First report of human immunodeficiency virus transmission via a blood donation that tested negative by 20-minipool nucleic acid amplification in Japan

The Japanese Red Cross (JRC) blood centers screen donated blood for infectious agents using serologic assays and nucleic acid amplification testing (NAT). A multiplex NAT for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus Type 1 (HIV-1) with a minipool (MP) format comprising 50 seronegative samples was started in 2000.[1] During the implementation of the 50-MP-NAT in 2003, HIV-1 was transmitted through fresh-frozen plasma (FFP) from one blood donor during the window period. To reinforce NAT screening, the pool size was decreased to 20 in 2004. Since 20-MP-NAT implementation in 2004, we have found 19 donations that were seronegative but positive for HIV in the 20-MP-NAT. The rate of HIV-infected donations that were positive only in the NAT was approximately 1 in 2.7 million. No transfusion-transmitted HIV infection (TT-HIV) has been reported in Japan since the 20-MP-NAT was introduced.

In November 2013, anti-HIV was detected in a blood sample from a repeat male blood donor aged in his 40s. Western blotting (New LAV Blot 1, Bio-Rad, Hercules, CA), real-time reverse transcription–polymerase chain reaction assay (Cobas TaqScreen HIV, Roche, Basel, Switzerland), and transcription-mediated amplification assay using a kit (Procleix Ultrio ABD, Novartis Diagnostics, Emeryville, CA) confirmed HIV-1 infection. A qualitative NAT for HIV-1 (Cobas TaqMan, Roche) detected a plasma HIV-1 viral load of 4.7×10^4 copies/mL. A cryopreserved sample of plasma from his previous donation in February 2013 was retested in accordance with the Japanese guidelines for lookback studies on blood products. Using individual donation (ID-) NAT, the Cobas TaqScreen HIV (plasma input volume, 850 µL; 95% limit of detection [LOD], 24.3 IU/mL) detected HIV-1 RNA in an archived blood sample from his previous donation, whereas the Procleix (plasma input volume, 500 µL; 95% LOD, 19.6 IU/mL) did not. Each of these NAT assays was performed as a single test. The low plasma volume in the archival sample did not allow for repeat analysis.

Red blood cell (RBC) and FFP components were prepared from the previous donation and transfused into two recipients. The RBCs were transfused to a female patient in her 80s. A pretransfusion sample and a posttransfusion sample collected 9 months after transfusion were HIV seronegative. The latter sample was also negative for HIV RNA. The FFP was transfused 8 months after donation to a male patient in his 60s, from whom a pretransfusion sample was seronegative for HIV. Serologic tests and NAT assay identified HIV-1 infection in this recipient at 34 days after transfusion, and the plasma HIV-1 viral load was 1.1×10^6 copies/mL (Fig. 1).

The viral sequences determined in blood samples from both the donor (postseroconversion donation) and the FFP recipient differed by only one among 341 nucleotides in the *env* region (99.7% identity) and by four of 2800 nucleotides in the *pol* region (99.9% identity). Such high genetic similarity among the sequences supported the notion that HIV had been transferred from the donor to the FFP recipient. Isolates of HIV-1 from the donor and recipient were Subtype B, which is the most common among individuals infected with HIV-1 in Japan. Major antiretroviral drug-resistant mutations were not detected in either the donor or the recipient. Sequencing the HIV-1 5′-long terminal repeat, which was the target region of our NAT screen, did not detect HIV-1 mutations that caused false-negative NAT results.[2]

To estimate the HIV-1 viral load in the implicated blood, the sensitivity of both the Cobas TaqScreen HIV and the Procleix was reassessed by probit analysis using serial threefold dilutions (four replicates per dilution) of postseroconverted plasma (4.7×10^4 copies/mL) from the donor, which revealed that the 95% LOD of both NAT screens was 10 copies/mL. The archived blood sample from the implicated donation was reactive in the Cobas, but not in the Procleix screen; therefore, we speculated that the viral load in the donor plasma was approximately at the detection limit of the two NATs. Thus, the estimated total amount of HIV-1 in the FFP (containing

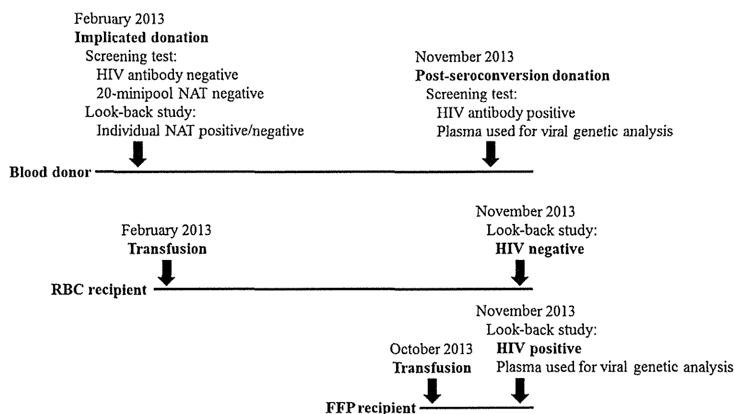

Fig. 1. Timeline of blood donations, transfusions, and infections.

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approximately 240 mL of plasma) that caused TT-HIV was fewer than 2400 copies.

The prevalence of HIV infections detected by NAT or antibody screening among blood donors during 2012 was 1.3 per 100,000 donations, whereas the prevalence among first-time blood donors has recently been five to six per 100,000 donations. This figure exceeded the frequency of individuals who were newly diagnosed with HIV infection among the general population aged 15 to 64 years in 2012 (1.2 per 100,000).[3] The donor described herein was probably in the very early stage of HIV-1 infection at the time of the implicated donation. That blood donation is being used for testing individuals with high-risk behaviors for HIV transmission is a concern. Questions given to blood donors about behaviors that confer risk for HIV are probably not being answered precisely. Methods of confirming whether or not donors understand the questions and the need to answer them precisely need to be improved.

This report describes the first known case of TT-HIV through a 20-MP-NAT–negative blood component in Japan. Our results indicate that ID-NAT using even the most sensitive methods available today might not detect HIV-1 in window period donations. Transfusion with FFP, but not RBC, components resulted in HIV-1 transmission in the TT-HIV case described herein. Similar cases with differential HIV transmission via blood components have been reported in other countries.[4] However, TT-HIV arising via an ID-NAT–negative blood component has not been identified in Japan or in any other country where donated blood is screened by NAT for HIV. We estimated that the risk of collecting a unit during the ID-NAT–negative HIV-1 window period is 2.75 per 5.5 million donations in Japan.[5] The JRC will introduce ID-NAT screening in August 2014, which should further reduce window period transmissions. However, even ID-NAT might not completely eliminate the window period of HIV-1 infection.

In the era of very high-sensitive ID-NAT or MP-NAT, TT-HIV will arise mainly in patients transfused with components with larger plasma volumes such as FFP and apheresis platelets, because these components contain a larger amount of HIV. Although pathogen inactivation technologies might help to reduce residual risk, the introduction of pathogen inactivation technology should be determined after carefully considering the balance among benefit, risk, and cost.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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N-Acetylcysteine for thrombotic thrombocytopenic purpura: is a von Willebrand factor–inhibitory dose feasible in vivo?

We read with interest the recent report by Li and colleagues[1] describing the first apparent successful use of *N*-acetylcysteine (NAC) in refractory thrombotic thrombocytopenic purpura (TTP). As we had previously described a case of NAC failure in a refractory TTP case that subsequently responded to bortezomib,[2] we sought to clarify whether our NAC dosing protocol differed from that utilized by Li and coworkers.[1]

As the only precedent for NAC dosing in TTP was preclinical,[3] we too adapted a clinical acetaminophen overdose protocol.[4] Our patient received 150 mg/kg NAC as a 1-hour bolus, followed by 50 mg/kg over 4 hours and then 100 mg/kg over the next 16 hours for the first day of dosing (with a cumulative exposure of 15 g in the first 24 hr). Twice-daily plasma exchange could not be interrupted

Establishment of culture systems for Genotypes 3 and 4 hepatitis E virus (HEV) obtained from human blood and application of HEV inactivation using a pathogen reduction technology system

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BACKGROUND: It has been demonstrated that the hepatitis E virus (HEV) can be transmitted via blood transfusion, and the risk of HEV transmission via transfusion has become a major global concern. An HEV culture system for blood-derived HEV has been sought to obtain valuable knowledge of the virus and the risk of HEV infection through blood products.

STUDY DESIGN AND METHODS: We endeavored to establish an HEV culture system using RNA-positive blood specimens for Genotypes (G) 3 and 4 and applied this system to evaluate tissue culture infectious dose (TCID). We applied this method to investigate the potential of the Mirasol pathogen reduction technology (PRT) system (Terumo BCT) to inactivate live HEV in contaminated platelet samples (PLTs). PLTs were spiked with cultured HEV G3 or G4 and then treated with the Mirasol PRT system. PLTs were examined before and after the treatment for HEV load using TCID titration.

RESULTS: We successfully established two strains for HEV production: the JRC-HE3 strain for G3 and the UA1 strain for G4. The Mirasol PRT system expressed more than 3 log inactivation for JRC-HE3 and more than 2 log inactivation for UA1.

CONCLUSION: The Mirasol PRT system inactivated greater than 2 to 3 logs of live HEV in PLTs and can potentially be used to lower the possibility of blood-borne HEV transmission. The G3 and G4 HEV inocula identified in this study and the hepatoma cell culture system provide a new means to assess HEV infectious titer and to evaluate other pathogen reduction strategies.

The hepatitis E virus (HEV), the causative agent of hepatitis E, was once believed to be transmitted orally and to be particularly localized in developing countries and regions.[1,2] However, a recent report demonstrated that HEV is spreading throughout the world, including industrialized nations.[3,4] HEV can also be transmitted by blood transfusion, and occasionally causes severe hepatitis,[5,6] so the risk of HEV infection via transfusion has become a major global concern.

HEV is roughly classified into four genotypes (G1-G4). Among these, G3 is the most widely distributed worldwide, while G4 has a tendency to cause severe hepatitis and is localized mainly in Asia.[7,8] For these reasons, the establishment of an HEV culture system, particularly for G3 and G4, has long been attempted, because it would be applicable to the assessment of technologies for blood product safety and as a tool to elucidate the HEV infection mechanism. Recently, two HEV cultivation systems using two cell lines—human hepatoma cells (PLC/PRF/5 cells) and human lung adenocarcinoma cells (A549 cells)—as the hosts, with HEV specimens derived from the feces or

ABBREVIATIONS: cDNA = complementary DNA; G = genotype; ORF = open reading frame; PRT(s) = pathogen reduction technology (-ies); TCID = tissue culture infectious dose.

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serum of HEV-infected individuals, have been established.[9,10] However, recent studies have demonstrated that the viruses obtained from the blood have an envelope-like structure that differs from the structure of those obtained from feces.[11] Therefore, in our view, it was reasonable to establish HEV cultivation systems using G3 and G4 HEV commonly isolated in Japan, from HEV RNA–positive blood specimens. Our aim was also to apply this system to perform an assay of HEV infectious titer, referred to as a tissue culture infectious dose (TCID), to evaluate the infection risk of HEV through blood or plasma products.

Although the risk of HEV infection via transfusion is now being recognized all over the world, an assay for viral infectivity using HEV proliferated in a cultured cell line originally isolated from HEV RNA–positive blood specimens did not exist. In addition, an assessment of the ability of a pathogen reduction technology (PRT) system to inactivate HEV virus in blood samples had never been performed before. We investigated the effect of the Mirasol PRT system on cultured G3 and G4 HEV originally derived from blood specimens and hereby report it.

MATERIALS AND METHODS

Establishment of HEV culture systems

HEV specimens

Ten plasma specimens and four serum specimens containing G3 or G4 HEV were employed. Plasma and serum were obtained from blood donors and patients who had been confirmed to be HEV RNA positive. The viral concentration of the specimens ranged from $10^{5.6}$ (HRC-HE21) to $10^{7.5}$ (JRC-HE3) copies/mL. The accession numbers of some specimens are also described in Table 1.

Cell culture and virus inoculation

The procedure for cell culture, HEV infection, and the maintenance of HEV-infected cells has been discussed in a previous report by Okamoto.[8] Briefly, human hepatoma cells (PLC/PRF/5 cells) and human lung adenocarcinoma cells (A549 cells; Health Science Research Resources Bank, Tokyo, Japan) were used in this assay. The cells were then infected with 2.5 mL of viral specimens for 2 hours at 37°C in a 5% CO_2 incubator. HEV-infected cells were maintained in a medium (maintenance medium) consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and medium 199 (Invitrogen, Tokyo, Japan), containing 2% heat-inactivated fetal calf serum, 30 mmol/L Mg^{2+} (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and 2.5 µg/mL amphotericin B (Wako Pure Chemical Industries, Ltd), and were cultured at 37°C in a humidified 5% CO_2 atmosphere using 10 mL of maintenance medium. Whole media were recovered weekly and replaced with fresh maintenance media. HEV RNA copies in the recovered media were quantified using real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis. The method for HEV RNA quantification is summarized below.

Confirmation of HEV infectivity against cells and method for quantifying HEV TCID

The acquisition of HEV infectivity was confirmed by detection of the viral progeny in the recovered cell culture supernatant. Given the establishment of HEV infection, the total number of viral RNAs existing in the recovered medium was overwhelmingly larger than the originally inoculated number. Viral TCID was investigated by limiting dilution analysis. The original viral solution for which the concentration of viral RNA (copies/mL) was already determined was serially diluted tenfold. The maximum dilution ratio against the original viral solution expressing infectivity on host cells was examined. The number of copies of HEV RNA required to express infectivity against host cells was calculated using both the original viral RNA concentration and the ratio of dilution.

TABLE 1. HEV specimens used for the establishment of the culture system

HEV strain	Source	Genotype	Log copies/mL (original concentration of specimen)	GenBank Accession Number for 412 bp (full genome)
HRC-HE21	Plasma	3	5.6	AB670957
HRC-HE22			5.8	AB670958
HRC-HE30			5.6	AB670966
HRC-HE80			6.0	AB671016
HRC-HE104			6.7	AB602891 (AB630970)
HRC-HE121			6.6	AB671054
HRC-HE159			5.7	AB671092
JRC-HE1			6.8	AB434144
JRC-HE3			7.5	AB434146 (AB630971)
JRC-HE8			5.9	AB434151
UA1	Serum	4	7.2	Not determined
UA2			6.9	
UA3			5.8	
SA1		Not determined	6.2	

Quantitation of HEV RNA

Quantitation of HEV RNA was performed following a previously reported method with minor modifications.[5] Briefly, RT-PCR was used to determine the number of copies of HEV RNA by targeting 75 nucleotides of a highly conserved sequence in the open reading frame (ORF) 2 region. The conditions for the RNA amplification stage were as follows: 25 μL of extracted RNA was incubated at 50°C for 30 minutes and at 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds and 60°C for 1 minute. Real-time RT-PCR was then performed using PCR and a Sequence Detection System (TaqMan and PRISM 7900, respectively, Applied Biosystems, Tokyo, Japan). The sensitivity of this system was determined to be 25 copies/mL, with a 95% confidence interval of 13 to 166 copies/mL by logistic analysis. The quantitation standard for HEV RNA was generated by transcribing the HEV complementary DNA (cDNA) of the HEV ORF2 region cloned into a plasmid (pCRII-TOPO, Invitrogen).

Full genome sequencing of HEV RNA

We referred to a previous report by Urayama and colleagues[12] for the method of full genome sequencing of HEV RNA. Total RNA was extracted either from 300 μL of HEV-positive blood specimens or from 200 μL of recovered cell culture supernatant. RT of HEV RNA was performed at 42°C for 1 hour. Consequently, nearly the full length of the cDNA was constructed. This cDNA was divided into two fragments: a 3′ fragment (3960 nucleotides) and a 5′ fragment (3160 nucleotides).

Phylogenetic analysis of HEV isolates

Complete or nearly complete sequences of HEV isolates were determined as previously described.[12] Sequences were aligned with the reported HEV strains using a computer program (ClustalW, Version 1.8, http://www.genome.jp/tools/clustalw/).

Photochemical inactivation of HEV in platelet samples

Preparation of platelets

Rejected platelets (PLTs), which are not clinically suitable to be used as blood derivatives because the alanine aminotransferase value exceeds the acceptance criteria, were used in this assay. All PLTs were collected by apheresis methods using equipment of the component collection system of Haemonetics (Braintree, MA), as well as TRIMA and TERUSYS of Terumo BCT (Lakewood, CO). All procedures were accomplished according to the manufacturers' protocols. The basic criteria for the volume and concentration of PLTs were 190 to 250 mL/bag and 1.9×10^{11} to 4.7×10^{11}/bag, respectively.

Treatment of PLTs with the Mirasol PRT system

Mirasol PRT treatment of PLTs was performed as previously described.[13] Briefly, 35 mL of riboflavin solution was added to a Mirasol PRT system kit to obtain a final concentration of 50 μmol/L, the unit was then exposed to a dose of UVB light (6.24 J/mL) and then left to rest for 30 minutes at an ambient temperature to prevent activation of the PLTs.

Sampling of HEV specimens from PLTs

Three milliliters of the pre-Mirasol-treated HEV PLT specimens was collected after the PLTs were thoroughly mixed with the virus in the dedicated bags, and the same volume of posttreated HEV specimens was likewise acquired. Titration samples for HEV infectivity were obtained from the supernatants of PLTs by centrifugation at 3000 rpm ($1750 \times g$) for 15 minutes under ambient atmosphere.

Confirmation of the noncytotoxicity against a cultured cell line caused by plasma from both pre- and post-Mirasol-treated PLTs

A cytotoxicity test against A549 cells was performed using virus-free control PLTs. This experimental condition was the same as the HEV infectivity assay, as described in the paragraph, "Cell culture and virus inoculation." Both pre- and post-Mirasol PRT-treated plasma obtained from the supernatant of PLTs were laid onto A549 cells for 2 hours. After the plasma was removed, the cells were cultured to confirm if any positive cell death or expression of a negative impact on cell growth were seen.

RESULTS

Establishment of the HEV culture systems

Selection of an HEV strain to establish a culture system using A549 cells

Fourteen G3- and G4-containing HEV specimens from plasma or serum (Table 1) were examined. All samples were diluted to $10^{4.8}$ copies/mL (total amount, $10^{5.2}$ copies in 2.5 mL) and then inoculated into A549 cells (Fig. 1). Consequently, only two HEV strains (JRC-HE3 for G3 and UA1 for G4) were found to be infectious. The HEV load of JRC-HE3 reached a plateau at an approximate concentration of 10^6 copies/mL (total amount, 10^7 copies in 10 mL of recovered medium) at around 42 days (6 weeks) postinoculation. On the other hand, UA1 reached a plateau at $10^{3.3}$ copies/mL at 60 days postinoculation.

HEV cultivation of JRC-HE3 using PLC/PRF/5 cells to procure a higher load of viral progeny

JRC-HE3 was diluted to $10^{5.5}$ copies/mL ($10^{5.9}$ copies in 2.5 mL) and then inoculated into alternative cells, namely, PLC/PRF/5 cells (Fig. 2). The viral concentration increased until 91 days (13 weeks) postinoculation and reached a plateau at an approximate concentration of 10^8 copies/mL (10^9 copies in 10 mL of recovered medium;

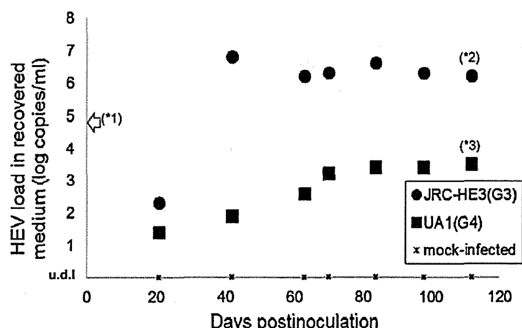

Fig. 1. Selection of HEV strains for the establishment of a culture system using A549 cells. The initial concentrations of the HEV strains used were all $10^{4.8}$ copies/mL ($10^{5.2}$ copies in 2.5 mL; *1). The concentrations of JRC-HE3 for G3 and UA1 for G4 in the recovered culture medium at 112 days postinoculation were $10^{6.2}$ copies/mL ($10^{7.2}$ copies in 10 mL) (*2) and $10^{3.3}$ copies/mL ($10^{4.3}$ copies in 10 mL; *3), respectively. A549 cells were utilized as the host for this assay. u.d.l. = under the detection limit.

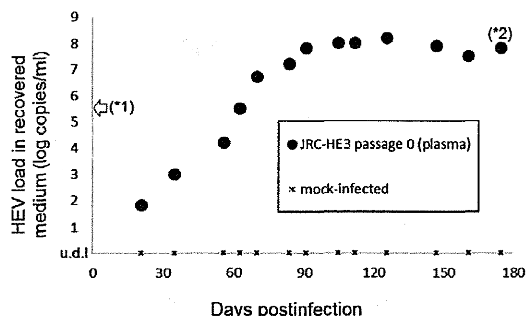

Fig. 2. Confirmation of a higher load of HEV progeny using PLC/PRF/5 cells. A high concentration of HEV progeny was obtained when PLC/PRF/5 cells were used. The HEV JRC-HE3 strain was inoculated at $10^{5.5}$ copies/mL ($10^{5.9}$ copies in 2.5 mL; *1). The load of JRC-HE3 in recovered culture medium at 175 days postinoculation was $10^{7.9}$ copies/mL ($10^{8.9}$ copies in 10 mL; *2). No viral progeny were detected in the mock-infected samples.

Fig. 2). It was possible to obtain a higher load of JRC-HE3 with PLC/PRF/5 cells than with A549 cells for virus proliferation.

Serial culture passage of JRC-HE3 using PLC/PRF/5 cells

HEV production resulting from different JRC-HE3 culture passages was examined using PLC/PRF/5 cells. For the first cultivation, an original HEV-positive-plasma specimen was used (Fig. 2, Passage 0). This means that the viral progeny referred to as Passage 1 was obtained from the result of Passage 0 infection. Each viral progeny from the specimens at 70 days (10 weeks) postinoculation was diluted to $10^{5.7}$ copies/mL before the next infection step (Fig. 3). The growth curves obtained for viral production were identical for all passages (Passages 1-4).

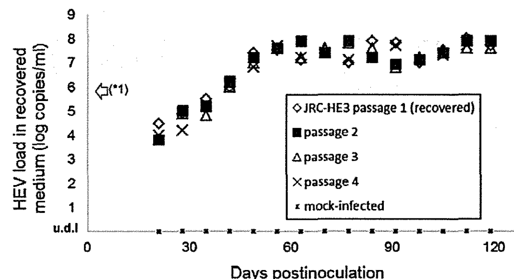

Fig. 3. JRC-HE3 serially passaged using PLC/PRF/5 cells. HEV samples in recovered culture media were diluted to $10^{5.7}$ copies/mL ($10^{6.1}$ copies in 2.5 mL; *1). Each viral sample from specimens at 70 days (10 weeks) postinoculation was inoculated into PLC/PRF/5 cells for each successive passage. There were no significant differences between the viral production curves from each passage. No viral progeny were detected in the mock-infected samples.

Genomic analysis of JRC-HE3 associated with culture passage and longer incubation of the virus

Full genome sequencing of JRC-HE3 was carried out on the original plasma (Passage 0) and progeny after long-term incubation (119 days [17 weeks], Passage 4), and genomic substitutions and amino acid alterations were investigated (Table 2). Consequently, only point substitutions at five bases in the ORF1 region and duplicate substitution at two bases in the ORF2 and ORF3 regions were found, respectively. These substitutions were demonstrated to result in alterations at four amino acid sites. Note that, due to triplet code degeneracy, some of the observed genomic base substitutions did not result in amino acid alteration.

Infectious titration of JRC-HE3 and UA1 using A549 cells and PLC/PRF/5 cells

Limited dilution assays were performed to determine the minimum HEV RNA load for infectivity against two cell lines. High loads of JRC-HE3 ($10^{9.4}$ copies/2.5 mL or $10^{8.5}$ copies/2.5 mL) were serially diluted 10-fold and inoculated into A549 cells and PLC/PRF/5 cells, respectively. Viral progeny were quantitatively investigated at 3, 6, and 9 weeks postinfection. Consequently, JRC-HE3 expressed its infectivity against A549 cells (Fig. 4A) and PLC/PRF/5 cells (Fig. 4B) even at 10^4- and 10^3-fold dilutions,

TABLE 2. HEV RNA and amino acid substitution associated with in vitro passaging and the longer incubation of HEV JRC-HE3 obtained during Passage 4 (Fig. 3)

ORF 1, five bases		Methyl transferase G137G [GGU(G) ⇒ GGC(G)]	
		Papain-like cysteine protease D452D [GAU(D) ⇒ GAC(D)]	
		Domain X W741P [UGG(W) ⇒ CGG(P)]	
		Domain X E871K [GAG(E) ⇒ AAG(K)]	
		Helicase F1124S [UUC(F) ⇒ UCC(S)]	
ORF 3 and ORF 2 (duplicative two bases)	ORF3 C20C [UGU(C) ⇒ UGC(C)]		ORF2, Capsid L8L [UUG(L) ⇒ CUG(L)]
	ORF3 P98P [CCG(P) ⇒ CCA(P)]		ORF2, Capsid A119T [GCU(A) ⇒ ACU(T)]

respectively. This indicates that $10^{5.4}$ and $10^{5.5}$ copies of JRC-HE3 are necessary to achieve infectivity against A549 cells and PLC/PRF/5 cells (Table 3). Thus, for both cell lines, 1 TCID of JRC-HE3 was estimated to be approximately $10^{5.5}$ copies. A similar experiment was performed using UA1 ($10^{7.5}$ copies/2.5 mL) along with A549 cells (Fig. 4C). Consequently, 1 TCID of UA1 was found to be approximately $10^{5.5}$ copies, although infectivity against PLC/PRF/5 cells could not be confirmed (data not shown). Based on the above-mentioned results, A549 cells may have to be used as the hosts when titration of both JRC-HE3 and UA1 are performed.

Photochemical inactivation of HEV in PLTs

The assays of HEV inactivation of both G3 (JRC-HE3) and G4 (UA1) in PLTs were performed thrice. The mean concentration of each strain in PLTs was consequently $10^{9.8}$ and $10^{7.9}$ copies per total volume (approx. 200 mL) of PLTs, respectively. HEV specimens collected from PLTs were serially diluted 10^{n}-fold (n = 0-6). Consequently, JRC-HE3 was found to express infectivity even if the original specimen (10^{0}-fold) was diluted 10^{3}-fold (Fig. 5A). This was clearly demonstrated by the fact that the viral progeny was found in the cell culture supernatant when the 10^{3}-fold–diluted specimens were applied. On the other hand, UA1 showed infectivity to a level of 10^{2}-fold dilution (Fig. 5C). No infectivity was recognized in any specimens after treatment with the Mirasol PRT system at the ninth or 10th week postinfection (Figs. 5B and 5D). In some cases, HEV RNA was detected in the cell culture supernatant in posttreated samples (Figs. 5B and 5D, white arrows). However, it would be reasonable to understand that these HEV RNA samples are derived from originally challenged HEV, not from virus progeny. All the data described in Fig. 5 are representative data of the results of each assay that was performed thrice. There was little difference between the assays. Based on these results, it was concluded that the Mirasol PRT system lowers the infectivity of G3 and G4 of live HEV, by more than 3 log (99.9%) and more than 2 log (99%), respectively (Table 4). In this experiment, A549 cells were utilized for HEV titration. The effect of HEV-free plasma treated or not treated with the Mirasol PRT system on the cells was also investigated. Consequently, no positive factors for cell death nor a negative impact on cell growth was observed.

DISCUSSION

Since HEV was recently categorized as a transfusion-transmittable infectious disease, the risk of HEV infection via blood transfusion has become a major global concern in transfusion medicine.[5,6] Hence, there has been a demand to establish an HEV culture system, especially for G3 and G4, which are frequently detected in Japan, China, and southeastern Asia,[7,8] as well as for a method to assay the HEV infectious titer, TCID. We therefore attempted to establish a culture system for G3 and G4 HEV and have successfully obtained two positive systems: the JRC-HE3 strain for G3 and the UA1 strain for G4. The concentrations of both viral strains in plasma or serum originally exceeded 10^{7} copies/mL; thus, it is plausible that the two strains may easily replicate and grow both in vivo and in vitro.

Although JRC-HE3 can infect both PLC/PRF/5 cells and A549 cells, the production efficiencies of the virions differed. A higher load of JRC-HE3 was inoculated into PLC/PRF/5 cells, and a higher concentration of approximately 10^{8} copies/mL of virus progeny was confirmed. In contrast, A549 cells generated progeny at a concentration of approximately 10^{6} copies/mL, with inoculations ranging from $10^{5.4}$ to $10^{9.4}$ copies in 2.5 mL. Since the PLC/PRF/5 cells are derived from human hepatoma, this cell line may be more suitable for HEV production than the A549 cells.

We examined the genomic alterations and amino acid substitutions of JRC-HE3 derived from PLC/PRF/5 cells after long-term culture. Full genome sequencing of JRC-

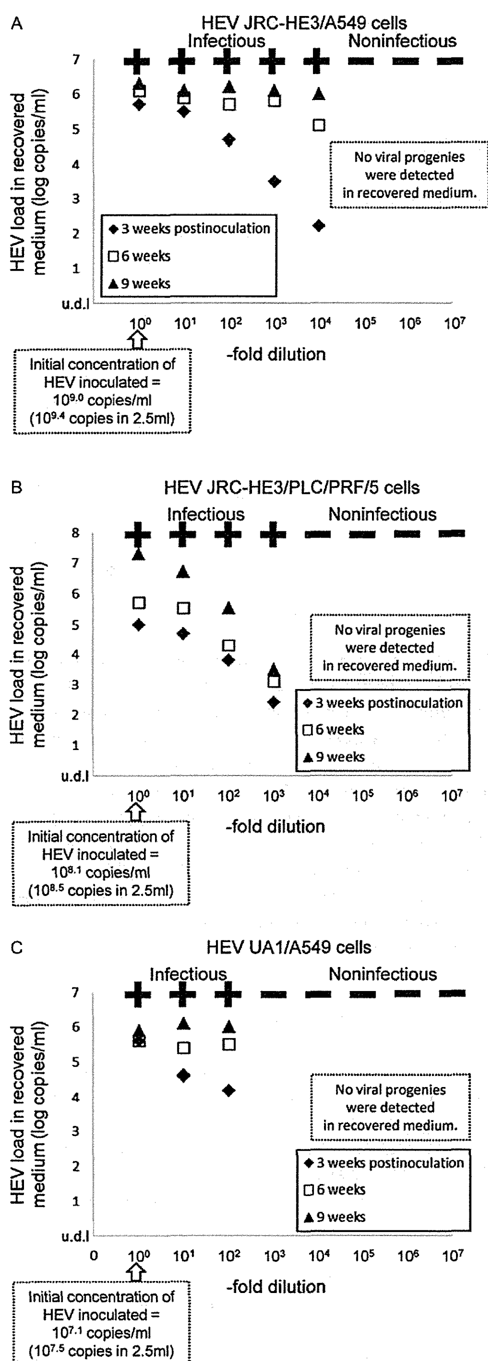

Fig. 4. (A) Titration of infectious HEV JRC-HE3 using A549 cells. A high HEV load of $10^{9.0}$ copies/mL was obtained by ultracentrifugation. The original HEV samples were serially diluted tenfold. Each diluted HEV sample was inoculated into A549 cells. The viral progeny in the recovered culture medium were examined every 3 weeks. Even the original HEV samples, diluted at 10^4, were found to be infectious. (B) Titration of infectious HEV JRC-HE3 using PLC/PRF/5 cells. A high HEV load of $10^{8.1}$ copies/mL was obtained by ultracentrifugation. Even the original HEV samples, diluted at 10^3, were found to be infectious. (C) Titration of infectious HEV UA1 using A549 cells. A high HEV load of $10^{7.1}$ copies/mL was obtained by ultracentrifugation. Even the original HEV samples, diluted at 10^2, were found to be infectious. u.d.l. = under the detection limit.

TABLE 3. Numerical relationship between two viral units: copies and TCID

Host cells	HEV strain	Copies per TCID
A549	HEV JRC-HE3 G3	$10^{5.4}$
PLC/PRF/5		$10^{5.5}$
A549	HEV UA1 G4	$10^{5.5}$
PLC/PRF/5		Could not be determined

HE3 revealed few variations. It may be concluded that JRC-HE3 has easily become habituated to in vitro cultivation. If the sequences of the viral genome would drastically change under long-term incubation, the efficacy of HEV production and viral infectiousness (infectious titer) may be negatively impacted. Based on these data, it could be concluded that HEV can stably and consistently be produced by this culture system, and its infectivity may remain almost the same in all situations. This consideration can be supported by the fact that the obtained growth curves demonstrating viral production were identical at a high level up to 120 days postinfection, regardless of the passage of the virus. Currently, a similar trial is being performed using UA1 produced by A549 cells.

In the first half of this article, we conclusively established an HEV culture system using HEV RNA–positive plasma or serum sample obtained from G3 or G4 HEV-infected individuals. Using the system established here, we attempted to undertake photochemical inactivation of HEV in PLTs using the Mirasol PRT system.

Log reduction values (LRVs) of more than 3 log for JRC-HE3 and more than 2 log for UA1 were achieved despite the fact that this agent is a nonenveloped virus, which are traditionally resistant to chemical inactivation. For the inactivation assay of JRC-HE3, a relatively higher concentration of the sample was obtained using PLC/PRF/5 cells as the virus producer. On the other hand, UA1 was not propagated in PLC/PRF/5 cells, but only in A549 cells. Therefore, more than 2-log reduction was marginally demonstrated due to the lower titer of the applied HEV.

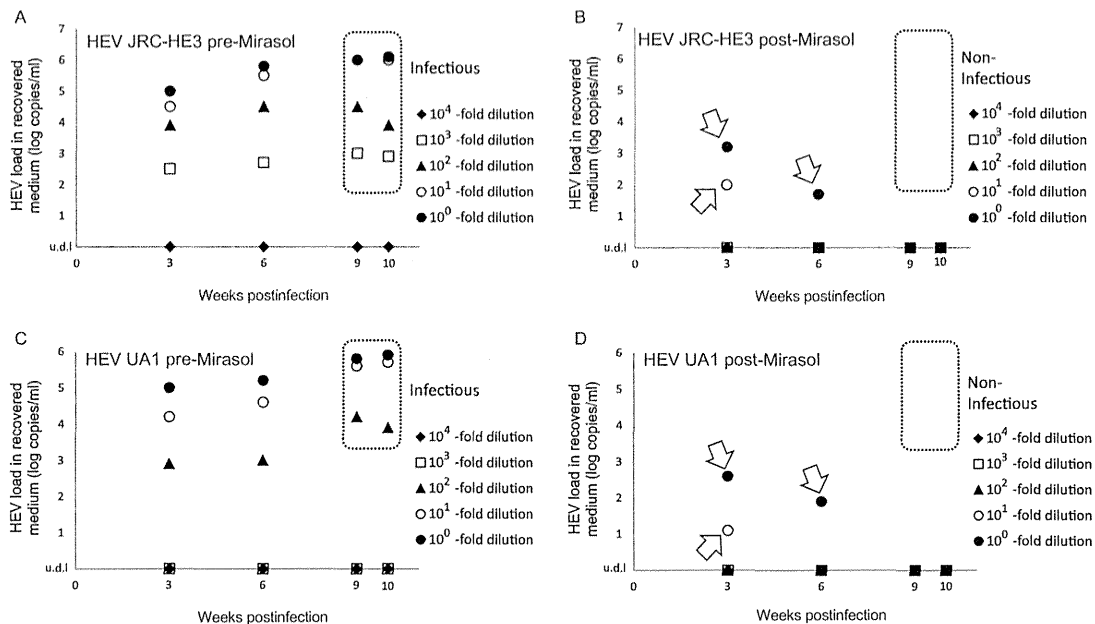

Fig. 5. (A and B) Inactivation of the live HEV JRC-HE3 in PLTs by the Mirasol PRT system. The change in the HEV load in the supernatant of A549 cell culture was investigated. The assayed HEVs were (A) Mirasol pretreated and (B) Mirasol posttreated HEV, respectively. The establishment of HEV infection was consequently confirmed at 9 and 10 weeks postinfection. The concentration of JRC-HE3 in PLTs was consequently $10^{9.8}$ copies per total volume of PLTs. JRC-HE3 expressed infectivity even when the original specimens were 10^3-fold diluted (A). No infectivity (viral progeny) was recognized in any Mirasol PRT system posttreated specimens of (B). HEV-RNAs detected in (B, white arrows) are derived from the originally challenged HEV, because the HEV loads in recovered medium were gradually decreasing. The Mirasol PRT system lowered the infectivity of JRC-HE3 more than 3 log (99.9%). These are the representative data of triplicate assays. (C and D) Inactivation of the live HEV UA1 in PLTs by the Mirasol PRT system. The HEVs assayed were (C) Mirasol pretreated and (D) Mirasol posttreated HEV, respectively. The concentration of UA1 in PLTs was consequently $10^{7.9}$ copies per total volume of PLTs. UA1 expressed infectivity even when the original specimens were 10^2-fold diluted (C). No infectivity (viral progeny) was recognized in any Mirasol PRT system posttreatment specimens (D). The Mirasol PRT system lowered the infectivity of UA1 more than 2 log (99%). These are the representative data of triplicate assays.

TABLE 4. Inactivation of the live HEV and HAV in PLTs by the Mirasol PRT system

HEV and HAV strain	Log reduction value (% of reduction value)
HEV JRC-HE3 G3	More than 3 log (more than 99.9%)
HEV UA1 G4	More than 2 log (more than 99%)
(Reference data)	
HAV VR-1402 GIB	1.8 log (98.4%)

The limited load of HEV obtained from in vitro cultivation precluded evaluation of the robustness of the measured LRV. If higher efficacy of viral infection against the hosts were attained, it could also be possible to acquire more robust data.

Of note, a 1.8-log (98.4%) reduction of LRV for the hepatitis A virus (HAV) strain of VR-1402 was previously obtained in our laboratory and an identical value was also reported by Prowse.[14] Thus, HEV inactivation is more efficient than that for HAV. VR-1402 has frequently been used for inactivation assays of HAV and is the usual model viruses for nonenveloped and relatively smaller-sized RNA viruses including HEV, because it is easy to titrate. We believe that the system we described here may be applied widely for infectivity studies of small, nonenveloped viruses, instead of VR-1402 as a model.

The Intercept blood system, similar to the Mirasol PRT system, is also well known as a photochemical method for PRT of blood products. Recently, two cases of HEV transmission caused by the transfusion of plasma treated with the Intercept were reported by Hauser and coworkers.[15] The primary mechanism of viral inactivation by the Intercept process has been described to involve an intercalation of amotosalen (the photochemical agent

employed in this system) into virus genome. This psoralen derivative is excited by UVA light, and the photochemical reaction that ensues subsequently induces a covalent bond between amotosalen and the DNA or RNA of the pathogen, which disrupts viral replication processes. Although the viral load in the transfused plasma that caused HEV infection was not described in the literature, these cases may prove that this inactivation mechanism by the Intercept process may be ineffective for HEV inactivation. It is also possible that the Mirasol system might inhibit certain step(s) essential for the viral life cycle and eventually induce the reduction of HEV infectious titer by at least 2 to 3 log. To fully determine which technology is more efficient for inactivating HEV more information regarding factors associated with HEV pathogenicity, such as the maximum HEV RNA concentration found in donated plasma and the minimum viral load required for the establishment of transfusion-transmitted HEV infection, would be needed. In relation to this, a concentration of more than 10^7 copies/mL has been detected in plasma collected for blood screening.[16] It is therefore possible that transmissions with either technology could still occur if viral titers exceed the capacity of the respective processes to inactivate all infectious particles that are present.

In conclusion, our results show that the Mirasol system has a possibility for the reduction of the risk of HEV transmission caused by blood transfusion. Further studies to determine the relationship between viral load and the pathogenesis of hepatitis E are necessary to establish whether the Mirasol system is indeed effective for preventing HEV transmission under clinical conditions. Since the concern about HEV transmission by blood transfusion has been rapidly increasing worldwide, the effect of PRTs on HEV should be further investigated.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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Antibody against immunoglobulin E contained in blood components as causative factor for anaphylactic transfusion reactions

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BACKGROUND: Determining the mechanism underlying the development of transfusion reactions is important in transfusion therapy. Two bags of fresh-frozen plasma obtained from a donor (index donor) were implicated in two cases of anaphylactic transfusion reactions.

STUDY DESIGN AND METHODS: The serum prepared from the index donor plasma transfused into the second patient (Patient 2) was evaluated using cord blood–derived mast cells (CBMCs) incubated with Patient 2 plasma. The component in the serum required for the degranulation was determined and quantified by chromatography in combination with degranulation assay, Western blot analysis, and enzyme-linked immunosorbent assay. The component in the plasma required for CBMC sensitization was determined using human immunoglobulin (Ig)E or normal plasma in place of Patient 2 plasma in the assay. Sera collected from the index donor between 2001 and 2008 were examined for the CBMC degranulation factor.

RESULTS: The donor serum activated CBMCs incubated with Patient 2 plasma. The IgG fraction of the donor serum induced degranulation of CBMCs sensitized with IgE or plasma containing a normal IgE concentration. The IgG anti-IgE at a concentration higher than 2200 ng/mL, which showed CBMC degranulation activity, was detected in the donor sera for at least 7 years.

CONCLUSION: Transfusion of a high concentration of the anti-IgE in the donor plasma was suggested to induce mast cell degranulation in the patients leading to the development of anaphylactic transfusion reactions. Antibodies existing in not only the patient circulation but also the transfused blood might cause transfusion-induced anaphylaxis.

Transfusion reaction cases that occurred throughout Japan have been reported voluntarily to the Japanese Red Cross Society (JRCS) since 1993 to improve transfusion safety.[1] Among approximately 1500 cases of transfusion reactions reported each year, more than 200 cases have been classified as severe anaphylaxis, which is generally termed anaphylactic transfusion reactions.[2] JRCS has also been collecting patient serum or plasma samples as well as the remaining blood components implicated in transfusion reactions to analyze their adverse effects. However, the cause of the reactions remains unclear in most cases.

Mast cells are considered to play a key role in anaphylaxis.[3] That is, the cross-linking of Fcε receptor I (FcεRI) via the complex of an antigen and immunoglobulin (Ig)E molecules on the cell surface stimulates the release of several enzymes and chemical mediators such as tryptase and histamine from the cells. These phenomena are closely related to the development of anaphylaxis and increased concentrations of serum or plasma tryptase are considered as a marker of anaphylaxis.[4,5] The serum or plasma tryptase concentration in patients who developed anaphylactic transfusion reactions was observed to

ABBREVIATIONS: CBMC(s) = cord blood–derived mast cell(s); FcεRI = Fcε receptor I; hIgE = human immunoglobulin E from plasma; JRCS = Japanese Red Cross Society.

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increase significantly after the onset of the reactions, suggesting that mast cell degranulation is triggered during these transfusion reactions.[6] We previously reported two cases of anaphylactic transfusion reactions associated with the transfusion of platelet (PLT) concentrates that contained oligomeric IgE capable of inducing the degranulation of cord blood–derived mast cells (CBMCs).[7]

JRCS was notified that another two bags of blood components obtained from a single donor were involved in two cases of anaphylactic transfusion reactions. In our preliminary analysis, the degranulation of CBMCs incubated with plasma from one of these patients upon stimulation with the serum sample prepared from one of the blood components was observed. In this study, we examined these samples by an in vitro degranulation assay using CBMCs and plasma protein purification techniques similar to those performed in a previous study.[7] We propose a new mechanism by which the adverse reactions were elicited in these cases.

CASE REPORTS

Two cases of anaphylactic transfusion reactions involving two bags of fresh-frozen plasma (FFP) obtained from a donor were reported to JRCS.

Index donor

The index donor was a 64-year-old female in 2004. She donated more than 100 times between 1994 and 2008 and at least 18 bags of blood components including FFP prepared from her blood were transfused into recipients. Two of them were implicated in anaphylactic transfusion reactions that developed in two different patients described below. She had no history of allergy. The concentrations of IgE and IgG in her serum prepared from the FFP used for the second patient (Patient 2) were determined to be 24.2 ng/mL and 10.5 mg/mL, respectively, which were normal concentrations (lower than 415 ng/mL and 8.70-17.0 mg/mL,[8,9] respectively). We examined retrospectively the hemovigilance data that had been accumulated in JRCS since 1993 by searching for reports concerning the blood component prepared from her blood. However, no cases were reported except these two cases of anaphylactic transfusion reactions. She had not donated since 2009 and currently she cannot donate owing to the age limitation for blood donation.

Patient 1

Patient 1 was a 53-year-old female suffering from pulmonary hypertension, renal failure, anemia, and thrombocytopenia. She had been diagnosed with thrombotic thrombocytopenic purpura and had been treated with plasma exchange four times without any transfusion reactions. She had no history of allergy. She received her fifth plasma exchange using five sequential bags of FFP in 2004. Within 10 minutes after the start of transfusion of the fifth bag of FFP, which was donated by the index donor on April 17, 2004, her systolic blood pressure decreased from 136 to 30 mmHg, and her pulse rate also decreased to 30 beats/min; she then stopped breathing. She was considered to have developed anaphylactic transfusion reaction from her clinical manifestation.[10,11] The IgE concentration in the pretransfusion plasma sample of the patient was 77.9 ng/mL. Her posttransfusion sample was not available for testing.

Patient 2

Patient 2 was a 62-year-old female with thrombotic thrombocytopenic purpura associated with systemic lupus erythematosus and underwent plasma exchange in 2005 using a bag of FFP obtained from the index donor on October 26, 2004. She had no history of allergy and she did not develop any adverse reactions during her transfusion therapies with four bags of FFP and a bag of PLT concentrate before the transfusion of FFP from the index donor. Ten minutes after the start of transfusion of FFP from the index donor, she started to feel throat pain and to wheeze, her forehead turned red, and her blood pressure dropped from 150/87 to 86/47 mmHg. Her oxygen saturation level measured by pulse oximetry was 86%. She was considered to have developed anaphylactic transfusion reaction from these clinical manifestations.[10,11] Her plasma IgE concentration decreased from 177 ng/mL before the transfusion to 90.7 ng/mL after the transfusion. The tryptase concentration in her plasma increased from 4.0 to 20.5 μg/L (normal range in healthy individuals, 2.1-9.0 μg/L),[12] indicating mast cell activation.

MATERIALS AND METHODS

Plasma and serum

Plasma samples were collected from Patient 1 before the transfusion and from Patient 2 before and after the transfusion. An aliquot of plasma from the bag of FFP transfused into Patient 2 was incubated with 40 mmol/L $CaCl_2$ at 37°C for 2 hours and then at 4°C overnight to prepare serum. Seven serum samples were also collected from the index donor when she donated her blood between 2001 and 2008. Control samples were collected from healthy donors. All the samples were dialyzed against or buffer exchanged with saline for the degranulation assay. IgE and IgG concentrations in the samples were determined by peak-rate nephelometry using an immunochemistry system (IMMAGE 800, Beckman Coulter, Tokyo, Japan).

CBMCs

CBMCs, which are generally used for the in vitro study of mast cells,[13] were prepared for degranulation assay.

Umbilical cord blood was obtained from the Japanese Red Cross Kanto-Koshinetsu Cord Blood Bank. Written informed consent was obtained from mothers for the use of the cord blood in transplantation- or transfusion-related studies in cases when the number of cord blood cells is not sufficient for transplantation. The differentiation of cord blood cells into mast cells was induced as previously described.[7]

CBMC degranulation assay

Mast cell degranulation activity in the test sample was determined from the percentage of tryptase or histamine release from activated CBMCs during the degranulation assay. CBMCs suspended in the culture medium were plated at 5×10^3 cells per well in a 96-well plate with 80 µg/mL sodium heparin and 20% (vol/vol) of the pretransfusion plasma from Patient 2, four plasma samples from healthy donors, or the index donor serum in a final volume of 50 µL. The cells were then incubated at 37°C in 5% CO_2 for 6 hours. Subsequently, 60 µL of a sample solution containing the index donor serum or a healthy donor serum in 0.18% (wt/vol) dextrose, 2.6 mmol/L $CaCl_2$, 1.8 mmol/L $MgCl_2$, and 0.18% (wt/vol) bovine serum albumin was added to each well. After incubation at 37°C for 40 minutes, the supernatant of each well was collected. The tryptase and histamine concentrations in the supernatant were measured using an allergy and autoimmunity analyzer (UniCAP 100, Phadia, Tokyo, Japan) and a histamine enzyme immunoassay kit (A05890, Bertin Pharma, Yvelines, France), respectively. The percentage of tryptase or histamine release was calculated as described previously.[7] To examine the effects of IgE and IgG on mast cell degranulation activity, CBMCs were incubated with 35.4 ng/mL human IgE from plasma (hIgE; 16-16-090705, Athens Research & Technology, Athens, GA) or human IgG from serum (hIgG; I4506, Sigma-Aldrich Japan, Tokyo, Japan). The CBMCs were then washed and incubated in a 96-well plate containing the sample solution described with or without 10,000 ng/mL hIgE or hIgG. The mast cell degranulation activity in the sample was then assessed.

Fractionation of index donor serum

To separate the components that induce CBMC degranulation from the other components of the serum, the index donor serum was fractionated by gel filtration and affinity chromatography. Gel filtration chromatography was carried out using 2 mL of the index donor serum on an column packed with prep grade (HR 16/50 and Superose 12, respectively, GE Healthcare Japan, Tokyo, Japan) at 4°C. The IgG fractions of the index donor serum and healthy donor serum were obtained using a protein G column (17-0404-01, GE Healthcare Japan) in accordance with the manufacturer's protocol. The unbound fraction was collected as the pass-through fraction. Chromatographic fractions were assessed to determine mast cell degranulation activity using CBMCs sensitized with Patient 2 plasma and IgG concentration.

Detection of IgG anti-human IgE

In the Western blot analysis to detect the IgG anti-IgE, hIgE was blotted onto a transfer membrane (Immobilon-P, Nihon Millipore, Tokyo, Japan). The membrane was incubated with the index donor serum, healthy donor serum, or the IgG fraction of these sera, and then the IgG anti-IgE was detected using a horseradish peroxidase–conjugated anti-human IgG antibody (HRP–anti-IgG; 054220, Life Technologies Japan, Tokyo, Japan) and Western blotting detection reagents (ECL Plus, GE Healthcare Japan). hIgE on the membrane was visualized using a gold staining kit (Protogold, British BioCell International, Cardiff, UK). The IgG anti-IgE was quantified by enzyme-linked immunosorbent assay (ELISA). The test serum samples from the index donor or healthy donor were incubated in a 96-well plate (Nunc Immuno Plate PolySorp, Thermo Fisher Scientific, Kanagawa, Japan) coated with hIgE. The IgG anti-IgE in each well was detected using HRP–anti-IgG with 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD). A humanized monoclonal IgG anti-human IgE, omalizumab (Novartis Pharma, Tokyo, Japan), was used to generate a standard curve for quantification (detection range, 100-40,000 ng/mL).[14]

Statistical analysis

The percentage of tryptase release and the concentration of the IgG anti-IgE were determined in two and four independent experiments, respectively. The mean percentage of tryptase release was compared between samples by the two-tailed t test for independent samples using computer software (Excel 2010, Microsoft Corp., Redmond, WA). A p value of less than 0.05 was considered significant.

RESULTS

Index donor serum–induced degranulation of CBMCs sensitized with Patient 2 plasma

The index donor serum induced the degranulation of CBMCs incubated with Patient 2 plasma (Fig. 1). No significant difference was observed between the tryptase concentrations in the culture supernatants of CBMCs incubated with 20% (vol/vol) Patient 2 plasma only and in that incubated without it (data not shown). However, the CBMCs sensitized with Patient 2 plasma released tryptase when incubated with 2.5 to 40 µL of the index donor serum in 110 µL of assay solution in a dose-dependent manner; CBMCs incubated without Patient 2 plasma were

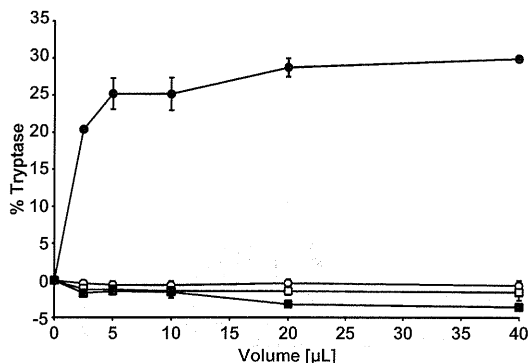

Fig. 1. Degranulation of CBMCs induced by index donor serum. CBMCs were incubated with (filled symbols) or without (open symbols) Patient 2 plasma followed by indicated volumes of index donor serum (●, ○) or healthy donor serum (■, □). The mean ± SD percentage of tryptase release is plotted.

not activated by the index donor serum. These results indicate that CBMCs were activated by the combination of Patient 2 plasma and the index donor serum.

CBMC degranulation induced by IgG fraction of index donor serum

Chromatographic fractionation was performed to separate the components of the index donor serum and identify those that activated CBMCs. Gel filtration fractions A37 to A49 induced the histamine release from CBMCs sensitized with Patient 2 plasma to a greater extent than the other fractions (Fig. 2A). These fractions contained a high concentration of IgG, suggesting that IgG is involved in the activation of CBMCs. The IgG fraction obtained from the index donor serum, as well as the index donor serum containing the same amount of IgG (3.56 μL), induced the degranulation of CBMCs sensitized with Patient 2 plasma (Fig. 2B). Thus, it is considered that the factors in the index donor serum that activated Patient 2 plasma–sensitized CBMCs were in the IgG fraction.

Requirement of human IgE for CBMC sensitization

Next, the components in Patient 2 plasma required for CBMC sensitization were determined. CBMCs washed after incubation with Patient 2 plasma were activated by the index donor serum (data not shown). Hence, IgE may be necessary for CBMC sensitization because IgE binds to FcεRI on the CBMC surface and is not removed by washing.[15] Indeed, CBMCs incubated with hIgE at the same concentration as that of IgE in Patient 2 plasma and then washed were activated by the index donor serum and the IgG fraction of the serum (Figs. 3A and 3B). Besides Patient 2 plasma, other plasma samples could also sensitize CBMCs to the index donor serum (Fig. 3C). CBMCs incubated with plasma with an IgE concentration similar to that in Patient 2 plasma were activated by the index donor serum, whereas CBMCs incubated with plasma containing a low IgE concentration or an extremely high IgE concentration were not activated by the serum. These results suggest that normal IgE generally contained in human plasma could contribute as a factor for CBMC sensitization at suitable concentrations in place of Patient 2 plasma.

IgG anti-IgE in index donor serum activating IgE-sensitized CBMCs

As shown in Figure 3C, CBMCs were not activated by the index donor serum in the presence of a high IgE concentration. Moreover, CBMC degranulation induced by the IgG fraction of the index donor serum was inhibited by the addition of an excess amount of hIgE (Fig. 4A), suggesting that the IgG fraction recognized IgE. Western blot analysis revealed that IgG in the index donor serum bound to hIgE (Fig. 4B). Hence, it is considered that the index donor serum contained the IgG antibody against human IgE and this antibody triggered CBMC degranulation by the cross-linking of IgE-bound FcεRI on the cells.

IgG anti-IgE in index donor serum detected over 7 years

The IgG anti-IgE in the index donor serum samples collected between 2001 and 2008 was examined to determine the relationship between the presence of the antibody and the occurrence of anaphylactic transfusion reactions (Table 1). The IgG anti-IgE was detected in all eight samples by Western blot analysis. The concentration of the antibody was calculated to be in the range between 2200 and 8900 ng/mL by ELISA. All the test samples activated hIgE-sensitized CBMCs. These results indicate that the index donor had continuously produced the IgG anti-IgE over a long period of at least 7 years. CBMCs incubated with Patient 1 plasma were also degranulated after incubation with the index donor serum obtained from FFP that was from the blood donated on April 17, 2004, and that was transfused to Patient 1 (data not shown).

DISCUSSION

Mast cells in transfusion recipients are activated and degranulate during anaphylactic transfusion reactions,[6] although the activation mechanism remains unclarified in most cases. In the cases presented in this study, the sera from the transfused FFP showed degranulation activity against CBMCs sensitized with the patient plasma and the

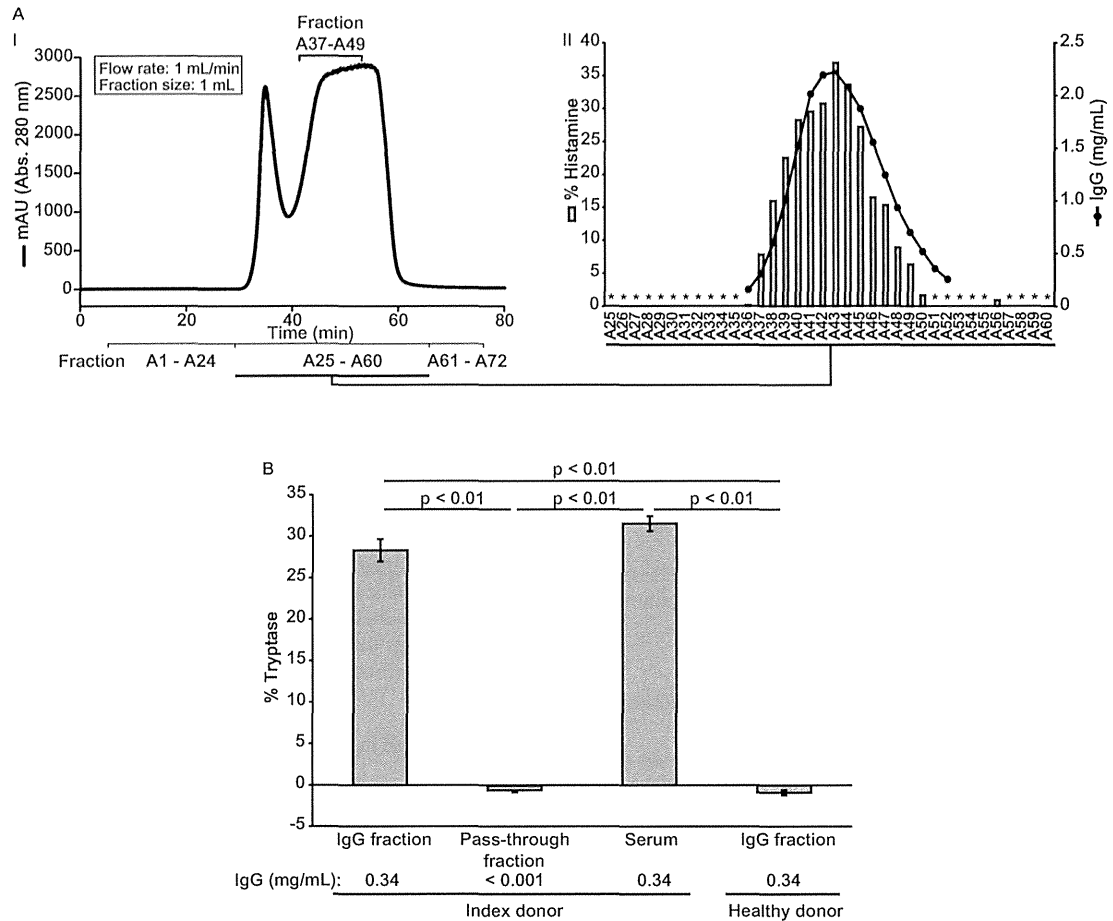

Fig. 2. Mast cell degranulation activity in fractions separated from index donor serum by chromatography. (A) The index donor serum was fractionated by gel filtration chromatography. (I) Chromatogram. (II) Elution patterns of mast cell degranulation activity and IgG. The histogram shows the percentage of histamine release during degranulation assay of 50 μL of each fraction. *The percentage of histamine release was not higher than 0%. The solid line represents the IgG concentration in each fraction. (B) CBMC degranulation assay was performed using the indicated samples. The concentrations of IgG from the test samples in the assay solutions are indicated at the bottom of the panel. p values indicating significant differences are shown. The mean ± SD percentage of tryptase release is plotted.

factor responsible for CBMC degranulation was determined to be the IgG anti-human IgE in the donor plasma. It was suggested that the antibody was the causative factor for the anaphylaxis.

Autoantibodies against human IgE are found in patients with various diseases such as allergic disorders,[14,16-19] systemic lupus erythematosus,[20] parasite infection,[21] systemic sclerosis,[22] Crohn's disease,[23] and visceral leishmaniasis.[24] The IgG anti-IgE was also detected in normal individuals in some studies.[14,23] Generally, no more than 300 ng/mL IgG anti-IgE was observed in patient serum samples.[17-22] However, another report showed that the IgG anti-IgE concentrations in two of 96 individuals were more than 4000 ng/mL.[14] The index donor was reported to have no history of allergic, autoimmune, or other diseases. Despite that, the IgG anti-IgE was detected in the index donor sera at relatively high concentrations ranging from 2200 to 8900 ng/mL. In our preliminary study to detect the IgG anti-IgE in approximately 250 serum samples prepared from the blood components, only one serum sample showed a high IgG anti-IgE concentration of 1200 ng/mL; however, that serum sample

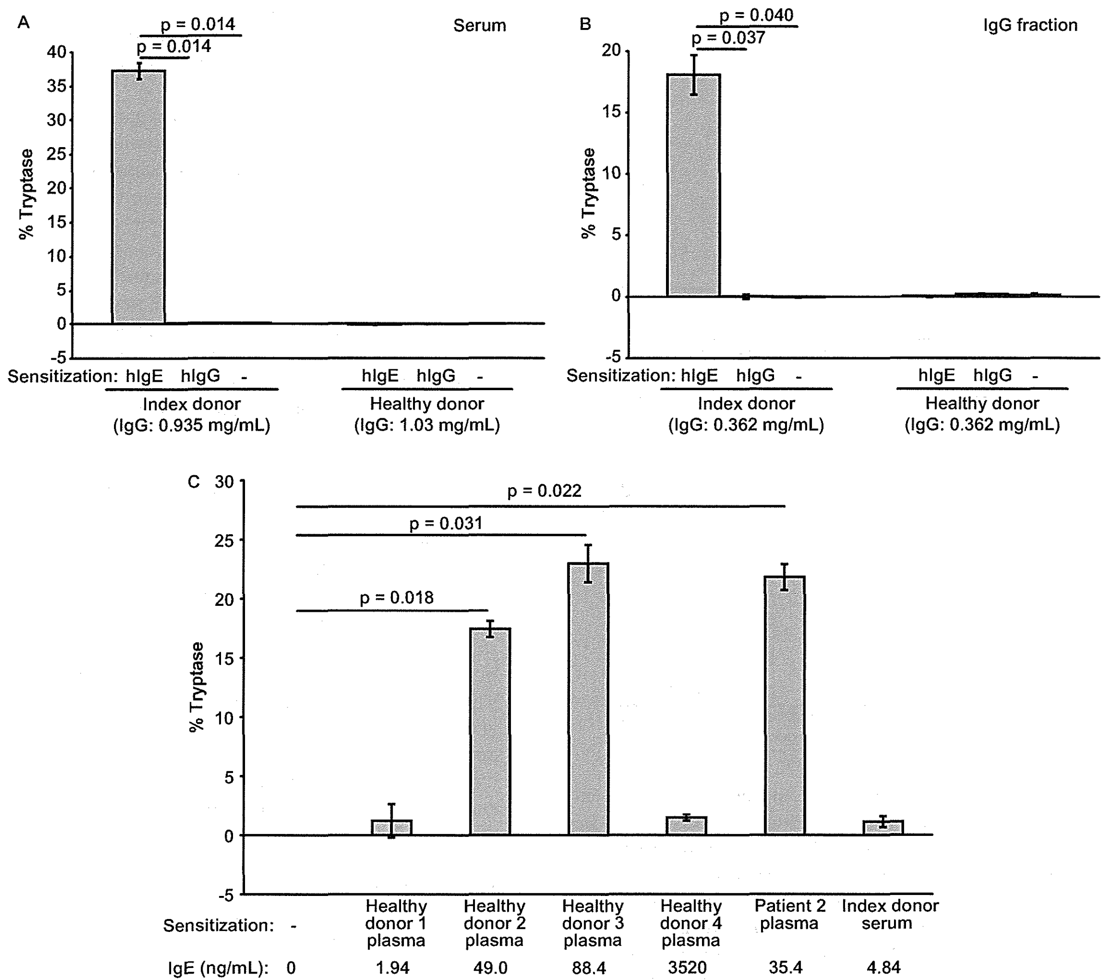

Fig. 3. Incubation of CBMCs for degranulation induced by index donor serum. CBMC degranulation assay was performed. (A and B) CBMCs incubated with 35.4 ng/mL hIgE or hIgG as the control were washed and then used for the degranulation assay of the indicated serum samples (A) and the IgG fractions of these samples (B). The IgG concentrations in the assay solutions are indicated at the bottom of the panel. (C) CBMCs were incubated with indicated plasma or serum, and then the degranulation assay was performed using 10 μL of the index donor serum. The IgE concentrations at which CBMCs were incubated are indicated at the bottom of the panel. p values indicating significant differences are shown. The mean ± SD percentage of tryptase release is plotted.

did not activate IgE-sensitized CBMCs. Thus, it is suggested that the blood component with a high concentration of the IgG anti-IgE possessing mast cell degranulation activity similarly to the index donor plasma is very rare.

The concentration of IgE that sensitizes the cells seems to regulate whether CBMCs are induced to degranulate by the index donor serum. CBMCs were activated by the index donor serum when incubated with plasma containing appropriate IgE concentrations, as shown under Results, and when incubated with hIgE at tens to a thousand of nanograms per milliliter but not at lower or higher than these concentrations (data not shown). The results suggest that at a low IgE concentration, the amount of IgE molecules on CBMCs was not sufficient to react with the IgG anti-IgE and that at an excessively high concentration of IgE, by forming an antigen–antibody complex, the IgE molecules in the assay solution inhibited the binding of the IgG anti-IgE to IgE on the CBMC surface to induce degranulation. Indeed, the IgE concentration in Patient 2 plasma decreased by half

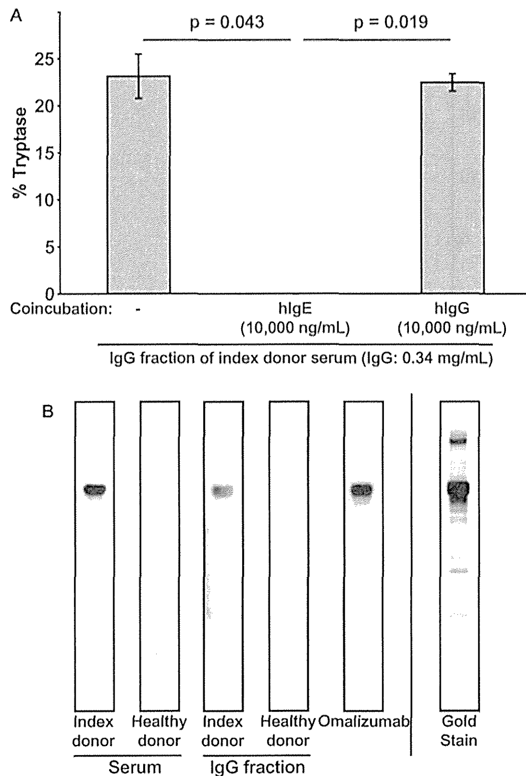

Fig. 4. Assessment of IgG anti-IgE in index donor serum activating IgE-sensitized CBMCs. (A) hIgE-sensitized CBMCs were washed and then used for the degranulation assay of the IgG fraction of the index donor serum (IgG: 0.34 mg/mL) coincubated with or without 10,000 ng/mL hIgE or with hIgG as the control. p values indicating significant differences are shown. The mean ± SD percentage of tryptase release is plotted. (B) The IgG antibody against IgE in the index donor serum was detected by Western blot analysis. The hIgE-blotted membrane was incubated with the samples indicated at the bottom of the panels.

after the occurrence of transfusion reactions. It might be suggested that the transfused IgG anti-IgE was consumed in part during its complexation with circulating IgE in the patient's blood.

In addition to the concentration of patient plasma IgE, a rapid transfusion rate might be required in the development of anaphylactic transfusion reactions. Both patients who developed anaphylactic transfusion reactions were treated by plasma exchange, in which the transfusion rate was considered to be much higher than that for supplementation of the blood coagulation factor. The rapid infusion of a high concentration of anti-IgE might facilitate the induction of the degranulation of the patients' mast cells. This may explain why no transfusion reaction cases other than the two cases presented in this study have been reported, despite the fact that many bags of blood components were prepared from the index donor blood.

In summary, the results suggest that the IgG anti-IgE in the transfused plasma caused anaphylactic transfusion reactions in these two patients. Thus, we propose a possible mechanism underlying these transfusion reactions. That is, the transferred antibody against a recipient's antigen causes anaphylactic transfusion reactions. An existing antibody in the patient blood that recognizes a substance in the transfused blood component has been proposed as a causative factor for anaphylactic transfusion reactions. However, concerning transfusion-related acute lung injury (TRALI), transferred antibodies, such as antibodies to human leukocyte antigens (HLA) and human neutrophil alloantigens (HNA) are considered as causative factors activating various cells in transfusion recipients.[25] The concentration of the transferred HLA antibody is suggested to be related to the development of TRALI.[26] Similarly, anaphylactic transfusion reactions could be induced in some patients in the presence of an adequate concentration of a transfused antibody against a specific antigen on the patient's cells, such as the IgG anti-IgE found in this study. Identification of causative antibodies in blood components would help reduce the risk of these transfusion reactions.

TABLE 1. IgG anti-IgE in index donor sera collected on various dates

	IgG anti-IgE				
		Concentration*		% Tryptase release†	
Collection date	Western blot analysis	Mean (ng/mL)	SEM	Mean (%)	SD
February 22, 2001	+	3200	210	30.7	2.0
August 23, 2002	+	3200	98	31.3	1.1
September 1, 2003	+	3000	230	30.8	2.4
April 17, 2004 (Case 1)	+	2300	130	23.3	2.1
September 17, 2004	+	2800	180	25.9	3.8
October 26, 2004 (Case 2)	+	2200	220	22.4	1.6
January 26, 2005	+	2500	250	21.1	3.0
July 11, 2008	+	8900	560	29.9	3.1

* The concentration of the IgG anti-IgE was measured by ELISA.
† For the degranulation assay, hIgE-sensitized CBMCs were activated with 10 μL of each of the index donor sera collected on the indicated dates.

CONFLICT OF INTEREST

The authors report no conflicts of interest or funding sources.

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第76回日本血液学会学術集会
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日本における輸血感染症のリスク

佐竹正博

Key words : Transfusion-transmitted infection, Emerging infectious disease, Blood screening

日本における輸血感染症の実態と残存リスク，これから有り得る輸血感染症について，その主なものを概説する。

1. 核酸増幅検査によるスクリーニング

B型肝炎ウイルス (HBV)，C型肝炎ウイルス (HCV)，ヒト免疫不全ウイルス (HIV) は，核酸増幅検査 (nucleic acid amplification testing, NAT) によるスクリーニングの対象となっている。日本赤十字社血液センターでは，従来はドナー血清を20本プールした検体を用いて検査を行っていたが，平成26年8月より，プールをしない個別検体での検査が開始された。核酸の抽出効率・試薬の感度はほぼ究極の段階にあり，これ以上の感度の上昇は検体の容量に依存することになる。この態勢下でなおこれらのウイルスによる感染がおこるのは，いわゆるウィンドウ期にある献血者の献血血液によるものがほとんどである。すなわち，献血者が感染を受けてから，その末梢血液中のウイルス濃度がNATの感度に達するまでの期間に献血された血液である。ウイルス抗原やウイルスに対する抗体が検出されるのはそのあとになる。

① HBV

HBVは感染性が非常に高いうえに，国民の間の侵淫率も高かった[1]。さらに末梢血中でのあり方にも特有のものがあり，これまでの輸血感染対策は複雑なものであった[2]。HBVゲノムの末梢血中の倍加時間は約2.6日[3]と非常に長く，それだけウィンドウ期間も長い。したがってその間に献血される頻度も高くなる。試算では，個別NAT陰性のウィンドウ期の献血血液は1年に約90本はあるだろうと考えられる[4]。これは輸血用血液製剤1バッグにHBVが1個以上入っているものの数であり，そのうちのごく一部が実際に感染を起こすものと思われる[5]。実際に血液センターが把握する輸血感染例は年に2, 3例である。もう一つの感染源は，HBV感染既往者の血液である。今日では，少年期以後の水平感染では治癒後HBVは体内から完全に駆逐されるという考えは間違いであり，肝細胞などにはHBVが残っていて，間歇的にあるいは持続的に末梢血中に低濃度のウイルスが放出されると考えられている[4,6,7]。近年では，極めて低濃度ながらこれらが原因となった輸血感染例はウィンドウ期血液による感染よりも多かった (Fig. 1)[4]。2012年，コア抗体陽性血液をすべて排除したことにより，このタイプの感染例はほとんどなくなったと考えられる。ただ，現行のコア抗体の感度以下の感染既往者の血液は存在するはずで，それらの血液による感染の可能性は残っている。

NATの高感度化により，出庫される血液製剤にHBVが混入したとしてもそれは極めて低濃度のものに限られる。このレベルになると，製剤の感染性は製剤1ユニット中の全ウイルス量に依存することが示されている[4]。したがって以後の感染は，赤血球製剤よりも，血漿量の多い新鮮凍結血漿や濃厚血小板に集中するであろうと推定される[4]。これまでは，HBVの高い感染性のために，プールNATの感度が感染性の有無を分けるレベルに達しておらず，そのほかの臨床的な要因が感染を規定していたと思われる。

血液製剤中に混入したHBVの量は非常に少なくなるため，輸血感染の後HBVマーカーが陽転したり実際に肝炎を発症するまでにはこれまでより時間がかかると思われる。遡及調査ガイドライン[8]で推奨されている輸血後3カ月では感染の有無をまだ断定できない可能性がある。したがって輸血時より遠く隔たった時期に肝炎を発症することが多くなり，フォローする医療機関の連携など，これまで以上に輸血後の患者のフォローに注意を必

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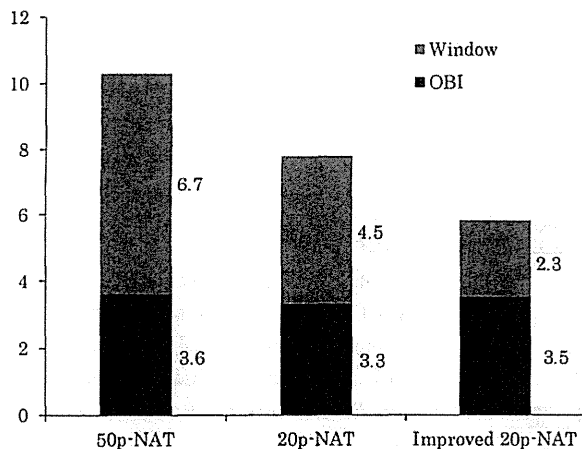

Fig. 1 Number of established Transfusion-Transmitted-HBV infections grouped according to pool-based NAT screening systems.
Window; Infection cases caused by window period-derived blood components.
OBI; Infection cases caused by occult HBV infection-derived components. OBI refers to anamnestic HBV infection stage typically represented by HBsAg-negative, HBcAb-positive, and HBV PCR-positive.
Intervals when indicated NAT systems were applied were Feb. 2000 - Jul. 2004 (4.5 y), Aug. 2004 - Jul. 2008 (4.0 y), and Aug. 2008 - Mar. 2010 (1.67 y), for 50 pool-NAT, 20 pool-NAT, and Improved 20 pool-NAT, respectively. Sensitivities of NAT systems used are 650, 260, and 76 copies/m*l*, for 50 pool-NAT, 20 pool-NAT, and Improved 20 pool-NAT, respectively. Vertical axis indicates the number of established transfusion-transmitted HBV infection cases per year.

要とする。

輸血後のB型肝炎と間違われやすいものに，HBVの再活性化がある。分子標的薬の投与や免疫抑制を行う患者はしばしば輸血も受けているので，時期的にどちらの肝炎であってもよい場合がある。再活性化による肝炎は重症で時に致命的であるので，どちらであるかを早急に鑑別し必要な治療を施さなければならない[9]。

② HCV

HCVの末梢血中での増殖速度は，倍加時間が約11時間と極めて短く[10]，たとえばHCVが1コピー/m*l*から個別NATの検出感度濃度に達するまで2日とかからない。たまたまこの短いウィンドウ期にあった個人が献血をするという確率は極めて低い。過去5年間の20プールNATの時期に，NATのみ陽性（抗体陰性）の感染初期の献血は37例であった。感染後のNAT単独陽性の時期の長さを90日とすると，2日間のウィンドウ期にある個人が献血する頻度は年間0.2回に満たない。プールNATの段階ですでにHCVの輸血感染はほとんど防がれていたと考えてよく，過去5年間に輸血によるHCV感染は1例のみであった。個別NAT下ではさらに少なくなることが期待される。しかも図に見るように，NATのみ陽性の献血（献血者が献血数カ月以内に感染の機会を有したことを示す）は年々減少してきている(Fig. 2)[11]。リスクを有することを自覚した人が献血をしないようになったか，あるいは民間医療を含めて観血的医療・施術に使用する器具や手技が，感染の伝播という点において改善されてきたことなどが考えられる（滅菌の徹底や使い捨て器具の使用など）。

③ HIV

HIVの末梢血中の倍加時間は20.5時間とされる[12]。20プールNAT下でのウィンドウ期は約2週間であったが，個別NAT下では約11日となる。日本では海外に比べて，献血者中のHIV抗体陽性者の割合が一般人口でのそれに比べて高い状態が続いてきた[13]。感染してから間もない抗体陰性・NAT陽性の献血者はここ14年間に27人であった。これらから計算すると，個別NAT陰性のHIV感染者の献血は1年に2例近くあることが推定される。実際2013年暮れに起きた輸血によるHIV感染事例は，HIVの輸血感染リスクが依然残っていることを示している[14]。この例においては，献血者がHIV感染後1, 2週で献血をし，その血液に由来するFFPの輸血によってひとりの患者に感染が起きた。献血者の保管検体の検査では3つのNATシステムのうち一つのみで検出することができた（ただしそのうちの一つは定量系）。世界的に，個別NAT陰性の血液によるHIV感染の報告はまだないが，今回の例は個別NATでも検出できない，感染性のある献血があり得ることを示している。同じ血液に由来する赤血球製剤では感染は起きなかった。ここでも感染性が輸注ウイルス量に依存することが示唆される。なおこの事例は，当該献血者が2度目の献血を行った際，HIV抗体が陽転しており，血液センターで前回の献血血液の保管検体を個別NATで調べた結果，HIV RNAがわずかに陽性であり，該当する血液を輸血された患者を至急遡及調査して判明したものである。

以上，NATでスクリーニングしている3つのウイルスについては，蓄積された検査データから残存リスクを推定することができる[15,16]。それはウイルスを含む献血の頻度としてあらわされ，上記のようにHBVやHIVに関しては依然として大きな数である。しかし実際の感染性はまた別の話であり，製剤の形態（PC，凍結品であるFFP，赤血球製剤の違い，血漿量の違いなど），献血者の感染ステージ，患者の免疫状態・全身状態などが複