◀ **Fig. 2** **a** FACS analysis using tetramer staining. To detect HBV specific CTLs in the PBMCs, we isolated PBMCs from 4 groups. The samples were stained with PE-conjugated anti-human HLA-A24 HBV core antibody and a PE-Cy5-conjugated anti-human CD8 antibody. **b** Frequencies of HBV-specific $CD8^+$ T cells and $CD4^+$ Tregs. The numbers of HBV-specific $CD8^+$ T cells and Tregs were analyzed by FACS at the time of diagnosis in HBV reactivation patients and prior to chemotherapy or antiviral therapy in the other groups. The *upper panels* show the percentages of HBV-specific $CD8^+$ T cells, among which the *left panel* shows the A2 core, the *center panel* shows the A24 core and the *right panel* shows A24 poly-specific $CD8^+$ T cells. The *middle panels* shows the percentage of IFN-γ producing $CD8^+$ T cells stimulated by peptides for A2 core, A24 core, and A24 poly, respectively. The *lower panels* show the percentages of Tregs, of which the *left panel* shows $CD4^+CD25^+$ Tregs and the *right panel* shows $CD4^+Foxp3^+$ Tregs. $*P < 0.05$, significant difference between the linked items. **c** Relationships between the frequencies of HBV-specific $CD8^+$ T cells and Tregs. The *scatter diagrams* show HBV reactivation patients (*white circle*) and other patients (*black dot*), respectively. The *left panel* shows the negative correlation between $CD4^+Foxp3^+$ Tregs and A2 core-specific $CD8^+$ T cells. The *center panel* shows the negative correlation between $CD4^+Foxp3^+$ Tregs and A24 core-specific $CD8^+$ T cells. The *right panel* shows the negative relationship between $CD4^+Foxp3^+$ Tregs and A24 poly-specific $CD8^+$ T cells

HBV-specific $CD8^+$ T cells to determine whether the $CD8^+$ T cell function in HBV reactivation patients was different from that in the other groups. Although PD-1 expression was higher on circulating HBV-specific $CD8^+$ T cells in ICHB and CHB patients, it was significantly lower on HBV-specific $CD8^+$ cells in HBV reactivation patients (Fig. 3). Curiously, PD-1 expression on HBV-specific $CD8^+$ T cells was low in resolved HBV patients compared with ICHB and CHB patients, indicating that HBV-specific $CD8^+$ T cells in resolved HBV patients may function in a similar manner to those in HBV reactivation patients.

It was also demonstrated that primary CD62L high expressing $CD8^+$ T cells were better at clearing LCMV infection compared with primary CD62L low expressing cells. In addition, CD62L high memory cells underwent robust expansion, and were efficient in preventing chronic LCMV infection [18]. Thus, to address the memory phenotype of cells we examined the expression of CD62L on HBV-specific $CD8^+$ T cells. However, we did not detect any significant differences in the expression of CD62L on $CD8^+$ T cells among the groups although CD62 expression in HBV reactivation patients had a tendency to be lower.

Longitudinal analysis of the frequencies of HBV-specific $CD8^+$ T cells and $CD4^+$ Tregs

To evaluate changes in the frequency of HBV-specific $CD8^+$ T cells during HBV reactivation, we monitored sALT levels, serum HBV DNA levels, percentages of HBV-specific $CD8^+$ T cells and numbers of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ cells in the six HBV reactivation patients. As shown in Fig. 4, when serum HBV DNA was

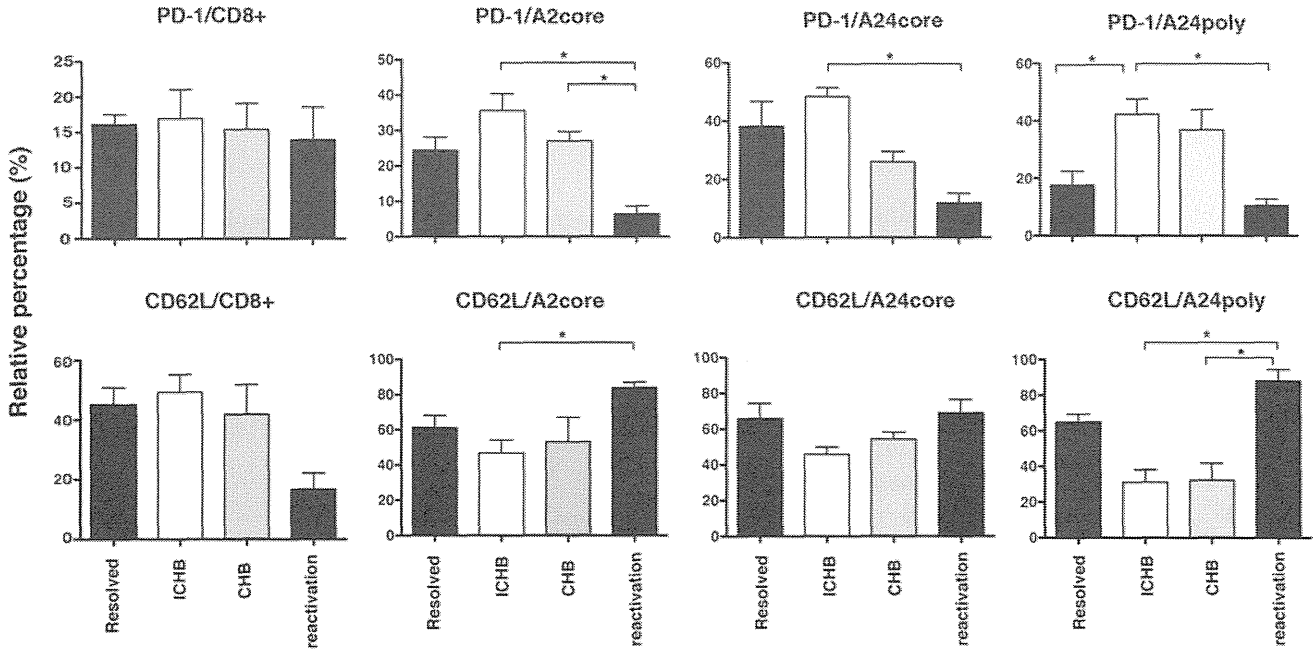

Fig. 3 PD-1 and CD62L expression in HBV-specific and total $CD8^+$ T cells. The PD-1 and CD62L expression levels in HBV-specific and total $CD8^+$ T cells were analyzed by FACS at the time of diagnosis in HBV reactivation patients and prior to chemotherapy or antiviral therapy in the other groups. The *left panel* shows the percentages of PD-1 or CD62L-positive cells in CD8 T^+ cells. The *three right panels* show PD-1 or CD62L-positive cells in HBV-specific $CD8^+$ T cells, respectively. $*P < 0.05$, significant difference between the linked items

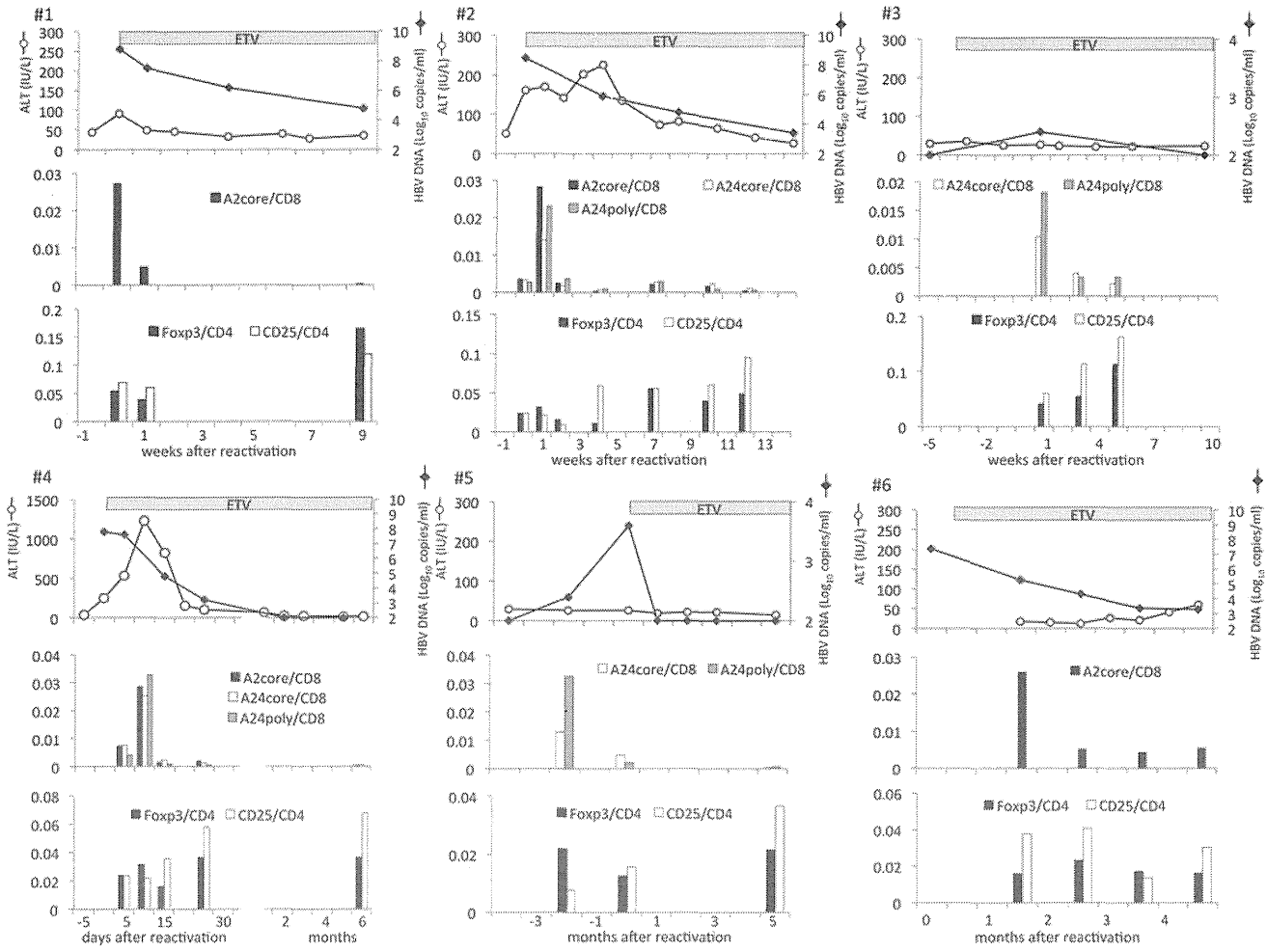

Fig. 4 Longitudinal analysis of the frequencies of HBV-specific $CD8^+$ T cells and Tregs. The *upper panels* show the kinetics of ALT (IU/L) (*white circle*) and HBV DNA (log_{10} copies/ml) (*black diamond*), the *middle panels* show the frequencies of HBV-specific $CD8^+$ T cells and the *lower panels* show the frequencies of Tregs, respectively. All patients were administered entecavir immediately following diagnosis

detected in resolved HBV patients, administration of entecavir was quickly started for all patients to prevent severe hepatitis. HBV-specific $CD8^+$ T cells were detected and reached their peak frequency levels at the onset of HBV reactivation in patients #1 to #6. Interestingly, a high percentage of HBV-specific $CD8^+$ T cells was observed in patient #4 compared with other patients and, consistent with this finding, the sALT level was markedly elevated to about 1200 IU/l, indicating that the number of HBV-specific $CD8^+$ T cells reflected the grade of liver damage, as previously reported [19]. Furthermore, when the numbers of HBV-specific $CD8^+$ T cells were maximal, the numbers of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells were minimal, indicating a negative correlation. Moreover, HBV-specific $CD8^+$ T cells decreased as the sALT level decreased, whereas $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells showed tendencies to increase. However, the number of HBV-specific $CD8^+$ T cells in patient #6 did not decrease throughout the time course, although this patient showed tendencies for higher numbers of $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells at the time of HBV reactivation. In this patient, the reduction in serum HBV DNA was slow and the sALT levels continued to be elevated. These findings suggest that $CD4^+$ Tregs may suppress an effective immune response against HBV.

Discussion

HBV reactivation is an almost universal event among patients with HBsAg undergoing bone marrow transplantation [20–22]. In retrospective analyses using sensitive serological and virological markers, a high proportion of people with anti-HBc antibodies without HBsAg in their serum also redevelop HBV DNA and HBsAg after bone marrow transplantation [23, 24]. In addition, the prolonged

impairment of immune-mediated control of intrahepatic HBV after extensive immunosuppression leads to reactivation of potential occult infection with HBsAg seroreversion [2]. Thus, although the risk of HBV reactivation during immunosuppression is well known, the mechanism for the induction of HBV reactivation is unclear.

In this study, we demonstrated that six patients with HBV reactivation showed increased numbers of HBV-specific $CD8^+$ T cells, similar to the case for self-limited acute hepatitis B, and that these T cells induced liver damage despite immunosuppression following treatment with an immunosuppressant and anti-cancer drug.

These findings are consistent with a previous report of a strong multifaceted CTL response in patients with acute hepatitis [7]. It is interesting to evaluate the function of antigen-specific $CD8^+$ T cells, including their proliferation and cytokine production, during immunosuppressive drug treatment, because a previous study showed that FK506 did not prevent the generation and proliferation of LCMV-specific T cells, but instead altered their differentiation so that these effector T cells lost their ability to control the virus [25]. Although we analyzed the role of $CD8^+$ T cells under the immunosuppressive status, it seems to be important to analyze macrophages, which produce TNF-α and IL-6 [25].

We found that the ratio of HBV-specific $CD8^+$ T cells was higher in resolved HBV patients than in ICHB and CHB patients, indicating that high viral loads suppress the frequency of these cells [26]. Furthermore, we showed that PD-1 expression on HBV-specific $CD8^+$ T cells was low in resolved HBV patients compared with ICHB and CHB patients, demonstrating that these cells can restore their function. These findings suggested that resolved HBV patients have numerous and functionally recovered HBV-specific $CD8^+$ T cells, and therefore, they may easily develop severe hepatitis once HBV reactivation is induced. This hypothesis was confirmed by a report that acute hepatitis in resolved HBV patients has a higher mortality rate than acute hepatitis in HBV-positive patients [23].

In addition, we found that the frequencies of HBV-specific $CD8^+$ T cells and $CD4^+Foxp3^+$ Tregs were reversible at the onset of HBV reactivation. These observations may imply that the reduction of $CD4^+Foxp3^+$ Tregs triggered the induction of antigen-specific CTLs. Although the effects of $CD4^+Foxp3^+$ Tregs are generally nonspecific or occur in a bystander manner, preferential inhibition of the antigen-specific T cell response has been observed in some cases, including human HBV infection [27]. In support of our results, Xu et al. [16] demonstrated that depletion of $CD4^+CD25^+$ Tregs led to an increase in HBV antigen-stimulated IFN-γ production and cellular proliferation of PBMCs in HBV-infected patients, and that coculture of $CD4^+CD25^+$ Tregs with effector cells significantly suppressed HBsAg-stimulated IFN-γ production and cellular proliferation. At the time of HBV reactivation in patient #6, when the number of $CD4^+Foxp3^+$ Tregs was increased, the reduction in serum HBV DNA was poor and liver damage was continuous. These findings suggest that a reduction in the number of $CD4^+Foxp3^+$ Tregs may induce an effective immune response.

We also observed that serum IL-7, IL-8 and MCP-1 were significantly higher in resolved HBV patients than in ICHB and CHB patients. However, the group of resolved HBV patients was quite miscellaneous and it remains unknown whether the differences among such cytokines and chemokines are responsible for HBV.

It has been demonstrated that IL-7 is required for T cell development and for maintaining and restoring the homeostasis of mature T cells. Administration of recombinant human IL-7 to patients resulted in widespread T cell proliferation, increased T cell numbers, modulation of peripheral T cell subsets and increased T cell receptor repertoire diversity [28]. Furthermore, IL-7 expression by hepatocytes directly controls T cell immune responses to Toll-like receptor signaling in vivo [29]. These observations suggest that IL-7 plays an important role for HBV-specific $CD8^+$ T cell proliferation and that a low level of IL-7 may be involved in the low frequencies of HBV-specific $CD8^+$ T cells in ICHB and CHB patients.

As previously reported, since rituximab therapy is a high risk factor for HBV reactivation, we examined a possible imbalance in serum Th1/Th2 cytokine secretion in HBV reactivation patients. As shown in supplementary Fig. 2, we analyzed the ratio of serum Th1/Th2 cytokines as follows: IFN-γ or IL-12 compared with IL-4 or IL-10. We observed a shift towards IL-10 compared with IFN-γ. However, when we compared IL-12 with IL-4 and IL-10, we observed a shift towards IL-12. Thus, we did not detect an obvious shift towards either Th1 or Th2 cytokines in the serum at the onset of HBV reactivation.

Finally, our study showed that HBV-specific $CD8^+$ T cells are increased at the onset of HBV reactivation despite an immunosuppressive status and declined following resolution of liver disease. In contrast, a reduced number of $CD4^+Foxp3^+$ Tregs was also observed and showed a negative correlation with the frequency of HBV-specific $CD8^+$ T cells. We plan to analyze additional resolved HBV patients prospectively and to clarify the relationships among $CD4^+Foxp3^+$ Tregs, HBV-specific $CD8^+$ T cells and liver damage.

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Conflict of interest The authors have no conflicts of interest to disclose.

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Residual risk of transfusion-transmitted hepatitis B virus (HBV) infection caused by blood components derived from donors with occult HBV infection in Japan

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BACKGROUND: Nucleic acid amplification testing (NAT) for hepatitis B virus (HBV) during blood screening has helped to prevent transfusion-transmitted HBV infection (TT-HBV) in Japan. Nevertheless, 4 to 13 TT-HBV infections arise annually.

STUDY DESIGN AND METHODS: The Japanese Red Cross (JRC) analyzed repository samples of donated blood for TT-HBV that was suspected through hemovigilance. Blood donations implicated in TT-HBV infections were categorized as either window period (WP) or occult HBV infection (OBI) related. In addition, we analyzed blood from 4742 donors with low antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis B surface antigen (anti-HBs) titers using individual-donation NAT (ID-NAT) to investigate the relationship between anti-HBc titer and proportion of viremic donors.

RESULTS: Introduction of a more sensitive NAT method for screening minipools of 20 donations increased the OBI detection rate from 3.9 to 15.2 per million, while also the confirmed OBI transmission rate increased from 0.67 to 1.49 per million. By contrast the WP transmission rate decreased from 0.92 to 0.46 per million. Testing repository samples of donations missed by minipools of 20 donations NAT showed that 75 and 85% of TT-HBV that arose from WP and OBI donations, respectively, would have been interdicted by ID-NAT. The ID-NAT trial revealed that 1.94% of donations with low anti-HBc and anti-HBs titers were viremic and that anti-HBc titers and the frequency of viremia did not correlate.

CONCLUSIONS: The JRC has elected to achieve maximal safety by discarding all units with low anti-HBc and anti-HBs titers that account for 1.3% of the total donations.

The prevalence of hepatitis B virus (HBV) surface antigen (HBsAg) in Japan is slightly higher than the average for developed countries. A recent screening of blood donors, local residents, and school pupils found an estimated national prevalence of HBsAg of 0.71%.[1] However, the prevalence was higher during the 1990s, being 1.5% among first-time blood donors aged in their 40s.[2] Taking into account horizontal transmission and a birth cohort effect, a relatively large cohort with historical HBV infection might persist among older individuals in Japan.

To prevent transfusion-transmitted HBV (TT-HBV) infection, Japanese Red Cross (JRC) blood centers introduced HBsAg screening for all blood donations in 1972. In 1989, antibody to hepatitis B core antigen (anti-HBc) testing was introduced to exclude donations by people with prior HBV infection. Because total elimination of anti-HBc–reactive donations might have seriously reduced the blood supply, donations with high antibody to hepatitis B surface antigen (anti-HBs) titers and those

ABBREVIATIONS: CLEIA(s) = chemiluminescence enzyme immunoassay(s); ID = individual donation; JRC = Japanese Red Cross; LOD = limit of detection; OBI = occult hepatitis B virus infection; PC(s) = platelet concentrate(s); S/CO = signal-to-cutoff ratio; TT-HBV = transfusion-transmitted hepatitis B virus infection; TTI = transfusion-transmitted infection; WP = window period.

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with low anti-HBc titers have been accepted, and only donations with low anti-HBs and high anti-HBc titers were excluded.

In addition to this serologic screening algorithm, the JRC implemented multiplex nucleic acid amplification testing (NAT) for HBV, hepatitis C virus (HCV), and human immunodeficiency virus Type 1 (HIV-1) in 1999.[3] Although NAT has greatly reinforced blood safety regarding TT-HBV infection, 4 to 13 TT-HBV infections continue to arise annually. While some occur as a result of transfusion with blood components obtained during the window period (WP), others arise due to components being derived from donors with occult HBV infection (OBI) defined as detectable HBV DNA in peripheral blood but no detectable HBsAg.[4,5] Although donations from donors with OBI have helped to maintain an adequate blood supply, such donations have also raised a concern about the risk of TT-HBV. Here, we describe the current status of TT-HBV under the NAT screening system as well as problems inherent in the current HBV screening algorithm, especially with regard to OBI-derived blood donations. We also discuss the feasibility of strategies that could increase HBV safety in countries such as Japan with a slightly elevated prevalence of HBV.

MATERIALS AND METHODS

Screening donated blood at JRC blood centers

The JRC blood centers are the only facilities authorized to handle blood collection, processing, testing, and delivery in Japan. Donated blood is screened at these centers for HBsAg, anti-HCV, anti-HIV-1 and -2, anti-human T-lymphotropic virus type 1, anti-*Treponema pallidum*, and human parvovirus B19 antigen. Whereas HBsAg-positive blood is rejected, HBsAg-negative samples are further tested for anti-HBc and anti-HBs (Table 1). Blood with a high anti-HBs titer (≥200 IU/L) is accepted irrespective of the anti-HBc titer and that with a low anti-HBc titer is also accepted irrespective of the anti-HBs titer. Blood with high anti-HBc and low anti-HBs titers (<200 IU/L) is disqualified. All blood had been serologically tested before 2008 using the agglutination method with the initial cutoff for a high anti-HBc titer being a dilution factor of 2^6, which was later revised to 2^5. All agglutination tests were replaced with chemiluminescence enzyme immunoassays (CLEIAs, CL4800 testing system, Fujirebio, Tokyo, Japan) in 2008 and the threshold for anti-HBc positivity is currently a signal-to-cutoff ratio (S/CO) of 12.0. This value was validated as being essentially equivalent to an agglutination titer of 2^5. Blood donations with elevated serum alanine aminotransferase (>60 IU/mL) are also rejected.

TABLE 1. HBV screening algorithm applied at JRC blood centers*

	Anti-HBc titer	
Anti-HBc reactive 4.9% 261,000, *49,000*	Low <2^6(2^5) or S/CO≥1.0 but <12.0	High ≥2^6(2^5) or S/CO ≥12.0
Anti-HBs ≥ 200 IU/L	Accepted 2.04% 108,000, *20,000*	Accepted 1.38% 73,000, *14,000*
Anti-HBs < 200 IU/L	Accepted 1.31% 69,000, *13,000*	Rejected 0.19% 10,000, *2,000*

* HBsAg-negative donations are tested for both anti-HBc and anti-HBs. Dilution factors for anti-HBc titers were applied for agglutination testing. Dilution factors in parentheses were applied between 1997 and 2007. The S/CO ranges are currently used for CLEIA. Ratios (%) of donations for each category are shown (2010 data). Observed number and number per million (italics) of donations are also included.

NAT

Samples that were qualified by the testing algorithm for anti-HBc and anti-HBs described above as well as by HBsAg testing are then screened using NAT. The JRC started NAT in 1999 using a real-time multiplex polymerase chain reaction system with a minipool format that originally comprised 500 samples (Ampli-NAT MPX system, Roche, Indianapolis, IN).[6] The pool size was decreased to 50 in 2000 and to 20 in 2004. The JRC implemented the Roche TaqScreen MPX system for NAT in 2008 with a pool size of 20, but with an approximately threefold increase in sensitivity because of the increased sample volume required for nucleic acid extraction and improvements in reagents. The screening sensitivity of HBV is 650, 260, and 76 copies/mL (50% limit of detection [LOD]; JRC data) for 50- (50p) and 20- (20p) sample pools using AmpliNAT and 20p using TaqScreen, respectively.

A trial screening using individual-donation NAT (ID-NAT) proceeded at the Tokyo Blood Center between December 2010 and May 2011 to verify the distribution of the rate of donations containing HBV DNA relative to anti-HBc titers and the residual TT-HBV risk that could arise from transfusion with blood donations that have low anti-HBc and anti-HBs titers. All available donations with both an anti-HBc titer between 1.0 and 12.0 S/CO and an anti-HBs titer of less than 200 IU/L were screened by ID-NAT using the Roche TaqScreen MPX system with a 50% LOD of 3.8 copies/mL (JRC data). The sensitivity of ID-NAT used in lookback studies (described below) was 13 copies/mL (50% LOD) using AmpliNAT until July 2008 and 3.8 copies/mL (50% LOD) using TaqScreen from August 2008.

Hemovigilance system

The JRC established a hemovigilance system in 1993 and has since collected reports on adverse effects caused by blood transfusion. Through blood screening the JRC obtains information about repeat donors who have

recently acquired infection.[7] Their previous donations are evaluated for transfusion-transmitted infection (TTI) risk by considering donation timing and performing ID-NAT on repository samples (lookback studies). If they are judged as harboring a TTI risk, the JRC notifies the relevant facilities that used the component at risk and requests that physicians investigate whether any patient who received a transfusion of the component has acquired the corresponding infection.

The JRC also obtains information about TTI in transfused patients through voluntary reports by physicians who are involved in blood transfusion at medical facilities.[7] Upon receiving such information, the JRC analyzes repository blood samples obtained from implicated donations using ID-NAT. The TTI risk of cocomponents derived from the implicated and previous donations provided by implicated donors is assessed. The JRC notifies the relevant medical facilities of the findings. Implicated blood components are interdicted if they have not yet been used for transfusion.

The JRC headquarters and central laboratory determine the causal relationship between the implicated donation and posttransfusion infection considering patient clinical course, results of virologic analysis including ID-NAT and sequence analysis, serologic viral markers, and donation timing. Even if all repository samples implicated for TTI are verified as being ID-NAT-negative, implicated donors are followed up for repeat donation thereafter for sero- or NAT conversion, because the possibility that the index donation was provided during the ID-NAT WP persists. All processes for lookback studies are defined in national guidelines[8] that describe in detail the test items and timing of testing for donated blood and at-risk patients in addition to the roles of the relevant physicians, blood centers, and blood authority.

Sequence analysis

The HBV genome sequence identity is assessed between implicated repository blood samples and patient samples by sequencing 1550 bp of the alpha region within the HBV pre-S and S regions using a genetic analyzer (ABI 3130XL, Life Technologies Japan, Tokyo, Japan). When the viral load is too low to sequence, viral nucleic acid is further extracted from larger plasma volumes if the accompanying plasma bag is available. When findings are ambiguous, HBV obtained from donor or patient samples is cloned, amplified, and sequenced.

Estimation of current risk of TT-HBV

Although universal pre- and posttransfusion testing of patient samples for TTI has been recommended, the likelihood that all transfused patients undergo this evaluation is low. Moreover, the JRC hemovigilance system described above is voluntary. Therefore, TTI might be underreported to JRC blood centers. The exact amount of TT-HBV infections that could occur under the current screening system must be defined to assess novel TT-HBV–mitigating strategies. This study therefore reevaluated the current risk of TT-HBV infection based on data obtained under current system.

The projected number of ID-NAT–positive donations derived from OBI donors was calculated using the ID-NAT positivity rate obtained in the ID-NAT trial screening described above and the number of donations with low anti-HBc and anti-HBs titers. The additional WP yield in donations determined by ID-NAT was calculated based on rates of detection of recently infected donors.[9,10] Assuming that the frequency of donation is constant at any time during the presymptomatic phase of acute infection, the yields by tests for an infection marker are in direct proportion to the length of time during which each test gives a yield. The potential ID-NAT yield (screening NAT negative) was calculated herein by multiplying the screening NAT yield by the ratio of the interval between ID-NAT detection and 20p-NAT detection (11.2 days) to that between 20p-NAT detection and HBsAg detection (9.7 days). The interval covered by each NAT strategy (11.2 and 9.7 days) was calculated using the value for the detection limit of each test (3.8, 76, and 1000 copies/mL for ID-NAT, 20p-NAT, and CLEIA detection, respectively) and the doubling time of HBV in human peripheral blood (2.6 days).[9,11] The number of donations that could appear in the ID-NAT–negative WP was similarly calculated separately for each component type taking into account both the interval between 1 copy/bag and ID-NAT detection deduced from the mean plasma volume of each component type and the number of each component issued to hospitals.

We estimated the number of TT-HBV infections with reference to our previous systematic lookback study.[7] The infectivity of ID-NAT–positive and screening NAT-negative components was calculated in that study as being 3% (95% confidence interval [CI], 0%-17.2%, n = 33) and 50% (95% CI, 28.2%-71.8%, n = 22) for OBI- and WP-derived components, respectively. The incidence rate for TT-HBV infections was thus obtained by multiplying the number of estimated at-risk donations deduced using the above method by the infection rates (0.03 or 0.5).

Statistical analysis

Data were statistically analyzed using computer software (SSRI for Windows, Excel Statistics Version 8, Social Survey Research Information Co. Ltd, Tokyo, Japan). Significance was determined using the chi-square test except for associations between total viral load in the components and alanine aminotransferase (ALT) levels in patients that were evaluated using the Mann-Whitney U test.

RESULTS

Reports of possible TT-HBV infections

The JRC blood centers received 789 reports of possible TT-HBV infections between 2001 and 2010 (Fig. 1). The number of such reports obviously increased in 2004 and 2005 because a nationwide systematic retrospective study started in 2004 that also identified patients with TT-HBV infection that would have previously been unrecognized. Causality was investigated in all but two of these possible TT-HBV infections. The possibility of TT-HBV infection was precluded in 97 (12.3%) of the 789 reported patients without testing repository samples based on evaluation of the patient's clinical course and the transfusion setting for each. For all of the remaining patients, repository samples were tested serologically and by ID-NAT to detect the HBV genome. Of the 789 initial reports, 98 (12.4%) were determined to be TT-HBV infections after the introduction of 50p-NAT. The HBV sequence identity was established between donor and recipient in 88 of these cases, and TT-HBV was determined considering other HBV markers and the clinical setting in the remaining 10. An HBV genome was not detected in repository samples for 587 (74.4%) potential TT-HBV infections. Although HBV was detected in four repository samples, HBV sequence identity was not confirmed between donors and recipients. Forty-two (43%) of the established TT-HBV infections were discovered through lookback studies that were started based on risk information provided by JRC blood centers. The remaining 56 (57%) were initially recognized by physicians at medical facilities. The number of established TT-HBV infections ranged from 4 to 13 per year between 2006 and 2010.

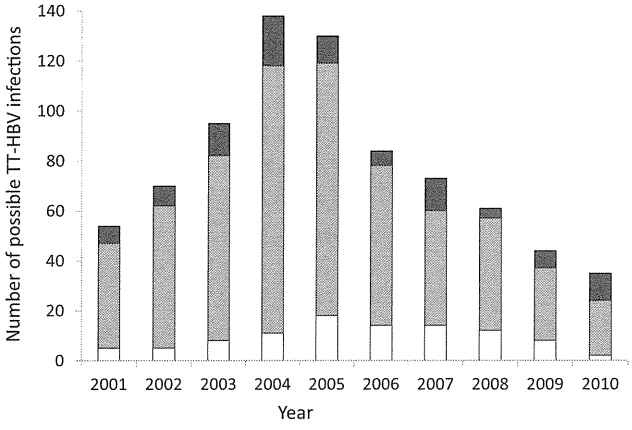

Fig. 1. Annual number of potential TT-HBV infections. (□) Patients in which possibility of TT-HBV was excluded (n = 97, 12.3%) without testing repository samples. (■, ▨) Patients in whom HBV DNA was identified or not, respectively, in the repository samples corresponding to a donation from the donor of the implicated blood components. Four patients are not included as HBV DNA sequence identity was not established.

Infection status of donors implicated in TT-HBV infection

The sensitivity of NAT screening improved through the three phases described above (50p-AmpliNAT, 20p-AmpliNAT, and 20p-TaqScreen). With the increased sensitivity of 20p-TaqScreen, the NAT yield of OBI donations increased from 3.9/million to 15.2/million, whereas the yield of WP donation decreased from 13.2/million to 5.7/million (Table 2). This was caused by the simultaneous introduction of CLEIA in 2008 for serologic screening including HBsAg detection, which effectively shortened the period that could be covered by 20p-NAT.

The established TT-HBV infections that occurred during each period were categorized based on the presence or absence of the HBV genome in the implicated component (that is, ID-NAT positive or negative) and the infection status of the donation (WP related [anti-HBc nonreactive] or OBI related [anti-HBc reactive]). Table 2 also shows the numbers of established TT-HBV infections associated with each group during each period. Figure 2 shows the incidence (per million donations) of estab-

TABLE 2. NAT yield and number of TT-HBV infections relative to three phases of screening NAT*

Screening system	50p-AmpliNAT	20p-AmpliNAT	20p-TaqScreen
Duration of screening period	Feb. 2000–Jul. 2004 (4.5 year)	Aug. 2004–Jul. 2008 (4.0 year)	Aug. 2008–Mar. 2010 (1.67 year)
Sensitivity of screening NAT (copies/mL)†	650	260	76
Sensitivity of ID-NAT used for lookback study (copies/mL)†	13	13	3.8
Number of donations tested	24,702,784	19,513,054	8,746,037
Confirmed WP donations (/million)	473 (19.1)	258 (13.2)	50 (5.7)
Confirmed OBI donations (/million)		76 (3.9)	133 (15.2)
Number of donations causing established HBV transmission			
ID-NAT–negative WP	5	6	1
ID-NAT–positive WP	28	12	3
ID-NAT–negative OBI	4 (1)‡	1 (0)‡	2 (1)‡
ID-NAT–positive OBI	13 (1)‡	12 (1)‡	11 (5)‡

* Yields by ID-NAT trial conducted from December 2010 are not included in the table.
† 50% LOD.
‡ Numbers of donations with anti-HBs of greater than 10 mIU/mL.

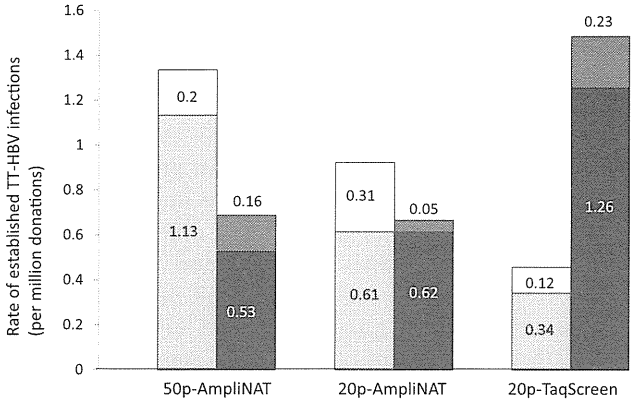

Fig. 2. Number of established TT-HBV infections grouped according to pool-based NAT screening systems. See Table 2 for intervals when indicated NAT systems were applied, sensitivities of NAT systems used, and actual yields for each category at each interval. (□, ▨) Infections caused by transfusion with ID-NAT–negative and -positive WP-derived components, respectively. (▩, ■) Infections caused by transfusion with ID-NAT–negative and -positive OBI-derived components, respectively.

lished TT-HBV infections relative to the three periods. The rate of infections caused by transfusion with a WP-derived component notably decreased with increasing NAT sensitivity, but that caused by transfusion with OBI-derived components rather increased despite the increased NAT sensitivity. Current NAT screening protocols indicated that TT-HBV infections occur more frequently due to transfusion with OBI- than with WP-related components (1.49/million vs. 0.46/million donations; Fig. 2). Nine TT-HBV infections occurred as a result of transfusion with blood components containing more than 10 mIU/mL anti-HBs during the past decade (Table 2). Two of them were caused by donations with negative ID-NAT.

The number of TT-HBV infections caused by transfusion with ID-NAT–negative components accounts for 15% (2/13) and 25% (1/4) of OBI- and WP-related TT-HBV infections, respectively, according to the current NAT system (Table 2). These infections involving ID-NAT–negative donations were determined as TTI by analyzing repository blood samples obtained before the index donation and/or by following up with the implicated donors after the index donation. Details of the clinical course of a typical TT-HBV infection caused by ID-NAT–negative OBI-related blood components are shown in Tables 3 and 4.

Impact of blood product on transmission rate

Table 5 shows the numbers of implicated donations by either ID-NAT negative or positive for groups categorized by the type of component and WP/OBI status. During the past decade, ID-NAT–positive donations have caused 79 TT-HBV infections. Transfusion with red blood cells (RBC), fresh-frozen plasma (FFP), and platelet concentrate (PC) was associated with infections in 42, 22, and 15 of them, respectively. Of 19 TT-HBV infections associated with ID-NAT–negative donations, 2, 4, and 13 were caused by transfusion with RBCs, FFP, and PC, respectively. Transfusion with blood components containing a larger plasma volume (FFP and PC, but not RBCs) caused more frequent TT-HBV infections among patients who received ID-NAT–negative donations (17/19, 89%) than among those who received ID-NAT–positive donations (37/79, 47%; $p < 0.01$), which could be a reflection of the plasma volume effect on infectivity.

Table 5 also shows that if ID-NAT had been implemented during the screening, 81% of established TT-HBV infections would have been avoided. The introduction of ID-NAT would have been the most (95%) and least (54%) effective for preventing TTI caused by RBC- and PC-related transfusions, respectively. Under the current 20p-TaqScreen system, 75 and 85% of TT-HBV infections arising from WP and OBI donations, respectively, are ID-NAT positive and will be interdicted by ID-NAT. In particular, the effect of ID-NAT will be 100% for OBI-related infections caused by RBC transfusion.

Outcomes of patients with TT-HBV infection

ALT levels during TT-HBV infection were determined in 68 transfusion recipients who developed TT-HBV infection. Table 6 shows the maximal ALT values relative to WP or OBI donations and ID-NAT–positive or -negative donations. Almost half (47%, 32/68) of the patients had maximal ALT values of more than 1000 IU/L. The proportion of patients with maximal ALT of more than 1000 IU/L was greater in OBI-related (61%, 19/31) than in WP-related (35%, 13/37; $p < 0.05$) infections. Total viral load in the implicated components did not significantly differ between patients with ALT values above and below 1000, which was true for both WP- and OBI-related infections. Although barely insignificant, total infused viral load tended to be lower in OBI- than in WP-related patients among groups with maximal ALT values of more than 1000.

Three patients with TT-HBV infection died of fulminant hepatitis after the introduction of NAT. One was caused by transfusion with PC derived from an ID-NAT–negative, WP-related donation (Genotype A with wild type precore region). The other two developed hepatitis after transfusion with RBCs derived from ID-NAT–positive, OBI-related donations (Genotype B with a G1898A precore mutation and Genotype C with a G1896A precore mutation).

ID-NAT screening trial in donations with low anti-HBc and anti-HBs titers

During a 6-month ID-NAT trial, 4742 (0.74%) of 640,628 blood donations at the Tokyo Blood Center with low anti-

HBc and anti-HBs titers were analyzed by ID-NAT. The number of donations analyzed by ID-NAT decreased as the anti-HBc titer increased (Fig. 3). HBV DNA was detected in 92 (1.94%) of the 4742 donations. Figure 4 shows the frequency of ID-NAT–positive donations relative to the anti-HBc titer. The frequency of ID-NAT positivity for HBV did not correlate with the anti-HBc titer and did not tend to increase with an increasing anti-HBc titer. The proportions of anti-HBs–positive (>10 mIU/mL) donations among those that were ID-NAT positive and negative were 77 and 75%, respectively, and did not significantly differ. The proportion of anti-HBs–positive donations increased with increasing anti-HBc S/CO values among ID-NAT–negative donations (67.5, 82.0, and 87.5% for anti-HBc S/CO 1.0-3.9, 4.0-7.9, and 8.0-11.9, respectively; $p < 0.01$ between any two groups). The frequency of ID-NAT positivity between males (1.8%) and females (2.4%) did not significantly differ. Eighty-three (90.2%) of the 92 ID-NAT–positive donors were at least 50 years of age. Fifteen had a viral load of less than 100 copies/mL, whereas quantitative NAT could not detect HBV DNA loads in samples from the remaining 77. The distribution of HBV genotypes among the ID-NAT–positive donations did not differ from that among the general Japanese population: Genotypes A, B, C, and D, n = 1, 24, 45, and 1, respectively (21 were undetermined).

TABLE 3. Representative TT-HBV infection caused by OBI-derived, ID-NAT–negative blood component: clinical course of a patient who received an implicated blood component.

Date	Clinical events and test results
Nov. 10, 2008	Surgery to treat head injury*
	HBsAg negative, anti-HBc negative, HBV DNA negative, preoperatively
	Transfused until Nov. 20 with 21 RBC units, 5 PCs, and 11 FFP† including one derived from the donation of Mar. 27, 2008, shown in Table 4
Mar. 05, 2009	AST 15, ALT 32
Mar. 25, 2009	AST 517, ALT 1273
Mar. 30, 2009	AST 1312, ALT 3110, HBsAg positive, IgM-anti-HBc positive
	Reported to JRC blood center
Mar. 31, 2009	AST 695, ALT 2396
Apr. 01, 2009	HBsAg negative, anti-HBs positive, HBV DNA positive

* Recipient was a teenage boy who was injured in a traffic accident.
† HBV DNA was not detected based on ID-NAT for the repository samples from these 37 blood components transfused. These results were obtained in the first lookback study performed in April 2009.

Estimation of current TT-HBV risk in Japan

From the frequency of ID-NAT–positive (1.94%) donations among those with low anti-HBc and anti-HBs titers (69,000/year or 13,000/million; see Table 1 and below), we calculated that 1339/year or 252/million donations should be ID-NAT positive among screening NAT-negative donations with low anti-HBc and anti-HBs titers. Using an infectivity rate of 3%[7] among components derived from OBI donations that were screening NAT negative and ID-NAT positive, we calculated that 40/year or 7.6/million OBI-related TT-HBV infections should arise. If TT-HBV infections related to OBI-derived ID-NAT-negative donations are taken into account, then the total number of TT-HBV infections should be 47/year or 8.9/million. This estimate was based on the observation that TT-HBV infection caused by ID-NAT–negative components during the

TABLE 4. Representative TT-HBV infection caused by OBI-derived, ID-NAT–negative blood component: HBV marker profile of blood donor responsible for the outcome shown in Table 3

Date of donation	Date of testing	Test results
Oct. 17, 2007*	Oct. 17, 2007 (screening)	Pool NAT negative, anti-HBc 2^4 (negative), anti-HBs negative
	Feb. 24, 2010 (repository sample tested in second lookback study)	ID-NAT negative
Mar. 27, 2008† (index donation)	Mar. 27, 2008 (screening)	Pool NAT negative, anti-HBc 2^4 (negative), anti-HBs negative
	Apr. 7, 2009 (repository sample tested in first lookback study)	ID-NAT negative (negative result reported to corresponding facility)
Feb. 05, 2010‡	Feb. 05, 2010 (screening)	Pool NAT negative, anti-HBc 15.4 S/CO§ (positive), anti-HBs negative
	Feb. 10, 2010 (donated blood sample tested in second lookback study)	ID-NAT positive (high probability of TT-HBV infection in Patient A reported to corresponding facility)

* RBCs derived from this donation were transfused to an HBsAg-negative patient. Patient continued to be HBsAg-negative until May 2008 when he died.
† FFP derived from this donation was transfused to patient shown in Table 3. Cocomponent (RBCs) processed from this donation was transfused to a patient who died of the primary disease soon after transfusion. Whether TT-HBV occurred remains unknown.
‡ This donation was rejected due to anti-HBc seroconversion and a second lookback study was conducted on the donation of October 17, 2007.
§ Because of very low HBV load in donated blood sample of February 5, 2010, HBV sequence was assessed in donor blood only at 193 bp (Nucleotides 475-667) of S region. HBV sequence in that region was identical except for nt. 654 between the blood samples from donor and patient on April 01, 2009.

TABLE 5. Blood components implicated in established TT-HBV infection*

	ID-NAT+/ID-NAT–					
	WP transmissions established			OBI transmissions established		
Screening period	RBCs	FFP	PC	RBCs	FFP	PC
50p-AmpliNAT	15/0	6/1	7/4	5/0	5/1	3/3
20p-AmpliNAT	8/2	0	4/4	7/0	5/1	0
20p-TaqScreen	2/0	0	1/1	5/0	6/1	0/1
Total	25/2	6/1	12/9	17/0	16/3	3/4

	WP plus OBI				All components		
	RBCs	FFP	PC	FFP + PC	WP	OBI	Total
50p-AmpliNAT	20/0	11/2	10/7		28/5	13/4	41/9
20p-AmpliNAT	15/2	5/1	4/4		12/6	12/1	24/7
20p-TaqScreen	7/0	6/1	1/2		3/1	11/2	14/3
	100%	86%	33%		75%	85%	82%
Total	42/2	22/4	15/13	37/17	43/12	36/7	79/19
	95%	85%	54%		78%	84%	81%

* Ratios (%) in the two bottom rows represent rates of ID-NAT–positive events or effectiveness of ID-NAT implementation.

TABLE 6. Maximal values for ALT in patients with TT-HBV infection and total viral load contained in implicated components

	ALT	
	<1000	>1000
WP* (n‡)	24 (7)§	13
OBI† (n‡)	12 (2)§	19
Total viral load (copies/bag)‖		
WP		
n‡	21	10
Min	40	100
Max	260,000	560,000
Median	1,400	9,100
Mean	20,460	74,790
OBI		
n‡	8	16
Min	60	40
Max	6,240	19,200
Median	630	1,470
Mean	1,440	3,750
ID-NAT status		
Positive‡	30	28
Negative‡	6	4
Component types		
RBCs‡	19	16
FFP‡	7	13
PC‡	10	3

* Patients transfused with WP-related components include 11, 8, and 18 patients with malignant hematologic disorder, solid tumor, and others, respectively.
† Patients transfused with OBI-related components include 7, 11, and 13 patients with malignant hematologic disorder, solid tumor, and others, respectively.
‡ Numbers of patients.
§ Numbers in parentheses, patients with maximal ALT values of less than 100 IU/L.
‖ Total viral load was calculated using viral concentrations in implicated donations and average plasma volume of each component type. When viral load was less than 100 copies/mL, total viral load in the component was calculated assuming that viral concentration is logarithmically distributed between 1 and 100 copies/mL.

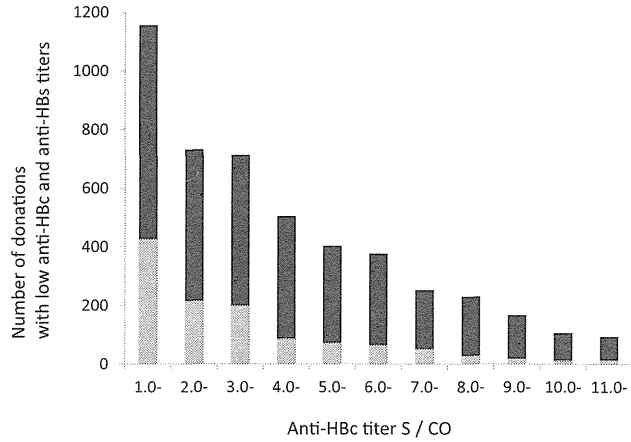

Fig. 3. Number of donations screened by ID-NAT trial categorized by anti-HBc titer. All donations tested had low anti-HBc (S/CO 1.0-11.9) and anti-HBs (<200 IU/L) titers and were qualified serologically based on algorithm applied at JRC blood centers. Donations verified to be ID-NAT-positive were disqualified. (■, □) donations with anti-HBs titers of more than and not more than 10 mIU/mL, respectively.

20p-TaqScreen period accounted for 15% (2/13) of all OBI-related infections (Table 2).

We estimated how many more WP-related TT-HBV infections would be prevented by introducing ID-NAT. The current screening NAT yield (30 donations/year or 5.7/million, Table 2) was multiplied by the ratio of the interval between ID-NAT and 20p NAT detection (11.2 days) to that between 20p NAT and HBsAg detection (9.7 days). We then deduced that 34.6/years or 6.6/million more viremic donations would be captured by ID-NAT. The number of ID-NAT–negative WP donations was calculated separately for each component type. Based on the plasma volume of each component (20, 200, 240, 450, and 120 mL for RBCs, PC, FFP-3, FFP-5, and FFP1.5,

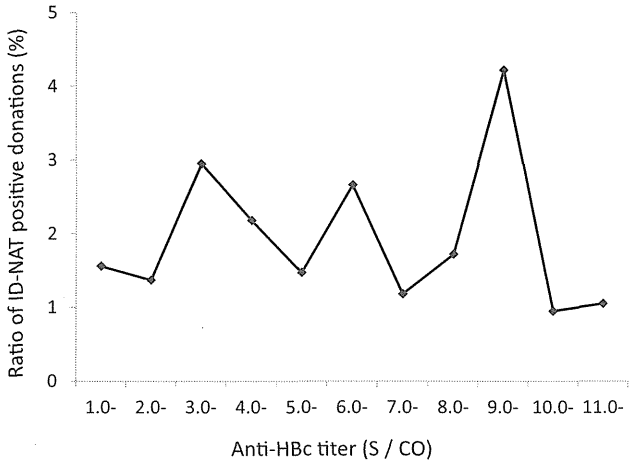

Fig. 4. Ratios (%) of ID-NAT–positive donations with low anti-HBc and anti-HBs titers relative to anti-HBc titer.

respectively), the deduced intervals between 1 copy/bag and ID-NAT detection were 16.3, 24.9, 25.5, 28, and 23 days, respectively. The ratio of the number of those components issued to hospitals is 6.3:2.2:2.1:0.7:0.1. The incidence of ID-NAT–negative WP donations calculated from these data was 59.5/year or 11.4/million. Adding ID-NAT–positive WP donations (34.6/year or 6.6/million), current risk related to WP donations amounts to 94.1/year or 18.0/million. The effect of ID-NAT on the reduction of all WP donation would be 37% (6.6/18.0). If the infectious risk (50%) of ID-NAT–positive, screening NAT-negative WP-related components is also applied to ID-NAT–negative WP-related components, the total number of WP-related TT-HBV infections would be 47.1/year or 9.0/million. Together, these estimates for WP- and OBI-related TT-HBV infections indicate that 94.1/year or 17.9/million TT-HBV infections are likely to occur in Japan.

DISCUSSION

Infection with HBV results in a wide spectrum of clinical manifestations ranging from asymptomatic liver dysfunction with only slightly elevated transaminase levels or acute self-limiting hepatitis to chronic hepatitis that in some patients progresses to cirrhosis, liver failure, or hepatic cell carcinoma. In rare cases, HBV infection can cause fulminant hepatitis that is associated with high mortality. Fulminant hepatitis in Japan is frequently associated with primary infection by HBV carrying precore or core-promoter mutations.[12,13] These HBV mutants are frequently found among chronic HBV carriers[14,15] who typically have an anti-HBc–positive serostatus. To prevent fulminant hepatitis arising as a result of blood transfusion,[16] the JRC incorporated anti-HBc testing into blood screening in 1989.

The agglutination method had been used for all serologic testing at JRC blood centers before 2008. Although this method was somewhat insensitive to HBsAg, it could semiquantify anti-HBc. Thus, the cutoff point for the anti-HBc titer had been set at 2^6, and donations with an anti-HBc titer of at least 2^6 and an anti-HBs titer of less than 200 mIU/mL were disqualified.[17] Although this anti-HBc testing had essentially prevented transfusion-transmitted fulminant hepatitis since 1989,[18] reports of fulminant or acute severe hepatitis continued for an additional 7 years. These conditions were attributed to transfusion with components with a 2^5 anti-HBc titer.[19] Consequently, the JRC lowered the anti-HBc cutoff from 2^6 to 2^5 in 1997. The agglutination method for serologic screening was replaced in 2008 with CLEIAs, which can also semiquantify anti-HBc. The policy described above is maintained in the algorithm for HBV screening with CLEIA; the range defined as a low anti-HBc titer includes S/CO values between 1.0 and 11.9, and donations with anti-HBc S/CO values within this range are currently accepted.

The highly sophisticated strategy of multiplex NAT was designed to decrease the incidence of TTI. Implementing HBV NAT into blood screening was important in Japan mainly because of the unsatisfactory sensitivity of the standard agglutination method to HBsAg. The JRC implemented multiplex NAT targeting HBV DNA, HIV RNA, and HCV RNA during 1999[6] and improved the sensitivity of the test at three points. The 98 infections described herein had been confirmed as TT-HBV since the introduction of 50p-AmpliNAT in 2000. The HBV genome was not detected in donor repository samples of 587 (74.4%) suspected TT-HBV infections. The JRC has informed the appropriate physicians of the ID-NAT results of viral detection that imply a low or high probability of TT-HBV infection.

In parallel with the increase in the screening NAT sensitivity, the incidence of WP-related TT-HBV infection has decreased as predicted, whereas that of OBI-related TT-HBV infection has not decreased (Fig. 2). To explain the increasing number of OBI-related TT-HBV infections, the increase in the sensitivity of NAT used in JRC laboratories for retrospective studies might have helped to identify TT-HBV infections, thus sustaining the number of OBI-related TT-HBV infections despite improvements in screening NAT sensitivity.[4]

This consideration could encourage the speculation that most of the 587 infection reports that had been excluded from established TTI (Fig. 1) based on negative results from repository samples might have been confirmed as TT-HBV had more sensitive NAT and a larger sample volume been analyzed. With regard to this notion, the outcomes of recent hemovigilance for TT-HBV are described below. During the 20p-Taqscreen period, the JRC received 61 clinical reports of possible HBV-TTI. Seventeen were determined as TTI, among which, three

repository samples were ID-NAT negative. Historical HBV infection was confirmed in 10 patients by retesting pretransfusion samples. Results from HBV tests of posttransfusion samples from five patients were false positive. The possibility of TT-HBV was ruled out in two patients related to ID-NAT–negative donations because repeated blood donations from two of two and three of three implicated donors were not sero- or NAT-converted. The remaining 27 patients related to ID-NAT–negative donations are inconclusive for TTI as follow-up studies have not yet been completed. Some of the 587 reported infections had been confirmed to be associated with passive anti-HBc transfer from infused components. Thus, it is unlikely that a considerable proportion of the infections excluded from the TTI category were real TT-HBV infections.

Among 19 patients with TT-HBV infections associated with ID-NAT–negative donations, 17 (89%) of them were caused by transfusion with FFP or PC that contained a larger plasma volume (120 to 450 mL) than RBCs (20 mL). In contrast, 37 (47%) were caused by FFP or PC among 79 infections associated with ID-NAT–positive donations (Table 5). This finding suggests that because HBV infectivity is extremely high, the relationship between infectivity and plasma volumes contaminated with HBV could only be established in the era of ID-NAT screening when the viral load in the donation is low enough to escape ID-NAT screening. This might explain why we could not previously establish such a relationship using viral loads around the sensitivity of the pool-based NAT system or serology.[7] If ID-NAT is introduced as routine screening, it will prevent 75 and 85% of WP- and OBI-related infections. In particular, all RBC-related TT-HBV could be prevented because of the small plasma volume involved. The finding also suggests that novel viral reduction technologies[20,21] could be an attractive strategy to decrease the incidence of TT-HBV because these technologies are presently more applicable to FFP or PC than to RBC.

The maximal ALT levels of patients with TT-HBV infection showed that transfusion with components harboring an extremely low HBV load that escaped NAT screening is not necessarily associated with mild clinical illness. This seems particularly true for OBI-related infection (Table 6). The frequency with which transfusion causes severe hepatitis (i.e., ALT > 1000) is significantly higher for OBI- than for WP-derived components. Moreover, OBI-derived components tend to cause severe hepatitis despite lower total viral loads compared with those in the WP-derived components. These findings should be further substantiated by analyzing samples from patients that are regularly obtained after transfusion because most of the maximal ALT values described in this article were found after occasional sampling.

Three patients died of TT-HBV fulminant hepatitis caused by transfusion with blood that had escaped NAT screening. Two of them were notably caused by transfusion of OBI-derived RBCs, and the other was caused by an ID-NAT–negative WP donation. Although a larger plasma volume might generally be required to establish TT-HBV infection under the NAT screening system, plasma volume or the total infused viral load might not be determining factors in fulminant hepatitis. Although viral genome mutations such as those in precore or core-promoter regions are frequently associated with the development of fulminant hepatitis in Japan,[12,13] other crucial factors have not clearly been demonstrated despite considerable investigation.

The JRC accepted 5.3 million donations in 2010, of which 4.9% (261,000) was anti-HBc reactive (Table 1), 0.19% (10,000) was rejected because of high anti-HBc and low anti-HBs titers. Another 3.4% (182,000) was accepted because of high anti-HBs titers (≥200 IU/L). The notion that blood components with an anti-HBs titer of more than 100 IU/L are not infectious is generally accepted.[22-25] The relationship between anti-HBs titer and TT-HBV infection will be discussed elsewhere (manuscript in preparation). Importantly, 1.3% of donations (69,000) with low anti-HBc and anti-HBs titers were accepted, and this category included all donations to which OBI-related TT-HBV infections were attributed. Our ID-NAT trial verified that 1.94% of the donations with low anti-HBc and anti-HBs titers were HBV DNA positive.[26] Accordingly, an estimated 1339/year or 252/million viremic OBI donors and 47/year or 8.9/million TT-HBV infections caused by OBI-derived components would be missed by the current screening algorithm. When estimates for WP-related TT-HBV infections are included, the calculated number of TT-HBV infections was 94.1/year or 17.9/million. Whole blood withdrawn from donors in Japan is split into RBCs and FFP, and the total number of components processed averages 23% more than the number of donations. However, because of outdating and rejection by testing or processing problems, the number of components finally issued by JRC becomes almost the same as the number of donations. Therefore, the calculated number of TT-HBV infections was not significantly influenced by the issue of splitting.

The considerable discrepancy between the estimated and established TT-HBV incidence per million (8.9 to 1.49 and 9.0 to 0.46 for OBI-related and WP-related infections, respectively) might be due to the following factors. A clinical manifestation of HBV infection is often unclear in patients transfused with blood components harboring a low viral load and low proliferative ability. Physicians might thus be likely to overlook infection under such circumstances. Medical practitioners are not compliant with national guidelines for lookback investigations. Indeed, only 30% to 40% of transfused patients were reportedly traced for TTI even after the guidelines were established.[27] A considerable proportion of patients who receive blood

transfusions die before TTI evaluation.[28] In fact, when we inquired about the outcomes of transfusions with components containing verified HBV at medical facilities, 99 (42%) of 238 patients who had been transfused with such components had already died (JRC data from 2009 to 2010). The transmissibility of ID-NAT–positive donations might require reevaluation because of the low numbers of patients analyzed in the previous study[7] (30 and 22 for OBI- and WP-related cases, respectively). The fact that a large proportion of elderly patients are immune to HBV due to prior infection might also contribute to the low figure for established TT-HBV and, finally, anti-HBs in cotransfused components neutralizes HBV. Classified WP donation that is anti-HBs positive and could be attributed to possible vaccine breakthrough infection or anti-HBc–negative chronic OBI could also be a factor influencing infectivity. However, we have not encountered any implicated WP donations with anti-HBs among established TT-HBV infections.

Because of the high probability of a residual risk of TT-HBV, novel strategies that reinforce the safety of blood components but do not damage the blood supply should be implemented. Transfusion with ID-NAT–negative infectious components currently cause 15 and 25% of OBI- and WP-related TT-HBV infections, respectively (Table 2), and screening with ID-NAT would interdict 85 and 75% of these infections, respectively (Table 5). With respect to this, the ID-NAT screening of only donations with low anti-HBc and anti-HBs titers that are currently qualified has been suggested.[29] However, screening with ID-NAT might not be as effective as expected. For example, the variability in viral load in individuals with OBI might allow persistent OBI-related TT-HBV infection; some individuals might have an intermittently elevated viral load.[30-33] Such donations could be identified as HBV positive only when the viral load exceeds the detection threshold of ID-NAT screening. Alternatively, the detection of intermittent viremia might reflect the stochastic phenomenon inherent in NAT technology, particularly at very low viral concentrations. Moreover, one report describes a donor in whom viral load increased in blood samples over a period of several years.[34] Nine among 48 blood donations from this donor were ID-NAT positive, and two of four ID-NAT–positive and three ID-NAT–negative blood transfusions had caused TT-HBV infections. The diverse fluctuation of viremia described above has supposedly hindered the efficient detection of viremic donations by pool-based NAT screening,[35] which is predictable even in the event of ID-NAT screening. Table 5 shows that ID-NAT is not sensitive enough in 16% of established OBI-related transmission events although most of those events are caused by FFP or PC transfusions and ID-NAT screened RBC transfusions are relatively safe. Moreover, although viremia is considered undetectable in most individuals with OBI, this assumption might be dependent on the sensitivity of the NAT used; a considerable number of donations might have viremia with a viral load below the ID-NAT detection limit.

Another strategy that might increase the safety of OBI-derived donations could be to accept only those OBI-derived donations with a profile that is safer than the current standards, if such a profile can be found and systematically applied. We initially expected to find that OBI donations with a very low anti-HBc titer would be safer based on ID-NAT. However, the finding from the ID-NAT trial was that the frequency of viremia does not correlate with anti-HBc titers in the range of S/CO 1.0 to 11.9. Therefore, we concluded that the risk of TT-HBV infection will not be mitigated by implementing a strategy that qualifies only donations with very low anti-HBc titers such as S/CO between 1.0 and 3.0.

We speculated during 2003 that more than 4% of donations would be disqualified if the anti-HBc cutoff were set at 2^1, that is, if all donations with low anti-HBc and anti-HBs titers are rejected. We thought that the loss of so many donations would cause catastrophic damage to the blood inventory and thus that cutoff was not implemented. However, based on current data, the number of donations received in 2010 with low anti-HBc and anti-HBs titers was 69,000, which accounts for 1.31% of all donations in Japan. Given this ratio, we consider that to eliminate all donations with low anti-HBc and anti-HBs titers is feasible. We verified that severe hepatitis is caused more often by OBI- than WP-derived blood. The fact that two patients died of fulminant hepatitis related to OBI-related donations is also serious. Rejecting this category of donations would eliminate nearly all those harboring a risk of OBI-related infection.[26] However, a slight, but distinct risk of TT-HBV infection might persist because a small fraction of OBI donors have an anti-HBc titer of less than 1.0 S/CO, and these donors as well as NAT WP donors present a TT-HBV risk.[36] A committee of the Ministry of Health, Labour and Welfare of the Japanese government has just discussed and authorized the implementation of a new policy in which all donations with low anti-HBc and anti-HBs titers would be rejected.

In conclusion, ID-NAT screening of donations with low anti-HBc and anti-HBs titers revealed that nearly 2% of these donations were associated with low-level viremia and that viremia was identified over the entire range of anti-HBc titers. Importantly, anti-HBc titer did not correlate with the frequency of viremia. The elimination of all donations with low anti-HBc and anti-HBs titers would be important to any strategy aimed at preventing OBI-related TT-HBV infections in countries such as Japan that have a slightly elevated HBV prevalence in blood donations. If this strategy is implemented, the only acceptable donors with OBI in Japan will be those with high anti-HBs titers (≥200 IU/L).

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CONFLICT OF INTEREST

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Transfusion Medicine | ORIGINAL ARTICLE

Analysis of 66 patients definitive with transfusion-associated graft-versus-host disease and the effect of universal irradiation of blood

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SUMMARY

Background: Transfusion-associated graft-versus-host disease (TA-GVHD) is a potentially fatal adverse reaction to blood transfusion. Although TA-GVHD was formerly considered to be rare and to occur only in immunocompromised patients, it was confirmed to occur even in immunocompetent patients in Japan, based on a definitive diagnostic test for TA-GVHD using highly polymorphic microsatellite repeat sequences. We clarify the clinical picture of TA-GVHD via definitive diagnosed cases and argue the validity of blood irradiation for TA-GVHD prevention.

Patients and methods: Two-hundred and ninety patients who were suspected of having TA-GVHD and referred to us for diagnostic testing from October 1992 to August 1999 were analysed for the associated clinical characteristics and risk factors. Effects of universal irradiation were followed up until 2010.

Results: Sixty-six of the 290 study patients were diagnosed as having definite TA-GVHD by microsatellite DNA analysis. Regarding the symptoms of patients with definite TA-GVHD, a fever of over 38 °C, erythema and leucocytopenia were found in virtually all of these patients. Among patients in whom human leucocyte antigen (HLA) typing was carried out, TA-GVHD almost always developed in HLA heterozygous patients following the transfusion of HLA homozygous blood. TA-GVHD was reported significantly more frequently in older patients. In this study, TA-GVHD was caused by the transfusion of HLA one-way match blood stored for 14 days.

Conclusion: No cases of TA-GVHD development have been confirmed since 2000, when the supply of irradiated blood products became widespread. No major problems have been encountered since the start of universal irradiation, more than 10 years ago.

Key words: definitive diagnosed cases, symptoms, TA-GVHD, universal irradiation.

Transfusion-associated graft-versus-host disease (TA-GVHD) is a potentially fatal adverse reaction to blood transfusion with a mortality rate of 90% or higher (Brubaker, 1983). TA-GVHD occurs when donor lymphocytes in blood components are allowed to engraft and proliferate in the recipient in response to host antigens recognised as 'foreign' (von Fliedner *et al.*, 1982; Leitman & Holland, 1985). Usually, such transfused lymphocytes are recognised by the recipient immune system and are rejected immediately. Therefore, TA-GVHD was considered to be rare and to occur only in immunocompromised patients. However, Aoki *et al.* reported the first case of TA-GVHD in an immunocompetent patient in 1984 (Aoki *et al.*, 1984). Since then, many cases of TA-GVHD in immunocompetent patients have been reported in Japan (Juji *et al.*, 1989). The clinical symptoms of the disorder include fever, erythematous rash, liver dysfunction, diarrhoea, bone marrow aplasia, agranulocytosis, pancytopenia and infection, which become very severe and progress very rapidly. Nearly all of these patients have died as a result of sepsis and/or multiple organ failure. No differences were found between the TA-GVHD symptoms in the immunocompromised patients reported thus far and those observed in our patients, almost all of whom appeared to be immunocompetent.

TA-GVHD can usually be diagnosed based on clinical course. However, in some cases, particularly in the early stages or in atypical cases of the disease, it is difficult to make a differential diagnosis of TA-GVHD, because its symptoms mimic those of other disorders such as drug allergies and bacterial infections. Previously, we developed a definitive diagnostic test for TA-GVHD using highly polymorphic microsatellite repeat sequences (Wang *et al.*, 1994; Uchida *et al.*, 1996). The results show the replacement of the patient's original peripheral blood

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DNA type by the donor's type, leading to the development of TA-GVHD. Sixty-six cases of TA-GVHD were identified using this test from October 1992 to August 1999.

TA-GVHD in immunocompetent patients occurs mainly when human leucocyte antigen (HLA) homozygous lymphocytes are transfused into an HLA heterozygous patient who shares one haplotype with the donor (Ito *et al.*, 1988). It was postulated that recipients are unable to recognise the donor lymphocytes as foreign because they are not HLA incompatible. This condition is deemed similar to TA-GVHD that occurs in mice when T cells are transfused from an inbred homozygous parent to their F1-hybrid offspring (Uchida *et al.*, 1994). Previously, it was very difficult to type HLA before and after the development of TA-GVHD, because pancytopenia in this disorder progresses so rapidly that there are not sufficient lymphocytes for HLA typing. Therefore, we typed HLA antigens mainly using polymerase chain reaction (PCR) amplification technologies (Mitsunaga *et al.*, 1992, 1998).

In April 1996, the Ministry of Health and Welfare sent information to transfusion implementation medical institutions throughout Japan regarding TA-GVHD prevention. This information indicated that the blood for all patients should be subjected to irradiation, when possible. The Japanese Red Cross Society (JRC) obtained approval to manufacture radiation-exposed blood from the Ministry of Health and Welfare in June 1998, and began to supply such blood immediately. Although four cases of TA-GVHD were confirmed in the first half of 1999, no cases have been reported up to the current time.

However, because some unclear points remain regarding TA-GVHD, we analysed the clinical symptoms, patient risk factors, underlying diseases, blood transfusion histories, patient outcomes and the HLA types of both patients and donors.

PATIENTS AND METHODS

Patients

Patients who were suspected of having TA-GVHD were studied. Those who developed a fever and/or erythema (erythroderma) post-transfusion were referred to us from throughout Japan for diagnostic testing, via the Japanese Red Cross haemovigilance network.

Two-hundred and ninety patients reported from October 1992 to August 1999 were analysed for clinical characteristics and risk factors.

Analysis of microsatellite DNA polymorphisms

The definitive diagnosis of TA-GVHD via microsatellite DNA polymorphism analysis methods has been confirmed, as described previously (Wang *et al.*, 1994; Uchida *et al.*, 1996). Microsatellite DNAs consist of repeating structures of two to four nucleotides, and the number of repeats differs from person to person. Individuals can be identified almost definitively by comparing five different microsatellite DNAs. The results show the replacement of the patient's original peripheral blood DNA type with the donor type, upon development of TA-GVHD. However, samples of patient blood cells obtained prior to transfusion are rarely stored for more than 2 weeks post-transfusion when TA-GVHD is suspected. On such occasions, we analysed PCR amplified fragments of DNA extracted from fingernail clippings to determine the patient's original DNA type (Uchida *et al.*, 1996). Several microsatellite DNA regions were amplified by PCR and the products were analysed via gel electrophoresis and silver staining.

HLA typing

HLA typing was generally performed by a PCR-restriction fragment length polymorphism (RFLP) method or a PCR-microtitre plate hybridisation (MPH) method, as described previously (Mitsunaga *et al.*, 1992, 1998). The DNA samples were the same as those used in the diagnostic test. At the beginning of this study, HLA DNA typing enabled the analysis of only HLA class II; however, the analysis of HLA class I has since become possible. Some patients were typed by conventional serological typing methods.

Blood irradiation

Blood components except for fresh frozen plasma were irradiated prior to transfusion at either blood centres or hospital transfusion services using gamma- or X-ray irradiators at a dose of 15–50 Gy.

RESULTS

Symptoms in definite TA-GVHD cases

Sixty-six of 290 patients were diagnosed as having definite TA-GVHD by microsatellite DNA analysis. In typical TA-GVHD cases, a fever of 38 °C or higher and erythema appear 1–2 weeks after transfusion. The erythema becomes generalised within a few days and erythroderma develops. Following fever and erythema, liver dysfunction develops, and diarrhoea and melena develop in roughly 30% of cases. Then, granulocytopenia, leucocytopenia, thrombocytopenia and reticulocytopenia appear. At this stage, the bone marrow is nearly depleted of nucleated cells and is myeloaplastic. Despite various therapeutic attempts, 65 of 66 patients died. Note that no further analysis was performed for TA-GVHD-negative cases.

Regarding the symptoms of patients with definite TA-GVHD, a fever of 38 °C or higher and erythema were found in all patients (100%). Liver dysfunction with an alanine aminotransferase (ALT) level of 100 IU L^{-1} or higher was noted in 56 patients (84·8%). Ten patients showed bilirubin level elevation although no elevation of ALT level was observed. Leucocytopenia [white blood cell (WBC) count of 1000 mm^{-3} or lower] was found in 63 patients (95·5%), even though nearly all patients were administered granulocyte-colony stimulating factor products. These symptoms did not differ from those of immune-compromised patients previously described by others.

Table 1. Patients' background

Underlying disease	Sex	Number of patients	Age	Surgery	Used blood	Storage period (days)	Outcome
Solid cancer	M	14	60–86	+	WB, CRC, MAP	1–11	Died (17–40 days)
	F	6	42–78	+	WB, CRC, MAP, AP	5–12	Died (16–36 days)
	M	1	87	–	MAP	10	Died (20 days)
	F	1	83	–	WB	4	Died (24 days)
Anti-CD3 + Cy A	M	4	47–73	+	WB, CRC, MAP	1–11	Died (24–42 days)
NM administration	M	1	60	+	MAP	8	Died (60 days)
Cardiovascular surgery	M	6	65–84	+	WB, CRC, MAP, AP	Unknown, 14	Died (14–38 days)
	F	3	69–80	+	WB + MAP	Unknown, 10	Died (22d–27 days)
Anti-CD3 + Cy A	M	1	70	+	WB + MAP	Unknown	Died (29 days)
Anti-CD3 + Cy A	F	3	63–73	+	WB + MAP + AP + hosp. B	Unknown	Died (19–33 days)
Anti-CD3 + Cy A plus NM administration	M	1	69	–	MAP	6	Died (35 days)
Severe trauma	M	4	20–74	+	MAP, hosp. B	Unknown, 5–10	Died (15–37 days)
	F	3	66–86	+	CRC, MAP, hosp. B	Unknown, 5	Died (14–30 days)
	M	1	46	–	MAP	7	Died (33 days)
Anti-CD3 + Cy A	F	1	92	+	MAP	5	Died (19 days)
Gastric ulcer	M	2	59, 73	+	WB, CRC, MAP	1–12	Died (15, 21 days)
	M	1	77	–	MAP	1–3	Died (30 days)
	F	1	85	–	WB + MAP	Unknown	Died (15 days)
NM administration	M	1	51	–	hosp. B	1	Died (175 days)
Prostate gland enlargement	M	3	72–84	+	MAP	4–8	Died (21–32 days)
Anaemia	F	1	72	–	MAP	Unknown	Died (24 days)
Anti-CD3 + Cy A	F	1	77	–	MAP	8	Died (25 days)
NM administration	F	1	77	–	MAP	6	Died (47 days)
Orthopaedic surgery	F	2	71–96	+	MAP	Unknown, 8	Died (16, 31 days)
Subarachnoid bleeding	F	1	74	+	MAP	7	Died (24 days)
Placenta previa							
Anti-CD3 + Cy A	F	1	32	+	CRC	3–4	Alive
SCID	M	1	7	–	CRC	2	Died (79 days)

AP, apheresis platelets; CRC, concentrated red cells; hosp. B, withdrawn blood in the hospital; MAP, mannitol, adenine and phosphate (MAP)-added red cell concentrate; WB, whole blood.

Risk factors of patients

Age and gender. The general characteristics of the 66 patients with definitive TA-GVHD are shown in Table 1. Forty-one (62·1%) of these patients were male. In an investigation regarding the transfusion situation of Tokyo in 1993, 55·9% (59 270/106 038) of transfusion patients were male. When the gender proportions of the patients receiving transfusions are taken into consideration, there is no significant difference in the development of TA-GVHD between genders. The TA-GVHD patient ages ranged from 7 to 96 years. Nine patients were 59 years of age or younger (13·6%), 18 were between 60 and 69 years (27·3%), 27 were between 70 and 79 years (40·9%) and 12 were older than 80 years (18·2%). Even when considering the age distribution of all blood recipients at that time, TA-GVHD occurred frequently in the elderly. There was a significantly small number of TA-GVHD patients aged in their 50s (3/66 cases; 4·6%) compared with all blood recipients (18 859/106 038, 17·8%) in the Tokyo in 1993 ($P < 0·05$). On the other hand, there were a large number of TA-GVHD patients aged older than 70 (39/66 cases, 59·1%) compared with all blood recipients (35 421/106 038, 33·4%) in the Tokyo in 1993 ($P < 0·05$).

Underlying patient diseases. The reason for transfusion in patients with definite TA-GVHD was surgical operations for solid cancers in 27 patients, cardiovascular surgery in 14 patients, severe trauma in 9 patients, bleeding from gastric ulcers in 5 patients, bleeding after prostate surgery in 3 patients, orthopaedic surgery in 2 patients and surgical operations for placenta previa and subarachnoid haemorrhage in 1 patient each. The amount of blood lost in these operations exceeded 1200 mL in all patients. Thus, the majority of these patients underwent surgery. Chronic anaemia in three patients and severe combined immunodeficiency (SCID) in one patient were due to internal medical causes. Only one patient was known to be immunocompromised (Table 1, SCID), and the remaining 65 patients were considered to be immunocompetent. There were no cases of malignant blood dyscrasia, although such cases have been reported in Western countries.

Patient blood transfusion histories. Fifty-six of the 66 patients (84·8%) developed TA-GVHD after receiving their first blood transfusion, whereas only two patients (3·0%) had a transfusion history. The remaining eight patients (12·1%) had unclear transfusion histories.

Patient outcome. Sixty-five (98·5%) of the patients with definitive TA-GVHD died, which includes all patients except for one who underwent caesarean section (Table 1, placenta previa), and was treated with anti-CD3 antibodies and cyclosporin A (Cy A). This therapy was, however, ineffective in other 11 patients who received the same treatment and medications (Table 1, anti-CD3 + Cy A).

It has been confirmed that nafamostat mesilate (NM), a serine-protease inhibitor used for the treatment of disseminated intravascular coagulation (DIC), inhibits the processes of target cell damage caused by cytotoxic T cells, transiently eliminates symptoms such as fever, skin eruption and liver dysfunction and improves patient granulocyte levels (Table 1, NM administration). However, these patients also eventually died after the cessation of NM administration.

Causative blood preparations

TA-GVHD observed in the majority of the patients (59 of 66) was caused by whole-blood (WB) preparations, concentrated erythrocyte preparations or a combination of the two. TA-GVHD in four patients was caused by a combination of apheresis platelets and red blood cell, and that in six patients was caused by fresh blood collected at the hospital. Because the majority of patients with TA-GVHD underwent surgical procedures that required transfusion with WB or red blood cell concentrates, the number of patients with TA-GVHD appeared to be greater for those blood preparations. Although fresh frozen plasma has not been reported as an unequivocal cause of TA-GVHD, other blood preparations for transfusion containing live lymphocytes have been reported as causes. No case has been reported in which TA-GVHD developed following the transfusion of only apheresis platelets.

The longest period of storage of these causative blood preparations was 10 days for WB preparations, 11 days for red blood cell concentrates (CRC) and 14 days for mannitol, adenine and phosphate (MAP)-added red cell concentrate (RC-MAP). Thus, the use of blood containing live lymphocytes that has been stored for less than 2 weeks is considered to be a risk factor. In seven patients, TA-GVHD developed despite the use of leucocyte-removal filters by bedside filtration (data not shown). All other patients had received transfusions of blood that had not undergone leucocyte removal.

HLA types of patients and donors

The HLA types of 20 cases of TA-GVHD for which analysis was possible are presented in Table 2. In almost all cases (17/19, 89·5%) of TA-GVHD in patients without apparent immunodeficiency in their primary disease, a one-way HLA match frequently occurs: namely, HLA homozygous blood is transfused into a heterozygous patient sharing one of its haplotypes. In one patient (case no. 10) with SCID, all six sites were different for HLA-A, B and DR. In case no. 8, HLA-DPB1 had a two-way mismatch, HLA-DQB1 was the same and HLA-DRB1 showed a one-way match. In another case, no. 12, only HLA-B had a two-way mismatch, HLA-A was the same and HLA-DRB1 showed a one-way match as expected. In case nos. 9 and 20, HLA-DRB1 and HLA-DQB1 were the same and only HLA-DPB1 showed a one-way match. Similarly, HLA-DRB1 was the same and HLA-DPB1 showed a one-way match in case no. 17. In another case, no. 11, HLA-A and HLA-B were completely the same and HLA-DRB1 showed a one-way match. In this case, the patient did not have leucocytopenia, but 92% of the patient's WBC were CD8-positive lymphocytes.

Adverse effect of universal irradiation of blood

More than 50 million bags of irradiated blood have been transfused since 1999. When critical transfusion side effects occur, the medical personnel are burdened with reports to the Ministry of Health, Labour and Welfare. Although hyperpotassaemia (Win *et al.*, 1997) and reduced flexibility (Cicha *et al.*, 2000) are known as disadvantages associated with irradiated red blood cells, no relevant increase in the numbers of cases of adverse effects has been reported including that of hyperpotassaemia.

DISCUSSION

In 1989, the Japanese Society for Blood Transfusion and the Japanese Society for Thoracic Surgery issued a questionnaire to thoracic surgeons, and found that TA-GVHD had occurred in 1 of 658·9 (96 of 63 257) cases of cardiac surgery in the period from 1981 to 1986 (Juji *et al.*, 1989). From 1991 to 1992, the JRC Research Group surveyed clinicians of all clinical departments on cases of probable TA-GVHD encountered since 1986, via questionnaire. In the 304 cases that were reported, 171 were clinically diagnosed as TA-GVHD (Takahashi *et al.*, 1994). This result suggests that TA-GVHD occurs not infrequently among patients who have undergone cardiovascular surgery. As mentioned above, the frequency of TA-GVHD development is high in Japan.

We developed a definitive diagnostic test using microsatellite polymorphism analysis, involving a comparison of DNA patterns from patient fingernails and peripheral blood after the onset of symptoms of TA-GVHD (Uchida *et al.*, 1996). The DNA patterns in the peripheral blood are completely identical to that in the fingernails in healthy individuals. When these two differ, the patient is diagnosed as having TA-GVHD. In this study, we reported the details of 66 patients with TA-GVHD, which was diagnosed via microsatellite analysis of a fingernail extract.

TA-GVHD occurred in both genders, but was seen more frequently in elderly patients, possibly owing to their lower