Table 1 Blood component containers routinely used in the blood transfusion service of the Fondazione Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

Blood component	Manufacturer	Code	Plastic/Plasticizer
Whole blood, RBC, FFP	MacoPharma	MQE6285LR	PVC/DEHP
Platelets (buffy coat method)	Terumo (Teruflex BP Kit)	2TETB00005	PVC/DEHP
		2TEIP00006	PVC/DEHP
Platelets (apheresis)	Fenwal	4R2340	Polyolefin
Peripheral blood stem cells (collection)	Terumo BCT	70620	PVC/DEHP
	Fresenius Kabi	9400421-301	PVC/DEHP
Cord blood (collection)	MacoPharma	MSC1202PU	PVC/DEHP
Components cryopreserved in liquid nitrogen	Fresenius Kabi	Z2002	Kapton-Teflon
	Miltenyi Biotec	200-074-400	Ethylene Vinyl Acetate
	Biosafe	FB-100.1	Ethylene Vinyl Acetate

Although we are aware of a general concern related to DEHP toxicity and plans to use alternative plastics [1], we do not have specific competences to positively exclude DEHP-containing containers from ours tenders. Moreover, besides the potential negative effects of DEHP, we also consider the apparently 'protective' integration of released DEHP into the RBC membrane, which reduces haemolysis of RBC suspended in additive solutions at the end of storage [2].

More recently, we read with interest reports suggesting the possibility to store RBC and platelets in non-DEHP containers with acceptable qualitative parameters at the end of storage [3, 4].

Question 2

Because of significant resource requirements for the validation of new procedures, in the absence of conclusive scientific evidence and mandatory regulatory requirements for the replacement of containers plasticized with DEHP, no programmes have been developed in our institution for replacing DEHP-containing plastics.

Question 3

We do not perform productive plasmapheresis collections. Moreover, more than 99% of our platelet needs are covered by processing buffy coats obtained from 450 ml whole blood units. Therefore, DEHP exposure of our apheresis blood donors is almost entirely limited to about 100 autologous or allogeneic peripheral blood stem cell collections per year. According to the above policies and procedures, we do not believe that donor exposure during apheresis represents a significant concern in our service.

Question 4

In our country, no norms prescribe the use of non-DEHP and/or non-PVC blood bags in specific patients groups. However, in compliance with EU directive no. 2007/47 and national decree no. 37/210 published on 21 March 2010, infusion systems containing phthalates must display this information and the potential risk for children and pregnant or lactating women. We never received questions or expressions of concern from our patients about the use of DEHP-plasticized PVC for storage of blood products.

Question 5

All intravenous infusion systems used in our hospital are DEHP free.

Question 6

In 2006, Greenpeace Italia has published a report on toxicity of various chemicals including phthalates (http://www.greenpeace.org/italy/Global/italy/report/2006/5/chimicaingrembo.pdf). Studies on health risks of phthalates have been performed by the Istituto Superiore di Sanità (National Institute of Health). (see http://www.iss.it/inte/ and http://www.iss.it/binary/publ/cont/09_33_web.pdf).

References

1 Sampson J, de Korte D: DEHP-plasticized PVC: relevance to blood services. *Transfus Med* 2011; 21:73–83.
2 AuBuchon JP, Estep TN, Davey RJ: The effect of the plasticizer di-2-ethyl phthalate on the survival of stored RBCs. *Blood* 1989; 71:448–452.
3 Dumont LJ, Baker S, Dumont DF, *et al.*: Exploratory in vitro study of red cell storage containers formulated with an alternative plasticizer. *Transfusion* 2012; 52:1439–1445.
4 Bhaskaran Nair CS, Vidya R, Ashalatha PM. Hexamoll DINCH plasticized containers for the storage of platelets. *Asian J Transfus Sci* 2011;5:18–22.

Elisabetta Raspollini & Stefania Villa
Centro Trasfusionale e di Immunoematologia
Fondazione Ca' Granda Ospedale Maggiore Policlinico
Via Francesco Sforza, 35

20122 Milano
Italy
E-mail: e.raspollini@policlinico.mi.it

Paolo Rebulla
Center of Transfusion Medicine, Cellular Therapy and Cryobiology
Fondazione Ca' Granda Ospedale Maggiore Policlinico
Via Francesco Sforza, 35
20122 Milano
Italy

M. Takanashi

Question 1
Yes, we currently use di(2-ethylhexyl) phthalate (DEHP)-plasticized PVC for storage of all of our blood components, which include red blood cells in MAP solution, apheresis platelet and fresh-frozen plasma. The storage period from collection for the blood components above is 21 days, 4 days and 1 year, respectively. We have never introduced non-DEHP/non-PVC plastics for collection or storage.

Question 2
No, we do not have any programme for replacing DEHP-containing plastics for blood containers with those that are DEHP free. We recognized that all of the blood components contain DEHP and mono(2-ethylhexyl) phthalate (MEHP) [1]. The Japanese Red Cross has added the statement below to the information circular (package inserts) of our blood components since July 2003: The plasticizer DEHP elucidates from the blood bag during storage, the amount depending on the storage period, although there have not been any reports of a health hazard caused by the elucidated DEHP.

Question 3
No, donor exposure to DEHP during apheresis is not a concern. The amount of DEHP exposure was calculated to be from 1/16 to 1/20 times less [2] than the parenteral TI value shown in a report by the US Food and Drug Administration [3].

Question 4
No, there is not a specific patient group for whom the use of non-DEHP/non-PVC blood components is prescribed. It was recommended by the Japanese Ministry of Health, Labour and Welfare that blood products in DEHP PVC bags are to be kept at a lower temperature and should be used within a short period, in the Pharmaceuticals and Medical Devices Safety Information, No. 182, of 2002 [4]. No questions or concerns about the DEHP content in blood products have been registered in recent years with the Japanese Red Cross, which is the only organization for blood services in Japan.

Question 5
There are choices for non-PVC or non-DEHP infusion tubes, extension tubes, three-way stopcocks and infusion-filter sets. The Japanese Ministry of Health, Labour and Welfare did recommend to use non-DEHP/non-PVC infusion tubes and feeding tubes for neonates and infants [4]. Hospitals with neonatal intensive care units generally choose non-PVC or non-DEHP products.

Question 6
No, recent concerns for health hazards are mostly for radiation (from the nuclear plant meltdowns), pesticides, genetically modified organisms or BSE. The report from the Japanese Ministry of the Environment also focused on the testing methods of biological systems [5]. It appears that medical devices are exempt from their attention because medical care is chosen on a risk-benefit bases, especially as blood transfusion is a life-saving procedure.

References

1 Inoue K, Kawaguchi M, Yamanaka R, *et al.*: Evaluation and analysis of exposure levels of di(2-ethylhexyl) phthalate from blood bags. *Clin Chim Acta* 2005; 358:159–166
2 Yoshizawa K. Standardizing analysis methods for safe blood for transfusion. Research report for a Science Research Grant from the Ministry of Health, Labour and Welfare. 200301268A, March 2004 (in Japanese) [cited 7 January 2013]. Available from: http://mhlw-grants.niph.go.jp/niph/search/NISR00.do
3 Center for Devices and Radiological Health. Safety assessment of di(2-ethylhexyl)phthalate (DEHP) released from PVC medical devices. U.S. Food and Drug Administration. Rockville, MD, 2002. [cited 17 December 2012]. Available from: http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM080457.pdf
4 Pharmaceuticals and Medical Devices Safety Information No. 182. Ministry of Health, Labour and Welfare, Pharmaceutical and Food Safety Bureau, October 2002. (in Japanese) [cited 4 January 2013]. Available from: http://www.info.pmda.go.jp/iyaku_anzen/PMDSI182d.html#12
5 Further actions to endocrine disrupting effects of chemical substances, -EXTEND 2010-. Ministry of the Environment, July 2010. [cited 4 January 2013]. Available from: www.env.go.jp/chemi/end/extend2010/extend2010_full.pdf (in Japanese), www.env.go.jp/en/chemi/ed/extend2010_full.pdf (tentative translation)

Minoko Takanashi
Japanese Red Cross Society, Blood Service Headquarters
2-1-67 Tatsumi

Koto-ku
Tokyo
135-8639 Japan
E-mail: m-takanashi@jrc.or.jp

D. de Korte

Question 1
Sanquin is using DEHP-plasticized PVC containers for refrigerated storage of red blood cell concentrates at 2–6°C and frozen storage of plasma for transfusion or fractionation at < −20°C. For storage of platelets at 20–24°C, we use non-DEHP-plasticized PVC. For adult dose platelet concentrates (PC), BTHC-plasticized PVC is used (PC volume about 340 ml, containing on average 320 x 10^9 platelets). For paediatric dose, PC DINCH-plasticized PVC is used (PC volume about 60 ml, containing 50–100 x 109 platelets). In the decision process on selection of type of container to be used for storage of red cells, concern relating to DEHP toxicity did not contribute; for platelet storage, the gas-exchange capacity of DEHP-plasticized PVC is not enough to warrant 7 days of storage for PC, the storage time Sanquin is using as a standard. With respect to the storage of red cells in non-DEHP containers, I think it will be possible to store red cells without shortening the current shelf-life of 35 days by use of alternative additive solutions and/or changes in storage conditions.

Question 2
We do not have an active programme for replacing DEHP-containing plastics, but are working together with suppliers to test alternatives. In these tests, the basic requirement is that alternatives should have minimally similar *in vitro* quality as our current products, before starting any type of *in vivo* evaluation. So far, we do not have specific requirements for *in vivo* quality, but in some initial discussions, it was clear that for real use, we would need a large amount of transfusions, in which we would focus on frequency of transfusion reactions, which should be similar or less compared with our current products. Our main concern with introduction of alternatives for DEHP-containing plastics would be defect rate of blood bag systems, which is currently very low for the systems in use, and might be increasing for alternatives, due to changes in production methods and less experience with the materials by the suppliers of blood bags.

Question 3
Donor exposure by apheresis is on the radar, but sofar, the amounts of DEHP to which a donor is exposed are not thought to be a threat to the donor. However, in contacts with suppliers, it is mentioned that replacement of DEHP-plasticized PVC tubing would be of interest.

Question 4
No specific patients groups are defined with respect to avoiding DEHP-plasticized PVC containers, but some clinicians are concerned about exposure to DEHP for neonates and interested in alternatives. From patients, neither from donors, no questions are received with respect to use of DEHP-plasticized PVC for blood products.

Question 5
We do not administrate IV fluids, but most, if not all, of our hospitals in the Netherlands are using DEHP-free infusion systems. No specific policies are set in the Netherlands for use of DEHP-containing plastics.

Question 6
To my knowledge, no organized activity on promoting use of non-DEHP plastics.

Dirk de Korte
Sanquin Blood Bank R&D
Plesmanlaan 125
1066 CX Amsterdam
The Netherlands
E-mail: d.dekorte@sanquin.nl

M. Lozano & J. Cid

Question 1
We do use currently DEHP-plasticized PVC for storage of red blood cells and plasma. Only the bag used for storing platelet concentrates is not containing DEHP.

Question 2
We do not have an active programme for replacing DEHP-containing plastics for red blood cells with those that are DEHP free.

Question 3
The exposure of donors to DEHP during apheresis currently is not matter of concern in our country.

Question 4
In Spain, there is no specific patient group for whom the use of non-DEHP is prescribed. We have not received any inquiry from patients about the issue of DEHP-plasticized PVC.

Question 5
We do not use DEHP-free infusion systems for the administration of IV fluids. We do not have in our hospital or country any specific policies with respect to the used of DEHP-containing plastics for medical devices.

Question 6
To our knowledge, in Spain, there is no advocate group actively promoting the use of non-DEHP plastics.

Miguel Lozano
Department of Hemotherapy and Hemostasis
University Clinic Hospital
Villarroel 170
08017 Barcelona
Spain
E-mail: mlozano@clinic.ub.es

Joan Cid
Department of Hemotherapy and Hemostasis
University Clinic Hospital
Villarroel 170
08017 Barcelona
Spain

H. Gullikson

Question 1
The blood bank system of Sweden is much decentralized and generally organized as hospital blood banks within 19 counties and 2 separate geographical regions. In 2011, there were 30 independent laboratory organizations in Sweden that also included blood banks. The blood banks of the 6 university hospitals of Sweden give support especially to minor blood banks within their geographical region. This situation implies that the selection and use of plastic material may vary significantly between different parts of Sweden. However, DEHP-plasticized PVC is generally used for the storage of red blood cells and plasma. For platelet storage containers, PVC-BTHC and polyolefin plastic material is used. Those specific materials were primarily selected to achieve better gas permeability to improve platelet storage conditions. We were also aware of the significant amounts of DEHP released from DEHP PVC storage containers during storage of platelets at +22°C.

Regarding red cells, it is a well-known fact that DEHP is integrated in the cell membranes and in that way stabilizes the membranes. Present red cell additive solutions will probably not be effective in neutralizing increasing levels of haemolysis in the absence of DEHP. There are some rooms for optimism that the next generation of red cell additive solutions will be more effective in this respect.

Question 2
Most counties in Sweden have programmes to reduce the use of PVC. Those are generally political decisions and imply that the use of DEHP also will be reduced in parallel. At present, there is a common ambition involving many Swedish counties to find other plastics, preferably non-PVC material without plasticizers for the production of blood bags. This ambition has resulted in an R&D project with financial support from EU's Life+ programme. The project involves commercial companies and blood banks in Sweden and is described at www.pvc-freebloodbag.eu. The requirements for *in vitro* and *in vivo* quality of products within accepted shelf-life will be the same as for presently used blood components and according to the EU regulations.

Question 3
Donor exposure to DEHP during apheresis and patient exposure during exchange transfusion of neonates is a concern. On the other hand, the awareness of the release of DEHP into blood components is very limited in Sweden.

Question 4
There are no such patient groups. Patients who express concern about DEHP-plasticized PVC are rare, possibly as a consequence of lack of awareness.

Question 5
Non-PVC infusion and transfusion sets are available and are used. Non-PVC/DEHP plastics should preferably be selected if available according to the policy described in Question 2.

Question 6
I am not aware of such groups.

Hans Gullikssson
Clinical Immunology/Transfusion Medicine C2 66
Karolinska University Hospital
SE-141 86 Stockholm
Sweden
E-mail: hans.gullikssson@karolinska.se

R. Cardigan

Question 1
We currently use DEHP-plasticized PVC for storage of all components except apheresis platelets. We are concerned

that moving to non-DEHP storage bags for red cells may have an adverse affect on the quality of red cells, at a time when there is a focus on improving their quality. It is possible that some alternatives may yield equivalent results to that of DEHP-containing bags when used in combination with alternative additive solutions. It is important that any alternatives are adequately validated in terms of effect on component quality, including studies *in vivo*, and what their toxicity profile is.

Question 2
We do not have an active programme for replacing DEHP-containing bags, but we are interested in developments in this area. Our current contracted supplier of blood bags is currently actively engaged in studies to develop alternatives to DEHP.

Question 3
We currently collect 80% of platelets by apheresis, but not significant amounts of plasma or red cell components. We are not aware of any data to suggest that exposure of donors to the plastics used in apheresis consumables constitutes a risk to the donor. However, there have been very few studies that have assessed DEHP levels in apheresis donors.

Question 4
We have not introduced non-DEHP bags for any specific patient groups. In general, we are not aware of any concerns being raised by patients, although we have had one recent inquiry (relating to possible allergy to DEHP rather than concern about reproductive toxicity).

Question 5
In the UK, administration sets for either blood components or IV fluids can contain DEHP. Some manufacturers do make DEHP-free sets. As far as we are aware, there is no national guidance on this topic and it would be for individual hospitals to decide which they use. We believe the majority of sets in use will contain DEHP, but we have not surveyed hospitals on this matter.

Question 6
We are not aware of any advocate groups in the UK promoting non-DEHP plastics.

Rebecca Cardigan
Head of Components Development
NHS Blood & Transplant
Cambridge CB2 0PT
UK
E-mail: Rebecca.cardigan@nhsbt.nhs.uk

C. Tooke & M. K. Fung

Question 1
Per the American Red Cross that supplies all of our blood products, our institution currently utilizes a combination of DEHP-plasticized and DEHP-free RBC units. We currently do not have a process that allows us to distinguish which units are DEHP free. All of our apheresis platelets are stored in DEHP-free bags. However, the tubing associated with these units does contain DEHP.

Question 2
To date, our institution does not have in place a method for the complete replacement of DEHP-containing transfusion products with DEHP-free alternatives. We are not aware of any initiative in the United States to completely replace DEHP-containing plastics for primary blood containers. However, DEHP-free syringes, and infusion sets have become available. Saline solutions can be obtained in DEHP-free plastic containers. The value of using DEHP-free syringes and infusion sets is of unknown value if the primary blood containers contain DEHP, and there had been sufficient time for leaching of DEHP into the blood product during storage with the primary blood container.

Question 3
Donor exposure to DEHP during apheresis procedures is of potential concern, especially in the adolescent and young adult populations, where cumulative effects of DEHP exposure may potentially pose risks to future reproductive capabilities. Several studies have demonstrated that DEHP doses in plateletpheresis donors meet or exceed both the tolerable daily intake (TI) and reference dose (Rfd) limits. However, still other studies have demonstrated that while these daily limits may be exceeded by plateletpheresis procedures, much of the DEHP is excreted and the amount of DEHP that donors retain falls within these reference intervals.

Question 4
It is recognized that certain patient populations, including neonates, young children and pregnant women, may be more vulnerable to DEHP exposure. Currently, federal laws in the United States have banned phthalate such as DEHP from the manufacture of children's toys and a variety of infant products, including teething rings, rubber nipples and bottles. However, at present time, there are no mandates in place in the United States for the reduction in healthcare-related DEHP exposure. In 2002, the United Stated Food and Drug Administration (USFDA) issued a public health notification regarding DEHP exposure,

strongly recommending (but not requiring) care providers to utilize DEHP-free devices when possible, especially when performing procedures on vulnerable populations. However, the USFDA has also noted that concern over DEHP exposure should not cause care providers to withhold necessary medical interventions in these populations. Additionally, in 2003, the American Academy of Pediatrics (AAP) published a technical report addressing concerns related to DEHP exposure, suggesting in conclusion that interventions to minimize DEHP exposure be undertaken for at-risk patient populations.

Our survey of academic medical institutions discovered that of fifteen academic institutions that responded to a survey, the majority (60%) cited both a general lack of knowledge and decreased concern regarding transfusion-associated DEHP exposure. Furthermore, 60% of respondents also reported being uncertain of specific patient populations who might benefit from DEHP-free products [1].

Question 5

Our institution has no policies in place that address the use of DEHP-plasticized medical devices. While there are no federal requirements related to the discontinuance or usage of DEHP-containing medical devices, the US Food and Drug Administration has issued a public health notification (see #4); currently, the utilization of DEHP-free alternative medical devices is determined by each individual institution.

Question 6

In the United States, there are many groups that advocate for DEHP-free medical devices, including Moms Rising, The Alliance for a Healthy Tomorrow, The Center for Heath, Environment and Justice, and HealthCare Without Harm. Each of these groups advocates for a reduction in DEHP from a variety of products; however, HealthCare Without Harm is the only group which also advocates specifically for DEHP-free medical devices. While their advocacy is not directed solely towards DEHP-free transfusion products, these products receive mention on their website (www.noharm.org), which lists approved DEHP-free alternatives to a vast array of medical and transfusion devices.

References

1 Tooke C, Fung M. Awareness and demand for plasticizer-free blood bags and I.V. tubing: a survey of U.S. academic medical centers. *Transfusion* 2012; 52(Suppl. S3):175A.

Chelsea Tooke & Mark K. Fung
Fletcher Allen Health Care
University of Vermont
111 Colchester Avenue
Burlington, VT
USA
E-mail: Mark.Fung@vtmednet.org

N. L. C. Luban

Question 1

In the USA, access to platelets and plasma-containing products stored in non-DEHP containers is common but dependent on which bag system(s) is used by the collection agency. One exception is platelets manufactured from whole blood collection where the primary collection bag contains DEHP, although the secondary bag(s) may be DEHP free. RBCs in non-DEHP containers are neither readily available nor used routinely. There is only one bag system that is FDA approved in the USA for RBC storage, the BTHC system (Fresenius-Kabi/Fenwal) but not commonly available. It is possible to store red cells in non-DEHP containers especially if patients receive relatively 'fresh' RBCs; total transfusion management with DEHP-free products is not possible in the USA.

Question 2

In the USA, manufacturers have responded by producing DEHP-free blood administration and syringe transfer sets, and heparin-coating of ECMO and some cardiovascular circuits as well as infant feeding tubes and enteral solution bags. Many institutions and healthcare delivery systems have replaced enteral feeding tubes and bags containing exposure total parenteral nutrition [1] to decrease enteral exposure. The complex medical care of sick infants and children still depends upon DEHP-containing plastic disposables including high flow cannulas and oscillator circuits and elements of CVS bypass circuitry.

Question 3

While there have been reports of repeat apheresis donors having increased levels of urinary DEHP and metabolites when compared with the general population, the DEHP dose/kg/day and Tolerable Index to dose ratio is low. In the USA, volunteer blood donors including apheresis donors are not considered an 'at-risk' population. Moreover, now that manufacturers utilize non-DEHP bags for primary collection and subsequent storage, the risk to recipients of apheresis collected platelets and plasma should be less than in historical studies, although documentation is lacking. Patients undergoing therapeutic apheresis procedures, especially those who require long-term repetitive support, have not been studied to my knowledge.

Question 4

Controversy continues despite multiple blue ribbon panels and safety assessments [2] as to the applicability of rodent studies to human toxicity. Nevertheless, certain patient groups considered at risk include premature and medically fragile neonates, those undergoing exchange transfusion, ECMO, and complex medical and/or surgical procedures, older children undergoing chronic transfusion, massive transfusion and cardiovascular procedures including ECMO. Note that there are no contemporaneous studies of such infants and children published in the last several years.

The consent process for blood transfusion is predominantly focused on major infectious and non-infectious risks. Short- or long-term plasticizer toxicity and endocrine disruption are not routinely mentioned.

Question 5

While there are no specific policies through US licensing agencies that prohibit the use of DEHP-containing plastics, the FDA and EPA have published materials and advised as to which populations may be at risk for toxicity, as outlined above [3]. Our institution does use DEHP-free infusion systems, catheters, ECMO circuits, transfer sets and enteral feeding tubes and bags and breast milk storage bags.

Question 6

Many advocacy groups support BPA-free and DEHP environments, be they medical or otherwise. Blood bags are of concern as one of many sources of exposure. These organizations include Health Care Without Harm [4] and other environmental coalitions.

References

1 Van Vliet ED, Reitano EM, Chhabra JS, *et al.*: A review of alternatives to di (2-ethylhexyl) phthalate-containing medical devices in the neonatal intensive care unit. *J Perinatol* 2011; 31:551–560.

2 http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM080457.pdf

3 http://www.fda.gov/medicaldevices/safety/alertsandnotices/ucm062182.htm

4 Health Care Without Harm. www.noharm.org/

Naomi L. C. Luban
Chief, Division of Laboratory Medicine, Director, Transfusion Medicine/The Edward J. Miller Donor Center, Vice Chair for Academic Affairs, Department of Pediatrics, Component Director, Clinical and Translational Science Institute-CN, Children's National Medical Center Pediatrics and Pathology
Division of Laboratory Medicine
Children's National Medical Center
George Washington University School of Medicine and Health Sciences
111 Michigan Avenue, NW
Washington, DC
20010 USA
E-mails: nluban@childrensnational.org; nluban@cnmc.org

R. Vassallo & R. Benjamin

Question 1

More than half of the whole blood bag sets used by the American Red Cross (ARC) contain DEHP-plasticized polyvinylchloride (PVC) in primary containers, secondary red blood cell (RBC) and plasma containers, tubing and connectors. Whole blood-derived platelets are stored in tri-(2-ethylhexyl)-trimellitate (TEHTM)-plasticized containers, but these sets all contain some DEHP, which is present at low concentrations in the platelet product. At present, approximately 45% of whole blood collected by the ARC is in sets whose RBC container is plasticized with butyryl-tri-n-hexyl citrate (BTHC) rather than DEHP. The primary bag, tubing and connectors in these sets all contain DEHP, and thus, low levels of DEHP are present in the RBC component. Apheresis platelets are stored in non-DEHP-containing bags (BTHC-plasticized PVC and polyolefin), but the circuit tubing contains DEHP. The better storage characteristics of platelets due to enhanced gas-exchange properties in non-DEHP containers have led to its uniform replacement as a plasticizer in all apheresis and whole blood-derived platelet storage bags. Concurrent apheresis RBC and plasma products as well as double red cell components collected on various apheresis platforms are stored in DEHP-plasticized PVC.

Currently, US Food and Drug Administration (FDA)-approved 'DEHP-free' whole blood collection sets are available from only one US manufacturer. The need for niche products and a broad supply base thus requires that some fraction of the approximately 40% of the US blood supply collected annually by the ARC be stored in DEHP-containing bags. Moreover, DEHP-limited blood products are infrequently requested by our hospital partners, validating this practice. RBCs with low concentrations of DEHP are currently collected in a set configuration whose only DEHP-free container is the additive solution RBC bag, although DEHP-free primary and plasma-storage bags are available at additional cost. DEHP is present in all these sets' tubing and connectors; however, so there is no truly DEHP-free blood product available in the United States.

Studies of non-DEHP-containing plastics for red cell storage have demonstrated equivalent or better *in vitro* metabolic parameters [1]. Limited *in vivo* studies suggest that red cells stored in some alternatives would meet

current US *in vivo* radiolabelled red cell standards [2]. Nevertheless, slightly higher haemolysis has been observed in every alternative storage system. While fractional haemolysis has not exceed the US 1% end-of-storage standard; thus far, the red cell membrane-stabilizing properties of DEHP have not been equalled by non-DEHP-plasticized products. RBC quality concerns aside, the key limiting factors to wider adoption of DEHP substitutes in the USA have been the lack of alternatives, need for supply diversification and the additional cost of these products.

Question 2

At present, low hospital demand (and low price tolerance) for slightly more expensive DEHP-limited red cell products has dampened manufacturers' enthusiasm to invest substantial sums in research and development, negotiating the regulatory approval process and retooling manufacturing processes. (Recouping these costs would inevitably result in higher RBC acquisition costs for hospitals). As such, blood collectors in the USA have limited options, and there is no programme within the ARC to replace DEHP-containing plastics. Without a regulatory mandate based upon evidence of causation of human disease, or cautionary principle concern backed by appropriate sources of funding, there is little impetus for change. When cost is removed from the equation, RBC quality is the only limiting factor in replacement of DEHP in blood component containers. The US standards for *in vitro* and *in vivo* properties of stored RBC products have been reviewed elsewhere [3]. Thus far, only BTHC has been introduced for RBC storage in the USA (receiving grandfathered approval before more stringent regulatory requirements were introduced). Another plasticizer, di-isonyl cyclohexane-1,2-dicarboxylic acid (DINCH), is currently being studied, with interest primarily generated by European regulatory efforts [1]. Currently, there are no US regulatory requirements stipulating the DEHP content of blood products.

Question 3

Donor exposure during plateletpheresis occurs when DEHP leaches from the circuit tubing. Parenteral intake has been quantified from 6.5 to 33 μg/kg per procedure, with higher observed values derived from 24-h urine collections [4]. This technique includes dietary and environmental sources over that period as well as apheresis-derived DEHP. Exposure of the US public from these former sources ranges from 1 to 30 μg/kg/day, so most apheresis procedures will not result in levels significantly above those associated with daily living. Apheresis exposure is also well below the US FDA's tolerable parenteral intake of 600 μg/kg/day for individuals undergoing medical procedures, although healthy donors cannot necessarily be compared with patients. Vulnerable adult populations (pregnant and recently postpartum females) are excluded from blood donation and thus are not exposed to DEHP via apheresis. Given the small, primarily theoretical, risk of low-level DEHP exposures (even as often as 24 times annually) and efforts to mitigate this quite low risk must be cost-effective. Replacement of DEHP with another plasticizer in cell separator kits, if not sufficiently inexpensive, would increase the cost of apheresis platelets. The introduction of safe, low-cost alternatives would be welcomed as an incremental safety initiative, but at present is not a practical option in the USA.

Question 4

DEHP is considered an endocrine-disrupting compound due to its anti-androgenic effects. In 2006, based upon animal studies, the US National Toxicology Program expressed serious concern for male infants undergoing intensive medical treatments [5]. Concern was also raised about male fetal/neonatal reproductive tract development in pregnant/breastfeeding women undergoing medical procedures associated with high-level DEHP exposure, and male infants below 1 year old exposed to high levels of DEHP. Less concern was attached to low-level *in utero* exposures or those after age 1, and there was minimal concern from typical background exposures. The US FDA has also recommended that non-DEHP-containing enteral/parenteral nutrition devices, IV/umbilical/wound/bladder catheters, ECMO circuits, fluid and blood bags, tubing, and dialysis equipment be used for male neonates, pregnant women carrying male foetuses, and peripubertal males [6]. Neonatal intensive care specialists have been the most vocal and in many hospitals have succeeded in redirecting equipment choices towards non-DEHP-plasticized PVC or alternative plastics. For high sales volume devices, market competition has tended to minimize cost differentials for non-DEHP products. Lower sales volumes and the high cost associated with regulatory reapproval of blood product quality in new non-DEHP containers have slowed similar progress in transfusion medicine. Advocacy in the USA remains primarily in the hands of healthcare providers and environmental organizations rather than patient groups. Lack of US labelling requirements, like those introduced in Europe, often makes it difficult to determine which products contain DEHP. Organizations such as Health Care Without Harm provide resources for hospitals to audit and replace DEHP-containing supplies and equipment. The US FDA released a Guidance for Industry in December 2012 recommending that DEHP be removed from (or justification be provided for why it cannot) products regulated by the Center for Drug Evaluation and Research (CDER) [7]. Whether this will eventually extend to blood products regulated by the Center for Biologics Evaluation and Research (CBER) is not clear.

Questions 5 and 6

As noted previously, the USA has no binding regulations governing DEHP-containing medical devices and products. The 2012 FDA guidance document contains non-binding recommendations, although in practice, such documents carry significant weight as potential standards of care. Many hospitals have voluntarily taken vigorous steps to eliminate DEHP, as much as possible, from their IV fluids, tubing, enteral nutrition equipment and NICU invasive devices. There are no guidelines at present that specifically address the DEHP content of blood products.

References

1 Dumont LJ, Baker S, Dumont DF, *et al.*: Exploratory in vitro study of red blood cell storage containers formulated with an alternative plasticizer. *Transfusion* 2012; **52**:1439–1445.

2 Seidl S, Gozda W, Reppucci AJ: The in vitro and in vivo evaluation of whole blood and red cell concentrates drawn on CPDA-1 and stored in a non-DEHP plasticized PVC container. *Vox Sang* 1991; **61**:8–13.

3 Hess JR: Scientific problems in the regulation of red blood cell products. *Transfusion* 2012; **52**:827–835.

4 Buchta C, Dettke M: Di(2-ethylhexyl)phthalate exposure of apheresis donors is procedure-related (letter). *Transfusion* 2006; **46**:1457–1459.

5 Center for the Evaluation of Risks to Human Reproduction: NTP-CERHR monograph on the potential human reproductive and developmental effects of di(2-ethylhexyl) phthalate (DEHP). National Toxicology Program, U.S. Department of Health and Human Services. Research Triangle Park, 2006.

6 FDA Public Health Notification: *PVC Devices Containing the Plasticizer DEHP.* US Food and Drug Administration, Bethesda, 2002. Available at: http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/PublicHealthNotifications/ucm062182.htm

7 Guidance for Industry Limiting the Use of Certain Phthalates as Excipients in CDER-Regulated Products. US Food and Drug Administration/CDER. Bethesda, 2012. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM294086.pdf

Ralph Vassallo
Northeast Division Chief Medical Officer
American Red Cross Blood Services
15601 Crabbs Branch Way
Rockville, MD
20855 USA
E-mail: ralph.vassallo@redcross.org

Richard Benjamin
Chief Medical Officer
American Red Cross Blood Services
15601 Crabbs Branch Way
Rockville, MD
20855 USA
E-mail: richard.benjamin@redcross.org

Bone Marrow Transplantation (2014) **49,** 228–235

www.nature.com/bmt

ORIGINAL ARTICLE

Risk factors and organ involvement of chronic GVHD in Japan

J Kanda[1], H Nakasone[1], Y Atsuta[2], T Toubai[3], H Yokoyama[4], T Fukuda[5], S Taniguchi[6], K Ohashi[7], H Ogawa[8], T Eto[9], K Miyamura[10], Y Morishima[11], T Nagamura-Inoue[12], H Sakamaki[7] and M Murata[13] on behalf of the GVHD Working Group of the Japan Society for Hematopoietic Cell Transplantation

Few studies have evaluated the risk factors for chronic GVHD and organ involvement associated with different graft types, including unrelated cord blood (U-CB). We retrospectively studied 4818 adult patients who received their first allogeneic transplantation and survived for at least 100 days. The incidence of chronic GVHD at 2 years was 37%. The following factors were associated with the development of chronic GVHD: female donor/male recipient, CMV-Ab seropositivity, matched related peripheral blood grafts vs matched related BM grafts, no *in vivo* T-cell depletion and the occurrence of grade II–IV acute GVHD. Among these factors, the association with acute GVHD occurrence was consistently significant across donor subtypes. The use of U-CB was not associated with chronic GVHD, but was associated with a low incidence of extensive chronic GVHD. Chronic GVHD patients who had received U-CB transplants showed less frequent involvement of the oral cavity (28% vs 55%), eye (12% vs 26%), liver (20% vs 44%), lung (11% vs 25%) and joint (0% vs 6%) than those with matched related BM grafts. In conclusion, we found that U-CB transplants were associated with a low incidence of extensive chronic GVHD and less frequent involvement of the oral cavity, eye, liver, lung and joints.

Bone Marrow Transplantation (2014) **49,** 228–235; doi:10.1038/bmt.2013.151; published online 30 September 2013

Keywords: chronic GVHD; unrelated cord blood; acute GVHD; risk factors

INTRODUCTION

Chronic GVHD is a serious complication that affects the survival and quality of life of long-term survivors after allogeneic hematopoietic SCT.[1–3] Various pre- and post-transplant risk factors associated with chronic GVHD have been identified, mostly in transplantations using BM and PBSC grafts from related or unrelated donors.[2,3] Several studies have reported a history of acute GVHD to be a strong risk factor that is consistently associated with chronic GVHD development.[4–8] Other identified risk factors include the following: female donor and male recipient,[4,6] use of PBSC grafts,[6,9–13] older patient,[4,6–8] older donor,[6,7] transplantation from a mismatched or unrelated donor,[5,6,14] diagnosis of CML[4,7,8] and absence of anti-thymocyte globulin (ATG) use.[15]

The number of unrelated cord blood (U-CB) transplantations performed has rapidly increased during the past decade. However, few studies have compared the incidences and risk factors of chronic GVHD and its organ-specific symptoms in adult patients receiving U-CB and other available grafts, including related or unrelated BM/PBSC grafts.[16,17] Therefore, we conducted a retrospective study using national registry data involving 4818 patients who underwent allogeneic transplantation. This study aimed to evaluate the incidence and risk factors of chronic GVHD, and the prevalence of chronic GVHD organ involvement in patients who received transplantation using various types of graft, including U-CB.

MATERIALS AND METHODS

Data collection

Data for 54 072 patients who had received auto-SCT or allo-SCT by December 31, 2009 were provided by the Transplant Registry Unified Management Program (TRUMP).[18] We included 4993 adult patients who had: (1) received allogeneic transplantation for hematologic malignancies; (2) received their first SCT; (3) used the same questionnaire form involving chronic GVHD organ involvement (skin, oral cavity, eye, liver, lung, joint, intestine/genitals and other manifestations; 2006–2009 for transplantations using BM or PBSC grafts and 2007–2009 for transplantations using U-CB units); (4) achieved neutrophil engraftment; (5) survived for at least 100 days; and (6) received the following: (a) a related BM or PBSC graft (R-BM/PB), (b) an unrelated BM (U-BM) or (c) a single U-CB unit. Donation of peripheral blood by unrelated volunteers was permitted for the first time in Japan in 2011. The following patients were excluded: (1) patients who received *ex vivo* T-cell-depleted grafts ($n = 26$) and (2) patients who lacked data on acute or chronic GVHD ($n = 149$). Thus, 4818 patients were included in this study, which was approved by the TRUMP Data Management Committees and by the institutional review board of the Nagoya University Graduate School of Medicine, where this study was performed.

Histocompatibility

Histocompatibility data for the HLA-A, HLA-B and HLA-DR loci were obtained through reports acquired from the institution where the transplantation was performed or from the cord blood bank. HLA

[1]Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama, Japan; [2]Department of Hematopoietic Stem Cell Transplantation Data Management/Biostatistics, Nagoya University School of Medicine, Nagoya, Japan; [3]Division of Hematology & Oncology, Department of Internal Medicine, University of Michigan Cancer Center, Ann Arbor, MI, USA; [4]Division of Clinical Oncology and Hematology, Jikei University School of Medicine, Tokyo, Japan; [5]Stem Cell Transplantation Division, National Cancer Center Hospital, Tokyo, Japan; [6]Department of Hematology, Toranomon Hospital, Tokyo, Japan; [7]Hematology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan; [8]Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan; [9]Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan; [10]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; [11]Division of Epidemiology and Prevention, Aichi Cancer Center, Nagoya, Japan; [12]Department of Cell Processing & Transfusion, Research Hospital, Institute of Medical Science, University of Tokyo, Tokyo, Japan and [13]Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan. Correspondence: Dr J Kanda, Division of Hematology, Saitama Medical Center, Jichi Medical University, 1-847 Amanuma-cho, Omiya-ku, Saitama City 330-8503, Japan.
E-mail: jkandajp@gmail.com
Received 25 April 2013; revised 10 August 2013; accepted 12 August 2013; published online 30 September 2013

matching was assessed using serological data for the HLA-A, HLA-B and HLA-DR loci in R-BM/PB or U-CB transplantations, and using allelic data for the HLA-A, HLA-B and HLA-DRB1 loci in U-BM transplantations.

Statistical analysis

The physicians who performed the transplantations at each center diagnosed and classified acute and chronic GVHD according to traditional criteria.[1,19] The reported type of chronic GVHD was reclassified according to the information on its organ involvement. 'Progressive onset' of chronic GVHD was defined as chronic GVHD transitioned from active acute GVHD, 'quiescent onset' as chronic GVHD after remission of acute GVHD and '*de novo* onset' as chronic GVHD without history or acute GVHD. The intensity of conditioning regimen was classified as myeloablative or reduced intensity on the basis of the Center for International Blood and Marrow Transplant Research report and the information from the questionnaire, as previously described.[20–23] We defined the following as standard-risk diseases: AML and ALL in first or second remission; CML in the first or

Table 1. Patient characteristics

Variable	*R-BM/PB*		*U-BM*		*U-CB*		P-*value*
	n = *1859*	%	n = *2215*	%	n = *744*	%	
Recipient age, years, median (range)	46 (16–74)		47 (16–73)		51 (16–82)		<0.001
Donor age, years, median (range)	43 (10–79)		35 (20–55)[a]		—	—	—
Recipient sex							
Female	789	42	916	41	334	45	0.238
Male	1070	58	1299	59	410	55	
Sex match between recipient and donor							
Match	965	52	1251	56	227	31	<0.001
Male to female	398	21	573	26	109	15	
Female to male	496	27	389	18	131	18	
Missing	0	0	2	0	277	37	
Disease							
AML	799	43	986	45	395	53	0.004
MDS	210	11	276	12	76	10	
CML	60	3	73	3	25	3	
ALL	385	21	439	20	123	17	
ATL	110	6	131	6	29	4	
NHL	206	11	214	10	70	9	
Other diseases	89	5	96	4	26	3	
Disease risk							
Standard	1058	57	1351	61	331	44	<0.001
High	724	39	780	35	390	52	
Missing	77	4	84	4	23	3	
Source of stem cells							
BM	842	45	2215	100	—	—	—
Peripheral blood	1017	55	—	—	—	—	
Cord blood	—	—	—	—	744	100	
HLA compatibility[b]							
Matched	1486	80	1507	68	53	7	<0.001
Mismatched	373	20	708	32	691	93	
Conditioning regimen							
Myeloablative	1202	65	1505	68	436	59	<0.001
Reduced intensity	649	35	696	31	308	41	
Missing	8	1	14	1	0	0	
GVHD prophylaxis							
CsA based	1367	74	469	21	311	42	<0.001
Tac based	449	24	1737	78	425	57	
Others/missing	43	2	9	1	8	1	
Use of in vivo *T-cell depletion*							
No	1741	94	2143	97	730	98	<0.001
Yes	118	6	72	3	14	2	
CMV Ab (recipient and donor)							
Both negative	127	7	150	7	151	20	<0.001
Either positive	1561	84	2003	90	535	72	
Unknown	171	9	62	3	58	8	
Acute GVHD							
Grade II–IV	665	36	897	41	338	45	<0.001
Grade III–IV	217	12	236	11	81	11	0.578
Follow-up of survivors (years), median (range)	2.0 (0.3–4.7)		1.9 (0.3–4.8)		1.7 (0.3–3.9)		<0.001

Abbreviations: ATL = adult T-cell leukemia; MDS = myelodysplastic syndrome; NHL = non-Hodgkin's lymphoma; R-BM/PB = related BM or PBSC; Tac = tacrolimus; U-BM = unrelated BM; U-CB = unrelated cord blood. [a]Data are missing in 20 patients [b]HLA matching was assessed by serological data for HLA-A, HLA-B and HLA-DR loci in transplantation using R-BM/PB or U-CB grafts, whereas it was assessed by allelic data for HLA-A, HLA-B and HLA-DRB1 loci in transplantation using U-BM grafts.

second chronic phase or in the accelerated phase; myelodysplastic syndrome (MDS) with refractory anemia or refractory anemia with ringed sideroblasts; adult T-cell leukemia (ATL) in CR; and Hodgkin's or non-Hodgkin's lymphoma (NHL) in CR or PR. Others were defined as high-risk diseases.

The probability of developing chronic GVHD was estimated on the basis of cumulative incidence curves.[24] Competing events for chronic GVHD were death or relapse without GVHD. Groups were compared using Gray's test.[25] The Cox proportional hazards model was used to evaluate the effect of confounding variables on chronic GVHD. The following possible confounding variables were considered: recipient age; recipient sex; sex mismatch between recipient and donor (match, male (donor)/female (recipient), or female (donor)/male (recipient)); disease (CML or others); disease risk before transplantation (standard or high risk); donor type (HLA-matched related BM (MR-BM), HLA-matched related PBSCs (MR-PB), HLA-mismatched related BM (MMR-BM), HLA-mismatched related PBSCs (MMR-PB), HLA-matched unrelated BM (MU-BM), HLA-mismatched unrelated BM (MMU-BM) and U-CB); type of conditioning regimen (myeloablative or reduced intensity); type of GVHD prophylaxis (CsA based or tacrolimus based); use of *in vivo* T-cell depletion (yes or no); anti-CMV Ab detection (negative for both recipient and donor, or positive for either recipient or donor), and presence of grade II–IV acute GVHD. Confounding factors were selected in a stepwise manner from the model with a variable retention criterion of $P<0.05$. Reported factors associated with chronic GVHD (recipient age, sex mismatch, donor type, use of *in vivo* T-cell depletion and the presence of grade II–IV acute GVHD) was additionally selected as confounding factors in the analysis of chronic GVHD risk. In the subset analysis, the same variables used in the analysis for the entire cohort were added to the final model. Furthermore, the following variables were also added for the specific group: donor age, presence of an HLA mismatch and the use of PBSCs for the R-BM/PB group; donor age and presence of an HLA mismatch for the U-BM group; and presence of an HLA mismatch for the U-CB group.

We also compared the prevalence of chronic GVHD presentation or organ involvement between MR-BM and other graft types using the χ^2 test. We further evaluated chronic GVHD-specific survival, which is defined as the time from the day of chronic GVHD diagnosis to the day of death in the absence of relapse, among patients who developed chronic GVHD. We also evaluated OS among those who developed chronic GVHD. The probability of developing chronic GVHD-specific survival or OS from the onset of chronic GVHD was estimated using the Kaplan–Meier method, and univariate comparison between groups was performed using the log-rank test. In the analysis of chronic GVHD-specific survival, patients who were alive without disease recurrence were censored at the time of their last follow-up visit and those who experienced disease recurrence were censored at the time of diagnosis of recurrence. The Cox proportional hazards model was used to evaluate the effect of presentation or of each organ's manifestation of chronic GVHD on chronic GVHD-specific survival, after adjusting for donor type and other confounding factors that were selected from the model in a stepwise manner using a variable retention criterion of $P<0.05$. We also evaluated the effect of chronic GVHD on relapse, where the occurrence of chronic GVHD was treated as a time-varying covariate.

All tests were two-sided, and *P*-values <0.05 were considered statistically significant, except for the comparison of prevalence of chronic GVHD organ involvement between MR-BM and other graft types, where *P*-values <0.008 was significant in consideration of multiple comparison. All statistical analyses were performed using Stata version 12 (Stata Corp., College Station, TX, USA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan),[26,27] which is a graphical user interface for R (R Foundation for Statistical Computing, version 2.13.0, Vienna, Austria).

RESULTS

Patient characteristics

Table 1 shows patient characteristics according to the stem cell source. The median age of recipients at the time of the transplant was 47 years (range, 16–82 years) for the entire cohort, and it was significantly higher for patients in the U-CB group. High-risk diseases were more prevalent in the U-CB group. The grafts used were MR-BM ($n=687$), MR-PB ($n=799$), MMR-BM ($n=155$), MMR-PB ($n=218$), MU-BM ($n=1507$), MMU-BM ($n=708$) and U-CB ($n=744$). CsA-based GVHD prophylaxis was received by 74% of the patients in the R-BM/PB group and by only 21% of the U-BM recipients. *In vivo* T-cell depletion was used for only 4% of the entire cohort (ATG, $n=197$; alemtuzumab, $n=7$). Grade II–IV and III–IV acute GVHD occurred in 39% and 11% of the cohort, respectively.

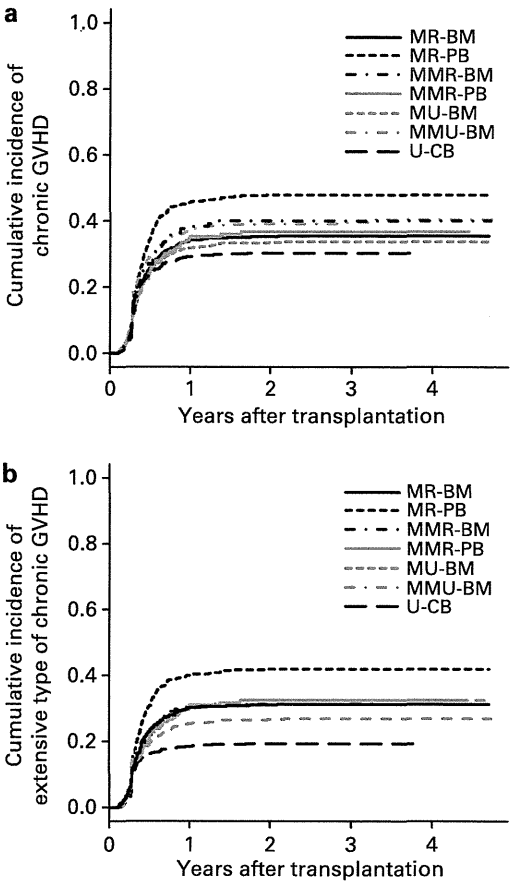

Figure 1. Cumulative incidence of chronic GVHD (**a**) and extensive type of chronic GVHD (**b**).

Chronic GVHD

The incidence of chronic GVHD at 2 years was 37% (95% confidence interval (CI), 35–38%) for the entire cohort, with a median onset of 120 days (range, 30–1203 days), 36% (32–39%) for the MR-BM group, 48% (44–51%) for the MR-PB group, 40% (32–48%) for the MMR-BM group, 37% (30–44%) for the MMR-PB group, 34% (31–36%) for the MU-BM group, 40% (36–44%) for the MMU-BM group and 30% (27–34%) for the U-CB group (Gray's test for the whole group, $P<0.001$; Figure 1a). Female/male mismatch between recipient and donor (hazard ratio (HR), 1.29; $P<0.001$), CMV Ab detection (HR, 1.26; $P=0.015$), the use of MR-PB vs MR-BM graft (HR, 1.49; $P<0.001$), the use of *in vivo* T-cell depletion (HR, 0.48; $P<0.001$) and the occurrence of grade II–IV acute GVHD (HR, 1.62; $P<0.001$) were significantly associated with chronic GVHD development (Table 2). The use of PBSC grafts was significantly associated with chronic GVHD development in the R-BM/PB group (HR, 1.42; $P<0.001$). The impact of CMV Ab positivity on chronic GVHD development was significant only for the U-CB group, but HR was consistently high across donor subtypes. The effect of sex mismatch was significant for the R-BM/PB group, but was not significant for the U-CB group. The effect of grade II–IV acute GVHD occurrence on chronic GVHD development was consistently significant across donor subtypes.

Table 2. Risk factors for chronic GVHD

Variable	*Chronic GVHD (Total)*			*Chronic GVHD (R-BM/PB)*			*Chronic GVHD (U-BM)*			*Chronic GVHD (U-CB)*		
	HR	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*	*HR*	*95% CI*	P-*value*
Recipient age, per 10 years	1.03	(0.99–1.06)	0.136	1.09	(1.01–1.17)	0.021	1.01	(0.96–1.07)	0.741	0.91	(0.83–1.00)	0.056
Donor age, per 10 years				1.01	(0.94–1.09)	0.730	1.04	(0.95–1.14)	0.429			
Sex match between recipient and donor												
Match	1.00			1.00						1.00		
Male to female	0.97	(0.86–1.10)	0.619	1.01	(0.83–1.23)	0.905	1.00	(0.84–1.19)	0.992	0.78	(0.51–1.19)	0.253
Female to male	1.29	(1.14–1.44)	<0.001	1.45	(1.23–1.71)	<0.001	1.16	(0.96–1.41)	0.127	1.12	(0.78–1.62)	0.535
CMV Ab (donor and recipient)												
Both negative	1.00			1.00			1.00			1.00		
Either positive	1.26	(1.05–1.52)	0.015	1.12	(0.82–1.54)	0.469	1.22	(0.90–1.66)	0.196	1.53	(1.07–2.21)	0.021
Type of donor and stem cell source												
MR-BM	1.00											
MR-PB	1.49	(1.26–1.75)	<0.001									
MMR-BM	1.21	(0.91–1.60)	0.187									
MMR-PB	1.31	(1.00–1.72)	0.054									
MU-BM	0.91	(0.78–1.07)	0.247									
MMU-BM	1.10	(0.92–1.31)	0.306									
U-CB	1.00	(0.81–1.23)	0.991									
Type of stem cell source												
BM				1.00								
PB				1.42	(1.23–1.65)	<0.001						
HLA disparity												
Match				1.00			1.00			1.00		
Mismatch				1.12	(0.92–1.36)	0.274	1.17	(1.00–1.36)	0.043	0.96	(0.55–1.69)	0.887
Use of in vivo *T-cell depletion*												
No		1.00		1.00			1.00			1.00		
Yes	0.48	(0.34–0.66)	<0.001	0.29	(0.18–0.45)	<0.001	0.85	(0.55–1.34)	0.490	0.35	(0.05–2.50)	0.293
Acute GVHD												
Grade 0–I		1.00		1.00			1.00			1.00		
Grade II–IV	1.62	(1.47–1.78)	<0.001	1.44	(1.24–1.66)	<0.001	1.73	(1.50–2.00)	<0.001	1.76	(1.34–2.31)	<0.001

Abbreviations: CI = confidence interval; HR = hazard ratio; MMR-BM = HLA-mismatched related BM; MMR-PB = HLA-mismatched related PBSCs; MMU-BM = HLA-mismatched unrelated BM; MR-BM = HLA-matched related BM; MR-PB = HLA-matched related PBSCs; MU-BM = HLA-matched unrelated BM; R-BM/PB = related BM or PBSC; U-BM; unrelated BM; U-CB = unrelated cord blood.

Extensive chronic GVHD

The incidence of extensive chronic GVHD at 2 years was 30% (29–31%) for the entire cohort, 32% (28–35%) for the MR-BM group, 42% (39–46%) for the MR-PB group, 31% (24–39%) for the MMR-BM group, 33% (26–39%) for the MMR-PB group, 27% (25–29%) for the MU-BM group, 32% (28–36%) for the MMU-BM group and 19% (17–22%) for the U-CB group (Gray's test for the whole group, $P<0.001$; Figure 1b). In addition to being a significant variable in the analysis of chronic GVHD, the use of reduced-intensity conditioning (vs myeloablative conditioning) was inversely associated with the development of extensive chronic GVHD (HR, 0.86; $P=0.019$; Table 3). Compared with MR-BM, MR-PB and MMR-PB were associated with the development of extensive chronic GVHD, whereas MU-BM and U-CB grafts were inversely associated with its development. Grade II–IV acute GVHD occurrence was the only significant variable consistently observed across all donor types.

Organ-specific chronic GVHD

Figure 2 shows the type of presentation and organ involvement associated with chronic GVHD. Among the 1716 patients who developed chronic GVHD, *de novo*, progressive and quiescent chronic GVHD presentations were observed in 467 (27%), 348 (20%) and 901 (53%) patients, respectively. Compared with the MR-BM group, progressive chronic GVHD was more frequently observed in the MMU-BM group (33% vs 15%), and quiescent chronic GVHD was more frequently observed in the U-CB group (62% vs 53%).

Limited type of skin involvement was more frequently observed in the U-CB group than in the MR-BM group (53% vs 29%). We examined the types of chronic GVHD (limited vs extensive) in patients with limited type of skin GVHD to evaluate the effect of limited type of skin GVHD on chronic GVHD type in the U-CB group. Accordingly, extensive chronic GVHD was observed in 73% of patients with limited type of skin GVHD in the MR-BM group, compared with 49% of patients in the U-CB group. Oral cavity (28% vs 55%), eye (12% vs 26%), liver (20% vs 44%), lung (11% vs 25%) and joint (0% vs 6%) involvement was less prevalent in the U-CB group than in the MR-BM group. There was no organ that was more frequently involved in the U-CB group than in the MR-BM group.

Progressive onset of chronic GVHD, extensive skin GVHD, intestinal or genital involvement and extensive type of chronic GVHD were significantly associated with lower chronic GVHD-specific survival rates in multivariate analysis, after adjusting for other confounders (Table 4). Lung involvement in GVHD was marginally significant. On the other hand, limited type of skin GVHD was associated with higher chronic GVHD-specific survival rates. Chronic GVHD-specific survival and OS curves showing a significant difference between the groups are shown in Figure 3 and Supplementary Figure 1. The impact of chronic GVHD on relapse is also an important issue. The occurrence of chronic GVHD

Table 3. Risk factors for extensive type of chronic GVHD

Variable	*Extensive chronic GVHD (Total)*			*Extensive chronic GVHD (R-BM/PB)*			*Extensive chronic GVHD (U-BM)*			*Extensive chronic GVHD (U-CB)*		
	HR	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*
Recipient age, per 10 years	1.10	(1.05–1.15)	<0.001	1.12	(1.03–1.21)	0.010	1.07	(1.00–1.15)	0.049	1.10	(0.96–1.26)	0.180
Donor age, per 10 years				1.02	(0.94–1.10)	0.662	1.08	(0.98–1.20)	0.136			
Sex match between recipient and donor												
Match	1.00			1.00			1.00			1.00		
Male to female	1.02	(0.89–1.16)	0.822	1.00	(0.81–1.24)	0.977	1.08	(0.90–1.31)	0.409	0.82	(0.49–1.37)	0.442
Female to male	1.32	(1.16–1.50)	<0.001	1.49	(1.25–1.77)	<0.001	1.25	(1.01–1.55)	0.042	0.88	(0.55–1.41)	0.608
CMV Ab (donor and recipient)												
Both negative	1.00			1.00			1.00			1.00		
Either positive	1.32	(1.06–1.64)	0.014	1.17	(0.83–1.64)	0.383	1.37	(0.95–1.97)	0.089	1.54	(0.97–2.44)	0.068
Type of donor and stem cell source												
MR-BM	1.00											
MR-PB	1.41	(1.19–1.58)	<0.001									
MMR-BM	1.08	(0.79–1.49)	0.614									
MMR-PB	1.35	(1.01–1.81)	0.042									
MU-BM	0.78	(0.66–0.93)	0.005									
MMU-BM	0.93	(0.77–1.13)	0.452									
U-CB	0.65	(0.51–0.83)	0.001									
Type of stem cell source												
BM				1.00								
PB				1.42	(1.21–1.66)	<0.001						
HLA disparity												
Match				1.00			1.00			1.00		
Mismatch				1.10	(0.88–1.36)	0.397	1.14	(0.96–1.35)	0.142	0.89	(0.45–1.76)	0.743
Conditioning												
Myeloablative	1.00			1.00			1.00			1.00		
Reduced intensity	0.86	(0.75–0.97)	0.019	0.90	(0.74–1.08)	0.255	0.88	(0.72–1.07)	0.206	0.64	(0.42–0.96)	0.031
Use of in vivo *T-cell depletion*												
No	1.00			1.00			1.00					
Yes	0.39	(0.26–0.58)	<0.001	0.23	(0.13–0.41)	<0.001	0.80	(0.46–1.37)	0.407			
Acute GVHD												
Grade 0–I	1.00			1.00			1.00			1.00		
Grade II–IV	1.74	(1.56–1.93)	<0.001	1.52	(1.30–1.78)	<0.001	1.91	(1.62–2.26)	<0.001	2.02	(1.43–2.86)	<0.001

Abbreviations: CI = confidence interval; HR = hazard ratio; MMR-BM = HLA-mismatched related BM; MMR-PB = HLA-mismatched related PBSCs; MMU-BM = HLA-mismatched unrelated BM; MR-BM = HLA-matched related BM; MR-PB = HLA-matched related PBSCs; MU-BM = HLA-matched unrelated BM; R-BM/PB = related BM or PBSC; U-BM; unrelated BM; U-CB = unrelated cord blood.

was significantly associated with lower incidence of relapse than the absence of chronic GVHD for the total cohort (HR 0.88, $P = 0.018$). However, we did not find any significant different impact of type, onset and organ involvement of chronic GVHD on relapse among those with chronic GVHD.

DISCUSSION

In the present study, we extensively analyzed the risk factors for chronic GVHD, particularly focusing on donor graft sources and organ involvement, using recently obtained national registry data that included a large number of U-CB transplantations. In addition to confirming previously reported chronic GVHD risk factors, we observed a lower incidence of extensive chronic GVHD in recipients of U-CB than in recipients of MR-BM. Moreover, in patients with chronic GVHD, oral cavity, eye, liver, lung and joint involvement was substantially lower in the U-CB group than in the MR-BM group.

Grade II–IV acute GVHD occurrence was a strong risk factor for chronic and extensive chronic GVHD, regardless of the donor type, which is consistent with previous findings.[4–7] The mechanism through which chronic GVHD develops is considered to be different from that of acute GVHD,[28] and the underlying mechanism by which acute GVHD strongly influences chronic GVHD development remains unknown. Acute GVHD causes thymic epithelial damage and functional deterioration, leading to a decrease in thymic output, represented by low T-cell receptor excision circle levels.[29] The association between low T-cell receptor excision circle levels and occurrence of chronic GVHD was reported in HLA-identical sibling transplantation,[30] which may partly explain the association between the history of acute GVHD and the development of chronic GVHD. The combination of female donor/male recipient was significantly associated with the development of chronic GVHD, which is also consistent with previous studies.[4,6] In the subset analysis, the combination of female donor/male recipient was significant for the R-BM/PB group, but not significant for the U-CB group. T cells transplanted from adult female donors can be activated by exposure to Y-chromosome-associated proteins and may cause chronic GVHD, but those from female U-CB units may be less activated against them.[31] Studies on the effect of the CMV Ab on chronic GVHD development have previously yielded controversial results.[2,32] In this study, we observed a significant impact of CMV seropositivity on the incidences of chronic GVHD and extensive chronic GVHD. However, the presence of antigenemia itself was not a significant factor in univariate analysis (data not shown); therefore, the mechanism through which CMV Ab affects chronic GVHD development remains unknown. We also confirmed that the use of a PBSC graft vs a BM graft constituted a strong risk factor for chronic and extensive chronic GVHD development in the R-BM/PB group. On the other

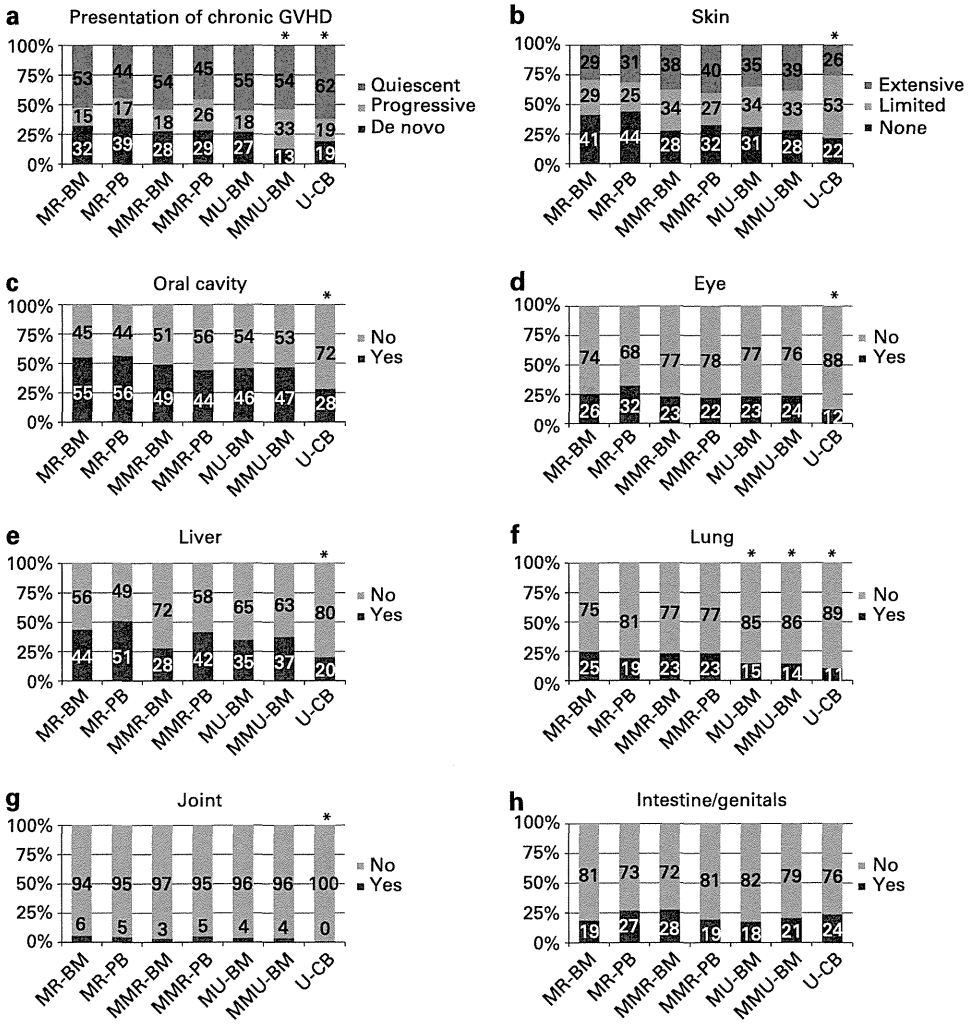

Figure 2. Presentation (**a**) and organ involvement (**b**–**h**) of chronic GVHD according to type of donor and stem cell source. Prevalence was compared between MR-BM and MR-PB, MMR-BM, MMR-PB, MU-BM, MMU-BM or U-CB. *$P<0.008$.

hand, the use of ATG was associated with a lower incidence of chronic GVHD, particularly in the R-BM/PB group. Contrary to previous reports, HLA disparity did not have a strong effect on chronic GVHD development in the R-BM/PB group. In addition, the use of MU-BM grafts was significantly associated with a lower incidence of extensive chronic GVHD. These findings may indicate that GVHD prophylaxis was intensified according to the acknowledged risk of GVHD. Therefore, we performed the same analysis after excluding the use of ATG or in the subgroup of patients who used tacrolimus or CsA as GVHD prophylaxis. However, we obtained the same result, which suggests that some other factor, such as the timing of immunosuppressive agent tapering, may be affecting the results.

In the analysis of chronic GVHD-specific survival, extensive type (vs limited type), progressive onset (vs *de novo* onset), extensive skin involvement (vs none), no skin involvement (vs limited involvement), and intestinal or genital involvement were associated with lower chronic GVHD-specific survival rate. The impact of quiescent onset chronic GVHD has been controversial,[2,33] but chronic GVHD-specific survival in the patients showing quiescent onset chronic GVHD was almost comparable to those showing *de novo* onset in line with several recent reports.[5,34] Although oral involvement was not associated with lower chronic GVHD-specific survival, which is compatible with a previous report,[35] intestinal or genital involvement was associated with lower survival rate. The use of U-CB was not associated with chronic GVHD-specific survival, even when only patients with extensive chronic GVHD were considered (data not shown). This finding suggests that chronic GVHD, if it occurs, does not behave differently regardless of the stem cell source. On the other hand, oral cavity, eye, liver, lung and joint involvement were substantially lower in the U-CB group, which contributed to the significantly lower incidence of extensive GVHD in the U-CB than in the MR-BM group. The high incidence of early TRM, such as that involving graft failure and infection, is considered a disadvantage of U-CB transplantations. However, if a patient survives the first few months following U-CB transplantation without treatment-related complications, the risk of extensive GVHD and GVHD-associated treatment-related complications would then be lower than in other transplantations. The low incidence of chronic GVHD would also contribute to the early discontinuation of immunosuppressive agents, which would allow or even promote immune reconstitution in long-term survivors of U-CB transplantation. Therefore, the choice of using U-CB as an alternative graft source might be prioritized if early treatment-related complications can be avoided through new approaches to ensure engraftment and enhance early immune reconstitution.

Table 4. Impact of type, presentation and organ involvement of chronic GVHD on chronic GVHD-specific survival

Characteristics	*Chronic GVHD-specific survival*		
	HR	*95% CI*	*P-value*
Type of chronic GVHD			
Limited	1.00		
Extensive	2.60	(1.67–4.05)	<0.001
Presentation of chronic GVHD			
de novo	1.00		
Progressive	1.73	(1.10–2.72)	0.017
Quiescent	0.76	(0.51–1.13)	0.173
Skin			
None	1.00		
Limited	0.58	(0.41–0.83)	0.002
Extensive	1.34	(1.01–1.78)	0.043
Oral cavity			
No	1.00		
Yes	0.97	(0.76–1.25)	0.840
Eye			
No	1.00		
Yes	1.03	(0.78–1.35)	0.859
Liver			
No	1.00		
Yes	1.17	(0.91–1.51)	0.225
Lung			
No	1.00		
Yes	1.29	(0.96–1.74)	0.091
Joint			
No	1.00		
Yes	0.93	(0.52–1.66)	0.795
Intestine/genitals			
No	1.00		
Yes	2.15	(1.66–2.78)	<0.001
Others			
No	1.00		
Yes	1.34	(0.85–2.11)	0.206

Abbreviations: CI = confidence interval; HR = hazard ratio. Hazard ratios were adjusted by type of stem cell source, recipient age, disease risk and grade II–IV acute GVHD.

Several limitations of this study should be noted. First, in this study, acute and chronic GVHD were diagnosed on the basis of traditional criteria, whereas chronic GVHD was diagnosed and classified on the basis of NIH criteria in recent studies.[36–39] Therefore, our results cannot be compared with those reported in other studies. In addition, it is possible that late onset acute GVHD was classified as chronic GVHD or early onset of chronic GVHD was defined as acute GVHD. This may bias the association between acute and chronic GVHD. Second, there is a possibility that chronic GVHD that developed a few years after SCT was not reported or was missed. Furthermore, detailed information on the clinical course of GVHD and on the onset of each chronic GVHD organ manifestation was not available; therefore, chronic GVHD-specific survival should be cautiously interpreted. Fourth, because organ involvement of chronic GVHD was not defined in detail in this large retrospective studies, there is a possibility of misclassification regarding organ involvement. Further, the information on intestinal or genital involvement was not separately collected in the questionnaire. Lastly, incidence of chronic GVHD in the present study was relatively low as compared with that in Caucasian cohorts, suggesting that the genetic differences between races may affect occurrence of chronic GVHD. Therefore, the results should be cautiously interpreted when the result is applied for non-Asian populations.

In conclusion, extensive chronic GVHD was less frequently observed in the U-CB group. In addition, among patients who developed chronic GVHD, oral cavity, eye, liver, lung and joint involvement were less frequently observed in the U-CB group. Although limited type of skin GVHD was frequently observed, it remains within the range of limited chronic GVHD. Therefore, the quality of life may be better for long-term survivors of the U-CB group than those of the MR-BM group or the other groups. Progressive onset, extensive chronic GVHD or intestinal or genital involvement was associated with lower chronic GVHD-specific survival, which suggests the need to intensify treatment for patients with these chronic GVHD characteristics. Finally, a prospective study using NIH criteria is needed to compare the

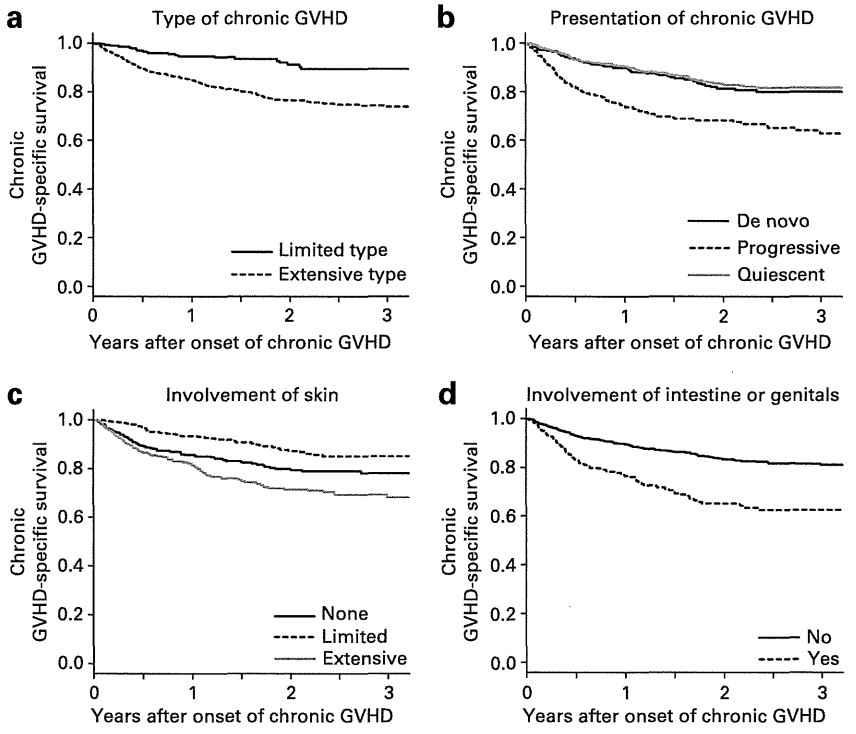

Figure 3. Chronic GVHD-specific survival stratified by type (**a**), presentation (**b**), involvement of skin (**c**) and involvement of intestine or genitals (**d**).

incidences of patients with chronic GVHD between Japan and other countries.

CONFLICT OF INTEREST

The authors declare no conflict interest.

ACKNOWLEDGEMENTS

We thank all the physicians and data managers at the centers who contributed valuable data on transplantation to the Japan Society for Hematopoietic Cell Transplantation (JSHCT), the Japan Marrow Donor Program (JMDP), the Japan Cord Blood Bank Network (JCBBN) and TRUMP. We also thank the members of the Data Management Committees of JSHCT, JMDP, JCBBN and TRUMP for their assistance. This work was supported in part by SENSHIN Medical Research Foundation (JK).

REFERENCES

1 Sullivan KM, Agura E, Anasetti C, Appelbaum F, Badger C, Bearman S *et al.* Chronic graft-versus-host disease and other late complications of bone marrow transplantation. *Semin Hematol* 1991; **28**: 250–259.
2 Lee SJ, Vogelsang G, Flowers ME. Chronic graft-versus-host disease. *Biol Blood Marrow Transplant* 2003; **9**: 215–233.
3 Subramaniam DS, Fowler DH, Pavletic SZ. Chronic graft-versus-host disease in the era of reduced-intensity conditioning. *Leukemia* 2007; **21**: 853–859.
4 Carlens S, Ringden O, Remberger M, Lonnqvist B, Hagglund H, Klaesson S *et al.* Risk factors for chronic graft-versus-host disease after bone marrow transplantation: a retrospective single centre analysis. *Bone Marrow Transplant* 1998; **22**: 755–761.
5 Atsuta Y, Suzuki R, Yamamoto K, Terakura S, Iida H, Kohno A *et al.* Risk and prognostic factors for Japanese patients with chronic graft-versus-host disease after bone marrow transplantation. *Bone Marrow Transplant* 2006; **37**: 289–296.
6 Flowers ME, Inamoto Y, Carpenter PA, Lee SJ, Kiem HP, Petersdorf EW *et al.* Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood* 2011; **117**: 3214–3219.
7 Ozawa S, Nakaseko C, Nishimura M, Maruta A, Cho R, Ohwada C *et al.* Chronic graft-versus-host disease after allogeneic bone marrow transplantation from an unrelated donor: incidence, risk factors and association with relapse. A report from the Japan Marrow Donor Program. *Br J Haematol* 2007; **137**: 142–151.
8 Pavletic SZ, Carter SL, Kernan NA, Henslee-Downey J, Mendizabal AM, Papadopoulos E *et al.* Influence of T-cell depletion on chronic graft-versus-host disease: results of a multicenter randomized trial in unrelated marrow donor transplantation. *Blood* 2005; **106**: 3308–3313.
9 Cutler C, Giri S, Jeyapalan S, Paniagua D, Viswanathan A, Antin JH. Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: a meta-analysis. *J Clin Oncol* 2001; **19**: 3685–3691.
10 Eapen M, Logan BR, Confer DL, Haagenson M, Wagner JE, Weisdorf DJ *et al.* Peripheral blood grafts from unrelated donors are associated with increased acute and chronic graft-versus-host disease without improved survival. *Biol Blood Marrow Transplant* 2007; **13**: 1461–1468.
11 Nagafuji K, Matsuo K, Teshima T, Mori S, Sakamaki H, Hidaka M *et al.* Peripheral blood stem cell versus bone marrow transplantation from HLA-identical sibling donors in patients with leukemia: a propensity score-based comparison from the Japan Society for Hematopoietic Stem Cell Transplantation registry. *Int J Hematol* 2010; **91**: 855–864.
12 Stem Cell Trialists' Collaborative Group. Allogeneic peripheral blood stem-cell compared with bone marrow transplantation in the management of hematologic malignancies: an individual patient data meta-analysis of nine randomized trials. *J Clin Oncol* 2005; **23**: 5074–5087.
13 Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR *et al.* Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med* 2012; **367**: 1487–1496.
14 Lee SJ, Klein JP, Barrett AJ, Ringden O, Antin JH, Cahn JY *et al.* Severity of chronic graft-versus-host disease: association with treatment-related mortality and relapse. *Blood* 2002; **100**: 406–414.
15 Socie G, Schmoor C, Bethge WA, Ottinger HD, Stelljes M, Zander AR *et al.* Chronic graft-versus-host disease: long-term results from a randomized trial on graft-versus-host disease prophylaxis with or without anti-T-cell globulin ATG-Fresenius. *Blood* 2011; **117**: 6375–6382.
16 Narimatsu H, Miyakoshi S, Yamaguchi T, Kami M, Matsumura T, Yuji K *et al.* Chronic graft-versus-host disease following umbilical cord blood transplantation: retrospective survey involving 1072 patients in Japan. *Blood* 2008; **112**: 2579–2582.
17 Eapen M, Rocha V, Sanz G, Scaradavou A, Zhang MJ, Arcese W *et al.* Effect of graft source on unrelated donor haemopoietic stem-cell transplantation in adults with acute leukaemia: a retrospective analysis. *Lancet Oncol* 2010; **11**: 653–660.
18 Atsuta Y, Suzuki R, Yoshimi A, Gondo H, Tanaka J, Hiraoka A *et al.* Unification of hematopoietic stem cell transplantation registries in Japan and establishment of the TRUMP System. *Int J Hematol* 2007; **86**: 269–274.
19 Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J *et al.* 1994Consensus conference on acute GVHD grading. *Bone Marrow Transplant* 1995; **15**: 825–828.
20 Giralt S, Ballen K, Rizzo D, Bacigalupo A, Horowitz M, Pasquini M *et al.* Reduced-intensity conditioning regimen workshop: defining the dose spectrum. Report of a workshop convened by the center for international blood and marrow transplant research. *Biol Blood Marrow Transplant* 2009; **15**: 367–369.
21 Kanda J, Atsuta Y, Wake A, Ichinohe T, Takanashi M, Morishima Y *et al.* Impact of the direction of HLA mismatch on transplantation outcomes in single unrelated cord blood transplantation. *Biol Blood Marrow Transplant* 2013; **19**: 247–254.
22 Atsuta Y, Kanda J, Takanashi M, Morishima Y, Taniguchi S, Takahashi S *et al.* Different effects of HLA disparity on transplant outcomes after single-unit cord blood transplantation between pediatric and adult patients with leukemia. *Haematologica* 2013; **98**: 814–822.
23 Kanda J, Ichinohe T, Kato S, Uchida N, Terakura S, Fukuda T *et al.* Unrelated cord blood transplantation vs related transplantation with HLA 1-antigen mismatch in the graft-versus-host direction. *Leukemia* 2013; **27**: 286–294.
24 Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med* 1999; **18**: 695–706.
25 Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. *Ann Stat* 1988; **16**: 1141–1154.
26 Kanda Y. *Free Statistical Software: EZR (Easy R) on R Commander.* Available from http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html (Accessed on 1 February 2012).
27 Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013; **48**: 452–458.
28 Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet* 2009; **373**: 1550–1561.
29 Krenger W, Blazar BR, Hollander GA. Thymic T-cell development in allogeneic stem cell transplantation. *Blood* 2011; **117**: 6768–6776.
30 Clave E, Rocha V, Talvensaari K, Busson M, Douay C, Appert ML *et al.* Prognostic value of pretransplantation host thymic function in HLA-identical sibling hematopoietic stem cell transplantation. *Blood* 2005; **105**: 2608–2613.
31 Toubai T, Tawara I, Sun Y, Liu C, Nieves E, Evers R *et al.* Induction of acute GVHD by sex-mismatched H-Y antigens in the absence of functional radiosensitive host hematopoietic-derived antigen-presenting cells. *Blood* 2012; **119**: 3844–3853.
32 Bostrom L, Ringden O, Jacobsen N, Zwaan F, Nilsson BA. European multicenter study of chronic graft-versus-host disease. The role of cytomegalovirus serology in recipients and donors--acute graft-versus-host disease, and splenectomy. *Transplantation* 1990; **49**: 1100–1105.
33 Akpek G, Zahurak ML, Piantadosi S, Margolis J, Doherty J, Davidson R *et al.* Development of a prognostic model for grading chronic graft-versus-host disease. *Blood* 2001; **97**: 1219–1226.
34 Kuzmina Z, Eder S, Bohm A, Pernicka E, Vormittag L, Kalhs P *et al.* Significantly worse survival of patients with NIH-defined chronic graft-versus-host disease and thrombocytopenia or progressive onset type: results of a prospective study. *Leukemia* 2012; **26**: 746–756.
35 Horn TD, Rest EB, Mirenski Y, Corio RL, Zahurak ML, Vogelsang GB. The significance of oral mucosal and salivary gland pathology after allogeneic bone marrow transplantation. *Arch Dermatol* 1995; **131**: 964–965.
36 Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ *et al.* National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant* 2005; **11**: 945–956.
37 Arai S, Jagasia M, Storer B, Chai X, Pidala J, Cutler C *et al.* Global and organ-specific chronic graft-versus-host disease severity according to the 2005 NIH consensus criteria. *Blood* 2011; **118**: 4242–4249.
38 Mitchell SA, Jacobsohn D, Thormann Powers KE, Carpenter PA, Flowers ME, Cowen EW *et al.* A multicenter pilot evaluation of the National Institutes of Health chronic graft-versus-host disease (cGVHD) therapeutic response measures: feasibility, interrater reliability, and minimum detectable change. *Biol Blood Marrow Transplant* 2011; **17**: 1619–1629.
39 Sato T, Ichinohe T, Kanda J, Yamashita K, Kondo T, Ishikawa T *et al.* Clinical significance of subcategory and severity of chronic graft-versus-host disease evaluated by National Institutes of Health consensus criteria. *Int J Hematol* 2011; **93**: 532–541.

Supplementary Information accompanies this paper on Bone Marrow Transplantation website (http://www.nature.com/bmt)

Rapid T-cell chimerism switch and memory T-cell expansion are associated with pre-engraftment immune reaction early after cord blood transplantation

Cord blood (CB) contains immature immune cells and is thought to be less active in inducing allogeneic immune reaction than other sources of stem cells. However, a high incidence of immune-mediated complications has been reported, such as pre-engraftment immune reaction (PIR) and haemophagocytic syndrome (HPS) early after cord blood transplantation (CBT) (Kishi *et al*, 2005; Narimatsu *et al*, 2007; Frangoul *et al*, 2009; Takagi *et al*, 2009; Patel *et al*, 2010). In addition, we reported that human leucocyte antigen (HLA) disparity in the graft-*versus*-host (GVH) direction adversely affected engraftment kinetics when single calcineurin inhibitors were used for GVH disease (GVHD) prophylaxis (Matsuno *et al*, 2009). These observations suggested that the GVH reaction plays a critical role in engraftment. Here, we report the engraftment kinetics of donor-derived T cells using a multicolour flow cytometry-based method (HLA-Flow method) (Watanabe *et al*, 2008) and also describe the results of naïve/memory T-cell phenotype analyses early after CBT.

Between November 2009 and September 2010, 73 adult patients underwent single-unit CBT at Toranomon hospital. This study reports 41 patients who were eligible for chimerism analysis using the HLA-Flow method and survived more than 14 d after CBT. Characteristics of the patients and CB are summarized in Table SI. All patients provided written informed consent, and the study was conducted in accordance with institutional review board requirements. Peripheral blood was collected at 1, 2, 3, 4, and 8 weeks after CBT. Anti-HLA monoclonal antibodies in combination with lineage-specific antibodies were used to analyse the lineage-specific chimerism as previously reported (Watanabe *et al*, 2008). Anti-HLA antibodies specific for donor and recipient HLA in all patients are summarized in Table SII. At 2, 4, and 8 weeks after CBT, T-cell subsets were analysed using the following monoclonal antibodies: peridinin-chlorophyll-protein – cyanin 5·5 (PerCP-Cy5·5)-CD8, phycoerythrin – cyanin 7 (PE-Cy7)-CCR7, allophycocyanin (APC)-CD4, APC-Cy7-CD3 (BD Pharmingen, San Jose, CA, USA), and Pacific Blue-CD45RA (CALTAG, Carlsbad, CA, USA). Absolute numbers of $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), and naïve ($CD45RA^+CCR7^+$) and memory ($CD45RA^-CCR7^{+/-}$) T cells were calculated by multiplying the peripheral lymphocyte counts by the percentage of positive cells. PIR was characterized by non-infectious high-grade fever (>38·5°C) coexisting with skin eruption, diarrhoea, jaundice and/or body weight gain greater than 5% of baseline, developing 6 or more days before engraftment (Kishi *et al*, 2005; Uchida *et al*, 2011). Cumulative incidence of neutrophil engraftment, PIR, and GVHD were calculated using Gray's method. Intergroup comparisons were performed using the Mann–Whitney *U*-test.

We analysed lineage-specific chimerism for 32, 40, 40, 34, and 34 patients at a median of 8 (range, 7–11; week 1), 15 (14–20; week 2), 22 (21–25; week 3), 29 (28–36; week 4), and 57 (56–62; week 8) days post-transplant, respectively. Fig 1A shows representative results for $CD4^+$ T-cell chimerism. $CD4^+$ and $CD8^+$ T-cell chimerism results in all patients are shown in Fig 1B. Of 41 enrolled patients, 37 achieved neutrophil engraftment at a median of 19 d (range, 13–38 d). Thirty-nine patients achieved donor-dominant T-cell chimerism (>90%) by 3 weeks after CBT, whereas the remaining two patients, with recipient-dominant T-cell chimerism (>90%) at every point tested, developed graft failure because of early relapse (day 14 post-transplant) and rejection, respectively. Among the 39 patients who achieved donor-dominant T-cell chimerism, two died before engraftment due to non-relapse causes on day 28 (infection) and day 25 (diffuse alveolar haemorrhage), respectively. Among those with donor-dominant chimerism, 24 (63%) of 38 evaluable patients developed PIR at a median of 8 (6–11) days after CBT. Patients who achieved donor-dominant T-cell chimerism (>90%) at 1 week had a higher incidence of PIR compared to those who did not ($P = 0{\cdot}017$, Fig 1C). In a representative patient at 2 weeks after CBT, rapid conversion from naïve to memory phenotype was observed in both $CD4^+$ and $CD8^+$ T cells (Fig 2A). Fig 2B shows the relative proportion of naïve $CD4^+$ and $CD8^+$ T cells at 2, 4, and 8 weeks after CBT in 37 evaluable patients who achieved donor-dominant T-cell chimerism. Patients who developed PIR had significantly more lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ memory T cells, and $CD8^+$ memory T cells at 2 weeks after CBT compared with those without PIR (Fig 2C and data not shown).

Our data confirmed that a majority of patients achieved donor-dominant T-cell chimerism around 2 weeks after CBT. We also found that early recipient-type T-cell chimerism was closely associated with graft rejection. A remarkable finding was that a rapid recipient-to donor-dominant switch

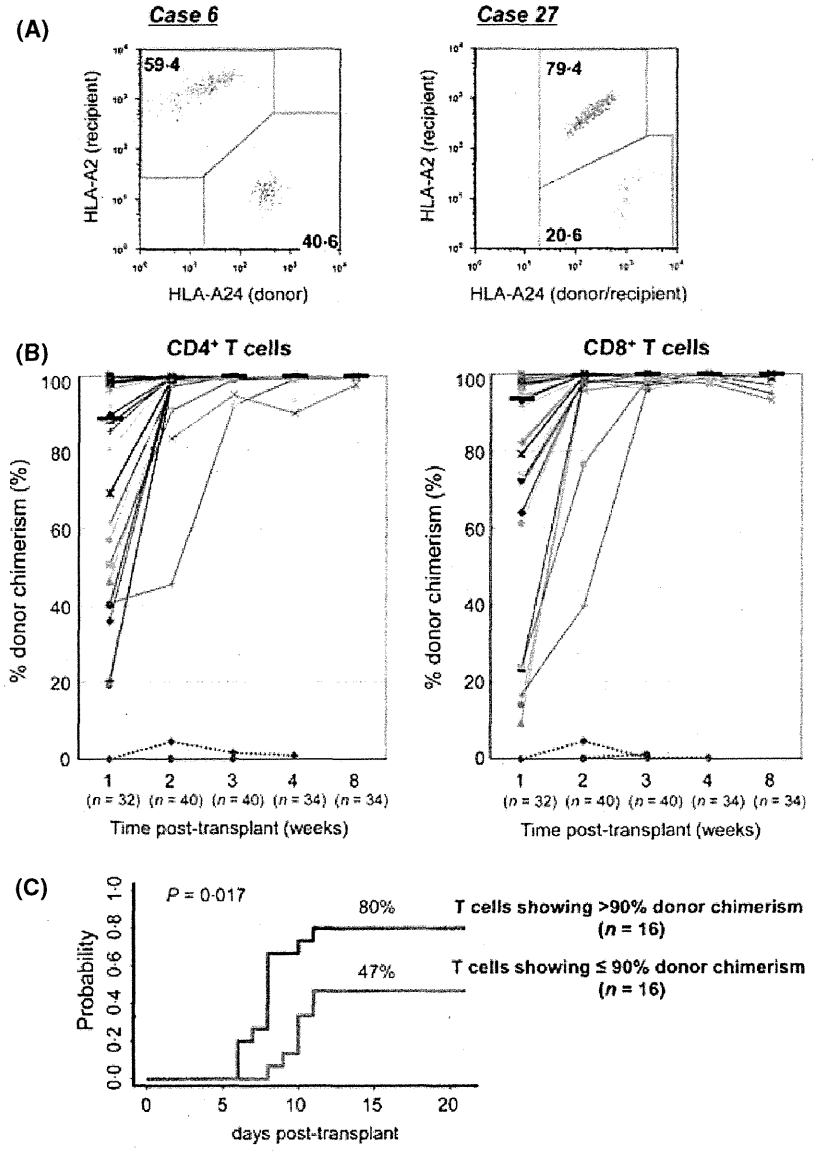

Fig 1. T-cell chimerism analysed by HLA-Flow method. (A) Chimerism analysis by the HLA-Flow method separated donor- vs. recipient-derived cells among $CD4^+$ T cells at 1 week after cord blood transplant (CBT). In Case 6, human leucocyte antigen (HLA)-A2 was recipient-specific and HLA-A24 was donor-specific. In Case 27, HLA-A2 was recipient-specific, whereas HLA-A24 was shared by both donor and recipient, indicating that HLA-A2-negative and HLA-A24-positive cells were donor-derived. (B) The median percentages of donor-derived $CD4^+$ T cells and $CD8^+$ T cells at 1 week after CBT were 88·9%, and 93·5%, respectively. Red dotted lines indicate recipient-dominant chimerism in two patients who developed graft failure. (C) Cumulative incidence of pre-engraftment immune reaction (PIR) according to chimerism status of T cells at 1 week after CBT

of T-cell chimerism at 1 week post-transplant was associated with a higher incidence of PIR, supporting a hypothesis that PIR could be an early variant form of GVH reaction caused by donor-derived T cells. CB T cells are naïve and do not include pathogen-specific effector T cells. Grindebacke *et al* (2009) demonstrated that about 80% of $CD4^+$ T cells kept the naïve phenotype during the first 18 months after birth. In contrast, we found a rapid conversion from naïve to memory phenotype at 2 weeks after CBT. In addition, PIR could be associated with peripheral expansion of donor-derived memory T cells. Recently, Gutman *et al* (2010) reported that $CD8^+$ T cells predominately expressed effector memory or effector phenotype early after double-unit CBT, reflecting an immune response of the dominant unit against the non-engrafting unit. These findings suggest that donor-derived naïve T cells will be activated by alloantigens and differentiate into mature cells early after CBT. Most of the present patients with PIR responded promptly after a

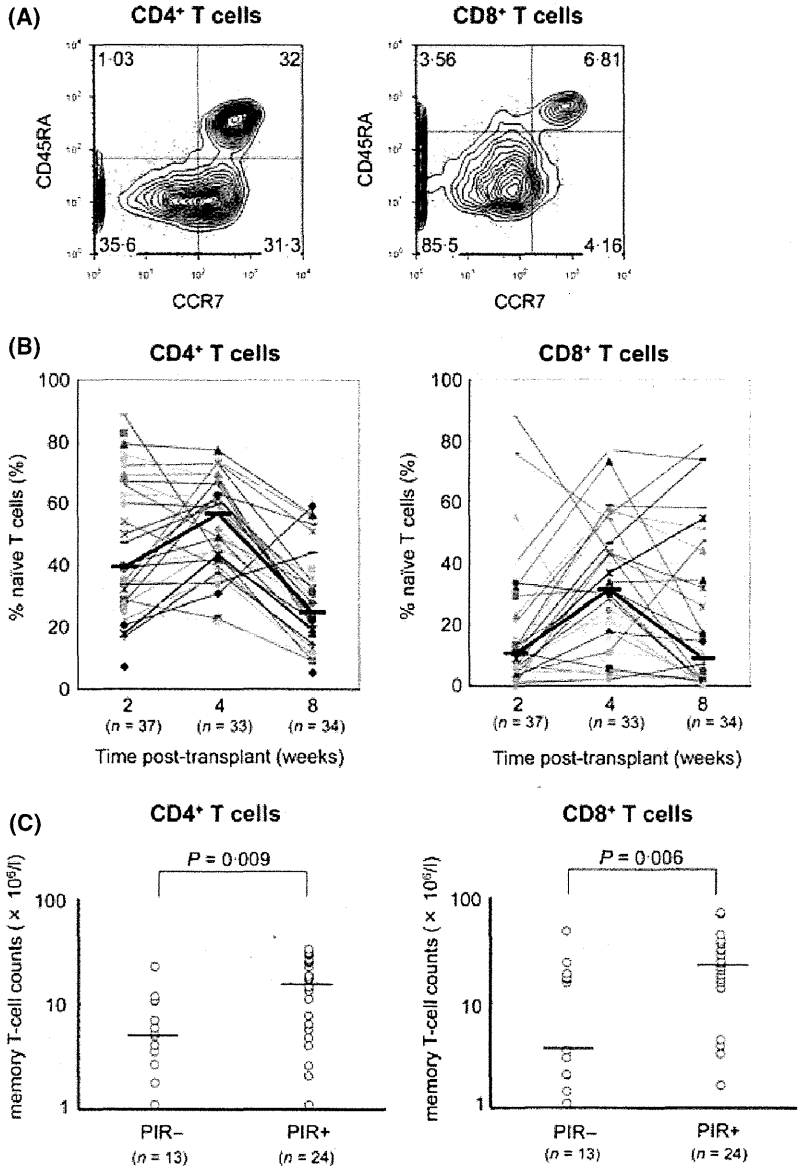

Fig 2. Conversion from naïve to memory T-cell phenotype. (A) A rapid conversion from naïve phenotype ($CD45RA^+CCR7^+$) to memory phenotype ($CD45RA^-CCR7^{+/-}$) in a representative sample at 2 weeks after cord blood transplant (CBT) (Case 5). (B) Relative proportion of naïve $CD4^+$ and $CD8^+$ T cells at 2, 4, and 8 weeks after CBT. Bold horizontal lines denote median values. (C) Memory T-cell counts at 2 weeks after CBT in patients with or without pre-engraftment immune reaction (PIR).

short course of steroid treatment, and none experienced graft failure due to HPS. This observation could be attributed to more intensive immunosuppression from adding mycophenolate mofetil to tacrolimus in the majority of patients (Uchida *et al*, 2011). Although neither the T-cell chimerism nor the memory T-cell counts affected the incidence of acute GVHD, steroid treatment for PIR could suppress the onset of acute GVHD. In conclusion, rapid T-cell chimerism switch and donor-derived memory T-cell expansion were associated with PIR, supporting a significant role of donor-derived T cells in the pathogenesis of the early immune reaction after CBT.

Acknowledgements

The authors thank Madoka Narita for data collection and her skilful secretarial assistance; Eri Watanabe, Mari Muto, and Stephanie Napier for their technical expertise. We also thank all physicians (Shigeyoshi Makino and Hideki Araoka), nurses, pharmacists (Yumiko Uchida and Tadaaki Ito), data managers (Naomi Yamada, Kaori Kobayashi and Rumiko Tsuchihashi), and support personnel for their care of patients involved in this study. This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Health, Labour, and Welfare (H21-Clinical Research-Ippan-020).