Table 2. Changes of HLA types in TA-GVHD patients

No.	HLA-A	HLA-B	HLA-DRB1	HLA-DQB1	HLA-DPB1
1	n.t	n.t	02 04	n.t	n.t
	n.t	n.t	02	n.t	n.t
2	n.t	n.t	02 12	n.t	n.t
	n.t	n.t	02	n.t	n.t
3	n.t	n.t	1101 1302	n.t	n.t
	n.t	n.t	1302	n.t	n.t
4	n.t	n.t	08 1502	n.t	0501 0901
	n.t	n.t	1502	n.t	0901
5	n.t	n.t	1501 1502	n.t	0501 0901
	n.t	n.t	1502	n.t	0901
6	n.t	n.t	0405 1502	0601	0402 0901
	n.t	n.t	1502	0601	0901
7	n.t	n.t	08032 1502	0601	0201 0901
	n.t	n.t	1502	0601	0901
8	n.t	n.t	1502 1302	0601	0501 0401
	n.t	n.t	1502	0601	0901 0201
9	n.t	n.t	0802 1502	0601 0402	0501 0901
	n.t	n.t	0802 1502	0601 0402	0901
10	2 11	48 55	9 12	n.t	n.t
	24 31	54 60	2 4	n.t	n.t
11	0201	46 39	0803 1501	n.t	n.t
	0201	46 39	1501	n.t	n.t
12	2402 2402	4002 52011	0901 1502	n.t	n.t
	24	61 38	0901	n.t	n.t
13	0201 2402	4601 52011	0901 1502	n.t	n.t
	2402	52011	1502	n.t	n.t
14	2402 3302	40 44	1101 1302	n.t	n.t
	3302	44	1302	n.t	n.t
15	2402 2601	52 39	1502 1201	n.t	n.t
	2402	52	1502	n.t	n.t
16	24 33	39 52	1501 1502	n.t	0201 0901
	24	52	1502	n.t	0901
17	24 33	52 39	1502	n.t	0901 0201
	24	52	1502	n.t	0901
18	24 2	52 38	1502 045	0601 0401	n.t
	24	52	1502	0601	n.t
19	2402 02	5201 59	1502 0405	0601 0401	n.t
	2402	5201	1502	0601	n.t
20	2 24	46 52	0803 1502	0601	0501 0901
	24	46 52	0803 1502	0601	0501

n.t, not tested.
The upper stage is a typing result in DNA extracted from the patient's nail. The lower stage is a typing result in DNA extracted from the patient's peripheral blood lymphocyte after developing symptoms of TA-GVHD.

immunocompetence (Linton & Dorshkind, 2004). The majority of patients underwent surgical operations. It has been reported that the numbers of natural killer and T cells are decreased following a surgical operation (Slade *et al.*, 1975).

Although the rate of first-time transfusion among patients receiving transfusions was not found, almost all the TA-GVHD patients (84·8%) in this study had no past transfusion history. Multiply transfused patients have anti-idiotypic antibodies to T-cell receptors, and this antibody may play a role in resistance to TA-GVHD (Nishimura *et al.*, 1992). Moreover, an experiment on rats indicated that a specific state of resistance to GVH reactions can be induced in F1 hybrid rats, as a consequence of inoculation with a small number of parental strain lymphocytes (Woodland & Wilson, 1977). Our patients included almost no blood dyscrasia patients; as such patients have received many transfusions, they may possess resistance to TA-GVHD.

There was no case in which TA-GVHD developed following the transfusion of only apheresis platelets. In Japan, only platelets of apheretic origin have been supplied, from the second half of the 1980s. Such platelets are characterised by little mixing of leucocytes, with less than 10^6 per bag. The scant mixing of leucocytes in platelets may be related to potential for PT-GVHD development.

It was confirmed that RC-MAP stored for 14 days caused TA-GVHD. All blood products that contain viable immunocompetent lymphocytes, including red cell concentrates, apheresis platelets and even fresh plasma, have been implicated in TA-GVHD. However, according to the JRC's experience (Takahashi *et al.*, 1994), no case associated with fresh frozen plasma has yet been documented.

TA-GVHD in immunocompetent patients usually results from the transfusion of blood from an HLA homozygous donor to a recipient heterozygous for a haplotype. Because cytotoxic T cells derived from blood products are not recognised as 'non-self' by the recipient's immune system in such cases, they are engrafted and injure the organs of the recipient. Because of this pathogenesis, TA-GVHD is fatal in most cases. The Japanese have a higher risk of TA-GVHD than any other population, as established previously (Takahashi *et al.*, 1991; Ohto *et al.*, 1992). This applies even when blood from unrelated donors is used, because the haplotype A24-B52-DR15 is as frequent as 7·5%. In this study, not only one-way match cases but also two-way mismatch cases (Table 2, nos. 8 and 12) were present. Because some form of immunodeficiency existed in the patients, it is considered that the donors' cells were not eliminated by those of the recipients, even given the presence of a two-way mismatch. In one patient with SCID, all six sites were different for HLA-A, B and DR. The SCID patient cannot eliminate a foreign substance.

There is no effective treatment once TA-GVHD has developed, and nearly all such patients die. A combination therapy of anti-CD3 antibodies with Cy A and steroids, which is used for treating rejection after renal transplantation, has been reported to be effective in some cases (Yasukawa *et al.*, 1994); however, recovery could not be achieved when a similar treatment was performed subsequently, and such reported cases are considered to be exceptional. The case of survival in this study involved a transfusion in a caesarean section. Medical treatment was started in early stages of symptom development, and the patient's young age as well as the immune state under pregnancy may have participated in the survival. On the other hand, NM administration was associated with a marked recovery of the patient's peripheral blood mononuclear cells, disappearance of body rash, body temperature normalisation, liver function improvement and an unusually long survival time (Table 1, NM administration, Yahagi *et al.*, 1997). Thus, the hope of a radical treatment is emerging by combining other therapies capable of damaging and eliminating the donor lymphocytes.

Prophylaxis is important, because no effective treatment has yet been established. In practice, the following measures should be taken: (i) strictly define indications for blood transfusion, and do not perform unnecessary transfusions; (ii) perform autologous transfusions for scheduled operations; and (iii) irradiate the blood to be transfused. Prophylaxis using a leucocyte-removing filter is not reliable (Akahoshi *et al.*, 1992). In our study, TA-GVHD developed in seven patients despite the use of third-generation leucocyte-removing filters by bedside filtration. Therefore, WBC-reduced components should be irradiated to prevent TA-GVHD. TA-GVHD can be prevented by the irradiation of blood components. In Japan, the use of irradiated blood components for transfusion was approved in 1998 by the Ministry of Health and Welfare. Since 2000, when the supply of irradiated blood products became widespread, we have had no cases of TA-GVHD when blood supplied by JRC Blood Centers is used. The irradiation dose was determined to be not less than 15 Gy, and not more than 50 Gy based on *in vitro* lymphocyte response tests to allogeneic cells (mixed leucocyte culture, MLC).

Irradiation of red blood cell bags is associated with an increased loss of potassium (K^+) from the cells. The transfusion of red blood cell bags with high amounts of K^+ has caused concern of an increased risk of cardiac arrest owing to transient hyperkalaemia. No major problems have yet been encountered since the implementation of universal irradiation, more than 10 years ago. This irradiation dose was confirmed to be suitable and safe. In Japan, the potassium adsorption filter is covered under the health insurance system. It is likely that the spread of usage of this filter has influenced the prevention of transfusion side effects.

Guideline V for Irradiation of Blood for the Prophylaxis of TA-GVHD by the Japan Society of Transfusion Medicine and Cell Therapy indicated that irradiation should be carried out for all the blood for transfusion, except for fresh frozen plasma. In 440 hospitals transfusing blood in the Tokyo in 2010, it was reported that all transfused blood was irradiated.

We conducted the analysis of 66 cases of definite TA-GVHD, and clarified the clinical picture, underlying diseases of patients and causative blood preparations of TA-GVHD. The irradiation to the blood products intended for transfusion was effective in the critical prevention of TA-GVHD, and its safety was high.

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CONFLICT OF INTEREST

The authors have no competing interests.

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Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors

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BACKGROUND: The current prevalence of cytomegalovirus (CMV) in Japan and the risk of CMV transfusion transmission are unknown in the era of seronegative leukoreduced blood components.

STUDY DESIGN AND METHODS: We measured CMV-specific immunoglobulin (Ig)M and IgG in 2400 samples of whole blood collected from 12 groups of blood donors categorized by sex and age at 10-year intervals from their teens to their 60s. We also tested for CMV DNA using polymerase chain reaction in the cellular fractions of all samples.

RESULTS: We found that 76.6% of blood donors were CMV seropositive. The seroprevalences among donors in their 20s and 30s were 58.3 and 73.3%, respectively. We detected CMV DNA in the cellular fraction of 4.3% of samples from donors in their 60s and in 1.0% of samples from donors younger than 60 years. None of the 562 seronegative samples was DNA positive. Furthermore, 14% of DNA-positive samples also contained DNA in the plasma fraction, and two of five such samples were derived from donors in their 60s. Leukoreduced plasma components derived from donations with CMV DNA in plasma samples also contained a relevant amount of CMV DNA.

CONCLUSION: The seroprevalence of CMV among Japanese blood donors of child-bearing age has not changed over the past 15 years. Latent CMV becomes reactivated more frequently among elderly donors than among younger donors. A proportion of them have free CMV DNA in their plasma fraction, which could not be diminished by leukoreduction. The risk of transfusion-transmitted CMV infection in blood with plasma CMV DNA should be determined.

Human cytomegalovirus (CMV; *Human herpesvirus 5*) ubiquitously infects humans and persists in a latent form for long periods. It can cause asymptomatic infection in the general population or a mononucleosis-like syndrome or transient hepatitis in some healthy individuals. However, it can cause serious morbidity and mortality in immunocompromised hosts, and congenital or perinatal CMV infection causes developmental abnormalities in newborns. Morbidity can arise due to either primary infection or CMV reactivation. The transfusion of blood contaminated with CMV could be a source of primary infection in seronegative patients. Thus, CMV-safe blood components are typically required for transfusing seronegative patients who will undergo marrow or organ transplantation, patients with immunodeficiency syndrome, or premature infants. Blood facilities have implemented serologic screening of donated blood for CMV-specific immunoglobulin (Ig)G to mitigate the incidence of transfusion-transmitted CMV infection (TT-CMV) in such patients. This is conducted universally or in response to requests from physicians and has largely prevented TT-CMV infection.[1]

Leukoreduction using white blood cell (WBC) filters has been widely implemented in blood facilities to help reduce the side effects of residual WBCs in blood components such as febrile reactions or alloimmunization against WBC antigen. Leukoreduction under good

ABBREVIATION: TT-CMV = transfusion-transmitted cytomegalovirus infection.

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manufacturing practices could also abrogate the transmission of WBC-associated virus such as CMV, Epstein-Barr virus, or human T cell leukemia virus. Thus, leukoreduced blood components have been advocated as an alternative to transfusion for patients at risk for CMV when seronegative blood is unavailable, although whether leukoreduced blood is as safe as seronegative blood in terms of TT-CMV risk remains a matter of debate.[2-5]

Breakthrough cases have been attributed to transfusion with CMV-seronegative, but CMV DNA–positive blood that might have been donated during a window period, namely, the preseroconversion viremic phase of acute infection.[6] This could justify using leukoreduced blood to avoid transfusion with blood obtained during window periods that serologic screening could miss.[7] Thus, seronegative leukoreduced blood components are currently regarded as the safest strategy to prevent TT-CMV. However, Ziemann and colleagues[8] recently reported that up to 2.9% of plasma derived from donors during the window period contains CMV DNA. Because leukofiltration might not efficiently remove free CMV from the plasma fraction, this would pose another TT-CMV risk that could not be overcome by combining the two strategies.

We screened blood samples (n = 2400) donated equally by male and female volunteers of all age categories using serologic assays and nucleic acid amplification testing (NAT) to assess the risk of CMV transmission in Japan, particularly through transfusion with leukoreduced and seronegative blood components. We established a national prevalence and demographic trend for CMV infection over a range of donor ages and found no blood samples that were both viremic and seronegative. We also found that the frequency of CMV DNA positivity was higher in samples from elderly than from younger donors.

MATERIALS AND METHODS

Blood samples

We sequentially selected whole blood samples at the Japanese Red Cross Tokyo Blood Center in November 2010, where whole blood and blood samples were collected from five prefectures around the greater Tokyo metropolitan area. The samples were allocated to 12 groups according to donor sex and age at 10-year intervals from the 20s to the 60s and from age 16 to 19 years. Each of the 12 categories comprised 200 blood samples. Whole blood collected into tubes containing ethylenediaminetetraacetic acid was separated by centrifugation, during which the separation media rose to the interface between the plasma and the cellular fraction and formed a hard gel. We could thus keep them frozen until use without the two fractions becoming mixed. The plasma fraction was analyzed by CMV serology and CMV NAT. After removing the remaining plasma and interface gel, the top portion of the cellular fraction was suspended in the same volume of phosphate-buffered saline for DNA extraction.

CMV serology assays

We tested CMV-specific IgG and IgM antibodies using automated microparticle enzyme immunoassays (EIAs) and an immunochemical automated analyzer (AxSYM CMV-G and CMV-M, Abbott Laboratories, Abbott Japan, Tokyo, Japan).

DNA extraction

We extracted DNA from the cellular fraction of blood samples using the automated DNA purification kits (QIAsymphony SP and QIAsymphony DNA Midi kits, Qiagen, Tokyo, Japan) according to the protocol provided by the manufacturer (DNA Blood 1000). The input and output sample volumes were 1200 and 200 μL, respectively. Plasma DNA was likewise extracted from samples that were positive for DNA in the cellular fraction using a virus and bacteria detection kit (QIAsymphony Midi kit, Qiagen) with its accompanying protocol (Virus Cellfree 1000). The input and output sample volumes were 1.0 mL and 60 μL, respectively.

CMV NAT

We detected CMV DNA using TaqMan PCR and an sequence detection system (ABI PRISM7900HT, Applied Biosystems, Tokyo, Japan) and artus CMV TM PCR kits (Qiagen) according to the manufacturer's instructions.

We also prepared an in-house TaqMan PCR to detect CMV DNA. This system amplifies a 58-bp fragment of the UL83 gene that encodes phosphorylated 65-kDa proteins (pp65). The forward and reverse primers were 5'-TGCC ATACGCCTTCCAATTC-3' and 5'-TGGCTACGGTTCAG GGTCA-3', respectively. The TaqMan probe, 5'-CGGT AGATGTCGTTGGC-3', was labeled with a reporter dye (6-carboxyfluorescein, FAM) at the 5' end and a minor groove binder at the 3' end. The amplification reagent was supplied with a probe PCR kit (QuantiTect, Qiagen). Each reaction mixture comprised 30 μL of master mix and 20 μL of extracted DNA (equivalent to 120 μL of original sample). The thermocycling protocol comprised 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The nucleic acid concentration was calculated by measuring the absorbance of the extracted DNA at 260 nm.

A validation study for PCR sensitivity included NATtrol NATCMV-0004 (ZeptoMetrix, Buffalo, NY) as the external reference CMV for both PCR analyses. The reference solution was serially diluted in 5% bovine serum albumin (BSA) and portioned into small tubes for PCR analysis over a period of 4 days. We tested CMV concentrations five times daily for each PCR procedure, for a total

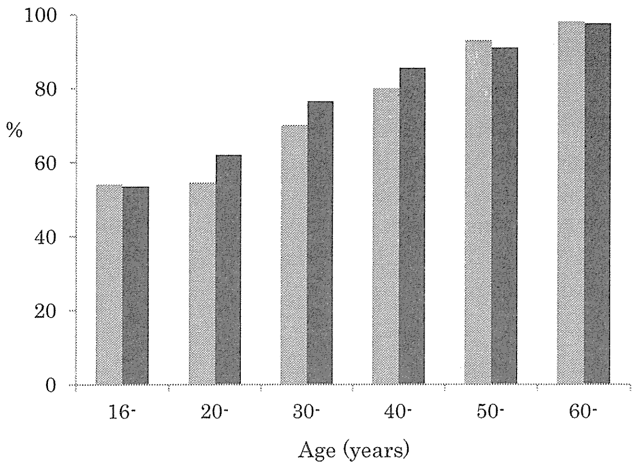

Fig. 1. Age distribution of CMV-specific IgG prevalences in (▒) men and (■) women.

of 20 replicates at each concentration. We then calculated the 95 and 50% limits of detection for each PCR using probit analysis. Correlation study between the reference solution and first World Health Organization international standard (NIBSC 09/162) revealed that 32.3 genome equivalents/mL (geq/mL) was equivalent to 1 IU/mL. Samples in which the PCR results were ambiguous were further analyzed using nested PCR targeting the UL139 sequence as described by Bradley and colleagues[9] with the modification for DNA polymerase (KAPA DNA polymerase, Nippon Genetics, Tokyo, Japan).

To adjust the amount of CMV DNA for the number of WBCs in the sample, we estimated the number of Exon 5 sequences of CD81 in specimens using real-time PCR.[10] CD81 was chosen as a marker of WBCs as it is present with two haploids in a cell. Amounts of CMV DNA are described as geq per 6.0×10^6 WBCs (geq/PBL unit) in this study. The lowest limit of quantitative CMV DNA detection was 40 geq/mL before adjustment for WBC numbers.

Statistical analysis

Data were analyzed using computer software (SSRI, Excel Statistics, Version 8, Social Survey Research Information, Tokyo, Japan; for Windows, Microsoft Excel 2007, Tokyo, Japan). Significance was determined using the chi-square test and t test. p values of less than 0.05 were considered significant.

RESULTS

We initially examined the prevalence of anti-CMV among Japanese blood donors. Figure 1 shows the prevalence of specific IgG among the age categories. The prevalence exceeded 50% even in male and female teenagers and steadily increased over time to reach nearly 100% in their 60s. Although not significant, the prevalence tended to be higher in females than in males aged from the 20s to the 40s. The increase in the prevalence was the highest between the 20s and 30s (15%; combined for both sexes) and gradually decreased with age to 5.8% between the 50s and 60s. The mean prevalence in the six age categories was 76.3%. The overall CMV prevalence adjusted for an assumed population with the age distribution of Japanese blood donors (Japanese Red Cross data, 2010) was 76.6%. The IgM prevalence was higher among females than males between the ages of 16 and 39 years ($p < 0.05$, Table 1). Seven donors were IgM positive and IgG negative, and four of them were teenagers.

We next examined the presence of CMV DNA in the cellular fraction of 2400 whole blood samples. A validation study showed that the 95 and 50% limits of CMV DNA detection for artus CMV TM PCR were 41.6 and 5.3 geq/mL, respectively, and those for the in-house PCR were 29.6 and 5.4 geq/mL, respectively (Table 2). Only samples that were positive for at least two PCR analyses including nested PCR targeting the UL139 sequence were defined as CMV DNA positive. We identified 37 samples that were positive for CMV DNA in the cellular fraction (Table 3). Four other samples were positive for only one PCR analysis and were defined as DNA indeterminate. Table 4 shows the relationship between DNA positivity and the serostatus of the specific antibody. We found DNA positivity in six (6.6%) of 91 samples that were both IgM and IgG positive and in 31 (1.8%) of 1740 that were only IgG positive. Although the samples that were positive only for IgM did not contain any that were DNA positive, the frequency of DNA positivity was significantly higher in six (6.12%) of 98 samples that were IgM positive with or without IgG than in those that were positive only for IgG ($p < 0.03$). Viral load was significantly higher in CMV DNA–positive samples that were both IgM and IgG positive (mean, 670 geq/PBL unit) than in those that were only IgG positive (170 geq/PBL unit, $p < 0.03$, t test). Notably, none of the 562 samples that were both IgM and IgG negative was also DNA positive.

Table 5 compares the distribution of 37 DNA-positive samples with age categories. The frequency of DNA positivity was significantly higher (17/400, 4.3%) among donors in their 60s than in any other age category (0.8%-1.3%, $p < 0.03$) from the teens to the 50s or the combined age category (1.0%, $p < 0.03$) from 16 to 59 years. The range of viral load in the 37 DNA-positive samples was between less than 40 and 3.4×10^3 geq/PBL unit (mean, 250 geq/PBL unit; median, 80 geq/PBL unit). The difference in viral load in the samples between donors aged less than 60 years (mean, 310 geq/PBL unit) and those in their 60s (mean, 170 geq/PBL unit) was not significant. The presence of DNA in the plasma fraction was further investigated in these 37 samples. Five (13.5%) of them were plasma DNA positive with a viral load between less than

TABLE 1. Prevalence of CMV-specific IgM among blood donors*

	Male		Female		Total	
Age (years)	Positivity	Percent	Positivity	Percent	Positivity	Percent
16-19	6	3.0†	13 (4)	6.5†	19 (4)	4.8
20-29	5 (1)	2.5†	15	7.5†	20 (1)	5.0
30-39	5 (1)	2.5†	13	6.5†	18 (1)	4.5
40-49	8	4.0	10	5.0	18	4.5
50-59	5 (1)	2.5	3	1.5	8 (1)	2.0
60-69	6	3.0	9	4.5	15	3.8
Total	35 (3)	2.9	63 (4)	5.3	98 (7)	4.1

* Numbers of donors positive only for specific IgM are shown in parentheses.
† IgM prevalence significantly higher among female donors than among male donors (16-19, 20-29, and 30-39 years); chi-square test ($p < 0.05$).

40 and 5.8×10^3 geq/mL (median, 170 geq/mL). These five were scattered across age categories with two being in their 60s. One sample obtained from a teenaged donor was IgM and IgG positive and the other four were positive only for IgG. We identified two more samples from donors in their 60s that were DNA positive with only one PCR analysis.

We interdicted three components of fresh-frozen plasma that had CMV DNA in the plasma fraction. All of them were derived from whole blood that was leukoreduced before storage. We also detected CMV DNA in all three plasma components. One component donated by the IgM- and IgG-positive teenaged male donor contained 9.7×10^3 geq/mL CMV DNA and the other two components that were positive only for IgG from donors in their 60s contained 1.9×10^2 and 1.6×10^3 geq/mL CMV DNA.

DISCUSSION

We investigated the prevalence of CMV among Japanese blood donors categorized by sex and age at 10-year intervals. The more than 50% prevalence of CMV infection among individuals aged between 16 and 19 years is in contrast with the approximately 30%[11] prevalence in other developed countries. The increase in the prevalence (15%) between donors aged in their 20s and 30s implies that young adults become infected with CMV at a rate of 1.5% per annum. This is similar to the annual rate of 1.69% observed between 1994 and 1999,[12] implying that the risk of CMV infection among females of child-bearing age that is directly related to symptomatic fetal CMV infection has not changed over the past 15 years. The reason for the sustained high prevalence in Japan is unclear, but prolonged breast-feeding and communal child care practices in Japan probably influenced the rates in younger donors. The prevalence in Japan has become almost maximal after the age of 60 years, which contrasts with the continuous lifelong primary infection found in other developed countries.[11] The CMV seroconversion rate (1.33%)[11] among German blood donors aged 30 to 35 years is close to the 1.5% rate of increase described above. However, care must be taken in comparing the present results with those of the German study because our results were generated from a cross-sectional study whereas the German findings were obtained through longitudinal follow-up of seronegative donors. Although insignificant, the prevalence in females tended to increase sooner than in males, a finding that is consistent with the higher prevalence of specific IgM in younger females than in younger males.[13,14]

We detected CMV DNA in the cellular fraction of 1.7% (41/2400) of all, or 2.2% (41/1831) of the seropositive, samples with or without specific IgM. This frequency was comparable to those reported by Greenlee and colleagues[15] and Roback and colleagues.[16] We found CMV DNA more frequently in samples that were IgM positive than in those that were only IgG positive (6.12% vs. 2.0%, $p < 0.03$), indicating that active CMV replication occurs more frequently during acute primary infection that is often accompanied by IgM positivity. None of the samples from the group of seven donors that was positive only for IgM was CMV DNA positive. This is reasonable because Ziemann and coworkers[17] detected CMV DNA only in 10% of 148 primary seroconverted blood donors. At that rate we would be unable to identify a single DNA-positive individual in our study population. The same authors showed that CMV DNA levels peak during the late phase of primary infection in newly seropositive donors.[8] Although whether a rationale exists for introducing screening for specific IgM in addition to IgG remains to be determined,[18] the chemiluminescence tests for CMV currently applied by the Japanese Red Cross detect only IgG. Although we have discussed seroprevalence and its relationship with the presence of DNA by interpreting IgM positivity as representing primary infection, reactivity for CMV-specific IgM measured by EIAs must be considered with caution. Several articles have reported frequent nonspecific reactions[19,20] and suggest including Western blot analyses or IgG avidity assays to ensure reactivity. Because of the small plasma volume of most of the donor samples, we were unable to apply these analyses. Thus, the above findings and our interpretations based on categories by IgM positivity might be inconclusive and require further investigation.

We found no DNA-positive samples among 562 that were seronegative, suggesting that the likelihood of donating DNA-positive blood during the window period[7] is very low in Japan. This finding is similar to that described by Roback and coworkers,[16] who found no CMV DNA positivity among 514 healthy, seronegative blood donors. However, these findings do not allow underestimation of

TABLE 2. Determination of sensitivity of two PCR systems by replicate testing and probit analysis

PCR	Ref* (geq/mL)	D 1	D 2	D 3	D 4	Total	95% LOD (geq/mL)	50% LOD (geq/mL)
Artus CMV	1	1/5	1/5	1/5	1/5	4/20	41.6	5.3
	5	5/5	3/5	2/5	5/5	15/20		
	10	5/5	5/5	4/5	4/5	18/20		
	50	5/5	5/5	5/5	5/5	20/20		
In house (UL83)	1	0/5	1/5	0/5	1/5	2/20	29.6	5.4
	5	5/5	3/5	5/5	4/5	17/20		
	10	5/5	5/5	4/5	5/5	19/20		
	50	5/5	5/5	5/5	5/5	20/20		

* NATtrol as CMV reference was diluted in 5% BSA.
artus = artus CMV TM PCR kits (Qiagen); D = day; in house (UL83) = in-house PCR targeting CMV UL83 sequence; LOD = limit of detection.

TABLE 3. CMV DNA–positive samples and PCR procedures

PCR results	Number of samples
UL83 positive and artus positive	29
UL83 repeatedly positive	2
artus repeatedly positive	2
UL83 positive and UL139 positive	3
artus positive and UL139 positive	1
Indeterminate*	4
Negative	2359
Total	2400

* Positive in only one PCR analysis.
artus = artus CMV TM PCR kits (Qiagen); UL139 = PCR targeting CMV UL139 sequence; UL83 = in-house PCR targeting CMV UL83 sequence.

the risk of TT-CMV caused by transfusion with window period–derived blood components because we did not focus on blood samples obtained at the time of acute primary infection when CMV replication is most likely to be active. In fact, Ziemann and colleagues[8] found that two (2.9%) samples were DNA positive among 68 plasma samples obtained from final seronegative donations during the course of seroconversion. Collectively, a risk of TT-CMV related to window period donation exists but the frequency seems very low.

The frequency of detecting CMV DNA was 4.3% among donors in their 60s, compared with 1.0% (0.8%-1.3%) in the population aged from 16 to 59 years. Considering that the specific IgG prevalence has already peaked by age 60 years in Japan, the notion that the DNA-positive individuals in their 60s were nonimmune to CMV and emitted CMV virions during the course of primary CMV infection is inconceivable. Latent CMV more likely became reactivated in those elderly individuals. The reactivation of CMV in elderly persons is thought to represent "immunosenescence" caused by chronic CMV infection.[21,22] The current concept of immunosenescence in relation to CMV infection is that terminally differentiated memory T cells accumulate with ageing in the limited peripheral "immunologic space," which causes a progressive decline in the generation of naive T cells that protect against new pathogens. In addition, a considerable portion of the accumulated memory T cells were specific for CMV.[23-25] Thus, CMV infection is considered a driving force or risk biomarker for the constitution of a skewed peripheral T-cell repertoire. Despite conflicting results and ideas about epidemiology and immunologic mechanisms, the clinical impact of the CMV infection on individuals who are not immunocompromised has remained a central question.[22] Whether or not all persons with CMV infection acquire skewed T-cell phenotypes with aging, the kinds of socioeconomic or physical factors that facilitate this process, and when this process starts to compromise the immune system should be addressed. We established statistical evidence of CMV reactivation occurring in the peripheral blood of voluntary blood donors in their 60s. Viral load did not significantly differ between donors in their 60s and those aged less than 60 years. Blood donors in Japan are supposedly healthy individuals who have all been qualified by questionnaires and consultation with physicians. Our results therefore suggest that CMV reactivation is a constitutional event in CMV carriers and starts to occur during the sixth decade of life, although the possibility remains that donors positive for CMV DNA recently might have had specific illnesses or behaviors that are related to CMV reactivation. The findings of animal experiments suggest that lytic viral reactivation is necessary to establish the peripheral T-cell repertoire skewed for CMV.[26] Stowe and colleagues[27] detected CMV in 57% of urine samples from elderly individuals (66 to 83 years) but in none of those from younger individuals (25 to 55 years). This would also suggest that CMV reactivation occurs more frequently among elderly, than younger, individuals, although they did not detect CMV in any blood samples from both groups. However, this might have resulted from the small sample size studied (11 elderly individuals compared with 400 aged ≥60 years in this study). The rather clear cutoff of the reactivation frequency between the 50s and 60s is reminiscent of a Swedish study[28] showing increased 10-year mortality

TABLE 4. Association between CMV serostatus and CMV DNA positivity

Serology status	Number of samples	DNA+ (n)	Ratio (%)	Viral load Mean/median Range (geq/PBL unit)
IgM–/IgG–	562	0	0	
IgM+/IgG–	7	0	0*	
IgM+/IgG+	91	6	6.6*	670/62† <40-3400
IgM–/IgG+	1740	31	1.8*	170/80† <40-920
Total	2400	37	1.5	

* DNA positivity ratio significantly higher in IgM+ than in IgM–/IgG+ samples (6 [6.1%] of 98 vs. 31 [1.8%] of 1740); chi-square test ($p < 0.03$).
† Viral load in IgM+/IgG+ samples significantly higher than that in IgM–/IgG+ samples; t test ($p < 0.03$).
DNA+ = DNA positive.

TABLE 5. Age distribution of CMV DNA positivity in cellular and plasma fractions*

Age (years)	Cellular fraction DNA+	Cellular fraction Ratio (%)	Plasma fraction, DNA+
16-19	4 (2)	1.0	1 (1)
20-29	5	1.3	1
30-39	5 (1)	1.3	0
40-49	3 (2)	0.8	0
50-59	3	0.8	1
16-59 total	20 (5)	1.0	3 (1)
60-69	17 (1)	4.3	2†
Total	37 (6)	1.5	5

* Numbers of donors specifically positive for both IgM and IgG are shown in parentheses.
† In addition to these two samples, two others from donors in their 60s were positive for one PCR analysis.
DNA+ = DNA positive.

rates among individuals with immune risk profiles at the age of 65 years but not at the age of 55 years.

We identified five (13.5%) samples that were positive for CMV DNA in the plasma fraction of 37 blood samples that contained CMV DNA in the cellular fraction. This result is comparable to the report by Ziemann and coworkers[8] in which 44% of blood samples from 82 recently seroconverted donors were CMV DNA positive in the plasma fraction. Drew and colleagues[6] also reported that three of 384 samples obtained from 192 seroconverted blood donors contained low plasma levels of CMV DNA. We quantified CMV DNA levels in three plasma products derived from donations that had CMV DNA in plasma samples. The DNA levels (1.9×10^2 to 9.7×10^3 geq/mL) were comparable to those measured in the plasma samples. Because all blood components including apheresis-derived plasma components are leukoreduced in Japan, this finding indicated that leukofiltration cannot reduce levels of free CMV DNA in the plasma fraction.

The identification of blood donations with plasma fractions containing CMV DNA raises concerns about the safety of blood components. The residual risk of TT-CMV under the current blood program that applies both seroscreening and universal leukoreduction could be focused on blood with plasma viremia that is provided during the window period because plasma viremia might not be appropriately managed by leukofiltration. This leads to the notion of Ziemann and colleagues[8] that leukoreduced components would be safer when obtained from seropositive donors at least 1 year after seroconversion. This is also based on the finding that plasma viremia has barely been detectable among donors who remain seropositive for more than 1 year. However, our study showed that a proportion of latently infected individuals presents with free CMV DNA in plasma fractions. Free CMV DNA in plasma could not be effectively diminished by prestorage leukoreduction, which was verified by assays of leukoreduced plasma products. Therefore, the strategy suggested by Ziemann and colleagues, while eliminating window period–related risk, might generate another risk associated with blood containing free plasma CMV DNA that is provided mainly by elderly donors. Although we identified only two samples from donors in their 60s that were plasma DNA positive, one of them related to a plasma product containing 1.6×10^3 geq/mL CMV DNA, a viral load that was comparable to that for window period donation provided by the teenager (9.7×10^3 geq/mL). Moreover, we found two other samples with possible plasma DNA among donors in their 60s, although they were DNA positive only for a single PCR analysis, suggesting low DNA concentrations. Whereas we found that whole blood CMV DNA positivity among donors in their 60s was 4.3%, that identified in an elderly US population with a mean age of 84.5 years was 42.3%.[29] Thus, since the frequency of whole blood CMV increases dramatically after 60 years, we can speculate that the frequency of plasma CMV also increases with age. In this context, serious problems could arise in countries that accept donors over 70 years of age if seropositive donations are accepted for transfusion into patients at risk. Although leukoreduced blood components have been advocated as an alternative when seronegative blood is not available, they might carry a higher risk of TT-CMV than seronegative blood, which might partly explain higher TT-CMV frequency among patients transfused with leukoreduced blood compared with seronegative blood.[2,4]

Further study is required to compare possible TT-CMV risks between persistently seropositive blood that might contain free CMV virions due to reactivation and seronegative blood that might incidentally contain such virions due to window period donation. Ziemann and coworkers concluded from a recent extensive study of more than 22,000 samples that TT-CMV risk is essentially comparable between window period donations among seronegative donors and donations with reactivation among long-term seropositive donors.[30] Before assessing the TT-CMV risk in Japan, the degree to which window period donation constitutes the blood donor population will need to be determined. Other basic issues also need to be resolved. Because we used techniques involving DNA amplification but not viral culture for plasma study, whether CMV DNA identified in plasma with this technique constitutes replication-competent virions remains unknown.[31,32] Whether plasma products containing free CMV virions is infectious through blood transfusion also needs to be resolved.[13,33] Whether blood components containing CMV virions possibly derived from reactivation in latently infected blood donors are as infectious as those derived from donations provided during acute primary infection also requires investigation. The clinical relevance of CMV neutralizing antibody that can be found in latently infected individuals also must be considered. Finally, the minimal infectious dose of CMV virions acquired through blood transfusion should be determined for each type of blood component.

In conclusion, the seroprevalence of CMV among a Japanese population of blood donors was 76.6%. The prevalence among donors in their 20s and 30s has not changed over the past 15 years. We detected CMV DNA in 1.7% of 2400 samples. None of the 562 seronegative samples was DNA positive. We detected CMV DNA more frequently in blood from donors aged in their 60s than from younger donors. Among DNA-positive samples, 14% contained DNA in the plasma fraction, and this frequency might be higher among donors in their 60s than younger donors. CMV DNA persists in a portion of seropositive blood even after prestorage leukoreduction and leukoreduced blood without seroscreening might not be as safe as seronegative blood in terms of TT-CMV risk. The risk of TT-CMV in blood with detectable CMV DNA in the plasma fraction should be determined.

CONFLICT OF INTEREST

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［報告］

Occult HBV carrier からの輸血による急性B型肝炎が強く疑われた１例

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The blood transfusion from an occult HBV carrier caused acute hepatitis B virus infection

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抄　録

Occult HBV carrierが原因と考えられる輸血後急性B型肝炎の１例を報告する。受血者は30代男性。27歳で特発性門脈圧亢進症と診断された。今回，食道離断術と摘脾術を受け，赤血球製剤３本，新鮮凍結血漿10本，血小板製剤１本の輸血を受けた。術前にHBV-DNAは検出されず，HBs抗体・HBc抗体ともに陰性であった。輸血後213日目の検査で急性B型肝炎と診断された。当該事例で使用された血液製剤全ての保管検体のHBV個別NATを施行した結果，新鮮凍結血漿の１検体が陽性であった。受血者血液と供血者血液のHBV-DNA解析により，両者の相同性は高いと判断された。両者のHBV GenotypeはCで，受血者のHBV CP/PreC領域はwild typeであった。この供血者は，当該献血の前後690日間に４回の献血（前１回，後３回）をしているが，当該献血以外は個別NATが陰性であり，HBc抗体弱陽性のoccult HBV carrierと考えられた。当該献血者の新鮮凍結血漿以外の血液製剤からのB型肝炎感染は認められなかった。

Key words: occult HBV carrier, acute hepatitis B,
transfusion-transmitted HBV infection

はじめに

Occult HBV carrier[1]とは，HBs抗原陰性，HBV-DNA陽性で，かつ，ウイルス量が200IU/mL以下の低濃度の感染状態である[2]。Occult HBV carrierからの輸血によるHBV感染は，1979年に初めて報告されている[3]。今回，Occult HBV carrierが原因と考えられる輸血後急性B型肝炎の１例を経験したので報告する

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症　例

受血者は30歳代男性。27歳時に特発性門脈圧亢進症と診断された。今回食道離断術と摘脾術を受け，赤血球製剤３本，新鮮凍結血漿10本，血小板製剤１本の輸血を受けた。表１に示すように，輸血前にHBV-DNAは検出されず，HBs抗体・HBc抗体ともに陰性であった。輸血後１カ月目までは肝機能異常は認めなかった。輸血199日後の検査で肝機能異常を認めた。輸血213日後の検査では，HBV-DNAは6.8 log copy/mLで，HBc抗体およびIgM-HBc抗体と，HBe抗原が陽性であった（表１）。この時点で急性B型肝炎と診断され，エンテカビルの内服を開始した。

感染経路を特定するために，使用された14本の血液製剤の保管検体でHBV個別NATを施行したところ，新鮮凍結血漿の１検体が個別NAT陽性であった。しかし，この検体ではウイルス量が少なく，コバスTaqMan法で定量下限値（20IU/mL）以下の陽性であり，また，ウイルスのDNA解析もできなかった。

当該献血者（60歳代男性）の遡及調査結果を表２に示す。当該献血の前後690日間に，個別NAT陽性の当該献血を含めて５回の献血があったが，当該献血以外は個別NAT陰性であった。HBc抗体価は，1.3から9.6 C.O.I.と1.0以上12.0未満であった。今回の遡及調査では，該当する献血日が2012年８月以前のため，HBc抗体価は12.0以上が陽性である。輸血によるB型肝炎を減少させるために，2012年８月からは，HBc抗体価は1.0以上が陽性となった。そのため，HBc抗体価1.0以上12.0未満の場合は，従来の陽性と区別して弱陽性とした。HBs抗体価は，CLEIA法で2.0から9.5mIU/mLと低値であった。HBs抗体価は200mIU/mL以上が陽性である。

個別NAT陽性となった当該献血から製造された赤血球製剤は，すでに使用されていたが，この受血者の輸血17カ月後のHBs抗原検査は陰性であった。また，個別NAT陰性となった当該以外の献血からの血液製剤によるHBV感染は認められなかった。

個別NAT陽性の保管検体では，ウイルス量が少なく，HBV-DNAの解析ができなかった。そのため，検体量が確保できる500日後に献血された新鮮凍結血漿製剤を用いてHBV-DNAの解析を行

表１　受血者検査結果

	輸血前	37日後	199日後	213日後
ALT（IU/L）	17	14	73	677
HBV-DNA	陰性	N.T.	N.T.	6.8 log copy/mL
HBs抗体	陰性	N.T.	N.T.	陰性
HBc抗体	陰性	N.T.	N.T.	陽性
IgM-HBc抗体	陰性	N.T.	N.T.	陽性
HBe抗原	陰性	N.T.	N.T.	陽性
HBe抗体	陰性	N.T.	N.T.	陰性

（N.T.検査せず）

表２　遡及調査結果

献血日	個別NAT	RCC	FFP	原料血漿	HBc抗体（C.O.I.）	HBs抗体（mIU/mL）
190日前	陰性	使用済		送付済	1.3	9.5
当該献血	陽性	使用済	当該製品		1.4	4.7
106日後	陰性	使用済		送付済	9.6	7.9
202日後	陰性	使用済		送付済	8.1	4.6
500日後	陰性	使用せず	PCR実施		5.6	2.0

HBc抗体価は12以上が陽性。HBs抗体価は200以上が陽性。

い，受血者と献血者由来のHBV-DNAを比較した。新鮮凍結血漿製剤検体もウイルス量は少なく，PreS/S領域を含むP領域の前半部1,550bp（nt. 2,333-3,215/1-667）はPCRで増幅できなかったので，S領域内の193bp（nt.475-667）について解析・比較した（図1）。新鮮凍結血漿製剤検体5mLから核酸を抽出・濃縮しS領域の増幅を試みたところ，12回行ったうちの1回から増幅産物が得られた。この領域の献血者由来HBV-DNAと受血者由来HBV-DNAの塩基配列（193bp）を比較したところ，2カ所で相違が認められた。その1カ所がコドン122の，サブタイプ特異抗原基であった。この点突然変異により，サブタイプが両者で異なっているが，サブタイプ自体容易に変異することが報告されている[4]。また，図2に示すように，データベース上で高い相同性を示した8株と塩基配列を比較したところ，献血者由来HBV-DNAと受血者由来HBV-DNAの両者にのみ特徴的な塩基配列が，nt.507，nt.547，nt.554の3カ所に認められた。以上より，当該献血者血液による輸血後B型肝炎であることが強く疑われた。両者のHBV-DNAはGenotype Cであった。献血者検体のCP/PreC領域はPCRで増幅できなかった。受血者由来HBV-DNAのCP/PreC領域の塩基配列はWild typeであった。

考　察

本事例の献血者は，HBc抗体弱陽性のHBV感染既往者である。輸血によるB型肝炎を認めた献血時の保管検体の個別NATで，コバスTaqMan法で定量下限以下の低濃度のHBVを保有していた。そして，上記献血の500日後に献血された新鮮凍結血漿製剤を用いてHBV-DNAの解析を行い，受血者のHBV-DNAとの相同性が高いと判断された。この500日後の献血時の保管検体は個別NATが陰性であったにもかかわらず，多量の検体から核酸を抽出・濃縮して解析を行い，献血者HBV-DNAの一部を同定し得た。以上より，本事例は，HBs抗原陰性，HBV-DNA陽性のoccult HBV carrierが感染源であることが強く疑われた。これはB型肝炎の流行地で多くみられるパターンで，慢性のHBV carrierで，HBs抗原陰性化し，HBc抗体のみ陽性となった“anti-HBc alone”の状態である[5]。

Occult HBV carrierからの輸血でHBVが感染する頻度は低い。急性B型肝炎のウインドウ期の感染率は81％で，occult HBV carrierからの感染率は19％とする報告がある[6]。受血者が免疫不全状態にあると感染のリスクが高いと報告されている[7]が，本事例では免疫不全状態ではなかった。並存するHBs抗体が低値であったことが，HBV感染成立に関与したと思われる[8]。

本事例では，個別NAT陰性の献血血液からHBV-DNAの解析が行われ，受血者のそれと相同性が高いと判断された。すなわち，個別NAT陰性であっても，HBVが存在することが示された。花田らは，個別NAT陰性のoccult HBV carrierからの献血血液を介したHBV感染が疑われる症例

```
       475                                          codon122: 受血者_Lys(d)  献血者_Arg(y)
受血者   1:TCCTCTAATT CCAGGAACAT CAACCACCAG TATGGGACCA TGCAAGACCT GCACGACTCC TGCTCAAGGA ACTTCTATGC TTCCCTCTTG TTGCTGTACA 100
献血者   1:.......... .......... .......... .......... ....G..... .......... .......... .......... .......... .......... 100
対象株   1:.......C.. .......... ....T..... C.C....... .......... ......T... .......... ..C......T .......A.. .......... 100

                                                             codon160:Lys(w)                      667
受血者 101:AAACCTTCGG CCGCAAACTG CACTTGTATT CCCATCCCAT CATCCTGGGC TTTCGCAAAA TTCCTATGGG AGTGGGCCTC AGTCCGTTTC TCC  193
献血者 101:.......... A......... .......... .......... .......... .......... .......... .......... .......... ...  193
対象株 101:.......... A..G...... .......... .......... .......... .....T..G. .......... .......... .......... ...  193
```

受血者：Genotype C / Subtype adw
献血者：Genotype C / Subtype ayw
対照株：Genotype C / Subtype adr

図1　S領域内の193塩基長の配列

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              nt.475                                    507                             547    554                  574
受血者       1:TCCTCTAATT CCAGGAACAT CAACCACCAG TATGGGACCA TGCAAGACCT GCACGACTCC TGCTCAAGGA ACTTCTATGC TTCCCTCTTG TTGCTGTACA 100
献血者       1:.......... .......... .......... .......... ....G..... .......... .......... .......... .......... .......... 100
AB030508.1   1:.......C.. .......... .......... C.C....... .......... .......... .......... ..C......T .......... .......... 100
AB221829.1   1:.......C.. .......... .......... ..C....... .......... ......T... .......... ..C......T .......... .......... 100
EU660229.1   1:.......C.. .......... .......... C.C...G... .......... .......... .......... ..C......T .......... .......... 100
FJ561020.1   1:.......C.. .......... .......... C.C....... .......... .......... .......... ..C......T .......... .......... 100
FJ622482.1   1:.......C.. .......... .......... ..C....... .......... .......... .......... ..C......T .......... .......... 100
GQ475351.1   1:.......C.. .......... .......... ..C....... .......... ......T... .......... ..C......T .......... .......... 100
GU079389.1   1:.......C.. .......... .......... C.C....... .......... ......T... .......... ..C......T .......... .......... 100
HM358180.1   1:.......C.. .......... .......... C.C....... .......... ......T... .......... ..C......T .......... .......... 100

              nt.575                                                                                         667
受血者       101:AAACCTTCGG CCGCAAACTG CACTTGTATT CCCATCCCAT CATCCTGGGC TTTCGCAAAA TTCCTATGGG AGTGGGCCTC AGTCCGTTTC TCC 193
献血者       101:.......... A......... .......... .......... .......... .......... .......... .......... .......... ... 193
AB030508.1   101:.......... A..G...... .......... .......... .......... ........G. .......... .......... .......... ... 193
AB221829.1   101:.......... A..G...... .......... .......... .......... ........G. .......... .......... .......... ... 193
EU660229.1   101:.......... A..G...... .......... .......... .......... .......... .......... .......... .......... ... 193
FJ561020.1   101:.......... A..G...... .......... .......... .......... ........G. .......... .......... .......... ... 193
FJ622482.1   101:.......... A..G...... .......... .......... .......... ........G. .......... .......... .......... ..T 193
GQ475351.1   101:.......... A..G...... .......... .......... .......... ........G. .......... .......... .......... ... 193
GU079389.1   101:.......... A......... .......... .......... .......... ........G. .......... .......... .......... ... 193
HM358180.1   101:.......... A..G...... .......... .......... .......... .......... .......... .......... .......... ... 193
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AB030508.1～HM358180.1　DDBJデータベースで高い相同性を示した株

図2　データベースで高い相同性を示した８株とのS領域内193塩基長の配列の比較

を報告している[9)]。しかし，本事例では，個別NAT陰性の赤血球製剤からのB型肝炎感染は認められなかった。また，個別NAT陽性の赤血球製剤からの感染も認めなかった。B型肝炎ウイルスの感染リスクは，輸血された血漿量すなわち輸血されたウイルス総量に比例すると考えられる[6)]。

日本赤十字社ではHBVの更なる安全対策として，2012年８月からHBc抗体の基準を厳しくし，HBc抗体価1.0（C.O.I.）以上かつHBs抗体価200mIU/mL未満のHBV感染既往献血者の血液を排除することとした。これにより，本事例のような既感染者由来occult HBV carrierからの輸血によるHBV感染は，さらに減少すると思われるが，引き続き医療機関と協力して，自発報告・遡及調査等の情報を共有しつつ，感染の拡大を防止することが重要である。

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Transfusion-transmitted hepatitis E in a patient with myelodysplastic syndromes

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Introduction

Patients with haematological diseases occasionally exhibit liver dysfunction during treatment. This liver dysfunction can have various causes such as therapy-related drugs and hepatitis B and C infections, although the cause is unclear in some cases. It was recently reported that some patients initially diagnosed with drug-induced liver dysfunction actually had hepatitis E[1]. Several cases of transfusion-transmitted hepatitis E infections have also been reported[1,2]. In Japan, screening for hepatitis E does not appear to be performed at the initial examination of patients with acute hepatitis. This might be because hepatitis E is believed to be orally transmitted and to occur mainly in developing countries and rarely in developed countries. However, hepatitis E is a zoonotic infectious disease. Cases of regional endemic hepatitis E virus (HEV) infection have been increasing in Europe, the United States, and Japan[1].

Although HEV usually causes self-limited acute hepatitis, it sometimes progresses to a chronic infection. Most cases of chronic infection occur in patients undergoing solid organ or haematopoietic stem cell transplantation, in those receiving anti-cancer or immunosuppressant drugs, and in patients with human immunodeficiency virus infection, in whom the condition may progress to liver cirrhosis[3]. HEV RNA persisted for a long period during treatment in a patient with T-cell lymphoma[4]. Reactivation of HEV hepatitis was reported after an allogeneic haematopoietic stem cell transplant in a patient with Philadelphia chromosome-positive acute lymphoblastic leukaemia[5]. On the other hand, a low risk of HEV reactivation after haematopoietic stem cell transplantation was also reported[6]. More studies on the risk of HEV reactivation are, therefore, required.

Here, we report the case of a patient with a myelodysplastic syndrome (MDS) who developed acute hepatitis due to transfusion-transmitted HEV infection. We also review the literature on the topic.

Case report

The patient was a 70-year old Japanese man who attended our hospital for Parkinson's disease in June 2001. In July 2001, he was referred to the Haematological Department because of thrombocytopenia. Haematological examinations revealed that he had pancytopenia with a white blood cell count of 2.9×10^9/L, haemoglobin level of 9.0 g/dL, and a platelet count of 36×10^9/L. Bone marrow findings showed 8.8% myeloblasts and trilineage dysplastic features. Chromosome abnormalities with [46,XY, -10, +marker] were detected in 15 of 22 mitotic bone marrow cells. He was, therefore, diagnosed with MDS. According to the French-American-British criteria, he was classified as having refractory anaemia with excess of blasts (RAEB)-1 and was given a score of intermediate-2 according to the International Prognostic Scoring System at that time. Because he was suffering from Parkinson's disease, he received combination therapy with oral vitamin K2 (menatetrenone, 45 mg/day) and vitamin D3 (alfacalcidol, 1 μg/day)[7] instead of chemotherapy. This treatment resulted in no progression to leukemic transformation over the next 10 years. However, the pancytopenia gradually worsened, and protein anabolic steroids (metenolone, 20 mg/day) were added to the treatment in 2009. Over the next 12 months, he received repeated red cell and platelet transfusions because of anaemia and haemorrhagic symptoms. Bacterial infections often occurred during medical home care, and his Parkinson's disease worsened. On April 28th, 2011, the patient was admitted to hospital with a lung abscess and aspiration pneumonia. He had a gastrointestinal bleed after admission to hospital and the volume of blood transfusions consequently increased. Although hepatic function was within the normal range on admission, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) began to increase from May 18th, peaking at 504 and 736 IU/L, respectively, on June 8th. Although these

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levels decreased transiently, they increased again from June 20th together with a rise in total bilirubin level. On June 22nd, the patient died of exacerbation of the lung abscess (Figure 1).

After the patient had died, the stocked plasma split from one of the donors of red blood cell (RBC) products given to our patient was screened for viruses before utilisation in plasma-fractionated products. The results revealed HEV RNA in the stocked plasma. We, therefore, performed complete examinations of the stocked donated blood and identified the HEV RNA-positive donor. The RBC product derived from this donor had been transfused into our patient on May 2nd. Serological examinations using the stored sera from this donor revealed an ALT level of 26 IU/L; the sera were negative for immunoglobulin (Ig)G anti-HEV and IgM anti-HEV assayed by enzyme immunoassay (IgG/IgM anti-HEV, Institute of Immunology, Tokyo, Japan) as well as hepatitis B virus (HBV) DNA and hepatitis C virus (HCV) RNA. The HEV RNA copy number quantitatively assayed with a TaqMan reverse transcription polymerase chain reaction (QIAGEN) was 1.2×10^3. These findings suggest that the blood had been donated during the "window period" of HEV infection in this donor.

We retrospectively investigated HEV RNA in our patient's stocked sera. As shown in Figure 1, HEV RNA began to rise on May 23rd, peaked on June 16th, and started to decrease on June 18th. The patient's serum from June 10th was found to be positive for IgG anti-HEV and IgM anti-HEV, whereas the sera stocked before May 30th were all negative for IgG anti-HEV and IgM anti-HEV. In addition, the results of viral examinations conducted when the patient presented with liver dysfunction were negative for HBV DNA, HCV RNA, cytomegalovirus and Epstein–Barr virus. These results strongly suggest that the patient had acquired transfusion-transmitted hepatitis E.

We then compared the sequence of the viral RNA detected from the stocked sera of the donor and patient using reverse transcription-polymerase chain reaction followed by direct sequencing. We also compared the sequence of the amplification products of open reading frame 1 (ORF1) (326 bp, nt 123-448) and ORF2 (412 bp, nt 5,987-6,398) of HEV between the patient's and donor's samples. Many different HEV sequences were determined among the sequences from the donor's sample. For precise analysis, the donor's HEV was cloned and sequenced. A total of 28 polymerase chain reaction products were obtained and seven HEV strains were isolated from the donor's stocked sera, all of which were genotype 3. The phylogenetic tree according to the ORF2 sequence is shown in Figure 2. Interestingly, the tree suggests that the donor carried two distinct phylogenies of HEV. Of these, one strain (isolate-1, in Figure 2) showed 100% sequence homology with the strain isolated from the patient's stocked serum with respect to both ORF1 and ORF2.

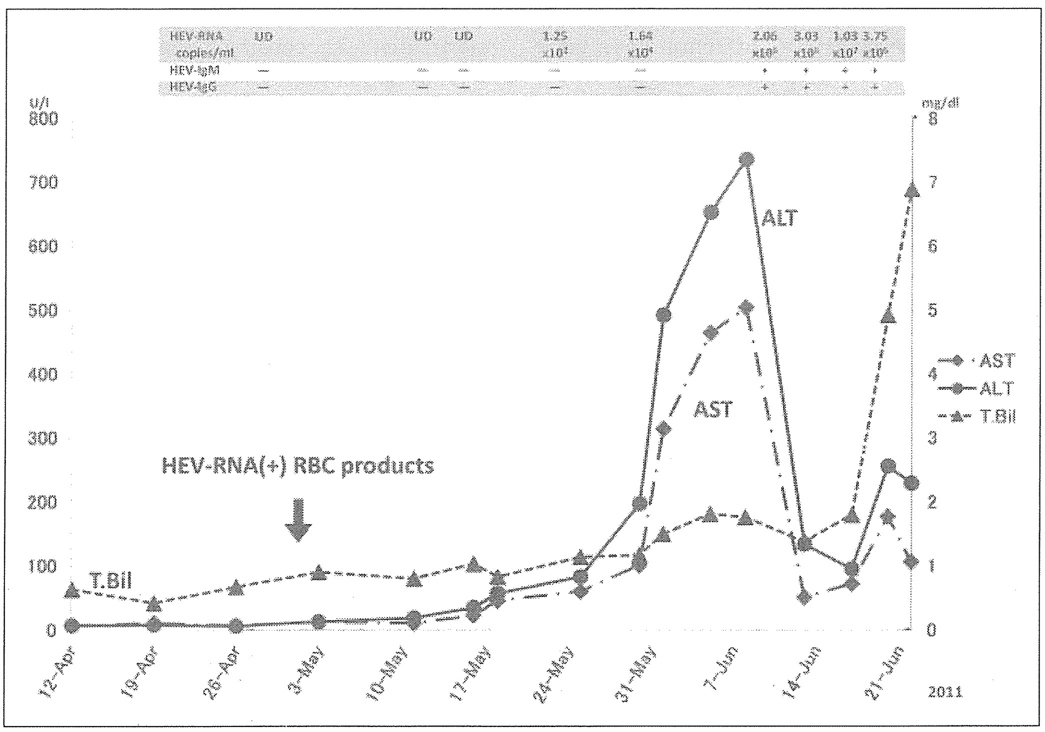

Figure 1 - Clinical course of the patient. (UD: undetectable)

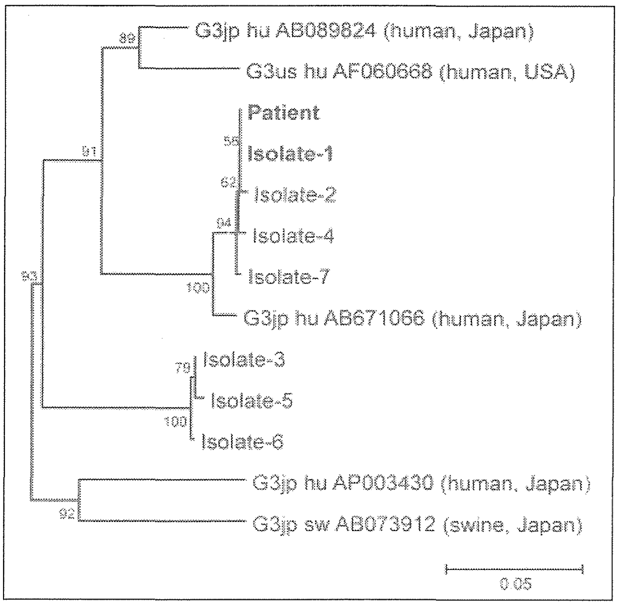

Figure 2 - Phylogenic analysis of ORF2 region Phylogenetic tree of HEV constructed by neighbor-joining method. A 100% homology is observed between the patient and isolate-1. Isolate-1 to -7: HEV clones from the blood donor.

Discussion

HEV is an RNA virus comprising approximately 7.2 kb and has four different genotypes[1]. HEV genotypes vary regionally. The epidemic form is related to genotypes 1 and 2, which cause severe acute disease and are spread via drinking water in developing countries. The regional endemic or autochthonous form is related to genotypes 3 and 4, which are zoonotic and cause mild asymptomatic disease that is spread via food in developed countries[2]. Cases of transfusion-transmitted HEV infection were recently reported in the United Kingdom, France, and Japan[8-10]. Among these, five patients including the present one had haematological diseases[4,8,11,12]. These cases comprised only male patients and the causative blood transfusion products were RBC and platelet products. The HEV genotypes were type 3 in four cases and type 4 in one case. In developed countries, most cases are reported to be of genotype 3, like the present case[2]. However, these cases are very likely to be regional endemic diseases rather than infections imported from developing countries.

Recent studies demonstrate that some cases of HEV infection were initially misdiagnosed as drug-induced liver dysfunction. A British study[13] found acute HEV infection in 13% (6/47 cases) of cases initially diagnosed as drug-induced liver dysfunction. Similarly, an American study reported HEV infection in 3% (9/318 cases) of cases initially diagnosed as drug-induced liver injury[14]. HEV RNA analysis was performed in four of these nine cases and revealed that all of the cases were caused by HEV genotype 3[14]. These data suggest that HEV infection should be included in the differential diagnosis of patients with liver dysfunction.

The prevalence rates of HEV vary among countries and even among regions within a country[15-19]. In Japan, out of 12,600 samples, 431(3.4%) were positive for IgG anti-HEV. The results of that study showed that the prevalence of IgG anti-HEV was significantly higher in eastern Japan (5.6%) than in western Japan (1.8%) ($P<0.001$), indicating marked regional variation[19]. Indeed, the infected donor involved in this case report was from Tokyo, which is in eastern Japan. Furthermore, the Hokkaido area is reported to have higher incidence of IgG anti-HEV than other eastern areas including Tokyo. Besides the high prevalence of HEV RNA[18], the incidence of HEV transmission is also high with a predominance of genotype 4, which is the viral genotype that causes severe symptoms[11,20]. Thus, HEV RNA screening of donated blood was experimentally initiated in Hokkaido in 2005.

Patients who have received many blood transfusions are reported to have a significantly higher incidence of markers of HEV infection (i.e., IgG/IgM anti-HEV and HEV RNA) than those who have received fewer blood transfusions[21]. Since HEV screening is not performed to prevent haematologically transmitted infections in developed countries, the frequency of transfusion-transmitted HEV infection might be underestimated. The present and previously reported cases indicate that any blood product, including RBC products[4,8], platelet products[11,12] and fresh-frozen plasma[10] can transmit HEV. However, the viral load required to induce transfusion-transmitted hepatitis E in recipients is unclear. Further investigation is required to clarify this point. Therefore, HEV screening for blood transfusion donors should be considered in areas in which the seroprevalence of HEV is high. However, the present case revealed that HEV can be transmitted via blood products from donors during the "window period". Moreover in two previous cases[11,12], blood products that transmitted HEV were positive for HEV RNA but negative for anti-HEV antibody. Thus, HEV RNA should be investigated at the onset of liver dysfunction in patients receiving frequent blood transfusions. Furthermore, HEV RNA screening among blood donors might be effective for the prevention of transfusion-transmitted HEV.

Keywords: hepatitis E virus, transfusion-transmitted infection, myelodysplastic syndromes.

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