strongly associated with HPAs, with these antigens being found in almost all NAIT cases. HPAs 1 through 16 have been identified from their association with NAIT.[3,5-18] The frequencies of NAIT cases due to HPAs differ among different populations. For example, the HPA-1a antibody is known to be the antibody most frequently responsible for NAIT in the United States, accounting for 86% of NAIT cases due to HPAs.[19] In contrast, the frequency of HPA-4b is extremely low in Caucasian populations, although two cases of HPA-4b–incompatible NAIT have been reported.[20,21] Recently, some severe cases of NAIT due to anti-HPA-9b (Max) have been reported in Europe and the United States.[22,23] However, to date, no NAIT case associated with anti-HPA-1a or -1b has been reported in Japan. HPA-4b–incompatible NAIT represents the majority (72%) of HPA-related NAIT cases in Japan,[24] and cases due to HPA-2b, HPA-3a, HPA-5b, and HPA-6b have also been reported.[22] Furthermore, before the present study, the low-frequency HPA-7 to HPA-13 antigens were found to be monomorphic in Asian (including Japanese) populations.[2,4,25]

In this study, we describe a case of NAIT from a hospital, for which requested analysis was performed. We detected a PLT antibody and identified a new alloantigen on the GPIIIa molecule. In addition, we investigated the frequency of the new antigen in the Japanese population.

CASE REPORT

A healthy 30-year-old woman (Hit), who had no significant medical history, gave birth to her second child (weight, 2310 g; PLT count, 12×10^9/L) by cesarean section after an uneventful 40-week and 5-day pregnancy. Nine hours after birth, the infant presented with severe petechiae and with a PLT count of 6×10^9/L. The infant was otherwise healthy (Apgar score, 7/8). The family to which this NAIT patient belongs will hereafter be referred to as Hit.

The mother's PLT count was normal (180×10^9/L). The infant received intravenous immunoglobulin G (IgG) and was discharged 18 days later, with a PLT count of 100×10^9/L. The woman's first child, whose father was the same as the second child's, was delivered with no specific clinical sign of bleeding.

During the preparation of this article, the mother gave birth to a third child. The third infant was also found to have NAIT caused by the same alloantibody and was treated by PLT concentrate transfusion.

MATERIALS AND METHODS

Blood samples and PLT serology

Blood samples from the mother, father, and infant—who were referred to us shortly after the child's delivery because of suspected NAIT—and subsequently from other family members, were analyzed. Informed consent was obtained from all participants. The blood samples of the parents and the infant were genetically typed for PLT alloantigens to detect incompatibilities.

The specific PLT antibodies were examined serologically using the mixed passive hemagglutination (MPHA) test as previously described.[26,27] For the characterization of PLT alloantibodies, panel PLTs were selected from Osaka blood donors with known HPA and O phenotypes. The maternal serum was tested with the paternal and infant PLTs (crossmatch) and 42 panel PLTs, including HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -6a, and/or -6b and with PLTs from other Hit family members.

The antigen specificities of the maternal Hit antibody were also examined by five-cell-lineage analysis using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA).[28,29]

Sera from the mother and infant were screened for HLA antibodies using commercially available kits (LABScreen PRA I and II, One Lambda, Inc., Canoga Park, CA).

Monoclonal antibody immobilization of PLT antigen and inhibition test using monoclonal antibodies to HPA molecules

We speculated that the Hit mother's serum included antibodies against one of the PLT-borne GP molecules. To confirm this speculation, a rapid modified monoclonal antibody-specific immobilization of PLT antigens (MAIPA) assay was performed using Hit serum and the father's PLTs.[30] The monoclonal antibodies (MoAbs) used

in the modified MAIPA were anti-CD49b (GPIa/IIa; Immunotech, Prague, Czech Republic), anti-CD42b (GPIb; Chemicon, Millipore Corp., Billerica, MA), anti-CD41a (GPIIb; Biolegend, San Diego, CA), and anti-CD61 (GPIIIa; Beckman Coulter, Inc., Fullerton, CA).

Additionally, an inhibition test was performed to specify the GP molecule on the PLTs. Aliquots of washed paternal PLT suspension were incubated with 20 μL of MoAb against GP proteins and/or 10 μL maternal serum. After three washes with phosphate-buffered saline (PBS), the PLTs were suspended in 50 μL of PBS-bovine serum albumin (BSA) and stained with 40 μL of fluorescence-labeled rabbit anti-human IgG (Dako Cytomation, Glostrup, Denmark) for 30 minutes. The labeled PLTs were washed twice and fluorescence intensity was measured by flow cytometry. The mean fluorescence intensity was calculated and compared between with and without the maternal serum. The MoAbs against GP proteins and other proteins expressed on the PLTs used in this study were as follows: CD36 (GPIV; Immunotech), CD41a (GPIIb; BD Biosciences), CD42a (GPIX; Immunotech), and CD61 (GPIIIa; BD Biosciences).

Genotyping of HPAs

Genomic DNA was extracted from white blood cells (WBCs) in 5 mL of ethylenediaminetetraacetate blood using a commercially available kit (QIAquick, Qiagen, Cologne, Germany). Genotyping of HPA-1 to -8 and -15 of the Hit family members was performed by polymerase chain reaction (PCR) with sequence-specific primers and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis.[31-33]

Sequence analysis of exon-specific PCR products

To analyze all the exons of the GPIIIa gene, these exons were amplified from the father's genomic DNA, using primers pairs generated from GPIIIa-specific intron sequences.[34] The PCR conditions used were as described previously.[34] The PCR products were purified on spin columns and directly sequenced using a commercially available kit (Big Dye Terminator, Applied Biosystems, Foster City, CA), with PCR primers used as sequencing primers, in a genetic analyzer (ABI 3130xl, Applied Biosystems). Furthermore, the PCR products were cloned using a cloning kit (TA, Invitrogen Corp., Carlsbad, CA) and some of the clones from the Exon 9 products were sequenced.

Genotyping of a T/C substitution of the GPIIIa gene by PCR-RFLP

For confirmation of the sequences, the nucleotide at nucleotide position (np) 1297 was also analyzed by PCR-RFLP using mismatch primers. The PCR was performed using the following primer set: FHPA01 (5′-GCATTG AGGCCAAGGTGCGAAGCTG-3′)/FHPA02 (5′-GGTGACCT GGACGATCAGGCTG-3′). FHPA01 is a mismatch primer. The sequence of the FHPA01 primer is identical to that of np 1271 to np 1295 of the GPIIIa gene, with the exception of np 1291 (G>A). The primer was designed to detect a C>T substitution at np 1297 by creating a restriction site for the restriction enzyme *Xmn*I, which recognizes GAANNNNTTC sequences. The PCR conditions were 95°C for 50 seconds and 68°C for 30 seconds, with a shuttle PCR of 35 cycles. The size of the PCR product was 98 bp. The amplified fragments were digested with *Xmn*I. When the amplified fragment contained a T nucleotide at np 1297, *Xmn*I cut the 98-bp fragment into 75- and 23-bp fragments.

Expression of allele-specific constructs in 293T cells

Cells of the 293T human kidney cell line (obtained from the American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cultured cells were cotransfected with mutated GPIIIa or nonmutated GPIIIa inserted into a pQCX1N vector (Clontech Laboratories, Inc., Mountain View, CA; pQ7new and pQ3a, respectively) and nonmutated GPIIb inserted into pQCX1P (Clontech Laboratories, Inc.; pQ2b), a mammalian expression vector containing neomycin and puromycin resistance genes. Cells lines were selected with 0.2 mg/mL neomycin and 0.6 mg/mL puromycin 3 days after infection. A suspension containing 4×10^5 transfectant cells was incubated with 20 μL of the mother's serum (or normal human serum as a negative control) for 15 minutes at room temperature. Labeled cells were then washed twice with PBS/0.2% BSA, costained with both 0.2 μL of anti-human IgG–phycoerythrin (Jackson ImmunoResearch Laboratories, West Grove, PA) and peridinin chlorophyll protein–conjugated MoAb CD61 (BD Biosciences) for 15 minutes, washed twice with PBS/0.2% BSA, and analyzed by selection for CD61-positive cells using a flow cytometry (FCM)-gating system. A suspension containing the same number of cells was also incubated with 10 μL of CD41a–fluorescein isothiocyanate MoAb (BD Biosciences) as a positive control, washed twice with PBS/0.2% BSA, and analyzed by a flow cytometer.

Screening for the Hit^a antigen in Japanese blood donors

Approximately 4500 Osaka blood donors were tested for Hit^a using the MPHA method with Hit serum.